An investigation of the kinase dependent functions of CRaf

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by

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Abstract

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Catherine Louise Noble

The CRaf protein kinase plays a key role in relaying proliferation, differentiation and survival signals from activated RAS or BRaf proteins to downstream effectors and is frequently hyperactivated in human cancers. Because of this essential role in signalling, CRaf kinase activity must be tightly regulated in the cell. A well-established model involves RAS-mediated translocation of CRaf to the plasma membrane where it is activated by a series of events including phosphorylation. Here, an important new mechanism of regulation that is distinct from the classical model has been discovered. Through the use of kinase defective craf<sup>D486A</sup> murine embryonic fibroblasts, or by the use of the Raf inhibitor sorafenib, we show that the principal function of the CRaf kinase activity is autophosphorylation of serine 621. This phosphorylation occurs <i>in cis</i>, does not involve MEK/ERK activation and is essential to ensure the correct folding and stability of the protein. This novel mechanism of regulation has significant implications for the control of CRaf activity in fundamental cellular processes in response to signalling from the oncoproteins RAS and BRaf. Indeed here it is demonstrated that CRaf promotes cell cycle progression, specifically the transition into S phase. Investigation of mutant craf cells demonstrated that this form of regulation is mediated in part by CRaf transcriptional repression of the CDK inhibitor p21<sup>CIP1</sup>. 
Acknowledgements

I would like to express sincere gratitude to my supervisor Prof. Catrin Pritchard for her invaluable advice, guidance and expertise. I would also like to thank all members of lab 3/43, both past and present. Particular thanks go to Susan, Vicky, Emma, Linda, Kath and Katerina for all their support, advice and invitations for coffee. I am as ever, especially indebted to my parents for their constant love and encouragement. Finally I want to say a special thank you to my husband James for his patience, love, friendship and humour. No one could have given as much unconditional support and encouragement. This thesis is dedicated to the memory of Aunty Pauline and Uncle Jim.
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Abbreviations

ATF-2 activating transcription factor 2
AMPK adenosine monophosphate-activated protein kinase
AP2 activator protein 2
Apaf-1 apoptotic protease factor-1
ASK1 apoptosis signal-regulating kinase 1
ATP adenosine triphosphate
Bim Bcl-2-interacting mediator of cell death
BrdU bromodeoxyuridine
cAMP cyclic AMP
CD95L CD95 ligand
CDC cell division cycle
CDK cyclin dependent kinase
cDNA complimentary deoxyribonucleic acid
C/EBPβ CCAAT/enhancer binding protein-β
CHIP carboxyl terminus of Hsc70 interacting protein
CK2 casein kinase 2
CKI cyclin dependent kinase inhibitors
CNK connector enhancer of KSR
cPLA$_2$ cytoplasmic phospholipase A$_2$
CR conserved region
CREB c-AMP-response element binding protein
CRD cysteine-rich domain
CT threshold cycle
DISC death-inducing signalling complex
DNA deoxyribonucleic acid
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<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>E2F</td>
<td>E2 promoter binding factor</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>eIF-4E</td>
<td>eukaryotic initiation factor 4E</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<tr>
<td>G0</td>
<td>gap 0</td>
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</tr>
<tr>
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<td>gap 2</td>
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<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
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<td>GDP</td>
<td>guanosine diphosphate</td>
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<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<tr>
<td>GPCR</td>
<td>G-Protein coupled receptor</td>
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<tr>
<td>G Protein</td>
<td>guanine nucleotide-binding protein</td>
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<td>guanosine triphosphate</td>
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<td>hbER</td>
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<td>HSP90</td>
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<tr>
<td>HVR</td>
<td>hypervariable region</td>
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<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early genes</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IMP</td>
<td>impedes mitogenic signal propagation</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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## Abbreviations

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<tr>
<td>kDa</td>
<td>kilodalton(s)</td>
</tr>
<tr>
<td>KSR</td>
<td>kinase suppressor of RAS</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LIF</td>
<td>leukaemia inhibitory factor</td>
</tr>
<tr>
<td>M</td>
<td>mitosis</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>mitogen-activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryo fibroblast</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
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<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
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<tr>
<td>MNKs</td>
<td>MAP kinase interacting kinases</td>
</tr>
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<td>MP1</td>
<td>MEK-partner 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSK</td>
<td>mitogen- and stress-activated kinase</td>
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<tr>
<td><em>neo</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>neomycin resistance</td>
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<td>nerve growth factor</td>
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<td>negative-charge regulatory region</td>
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<td>p90 ribosomal S6 kinase</td>
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<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
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<td>PARP</td>
<td>poly ADP ribose polymerase</td>
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<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>PI3K</td>
<td>phosphoinositide 3 kinase</td>
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<td>protein kinase A</td>
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<td>protein kinase B</td>
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<tr>
<td>PMA</td>
<td>phorbol-12-myristate-13-acetate</td>
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<td>protein phosphatase 2A</td>
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<td>qPCR</td>
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<td>RTK</td>
<td>receptor tyrosine kinase</td>
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<td>SOS</td>
<td>son of sevenless</td>
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<tr>
<td>SPRED</td>
<td>Sprouty, and related proteins with EVH1 domains</td>
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<td>serum response factor</td>
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1. INTRODUCTION

1.1 Cell signalling

Cell signalling is a method of communication between cells, necessary for them to regulate their development, proliferation, differentiation and organisation into tissues. This process involves the secretion of chemicals by one cell, which are detected by receptors of another cell; or the presentation of plasma membrane bound signalling molecules that contact neighbouring cells. Eukaryotic cells contain extensive protein networks, which allow cells to respond appropriately to an extracellular signal. Such networks are organised into signal transduction cascades, often with a high degree of cross-talk between them. A model signal transduction cascade would include a cell surface receptor which, when stimulated by extracellular ligand binding, would activate the first in a series of protein kinases and/or phosphatases and make use of other accessory proteins including GTP binding proteins and adaptor proteins. An example of such a cascade is the mitogen-activated protein kinase (MAPK) pathway.

1.1.1 The mitogen-activated protein kinase (MAPK) pathway

The MAPK pathway is a fundamental cell signalling cascade conserved among eukaryotes. Such pathways are involved in a diverse set of responses including proliferation, differentiation, adaptation to environmental stress, and apoptosis which occur through the regulation of transcription, metabolism and cytoskeletal rearrangements. The basic assembly of a MAPK pathway is composed of three kinases that are sequentially activated by phosphorylation (Figure 1.1). A receptor protein, which may be a G-Protein coupled receptor (GPCR) or a receptor tyrosine kinase (RTK), responds to an extracellular stimulus and activates the first protein in a core module of three conserved kinases; a mitogen-activated protein kinase kinase kinase (MAPKKK). The MAPKKKs are serine/threonine kinases that, when activated, phosphorylate and activate the next kinase in the module, a
A receptor protein responds to an extracellular stimulus and indirectly activates a MAPKKK. These are serine/threonine kinases which activate a MAPKK. MAPKKK recognise and phosphorylate a Thr-X-Tyr motif in the activation loop of a MAPK. Activated MAPK phosphorylates nuclear and/or cytoplasmic substrates on serine and threonine residues, thereby inducing cellular effects.
mitogen-activated protein kinase kinase (MAPKK). MAPKKs are dual-specificity kinases that recognise and phosphorylate a Thr-X-Tyr motif in the activation loop of a mitogen-activated protein kinase (MAPK), which is the final conserved kinase of the three-tiered kinase module. Activated MAPKs phosphorylate substrates on serine and threonine residues. The majority of MAPK substrates are transcription factors, but substrates also include other protein kinases, phospholipases, and cytoskeleton-associated proteins. The arrangement of the MAPK cascade allows amplification of the signal and perhaps, more importantly, the opportunity for further levels of regulation of the activity of each component of the cascade.

1.1.2 Mammalian cells possess multiple MAPK pathways

Multiple MAPK pathways exist in mammalian cells which are activated by distinct stimuli and mediate specific responses. MAPK cascades include the extracellular signal-regulated kinase 1 and 2 (ERK 1/2), p38MAPK, c-Jun N terminal kinase (JNK), and ERK5 pathways. Within any given pathway, several MAPKKKs, MAPKKs, and MAPKs can operate. For example, there are three isoforms and ten different splice variants of the MAPKs in the JNK cascade (Gupta et al., 1996). MAPK pathways are organised into modules, achieved by the tethering of the kinases to scaffold proteins as well as by direct interaction between the kinases. This modular organisation ensures segregation of the pathways from other cellular signalling events and also allows the use of any given kinase in more than one MAPK module without affecting the specificity of the response.

The best described MAPK signalling pathway in mammalian cells is the extracellular signal-regulated kinase (ERK) pathway and is focused on here. This cascade is predominantly activated by mitogenic stimuli. In this pathway, activated Ras binds to and brings about the activation of the MAPKKK, Raf, of which there are three isoforms: ARaf, BRaf and CRaf. Raf phosphorylates and activates MEK1 or MEK2, the MAPKKs (Marais and Marshall, 1996, Whitmarsh and Davis, 1996). MEK1/2 in turn phosphorylate and activate ERK1 or ERK2, the MAPKs. Activated ERKs can then translocate to the nucleus and activate transcription
factors by phosphorylation, thus regulating the expression of specific target genes. Additionally, ERKs also have cytoplasmic substrates, which when activated may influence gene expression directly or indirectly. ERKs cytoplasmic substrates include the protein kinase p90RSK (p90 ribosomal S6 kinase) (Sturgill et al., 1988). Nuclear substrates include the transcription factors Elk-1 (Marais et al., 1993), ATF-2 (Abdel-Hafiz et al., 1992) and c-myc (Seth et al., 1992).

1.2 The Raf proteins

1.2.1 Identification of the Raf family members

The Raf proteins are a family of serine/threonine kinases, that participate as the MAPKKKs in the ERK cascade. Three isoforms exist in mammalian cells: ARaf, BRaf and CRaf. The craf gene was originally identified as the cellular homolog of v-raf. This was the first oncogenic serine/threonine kinase to be discovered. It was found in the retrovirus MSV3611 which infects mice (Rapp et al., 1983) inducing fibrosarcomas and erythroleukaemias. V-Raf was later identified as a truncated version of murine CRaf, where the regulatory domain is deleted and the kinase domain is fused to retroviral Gag sequences. The avian homolog of v-raf was identified as v-mil, an oncogene present in the retrovirus Mill-Hill No. 2 (Jansen et al., 1983). The araf gene was later identified by its homology to v-raf (Huleihel et al., 1986) and braf was identified by its homology to v-mil (Marx et al., 1988) and also as an oncogene in NIH3T3 cells transformed with human Ewing sarcoma DNA (Ikawa et al., 1988). Subsequently it was found that the Raf kinases are highly conserved across many organisms, and have roles in a variety of developmental processes, as well as functioning in apoptosis and the regulation of the cell cycle. (Wojnowski et al., 2000).
1.2.2 Raf expression patterns

Historically it was believed that both the araf and braf genes had limited expression profiles and that craf was the only isoform to have ubiquitously expressed mRNA. araf mRNA was found to be expressed most highly in urogenital organs and braf mRNA expression was restricted to neuronal tissues (Barnier et al., 1995, Storm et al., 1990b). Therefore CRaf was studied to the greatest extent. However, due to the availability of improved antibodies for the Raf isoforms, subsequent studies have demonstrated that BRaf and ARaf are ubiquitously expressed throughout adult and embryonic mice (Luckett et al., 2000, Wojnowski et al., 2000).

ARaf is the smallest isoform, at approximately 68 kDa and CRaf is 74 kDa. BRaf is subject to alternative splicing involving exons 8b and 9b (originally named exon 10a). Additionally this is coupled with the presence of two different N termini which give rise to a variety of proteins ranging from 67-99 kDa, which have differing tissue-specific expression profiles in the adult mouse (Barnier et al., 1995, Storm et al., 1990b, Eychene et al., 1995). For example in the PC12 cell line, BRaf is observed as a 95kDa protein (Stephens et al., 1992). However two BRaf isoforms of 75 kDa and 77 kDa have been detected in mouse testis (Sithanandam et al., 1990). It has been demonstrated that this differential splicing can affect the ability of BRaf to activate MEK, suggesting that BRaf is subject to a secondary mode of regulation in comparison with ARaf and CRaf. The presence of exon 9b increases BRaf’s affinity and basal kinase activity towards MEK, however the presence of exon 8b has the opposite effect (Papin et al., 1998, Hmitou et al., 2007). The mechanism for this regulation was investigated by Hmitou et al (2007). They showed that exons 8b and 9b modulate the ability of the N-terminus to interact with and inhibit the BRaf kinase domain in an opposite manner. B-Raf activity has previously been reported to be down-regulated upon phosphorylation of S365 and S429 and interestingly these residues were found to be involved in the differential regulation of BRaf isoforms. By using phospho-specific antibodies and generating S365A and S429A mutants of the different isoforms the authors identified
that the presence of exon 8b favours phosphorylation of S365, whereas in isoforms containing exon 9b this residue is less efficiently phosphorylated, in correlation with its elevated activity. Phosphorylation of S429 differentially regulates the activity of the isoforms of BRaf to a lesser extent. However, this residue’s phosphorylation results in the activation of 8b-containing isoforms and the inhibition of 9b-containing BRaf isoforms (Hmitou et al., 2007).

1.2.3 Raf protein structure

The Raf proteins share a common architecture, consisting of three conserved regions; CR1, CR2 and CR3 (Figure 1.2). Contrastingly, the remainder of each protein’s sequence is highly variable (Bonner et al., 1986, Beck et al., 1987, Sithanandam et al., 1990). The CR3 region, located in the C-terminus contains the catalytic kinase domain and has been identified as the minimum transforming portion of the protein (Beck et al., 1987, Stanton and Cooper, 1987, Ikawa et al., 1988, Stanton et al., 1989, Heidecker et al., 1990), whereas the CR1 and CR2 regions are located in the N-terminus. The loss of this region in the virally acquired braf and craf oncogenes implied that this portion of the Raf proteins was responsible for suppressing the kinase domain. Indeed N-terminal truncations result in the Raf proteins’ constitutive activation and ability to transform NIH3T3 cells. Furthermore, when the regulatory domain and kinase domain are expressed as separate constructs, the regulatory domain can inhibit the kinase (Cutler et al., 1998). However investigation has shown that the activity of the kinase is not regulated by the N-terminus alone; the process of activation is far more complex and is described later.

The CR1 region in CRaf consists of residues 51-194 and contains three domains, including a putative zinc-binding domain (Beck et al., 1987, Ghosh and Bell, 1994). Two of the CR1 domains; the Ras binding domain (RBD), spanning residues 51-131
Figure 1.2 **Simplified structure of the Raf proteins.**

The Raf isoforms share conserved regions CR1 (blue), CR2 (green) and CR3 (red). The CR1 contains the Ras-binding domain (RBD) and the cysteine-rich domain (CRD), required for membrane recruitment. The CR3 contains the kinase domain. Significant regulatory phosphorylation sites are indicated.
(Scheffler et al., 1994, Vojtek et al., 1993), and the cysteine-rich domain (CRD), spanning residues 139-184 (Mott et al., 1996), bind to Ras. The binding of Ras to the CRD is independent of Ras GTP/GDP status. However the RBD only binds to Ras in its GTP-bound and active form. The RBD was identified by a yeast two-hybrid screen, in which several clones were isolated that coded for overlapping Ras-binding fragments of Raf (Vojtek et al., 1993). Subsequently a 81 amino acid sequence comprising of residues 51-131 of the RBD was identified as common to the clones and was sufficient for Ras.GTP binding (Emerson et al., 1994, Chuang et al., 1994, Herrmann et al., 1995). The X-ray crystal structure of the RBD of CRaf in complex with Rap1A (a member of the Ras family with an effector region identical to Ras and an overall sequence homology of 50%) bound to the GTP analogue GppNHp, was solved by Nassar et al. (1995). The crystal structure of a Raf isoform bound to Ras has not yet been solved, however the interaction is believed to be analogous to that occurring with Rap1A due to the similarity between Rap1A and Ras. The topology of the RBD was identified as similar to that of ubiquitin and as such was classified as an ubiquitin superfold. The interaction between CRaf and Rap1A appears to be mediated by a central anti-parallel β-sheet formed by the interaction of two β-strands from each protein. The binding of Ras is required for the localisation of Raf to the plasma membrane where Ras is predominantly located. Localisation to the plasma membrane is not sufficient for Raf activation but initiates the process. Therefore the N-terminus of the Raf proteins has a dual role in suppressing kinase activity and the regulation of the subcellular localisation of the protein.

The CR2 region encompasses residues 254–269 of CRaf. It is rich in serine and threonine residues, some of which have been identified as regulatory phosphorylation sites. For example phosphorylation of S259 allows the binding of the adapter/scaffold protein 14-3-3 which interferes with Ras binding (Dhillon et al., 2002, Light et al., 2002).
The CR3 region in CRaf consists of residues 335-627. This region encloses the negative-charge regulatory region (N-region) and the kinase domain and is the most homologous region between the three Raf proteins (Daum et al., 1994).

The N-region contains Y341, conserved in ARaf (Y302) but replaced in BRaf by D448. Phosphorylation of this residue is necessary to fully activate ARaf and CRaf at the plasma membrane. However, its absence in BRaf results in some important regulatory differences in this isoform which will be discussed later. S338 is conserved in all Raf proteins (S299 in ARaf and S445 in BRaf), and must be phosphorylated for full activity, but in BRaf, it is constitutively phosphorylated. Again this is a key difference, altering the regulation of BRaf.

The crystal structure of the BRAF kinase domain bound to the small molecule inhibitor BAY43-9006 was solved in 2004 (Wan et al., 2004). Its conformation is typical of a kinase domain (Figure 1.3); it is separated into N-terminal and C-terminal lobes separated by a catalytic cleft, where the ATP binding site is located. The N-terminal lobe is stabilised by an aspartic acid residue at position 447. This lobe contains a glycine loop, identified by the GXGXXG motif, which functions in the localisation of the phosphates of ATP. The C-terminal lobe contains the activation loop; this is a region spanning the conserved sequences DFG and APE which is involved in the binding of an ATP chelating metal. This element contains two phosphorylation sites T491 and S494, which are conserved in ARaf (T452 and T455) and BRaf (T598 and S601) and are necessary for the activation of CRaf, BRaf and most probably ARaf. The BRAF kinase domain was found to be atypical in one respect; there is an intramolecular interaction between the glycine rich loop and the activation loop. This displaces the activation loop and effectively holds the kinase domain in an inactive conformation (Wan et al., 2004). The BRAF activating phosphorylation site T599 (T491 in CRAF) is buried within the interface of this interaction. When this site is mutated to isoleucine, BRAF is strongly activated (Wan et al., 2004, Ikenoue et al., 2003).
Figure 1.3 The BRAF kinase domain crystal structure. (Taken from Garnett and Marais, 2004)

The BRAF kinase domain consists of residues 447-725. It is highly homologous to the kinase domains of ARAF and CRAF. Key features of the domain are indicated. The interaction that occurs between D447 of the N-region and R505 of the αC-helix is shown as a dotted red line. T598, the activation segment phosphorylation site, is coloured yellow. A portion of the activation segment is disordered and is indicated by the dashed magenta line. For clarity the inhibitor that BRAF was crystallized with, BAY43-9006, has been omitted.
This observation indicates that the presence of the bulky side chain of the isoleucine residue introduced into this position can disrupt the inhibitory interactions that occur between the activation loop and the glycine rich loop, freeing the activation loop and allowing the kinase to fold into the active conformation. Assuming ARAF and CRAF kinase domains are homologous to that of BRAF; an explanation can be suggested for how phosphorylation of residues within the activation loop can lead to activation of the kinase. In unstimulated cells the kinase domain is held in an inactive conformation because of the inhibitory interactions that occur between the activation loop and the glycine rich loop. However phosphorylation of the activation loop residues disrupts this interaction, which frees the activation segment and allows the kinase to fold into the active conformation.

1.3 Activation of the Raf proteins

1.3.1 Signalling through receptor proteins to Ras

There are many different receptor types capable of activating the ERK pathway, including RTKs, GPCRs, certain cytokine receptors which regulate intracellular protein tyrosine kinases and the engagement and clustering of integrins.

The pathway leading from RTKs to the activation of the ERK cascade is presently the best understood (Figure 1.4). RTKs include the epidermal growth factor (EGF) receptor, and the platelet derived growth (PDGF) receptor. Such receptors are monomers which span the plasma membrane. RTKs are activated upon a ligand binding to the extracellular domain, which induces receptor dimerisation and trans-autophosphorylation of tyrosine residues in the RTK cytoplasmic domain (Schlessinger, 1988, Lemmon and Schlessinger, 1994, Jiang and Hunter, 1999). The phosphotyrosine residues serve as binding sites for Src homology (SH2) or phosphotyrosine binding domains (PTB) of a variety of adaptor proteins including Shc. The binding of Shc to the RTK via its PTB permits tyrosine phosphorylation of Shc by the receptor itself or intracellular tyrosine kinases such as Src (Basu et al., 1994). The
RTKs become activated upon ligand binding which induces their dimerisation and trans-autophosphorylation of their cytoplasmic domains. This enables the binding of Grb2/Sos which activate Ras. Ras binds to CRaf, localising it to the plasma membrane where it is activated by a series of phosphorylation and dephosphorylation events. Active CRaf phosphorylates MEK, which in turn phosphorylates ERK. ERK has nuclear and cytoplasmic substrates.
phosphorylation of Shc allows the binding of another adapter protein, Grb2, which contains a SH2 domain and two SH3 domains (Sasaoka et al., 1994). The association between Shc and Grb2 is mediated by the SH2 domain of Grb2. Additionally Grb2 can also bind directly to the EGF receptor through its SH2 domain (Basu et al., 1994). The binding of Grb2 also recruits the guanine nucleotide exchange factor Sos (son of sevenless) which binds constitutively to the Grb2 SH3 domain (Sasaoka et al., 1994). Sos catalyses the exchange of Ras.GDP for GTP, activating Ras (Quilliam et al., 1995). The co-localisation of Sos with its target protein Ras, which is localised at the plasma membrane was initially thought to be the primary mechanism regulating the Sos-mediated nucleotide exchange of Ras (Rozakis-Adcock et al., 1993, Li et al., 1994, Matsuda et al., 1994). However, later studies have revealed that Sos exists in an auto-inhibited state and the direct interaction with Ras modulates its activity in a two-step manner. Firstly, the binding of Ras.GDP to Sos induces low GEF activity (Sondermann et al., 2004). Ras then binds with a higher affinity to the same site, causing a conformational change in Sos which relieves the steric hindrance at its active site and induces a high GEF activity (Margarit et al., 2003, Freedman et al., 2006). This mechanism ensures that cytoplasmic Sos remains inactive until recruited to the plasma membrane and to Ras. It also provides a feedback loop which affects the duration of Ras signalling (Boykevisch et al., 2006), furthermore phosphorylation of Sos by ERK induces its disassembly and terminates Ras activation. The activation of Ras leads to the activation of Raf, the MAPKKK in the ERK cascade.

1.3.2 Ras

Ras proteins are 21kDa membrane associated guanine nucleotide binding proteins. There are three human RAS genes which encode four homologous proteins, H-RAS, N-RAS, K-RAS4A and K-RAS4B. The two K-RAS isoforms are acquired through alternative splicing events. Together these RAS isoforms are the founding members of a large superfamily of RAS-related proteins.
Historically, Ras activation was thought to occur exclusively at the plasma membrane as has been described here. However, isolation of an activated signalling complex from endosomes suggested that Ras might also signal from other intracellular sites (Di Guglielmo et al., 1994, Baass et al., 1995). Activated Ras proteins have also been detected on intracellular membranes, including the Golgi and endoplasmic reticulum (Burke et al., 2001) (Chiu et al., 2002). Ras localisation is influenced by its processing and trafficking. The membrane trafficking of Ras is mediated by the sequence and post-translational modifications of its hypervariable region (HVR) in the C-terminal region. For example the HVR of K-Ras 4B contains basic residues, unlike H-Ras and N-Ras which contain cysteine residues that are palmitoylated (Hancock et al., 1990). K-Ras predominantly locates to the plasma membrane. However, when the basic region is phosphorylated or targeted by Ca²⁺/calmodulin, K-Ras relocates to the Golgi, endoplasmic reticulum and mitochondria (Fivaz and Meyer, 2005, Bivona et al., 2006). H-Ras and N-Ras shuttle between the Golgi and plasma membrane as a result of a constitutive depalmitoylation/repalmitoylation cycle (Goodwin et al., 2005, Rocks et al., 2005).

The Ras proteins function as molecular switches which are regulated GDP and GTP. They are activated upon an incoming growth factor signal. They exist in one of two structural and functional conformations: They may be GTP-bound and active or GDP-bound and inactive. The transition between these states is mediated by hydrolysing bound GTP to bound GDP and the dissociation of the nucleotide, allowing exchange of bound GDP for free GTP. The intrinsic rate of GTP hydrolysis and nucleotide exchange of Ras is too slow in order to allow efficient GDP–GTP cycling for physiological reactions, so accessory proteins are required to augment these reactions. Guanine-nucleotide-exchange factors (GEFs) such as Sos stimulate the release of bound nucleotides (Bar-Sagi, 1994). Because there is a higher cellular concentration of GTP than GDP, GEFs promote formation of active, GTP-bound Ras. The active conformation of Ras.GTP persists for only a short time because GTPase-activating proteins (GAPs) such as p120GAP and neurofibromin stimulate the intrinsic rate of
GTP hydrolysis and the formation of inactive, GDP-bound Ras (Lowy et al., 1991). The binding of GTP to Ras activates the protein by causing a conformational change in two mobile regions; the switch I region (residues 30–40) and the switch II region (residues 60–76), exposing a substrate binding site. The switch I region encompasses the effector domain (residues 32–40) which is needed for substrate binding and activation. Upon GTP binding this domain forms a loop on the surface of the protein which is accessible to and binds to substrate proteins.

All Ras substrates contain a RBD; at least three different sequences of approximately 100 amino acids have been identified as RBDs. Even though these sequences are not homologous, they each confer a similar topology of an ubiquitin superfold, characterized by a $\beta\alpha\beta\alpha\beta$ tertiary structure (Herrmann, 2003). Ras binds to an array of proteins including phosphoinositide 3 kinase (PI3K) (Rodriguez-Viciana et al., 1994) and members of the Ral-GNEF family (Wolthuis et al., 1998). However the best characterised effectors of Ras are the Raf family.

**1.3.3 CRaf as a Ras effector**

The first evidence that CRaf functions downstream from Ras was provided by experiments in which the proliferative and transformative effects of activated K-Ras and H-Ras were blocked by dominant negative, kinase inactive CRaf$^{K375W}$ mutants and craf antisense constructs (Kolch et al., 1991). Furthermore, a direct physical interaction was observed between Ras.GTP and the N-terminal region of CRaf (Vojtek et al., 1993, Koide et al., 1993, Van Aelst et al., 1993, Warne et al., 1993, Zhang et al., 1993). Specifically, Ras interacts with two N-terminal domains in CRaf; the RBD and the CRD (Brtva et al., 1995, Hu et al., 1995). The stronger of the two interactions is with the RBD, and was found to be essential for CRaf activation (Chuang et al., 1994). The Ras isoforms, each have different binding affinities towards the RBDs of each Raf isoforms (Weber et al., 2000), thus opening up the possibility of differential regulation. It has been shown that K-Ras4B has a stronger affinity
for CRaf than K-Ras4A, which binds more effectively than N-Ras, which is more effective than H-Ras (Voice et al., 1999). Ras is predominantly located attached to the inner leaflet of the plasma membrane (Hancock, 2003). Hence, the function of Ras binding is to localise CRaf to the plasma membrane where it becomes activated. This hypothesis was supported by experiments where the prenylation sequence (CAAX box) of Ras was added to the C-terminus of CRaf (Hancock et al., 1991). The prenylated plasma membrane targeted CRaf-CAAX chimera was found to have seven times greater kinase activity at similar levels of expression compared to wild-type CRaf (Leevers et al., 1994). The chimera was shown to be constitutively active and oncogenic in fibroblast transformation assays. However, its activation was increased upon stimulation with growth factors or direct binding of Ras.GTP (Mineo et al., 1997). The secondary interaction of Ras with CRaf CRD is independent of the GTP status of Ras and is not necessary for the membrane localisation of Raf. However it is crucial for the complete activation of CRaf (Luo et al., 1997).

There are other members of the Ras family that can interact with the Raf proteins. For example, TC21 can interact with CRaf and BRaf, but not ARaf. Similarly to Ras, oncogenic TC21 can translocate Raf to the plasma membrane where it can become activated (Rosario et al., 1999). In addition Rap1A can activate BRaf (Ohtsuka et al., 1996, Vossler et al., 1997), but antagonises Ras-dependent activation of CRaf (Cook et al., 1993) as discussed later.

1.3.4 Model for the regulation of CRaf

The Raf isoforms are subject to complex regulation as reflected by the numerous phosphorylation sites distributed throughout the proteins (Figure 1.2). Some of the sites are conserved in all three isoforms, indicating common mechanisms of regulation. However some are not, demonstrating that Raf isoforms can be regulated independently. As CRaf has been most intensively studied, knowledge focuses on this isoform. Although some of the
mechanisms appear to be conserved for ARaf and BRaf, there do appear to be important differences.

In unstimulated cells, CRaf is cytoplasmic (Wartmann and Davis, 1994) and bound to 14-3-3. The role of 14-3-3 in regulating CRaf is controversial; many studies suggest that 14-3-3 binding is essential for kinase activity (Fantl et al., 1994, Freed et al., 1994, Irie et al., 1994, Li et al., 1995, McPherson et al., 1999, Roy et al., 1998, Thorson et al., 1998, Yip-Schneider et al., 2000), however others suggest that it is not (Fu et al., 1994, Michaud et al., 1995, Suen et al., 1995). The discrepancy between these studies may in part be due to the presence of two main 14-3-3 binding sites in CRaf, S259 and S621, which appear to have opposing roles. Phosphorylation of S259 allows 14-3-3 binding to the CR2 domain; this appears to suppress CRaf activity. Indeed when 14-3-3 binding to CR2 was disrupted the basal kinase activity of CRaf was increased (Light et al., 2002). However phosphorylation of S621 allows 14-3-3 binding to the CR3 domain and appears to be essential for kinase activity. Peptide displacement studies have demonstrated that active CRaf can be inactivated by phosphopeptides that displace 14-3-3 and the addition of recombinant 14-3-3 reactivates CRaf (McPherson et al., 1999, Thorson et al., 1998, Tzivion et al., 1998). Furthermore similar results were obtained using the isolated kinase domain, indicating that binding to CR3 is essential for activity (Yip-Schneider et al., 2000). Since 14-3-3 proteins are dimeric and can simultaneously bind to two peptides (Yaffe et al., 1997), a model has been proposed whereby in unstimulated cells CRaf is phosphorylated on S259 and S621 and one 14-3-3 dimer binds to both CR2 and CR3 to keep CRaf in a closed, inactive conformation (Rommel et al., 1996, Tzivion et al., 1998). This enables the N-terminal domain of CRaf to bind to and suppress the kinase domain and interfere with Ras binding (Tzivion et al., 1998, Dhillon et al., 2002, Light et al., 2002).

14-3-3 binding must be disrupted to permit CRaf activation. The binding of Ras.GTP has been shown to disrupt 14-3-3 binding to CR2 in vitro (Clark et al., 1997, Rommel et al.,
Displacement of 14-3-3 from phospho-S259 appears to occur by an active process in which it is made accessible to conversion from the *cis* to the *trans* conformation by the action of the prolyl isomerase Pin1, which allows the residue to be preferentially recognised and dephosphorylated by the phosphatase PP2A (Abraham et al., 2000, Jaumot and Hancock, 2001, Kubicek et al., 2002, Ory et al., 2003). The binding of Ras enforces conformation changes in CRaf which are needed for its activation and possibly to expose a binding site for MEK (Terai and Matsuda, 2005). However it is believed that the most important function of Ras binding is the translocation of CRaf to the plasma membrane where it becomes activated. However, some studies have indicated that CRaf membrane localisation is not required for activation. An example is the activation of CRaf by phosphorylation of S338 by p21-activated kinase (PAK), which occurs independently of Ras and is discussed later (King et al., 1998, Chaudhary et al., 2000). Importantly the RBD and CRD of all the Raf isoforms are highly conserved, and both ARaf and BRaf have been shown to be recruited to the plasma membrane by activated Ras (Marais et al., 1997). Therefore it is presumed that recruitment to the membrane is also an important step in their activation, although further investigation is required.

### 1.3.5 Regulatory phosphorylations of CRaf

The kinase activity of CRaf has been observed to correlate with its phosphorylation status (Morrison, 1990). Treatment of cells with growth factors and mitogens causes CRaf hyperphosphorylation and concurrently the kinase activity of CRaf is enhanced in mitogen-treated cells. The phosphorylation of CRaf is believed to maintain the active conformation since treatment of active CRaf from insulin-stimulated cells with phosphatases drastically reduces the kinase activity (Kovacina et al., 1990). Therefore the evidence suggests a model whereby phosphorylation of CRaf may regulate its kinase activity in a reversible manner. Morrison et al. (1993) first identified several CRaf phosphorylation sites by tryptic phosphopeptide mapping and confirmed the identification of these sites by the use of site-directed mutagenesis. The authors documented three sites (S43, S259 and S621) which
were phosphorylated in vivo in mammalian cells and in Sf9 insect cells expressing human CRaf and one site phosphorylated in vitro (T268) which was subject to auto-phosphorylation (Morrison et al., 1993). Since these early studies, phosphorylation and dephosphorylation events have been shown to be crucial for the complete activity of the Raf proteins and again because of the historical focus on CRaf, knowledge of its regulation by phosphorylation is the most complete. However, the regulatory phosphorylations of CRaf are not completely understood and a degree of controversy exists. It is inevitable that further study will discover new phosphorylation sites or lead us to critically examine those currently identified.

S338, located just upstream of the kinase domain in a subdomain known as the N-region, was identified as a phosphorylation site by King et al (1998). Phosphorylation of S338 is absolutely required for CRaf activation in response to Ras activation; mutation of this site virtually abolishes activation of the kinase domain by mitogens (Mason et al., 1999). However, S338 phosphorylation may not be required for CRaf activation downstream of GPCRs (Oehrl et al., 2003).

PAK1 and PAK3 were originally identified as S338 kinases (King et al., 1998, Chaudhary et al., 2000). PAKs are serine/threonine specific protein kinases that are activated by membrane-bound CDC42 and RAC, which are part of the Rho family of GTPases. It was proposed that PAK1 and PAK3 phosphorylate S338 in a RAC/CDC42 and PI3K-dependent manner (Sun et al., 2000). PAKs certainly phosphorylate S338 in vitro, but it has been disputed as to whether they function in this manner in vivo. It is established that S338 phosphorylation occurs at the plasma membrane in response to activated Ras and growth factors. However PAKs phosphorylate S338 in the cytoplasm in a Ras-independent manner (Chaudhary et al., 2000, Marais et al., 1995, Chiloeches et al., 2001). In addition, PAK is not activated by growth factors that stimulate Ras-dependent S338 phosphorylation of CRaf (Zang et al., 2002, Chiloeches et al., 2001). Furthermore, dominant negative mutants of the PAK isoforms or CDC42 do not prevent growth factor stimulated phosphorylation of S338
and when PI3K activity is inhibited, growth factors still stimulate S388 phosphorylation (Chiloeches et al., 2001). Therefore, it is possible that there may be multiple S338 kinases operating downstream of distinct pathways. Indeed, it has been shown that casein kinase 2 (CK2) can phosphorylate S338 in a growth factor dependent manner (Ritt et al., 2007). This is a heterotetrameric serine/threonine protein kinase composed of two regulatory and two catalytic subunits (Pinna, 1997, Dobrowolska et al., 1999). CK2 was identified as a constitutive KSR1 (kinase suppressor of Ras) binding partner by Ritt et al. (2007) in co-immunoprecipitation assays. The authors demonstrated that CRaf was a substrate of CK2 by in vitro kinase assays. In these experiments they used the recombinant catalytic subunit of CK2 and affinity-purified, kinase-dead CRaf (to ensure that any in vitro phosphorylation detected could be attributed to CK2 activity and not Raf autophosphorylation). The phosphorylation site targeted by CK2 was identified as S338. However its phosphorylation was dependent upon prior phosphorylation of Y341 in agreement with previous reports which identified an unknown S338 kinase whose activity was dependent on Y341 phosphorylation (Mason et al., 1999, Chiloeches et al., 2001). Indeed CK2 could only phosphorylate CRaf that had been co-expressed with v-Src (to induce Y341 phosphorylation) or contained an aspartic-acid residue at the 341 site. The authors went on to examine S338 phosphorylation in vivo by inhibiting CK2 expression and activity. RNAi against CK2 subunits enabled the efficient depletion of CK2; however it resulted in a loss of cell viability. Whereas the use of the CK2 inhibitor 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole demonstrated that the inhibition of CK2 activity caused a significant reduction in the phosphorylation of S338 induced by PDGF treatment.

Both Y340 and Y341 are phosphorylated during the activation process of CRaf. These sites are located upstream of the kinase domain in the N-region. They were identified using the baculovirus-Sf9 expression system in which CRaf was co-expressed with activated tyrosine kinases (Fabian et al., 1993). The phosphorylation of Y341 is crucial for CRaf MEK kinase activity since treatment with tyrosine phosphatases inactivates the protein (Dent et al.,
SRC and SRC-family kinases have been implicated in the phosphorylation of Y341 \textit{in vitro} and in cell culture (Marais et al., 1995, Marais et al., 1997, Fabian et al., 1993, Chow et al., 1995, King et al., 2001, Tilbrook et al., 2001). It has been proposed that phosphorylation of these residues introduces a negative charge which is necessary to overcome the inhibitory function of the N terminus on the kinase domain (Tran and Frost, 2003). Indeed, the replacement of Y341 by a phosphomimetic aspartate residue results in strong activation of CRaf kinase activity and enables the transformation of BALB/3T3 cells (Fabian et al., 1993). However the role of phosphorylation of Y341 and the neighbouring Y340 is not fully understood. Studies in COS7 cells have demonstrated a requirement for the phosphorylation of Y340 and Y341 for activation of CRaf, because their mutation to phenylalanine blocked the activation of CRaf by oncogenic Ras and v-Src or by ligand stimulation (Marais et al., 1995, Marais et al., 1997, Barnard et al., 1998). Mutation of Y340 and Y341 to phenylalanine in the case of Raf\textsuperscript{FF} mice (see later for description), prevented CRaf from phosphorylating and activating MEK (Huser et al., 2001). Hence phosphorylation of these residues would seem to be critical for MEK kinase activity. The phosphorylation of Y341 has been shown to be critical in activating CRaf rather than the phosphorylation of Y340, which was not essential (Mason et al., 1999).

Additional activating phosphorylation sites include S497 and S499 which may be phosphorylated by (protein kinase C\textalpha) PKC\textalpha (Kolch et al., 1993) but this has since been disputed (Marais et al., 1998) and studies on the functional role of phosphorylation of these residues is limited (Barnard et al., 1998). T491 and S494 located within the activation loop of the kinase domain are also phosphorylated upon CRaf activation and their mutation blocks CRaf activation (Chong et al., 2001). Again data pertaining to their role and kinases is limited. S471 is a growth factor induced phosphorylation site; its mutation to alanine, cysteine, glutamic acid, or aspartic acid produced an inactive kinase, but substitution to threonine preserved some activity (Zhu et al., 2005).
S621 is the C-terminal 14-3-3 binding site and binding of 14-3-3 to phospho-S621 appears to be necessary for CRaf activity (McPherson et al., 1999, Thorson et al., 1998, Tzivion et al., 1998, Yip-Schneider et al., 2000). Phosphorylation of S621 was initially shown to be uncompromised in the kinase dead CRAF mutant, K375M-CRAF, expressed in insect cells using phospho-peptide mapping (Morrison et al., 1993), therefore implying S621 is not auto-phosphorylated and requires the presence of a second kinase to phosphorylate this residue. Subsequent studies proposed protein kinase A (PKA) and adenosine monophosphate-activated protein kinase (AMPK) as a S621 kinases (Mischak et al., 1996, Sprenkle et al., 1997). However these findings are controversial and studies using phospho-antibodies support a view that phosphorylation of S621 is mediated by autophosphorylation and that S621 is not phosphorylated in insect, HeLa, or Sf9 cells expressing kinase dead CRaf (Mischak et al., 1996, Hekman et al., 2004, Thorson et al., 1998). The role of S621 phosphorylation has been a subject of controversy. Claims that it acts as an activating site are supported by the observation that its mutation leads to loss of kinase activity (Thorson et al., 1998). However its phosphorylation correlates with the ability of PKA to inhibit the kinase domain of CRaf (Mischak et al., 1996), leading to counter claims that it has an inhibitory role. This phosphorylation site is central to the work presented here and will be discussed in more detail in Chapter 4.

Several sites are phosphorylated to suppress CRaf activity and the dephosphorylation of these negatively regulating residues is required for CRaf activation. S43, S233 and S259 are phosphorylated when cyclic AMP (cAMP) levels increase in cells. Therefore their phosphorylation appears to be mediated by the cAMP-dependent kinase PKA (Wu et al., 1993, Sidovar et al., 2000, Dumaz et al., 2002, Dhillon et al., 2002, Dumaz and Marais, 2003). Phosphorylation of S259 was also observed to occur due to the action of protein kinase B (PKB) (Zimmermann and Moelling, 1999). This investigation expressed PKB in insulin-like growth factor (IGF) stimulated HEK293 cells and subjected immunoprecipitated PKB to an in vitro kinase assay with CRaf N-terminal truncated mutants as substrate. The
authors demonstrated that the CRaf N-terminal truncation was phosphorylated by PKB whereas the S259A mutant was not. S259 has been shown to be dephosphorylated by PP2A, which associates with CRaf at the plasma membrane (Kubicek et al., 2002). Phosphorylation of this site is inhibitory as it serves as a binding site for 14-3-3. The other main 14-3-3 binding site is phospho-S621 located in the C-terminus. The phosphorylation of S233 forms a third 14-3-3 binding site (Dumaz and Marais, 2003). Phosphorylation of S43 appears to sterically hinder binding of Ras to CRaf at its N-terminal (Wu et al., 1993). Dhillon et al., (2002) suggested that PKA inhibition of CRaf was entirely mediated through phosphorylation of S259. However other evidence implies that S43, S233 and S259 all contribute to CRaf inhibition by PKA (Dumaz et al., 2002). The latter authors demonstrated that all three sites had to be mutated to overcome the inhibition mediated by PKA. Such mutants had strongly elevated basal kinase activity and were completely resistant to treatment with forskolin and 3-isobutyl-1-methylxanthine (IBMX), which stimulate PKA activity.

Two additional negatively regulating residues are S268 and T269. These sites are believed to be subject to autophosphorylation and targets for a ceramide activated kinase or KSR, respectively (Morrison et al., 1993, Zhang et al., 1997, Xing and Kolesnick, 2001). However the kinases responsible for their phosphorylation and their roles need further study as a kinase-dependent role for KSR is not clear. Additionally ERK has been shown to phosphorylate S29, S43, S289, S296, S301 and S642 (Dougherty et al., 2005), which introduces the possibility that the activation of CRaf may be terminated by a negative feedback loop mediated by ERK, which would therefore limit ERK signalling (Dougherty et al., 2005, Hekman et al., 2005). However, it has also been proposed that their phosphorylation mediates positive feedback of CRaf regulation by ERK since their phosphorylation was induced by growth factors and during cell cycle progression (Balan et al., 2006). These sites are not well conserved among ARaf and BRaf and so may mediate the isoform specific regulation of CRaf.
1.3.6 Requirement of lipids in the regulation of CRaf

Another key factor in the activation of CRaf is the role of membrane lipids in regulating the Ras/CRaf interaction and the membrane recruitment of CRaf. Gosh et al (1994) purified GST-CRaf truncations from E. coli by affinity chromatography and identified truncations containing the CRD to be bound to liposomes in a phosphatidylserine-dependent manner in vivo (Ghosh et al., 1994). Indeed the CRD contains an array of hydrophobic and positively charged residues which may provide sites for binding to anionic phospholipids. Further analysis showed that full-length CRaf can be bound to both phosphatidylserine and phosphatidic acid. Phosphatidic acid is produced from hydrolysis of phosphatidylcholine by phospholipase D, an enzyme which is activated by numerous mitogens. The phosphatidic acid binding site was mapped to 35 amino acids located at the CRaf C-terminus. The inhibition of phosphatidic acid formation in cells by treatment with 1% ethanol was found to significantly reduce the translocation of CRaf from the cytoplasm to the membrane (Ghosh et al., 1996). This suggests a potential role of phosphatidic acid in the regulation of CRaf translocation. Furthermore activation of phospholipase D by insulin and the resultant generation of phosphatidic acid appeared to stimulate the activation of the MAPK cascade. This activity was inhibited by treatment with Brefeldin A, which prevents insulin-dependent production of phosphatidic acid. The subsequent addition of phosphatidic acid reversed this inhibition. Translocation to the membrane was independent of Ras, although phosphatidic acid did not activate CRaf in vitro or in vivo, indicating that Ras was still required for CRaf activation (Rizzo et al., 2000).

1.3.7 Activation of B RAF

The regulation of ARaf appears to mirror CRaf in its need for Ras binding and phosphorylation of the tyrosine residue corresponding to Y341 in CRaf (Marais et al., 1997). However, the model for CRaf activation cannot be wholly conserved for B RAF, as there are key differences between the structures and sites of phosphorylation of the proteins. Indeed BRaf is capable of becoming strongly activated by oncogenic Ras alone; however the
maximal activation of CRaf and ARaf requires secondary inputs (Marais et al., 1997). The main differences between BRaf and CRaf are within the N-region. The equivalent residues to CRaf Y340 and Y341 in BRaf are aspartic acids at positions 448 and 449. Due to their negative charge, these residues mimic phosphorylated residues, with the result that BRaf does not require an equivalent phosphorylation event to CRaf phosphorylation of Y341 for full activation (Mercer and Pritchard, 2003). Indeed, substitution of D448 and D449 for the non-polar amino acids phenylalanine or alanine reduced BRaf basal kinase activity. However it did not totally block its ability to respond to oncogenic Ras (Marais et al., 1997, Mason et al., 1999). Additionally, a conserved serine residue at position 446 in BRaf (equivalent to S338 in CRaf) is constitutively phosphorylated. This was observed using a phospho-specific antibody designed against phospo-S338 in CRaf, but due to the conservation of sequence between CRaf and BRaf, the antibody was able to bind to phospho-S446 in ectopically expressed BRaf in COS cells and in endogenous BRaf in PC12 cells (Mason et al., 1999). Hence unlike CRaf, again BRaf does not require a phosphorylation event for maximal activation. The presence of the constitutively phosphorylated S446 residue and the aspartic acid residue at 449 in BRaf result in this isoform being primed for activation and contributes to this isoform's higher basal kinase activity, which is 15-20 times greater than that of CRaf (Mason et al., 1999).

In analogy with CRaf, unstimulated BRaf is located in the cytoplasm. However, BRaf appears to have a more open conformation due to the presence of phospho-S446, D449 and the possible inclusion of amino acids encoded by exon 10a (Mercer and Pritchard, 2003). It is unclear if inactive BRaf is stabilised in its inactive conformation by a 14-3-3 dimer: 14-3-3 can bind to phospho-S729 and potentially to a second site at phospho-S365, phosphorylated by PKB (Guan et al., 2000). Phosphorylation of this residue has been shown to inhibit BRaf activity. However it has not been shown that BRaf is naturally phosphorylated on this site in quiescent cells, nor has it been shown that dephosphorylation of this site is necessary for BRaf activation. Additional PKB phosphorylation consensus sites are located
at S428 and T439 in BRaf but it is not known whether these sites are phosphorylated in vivo.

Upon activation, Ras binds to BRaf. The interacting amino acids at the Ras/Raf interface are conserved in BRaf and it has been shown that both BRaf and CRaf bind with equal efficiency to HRas in vitro (Shinkai et al., 1996, Okada et al., 1999). This binding event results in the translocation of BRaf to the plasma membrane, leading to its activation (Marais et al., 1997). In analogy with CRaf, phospho-S365 may be dephosphorylated, possibly by PP2A. Additional activating phosphorylation events take place at the activation loop residues, homologous to CRaf, T599 and S602 (Zhang and Guan, 2000), resulting in full BRaf activation. However the kinases responsible for these phosphorylation events have not been identified. Counter to this proposed mechanism, a membrane-free complex of BRaf and 14-3-3 has been shown to be activated in vitro by Ras (Yamamori et al., 1995). This serves to highlight the differences between the activation process of CRaf and BRaf owing to the differences in their structures, as CRaf requires serine and tyrosine phosphorylation events at the plasma membrane for full activation and cannot be activated by Ras alone (Marais et al., 1997).

1.3.8 Raf dimerisation

The Raf proteins were shown to be capable of forming homodimers by using CRaf chimeras which were constructed to include regions that would bind bivalent drugs (Farrar et al., 1996, Luo et al., 1996). Such studies indicated that dimerisation stimulated CRaf activity. Hence it was proposed that dimerisation could potentially occur in the mechanism of Raf activation by Ras. In addition wild-type CRaf and BRaf have been shown to heterodimerise in vivo (Weber et al., 2001). The authors demonstrated that BRaf and CRaf ectopically expressed in HEK293 cells co-immunoprecipitate, but this is dependent on active Ras. It was found that the CRaf C-terminus constitutively associated with BRaf in the presence of active Ras, whereas the N-terminus did not. Furthermore, the association of CRaf and BRaf
was reduced when S621 in CRaf was substituted for alanine. However no change was observed using CRafS259A mutants. Hence 14-3-3 binding to phospho-S621 was suggested to be important for formation of the heterodimers. (Weber et al., 2001, Garnett et al., 2005, Rushworth et al., 2006). The identification of Raf heterodimers led to the possibility that CRaf and BRaf may cross-regulate each other. Indeed heterodimerisation with BRaf contributes to CRaf activation in response to normal signalling events (Garnett et al., 2005, Rushworth et al., 2006). The activation of CRaf in this manner still requires phosphorylation of the activation segment, but it is unknown if BRaf directly phosphorylates CRaf, recruits another kinase or induces CRaf to autophosphorylate. Additionally, oncogenic BRaf mutants, unable to phosphorylate MEK in vitro can still activate the ERK cascade in vivo by activating CRaf (Wan et al., 2004). Oncogenic BRaf proteins interact constitutively with CRaf in a Ras-independent manner, whereas binding between wild-type BRaf and CRaf is induced by Ras activation (Garnett et al., 2005). The BRaf/CRaf heterodimer has been shown to have vastly elevated kinase activity compared to the respective monomers or homodimers (Rushworth et al., 2006). As yet it is unknown if ARaf can also form heterodimers, or if CRaf can modulate BRaf activity in a physiological setting. However the isolated auto-inhibitory domain of CRaf can interact with and inhibit the catalytic domain of BRaf (Tran et al., 2005).

1.4 Associated proteins

CRaf has been found to interact with a number of proteins in addition to its upstream activator Ras and its downstream substrates including MEK, indicating that CRaf is part of a large signalling complex. Such proteins mediate various functions and include KSR, MEK-partner 1 (MP1), Sef, suppressor of RAS8 (SUR8), connector enhancer of KSR (CNK), Erbin, Sprouty, and related proteins with EVH1 domains (SPRED), Raf inhibitory protein (RKIP), 14-3-3, heat shock protein 90 (HSP90) and p50 (Cdc37).
1.4.1 Scaffolding proteins

A method employed by the ERK cascade to both regulate and achieve fidelity of signalling is the use of scaffold proteins. Such proteins recruit selected signalling components, co-localising them, so that signal propagation through the cascade is favoured. They provide specificity to the signalling cascade by insulating the module from additional stimuli and by regulating the subcellular localisation of the components of the cascade. KSR1, MP1 and Sef all act as such scaffolding proteins.

KSR functions as a scaffold for the ERK cascade and facilitates activation at the plasma membrane. KSR was first identified in *Drosophila melanogaster* and *C. elegans* as a positive regulator of Ras/ERK signalling, and two family members (KSR1 and KSR2) are found in mammals. Experiments in *Drosophila* showed that KSR binds to Raf and MEK simultaneously and enhances MEK activation (Roy et al., 2002). In unstimulated cells, KSR binds to MEK, but upon mitogenic stimulation it also forms interactions with CRaf and ERK, so providing a scaffold that brings the components of the cascade together and hence enhances signal transmission. KSR also binds to additional proteins including 14-3-3, p50 and HSP90 (Stewart et al., 1999), and therefore can be thought of as a scaffold which assembles a large signalling complex around the ERK cascade module. KSR is related to CRaf, but its kinase domain contains mutations at critical sites, necessary for activity in other kinases and most investigations have found that KSR lacks kinase activity (Morrison, 2001). Knockout of KSR in the mouse did not affect CRaf activation, but resulted in impaired MEK and ERK activation (Nguyen et al., 2002). KSR localisation is regulated by phosphorylation of serine 392. This allows the binding of 14-3-3 and sequestration of KSR in the cytoplasm by masking the cysteine-rich C1 domain of KSR required for membrane targeting (Muller et al., 2001). Cytoplasmic KSR also binds to the E3 ubiquitin ligase IMP (impedes mitogenic signal propagation). RTK signalling induces the dissociation of IMP by the binding of activated Ras, promoting IMP ubiquitination (Matheny et al., 2004). Activated Ras indirectly causes the dephosphorylation of the 14-3-3 binding site. The release of 14-3-3 from this site
exposes the membrane targeting C1 domain (Zhou et al., 2002), as well as the docking site for ERK1/2 (Jacobs et al., 1999). As a result KSR localises to the plasma membrane, where it interacts with CRaf, co-localising it with constitutively bound MEK and recruited ERK.

MP1 is a small ERK scaffold that localises to late endosomes through a constitutive interaction with p14, an adaptor protein that resides on the cytoplasmic face of endosomes (Wunderlich et al., 2001). It selectively promotes the activation of ERK1, because it interacts only with MEK1 and ERK1, but not MEK2 or ERK2. The presence and localisation of the p14/MP1 complex has been shown to be necessary for full ERK activation in response to EGF stimulation at the endosome (Teis et al., 2002, Teis et al., 2006).

Sef is another ERK scaffold with unique subcellular targeting properties. This is a transmembrane protein that resides on the Golgi apparatus (Torii et al., 2004). Sef binds to activated MEK, allowing it to phosphorylate ERK. Activated ERK remains associated with the MEK/Sef complex and is thus prevented from entering the nucleus. This has the effect of restricting ERKs substrates to the cytoplasmic cohort.

1.4.2 Modulators of the ERK cascade

Modulators can be described as proteins which regulate the duration and intensity of ERK signalling, regulate inhibition, and/or mediate crosstalk with other signalling cascades. Such functions are important for achieving the correct biological outcome from the wide array of extracellular signals which are transduced through the ERK cascade.

SUR8 and CNK can be described as positive modulators. Over-expression of SUR8 enhances Raf activation by promoting the Ras/Raf interaction (Li et al., 2000) SUR8 is believed to enhance the strength of ERK signalling by facilitating events that promote Raf activation. For example it has been show to indirectly contribute to Raf activation by promoting the dephosphorylation of the inhibitory N-terminal 14-3-3 binding site, serine 259
on CRaf (Rodriguez-Viciana et al., 2006). There are at least 5 CNK isoforms found in mammals. The ubiquitously expressed isoform, CNK1, enhances CRaf activation by co-localising the Src tyrosine kinase with membrane-localised CRaf, thereby promoting the phosphorylation of CRaf at its N region by Src (Ziogas et al., 2005).

Negative modulators of the ERK cascade include Erbin, Sprouty, SPRED and RKIP. Erbin is a member of the LAP (leucine rich-repeat and PDZ domain) protein family. It interacts with Ras and prevents the binding of Raf to activated Ras (Huang et al., 2003, Dai et al., 2006). The Sprouty proteins can bind to and sequester the Grb/Sos complex, and thus prevent Ras activation (Hanafusa et al., 2002). Additionally Sprouty and SPRED proteins can interact with the Raf kinase domain, interfering with the phosphorylation of Raf on activating sites (Wakioka et al., 2001, Sasaki et al., 2003). RKIP was shown by over-expression experiments to inhibit the ERK cascade. It is very selective in its inhibition of MEK phosphorylation by Raf. It does not affect the phosphorylation of ERK by MEK, the autophosphorylation of Raf, the phosphorylation of an artificial Raf substrate, or the phosphorylation of MEK by MEKK-1. RKIP prevents MEK activation by Raf by interacting with the kinase domains of MEK and Raf, preventing the Raf/MEK interaction (Yeung et al., 2000, Trakul and Rosner, 2005, Yeung et al., 1999). The binding of RKIP to CRaf is regulated by growth factors, upon stimulation CRaf is released from RKIP. This may be due to the conformational changes which occur upon CRaf activation.

1.4.3 Chaperones of the ERK cascade

Chaperones can be described as proteins which assist in the correct folding of a substrate protein, maintaining its stability, and/or function in the assembly of multi-protein structures around their substrate protein. 14-3-3, HSP90 and HSP70 are chaperones of Raf.

Members of the 14-3-3 family are conserved phosphoserine-specific adapter proteins which are ubiquitously expressed and form dimers (Tzivion et al., 2001). They are capable of
regulating cell-cycle checkpoints, proliferation, differentiation and apoptosis (Fu et al., 2000). The association between CRaf and 14-3-3 was identified by several groups (Fantl et al., 1994, Freed et al., 1994, Fu et al., 1994, Irie et al., 1994). Both 14-3-3β and 14-3-3ε isomers have been shown to bind to CRaf (Force et al., 1994). It is not yet fully understood what roles the binding of 14-3-3 plays. Different investigators have shown that 14-3-3 binding enhances, suppresses, or has no effect on CRaf kinase activity (Fantl et al., 1994, Freed et al., 1994, Irie et al., 1994, Michaud et al., 1995). Investigations in the diverse model systems of yeast, *Xenopus laevis* oocytes, mammalian PC12 cells and *Drosophila melanogaster* indicate that 14-3-3 binding stimulates CRaf signalling (Irie et al., 1994, Freed et al., 1994, Fantl et al., 1994, Li et al., 1995, Kockel et al., 1997, Li et al., 1997b). Despite this, purified 14-3-3 was unable to activate CRaf *in vitro*. This is understandable given the complex mode of CRaf regulation and so it was hypothesised that 14-3-3 may enhance signalling by coupling CRaf to upstream activators or downstream substrates (Li et al., 1995). Indeed the X-ray structure of 14-3-3 provides evidence to support this. The 14-3-3 dimer forms a groove which can accommodate the simultaneous binding of two proteins (Liu et al., 1995). Further evidence includes experiments where inactive CRaf was found associated with ectopically expressed 14-3-3 mutants unable to form dimers, whereas active CRaf was associated with the endogenous wild-type 14-3-3 dimer (Luo et al., 1995). The 14-3-3 dimer binds to both the inactive and active conformations of CRaf. In unstimulated cells, CRaf binds to a 14-3-3 dimer via two serine residues; S259 and S621. As mentioned above this binding holds CRaf in an inactive, closed conformation (Tzivion et al., 1998). Active CRaf also associates with 14-3-3, which enables it to bind to a variety of proteins including Bcr, PKC and the cell cycle regulator Cdc25 (Conklin et al., 1995). Although the binding sites for 14-3-3 are conserved in the Raf isomers, the binding of 14-3-3 to ARaf has not yet been reported. However an interaction between BRaf and 14-3-3 has been shown to occur *in vitro* (Papin et al., 1996).

HSP90 is a ubiquitous chaperone which assists and maintains the folding of many proteins including CRaf. The chaperone activity of HSP90, in conjunction with other chaperones
including HSP70 and p50\textsuperscript{cdc37}, is responsible for establishing the correct tertiary structure of CRaf. Treatment with the drug geldanamycin prevents the interaction of HSP90 with its client proteins and leads to the ubiquitination and degradation of such proteins including CRaf (Schulte et al., 1995, Schulte et al., 1997). The association of CRaf with HSP90 is mediated by p50 (Silverstein et al., 1998). Indeed the formation of a CRaf/p50/HSP90 complex would seem to be essential for CRaf activity and proper ERK signalling. This is demonstrated by impaired ERK cascade signalling when a p50 mutant with defective HSP90 binding was over-expressed, whereas co-expression of wild-type p50 and CRaf enhanced Raf activation (Grammatikakis et al., 1999). The HSP90/p50 complex may have further roles in acting as a modulator of CRaf. A complex containing BRaf and HSP90 has been identified (Jaiswal et al., 1996), but as yet no interaction has been found with ARaf.

1.5 Substrates of the Raf Proteins

1.5.1 MEK and ERK

The link between Ras and the activation of ERK1/2 was established by the expression of oncogenic Ras activating ERK1 and ERK2 (Leevers and Marshall, 1992, Wood et al., 1992). Furthermore, a dominant negative Ras mutant prevented ERK activation in response to growth factors (Wood et al., 1992, Thomas et al., 1992, de Vries-Smits et al., 1992). Raf was also shown to induce the activation of ERK in \textit{v-raf} transformed cells and in cells transfected with activated CRaf (Dent et al., 1992, Howe et al., 1992).

The link between the Raf proteins and ERK1/2 are MEK1/2, which are the only commonly accepted substrates for the Raf proteins. It has been shown that all three Raf proteins can phosphorylate both MEK1 and MEK2 \textit{in vitro} (Mercer and Pritchard, 2003). Other kinases including MEK kinase-1, mos or Tpl-2 can also phosphorylate the same activating MEK1/2 residues. However it would appear that the Raf proteins are the main activators of MEK1/2 in most cell types (Schaeffer and Weber, 1999).
MEK1 and MEK2 are highly homologous in their amino acid sequence and function as dual threonine/tyrosine kinases. Both MEK1 and 2 contain a proline rich sequence, which is believed to be needed for recognition and activation by the Raf isoforms (Catling et al., 1995). As this sequence is not present in other MAPKKs, it provides a basis for the specific signalling from Raf proteins to MEK1/2. Specifically, MEK proteins are activated by phosphorylation on two serine residues within their activation segments (S217 and S221 in MEK1). Activated MEK1/2 then phosphorylates ERK1/2 in the continuation of the ERK cascade. Phosphorylation occurs in the activation loop of ERK1/2 on a threonine residue and tyrosine residue, separated by one amino acid (T202/Y204 in ERK1). This dual phosphorylation event causes a conformational change in the activation loop and the neighbouring regions of the ERK kinase domain that results in >1000-fold increase in ERK activity.

The subcellular localisation of MEK and ERK is important in the determination of the specific response to the activation of the MAPK cascade. For example forcing the nuclear localisation of an ERK2–MEK1 chimera resulted in increased transcriptional activity (Robinson et al., 1998). However, forcing association of ERK with the plasma membrane resulted in the reduction of this activity (Hochholdinger et al., 1999). In unstimulated cells MEK and ERK appear to be cytoplasmically located. MEK1/2 contain a nuclear export signal in the N-terminal domain (Fukuda et al., 1996, Jaaro et al., 1997) and in addition residues of the C-terminus may also be involved in this localisation (Cha et al., 2001). The interaction of MEK and ERK with scaffold proteins is also important for their cytoplasmic location (Chuderland and Seger, 2005). For example phosphoprotein enriched in astrocytes (PEA-15) binds to ERK in the cytoplasm, preventing its nuclear transport (Formstecher et al., 2001). In stimulated cells MEK and ERK are released from their cytoplasmic anchors, allowing them to translocate into the nucleus (Jaaro et al., 1997, Chen et al., 1992). Both MEK and ERK lack nuclear localisation signals, and ERK nuclear translocation has been shown to require a direct interaction with nuclear pore proteins (Matsubayashi et al., 2001,
Whitehurst et al., 2002). MEK1/2 are believed to be exported from the nucleus by the exportin system that utilises their NES sequence shortly after stimulation (Jaaro et al., 1997). However ERK1/2 remain in the nucleus longer, possibly due to their association with other nuclear proteins (Lenormand et al., 1993).

Since the two MEK and the two ERK genes are co-expressed and evolutionally conserved these genes may have evolved functional diversification. Indeed mek1 and mek2 knockout mice have been generated which imply the presence of differential functions of MEK1 and MEK2. The targeted knockout of mek1 causes embryonic lethality at E 10.5 days, due to reduced angiogenesis in the labyrinthine region of the placenta (Giroux et al., 1999). However mek2−/− mice are viable and fertile (Belanger et al., 2003). Furthermore specificity of interaction with the Raf isoforms indicates a functional diversification. For example Ras and CRaf were identified to form a signalling complex with MEK1, but not MEK2 (Jelinek et al., 1994) and in HeLa cells stimulated with EGF, CRaf was shown to activate both MEK1 and MEK2, whereas ARaf only activated MEK2 (Wu et al., 1996). Finally the reduction in MEK1 expression results in a G2/M arrest whereas reduction of MEK2 has no effect (Liu et al., 2004).

Knockout mice have also been generated which suggest that ERK1 and ERK2 are not functionally equivalent. erk1−/− mice are viable, fertile, and of normal size, but show deficits in thymocyte maturation (Vanhouette et al., 1999). However, the targeted knockout of the erk2 gene results in embryonic lethality by E 11.5 and severe abnormalities of the placenta. In these animals, the labyrinthine layer of the placenta is very thin and few foetal blood vessels are observed (Hatano et al., 2003). This indicates that ERK1 cannot compensate for the loss of ERK2.

Other studies have implied a inhibitory role for ERK1 on ERK2 signalling (Vantaggiato et al., 2006, Mazzucchelli et al., 2002). These investigations report that the loss of ERK1 in the
mouse results in enhanced ERK2 activity in primary neurones resulting in an increased rate of learning and improved long-term memory (Mazzucchelli et al., 2002). Furthermore MEFs isolated from ERK1 knockout mice proliferated faster than control cells and although ERK2 expression was not altered, activation of ERK2 was increased and more sustained (Vantaggiato et al., 2006). Similar results were obtained by the knockdown of ERK1 expression using shRNA directed against erk1. However when ERK2 was knocked down in wild-type MEFs using shRNA, cells proliferated poorly. These data would seem to indicate that the signal to proliferate is mediated by ERK2, whereas ERK1 has some type of inhibitory function, by antagonising ERK2 activity. This hypothesis is supported by an experiment using NIH 3T3 cells which express apparently similar levels of ERK1 and ERK2 and Ras-induced colony formation was inhibited by knock-down of ERK2. However the over-expression of ERK1 inhibited Ras-induced transformation whereas over-expression of ERK2 did not. Importantly, the use of a kinase-dead ERK1 mutant indicated that this inhibition did not require ERK1 kinase activity (Vantaggiato et al., 2006). This led to the hypothesis that ERK1 and ERK2 compete for their upstream kinase MEK, but have different cellular targets, and hence functions.

1.5.2 MEK kinase activation efficiency

Historically, as CRaf was believed to be the only Raf isoform ubiquitously expressed, it was thought that CRaf was the major MEK1/2 activator. However, it has been demonstrated in diverse cell types, including fibroblasts, neuronal tissue and lymphocytes, that BRaf has a far greater efficiency for activating MEK1/2 than the other Raf isoforms (Huser et al., 2001, Catling et al., 1994, Reuter et al., 1995, Jaiswal et al., 1994, Eychene et al., 1995, Jaiswal et al., 1996, Traverse and Cohen, 1994). Furthermore in mice with targeted mutations of the raf genes it has been shown that CRaf is dispensable for MEK/ERK activation and that BRaf appears to be the primary MEK/ERK activator in most tissues and cell types (Wojnowski et al., 2000, Huser et al., 2001, Mikula et al., 2001). Additionally, ERK activation has been shown to be reduced in BRaf deficient, but not in ARaf or CRaf deficient cells (Mercer et al.,
2002, Huser et al., 2001, Pritchard et al., 2004). BRaf has a higher basal kinase activity than the other isoforms. This is believed to be because of key differences in the primary structure of BRaf at residues 446, 448 and 449 as mentioned above (Mason et al., 1999, Zhang and Guan, 2000). Furthermore, comparison of the three Raf kinases has shown that BRaf binds most strongly to MEK (Papin et al., 1996). This evidence for the role of BRaf as the primary MEK kinase is supported by genetic analysis, which shows that the Raf proteins from lower organisms such as C. elegans and Drosophila are more similar to BRaf than the other two mammalian isoforms (Baccarini, 2005). This suggests that BRaf may be the classic MEK kinase, whereas CRaf and ARaf may have diverged to perform other functions.

1.5.3 Downstream of the ERK cascade

ERK1/2 are proline directed kinases, which have been shown to have specificity for substrates containing the general consensus sequence Pro-X-Ser/Thr-Pro (Alvarez et al., 1991). ERK1/2 may function in feedback regulation as two of their identified substrates are CRaf (Anderson et al., 1991) and MEK (Matsuda et al., 1993). ERK1/2 have been shown to hyperphosphorylate specific sites and desensitise CRaf, preventing additional activation events. This negative regulation of CRaf, together with the ability of MEK1 to activate CRaf, may be important in finely tuning the duration and strength of the ERK1/2 response, hence leading to the correct biological response. Indeed the accurate regulation of ERK is crucial; for example in neuronal cells, transient ERK activity leads to proliferation, whereas sustained activity leads to differentiation (Marshall, 1995). However in fibroblasts the intensity of the signal also seems to be important. Proliferation induced by PDGF requires sustained ERK activity in fibroblasts (Weber et al., 1997), but manipulation of Raf to produce a strong signal causes cell cycle arrest by the induction of p21\textsuperscript{CIP1} (Woods et al., 1997, Kerkhoff and Rapp, 1998). The termination of ERK signalling involves the dephosphorylation of the threonine and tyrosine residues by phosphatases including MKP-1, MKP-3, and MKP-4 (Franklin and Kraft, 1997, Groom et al., 1996, Muda et al., 1997, Muda et al., 1996). MKP-
3 and MKP-4 are primarily located in the cytoplasm, whereas MKP-1 is nuclear; this introduces the possibility of differential regulation of ERK, dependent upon its location.

Activated ERK1/2 can translocate to the nucleus and activate transcription factors, thus regulating the expression of specific target genes. ERK1/2 also have cytoplasmic substrates which, when activated, may influence gene expression directly or indirectly (Figure 1.5).

1.5.4 Cytoplasmic substrates of ERK

Cytoplasmic substrates for ERK include p90<sub>RSK</sub> (Sturgill et al., 1988). There are four human isoforms of these proteins, which have roles in transcriptional regulation, cell survival and control of the cell cycle. Upon activation, the p90<sub>RSK</sub> isoforms phosphorylate their downstream targets. p90<sub>RSK</sub> substrates include transcriptional regulators such as the c-AMP-response element binding protein (CREB) (Xing et al., 1996), c-Fos (Chen et al., 1993), and the serum response factor (SRF) (Rivera et al., 1993). p90<sub>RSK</sub> may also promote cell cycle progression by the phosphorylation of p27<sup>KIP1</sup>, a cyclin dependent kinase inhibitor (Fujita et al., 2003).

MAP kinase interacting kinases (MNKs) are another set of ERK1/2 substrates. These proteins, once activated, phosphorylate the eukaryotic initiation factor 4E (eIF-4E), which lead to the recruitment of ribosomes and protein synthesis initiation factors to mRNA (Waskiewicz et al., 1997). Cytoplasmic ERK substrates also include the mitogen- and stress-activated kinases (MSKs), which are involved in transcriptional regulation by activating CREB and ATF-1.

The ERK proteins also function in cell shape and motility through interactions with integrins (Hughes et al., 1997), direct phosphorylation of myosin light chain kinase (MLCK) (Klemke et al., 1997) and the activation of calpain II (Glading et al., 2000). Furthermore ERK1/2 can phosphorylate cytoskeletal proteins such as tau which is a microtubule associate protein.
Figure 1.5 **Cytoplasmic and nuclear substrates of ERK1/2.**

Activate ERK1/2 targets cytoplasmic substrates as shown in the figure, which may influence gene expression directly or indirectly. In addition activated ERK1/2 can translocate to the nucleus and activate transcription factors, thus directly regulating the expression of specific target genes.
(Drewes et al., 1992); its phosphorylation regulates cytoskeleton rearrangements and overall cellular morphology.

ERK can also phosphorylate enzymes at the cell membrane including the EGF receptor (Northwood et al., 1991, Takishima et al., 1991) and cytoplasmic phospholipase A₂ (cPLA₂) (Lin et al., 1993, Nemenoff et al., 1993). The phosphorylation of cPLA₂ activates it, causing increased arachidonic acid release and formation of lysophospholipids from membrane phospholipids (Lin et al., 1993). Therefore the activation of ERK1/2 brings about the activation of a range of other signalling pathways, elicited from the cytoplasm.

1.5.5 Nuclear substrates of ERK

As described above, ERK1/2 can modulate gene expression via intermediate kinases including p90RSK and MSK. However ERK can also translocate into the nucleus to target nuclear substrates and hence directly regulate gene expression.

ERK1/2 phosphorylates members of the ETS family of transcription factors known as ternary complex factors (TCFs) such as Elk-1 (Gille et al., 1992, Wyke et al., 1995). Upon phosphorylation, TCFs form complexes with serum response factor (SRF) proteins. The function of SRF is to recruit TCF to the serum response element (SRE), a region located in the promoter of many immediate early genes (IEGs), including c-fos, which promotes their transcription (Marais et al., 1993) (Figure 1.6). In addition the protein product of c-fos forms a heterodimeric complex with c-Jun, which is termed AP1. This is a dimeric transcription factor complex which binds to AP1 binding sites located in the promoter region of many genes. It is formed upon heterodimerisation of a c-Fos and c-Jun family member or the homodimerisation of c-Jun family members. The Jun family member is required for DNA binding. Importantly the AP1 complex is also regulated by ERK-mediated phosphorylation of c-Jun and c-Fos (Pulverer et al., 1991). Furthermore the AP1 complex regulates the
ERK1/2 control the expression of immediate early genes.

ERK1/2 phosphorylates ternary complex factors (TCFs) such as Elk-1. Upon their phosphorylation, TCFs form complexes with serum response factor (SRF) proteins. The SRF recruits TCF to the serum response element (SRE), a region located in the promoter of genes including c-fos. c-Fos forms a complex with c-Jun, which is known as AP1, a transcription factor complex which binds to AP1 binding sites located in the promoter region of many genes including c-fos and c-jun. The AP1 complex is also regulated by ERK-mediated phosphorylation of c-Jun and c-Fos. In this way ERK can regulate AP1, which in turn regulates the transcription of numerous genes including c-fos and c-jun, leading to an increase in their expression by a positive feedback mechanism.
transcription of numerous genes including \textit{c-fos} and \textit{c-jun}, leading to a positive feedback mechanism of their expression.

ERK1/2 have been observed to phosphorylate a number of other IEG products. These include ATF-2 (Abdel-Hafiz et al., 1992) and \textit{c-myc} (Alvarez et al., 1991, Gupta et al., 1993). Other transcription factors phosphorylated by ERK include the TNF Ets-2 (McCarthy et al., 1995, McCarthy et al., 1997). The early response genes, \textit{c-fos}, \textit{c-jun} and \textit{c-myc} among others function to regulate the delayed response genes including cyclins and cyclin dependent kinases which are required for transition through the cell cycle and will be discussed later.

1.6 Other Raf protein binding partners

Although the commonly accepted substrate for the Raf proteins are MEK1/2, the Raf proteins have been shown to interact with other proteins. BRaf has been shown to bind to the \(\alpha\) subunit of the 11S proteasome regulator (Kalmes et al., 1998). It is postulated that since this interaction occurs in a region of the \(\alpha\) subunit which is important for its proteasomal-activating function, BRaf may be involved in the regulation of proteasomal activity. ARaf has been shown to bind to hTOM and hTIM, which may function in the mitochondrial transport of ARaf (Yuryev et al., 2000). ARaf has also been shown to interact with the \(\beta\) subunit of CK2 (Boldyreff and Issinger, 1997). CRaf has an array of binding partners (detailed in the review by (Kolch, 2000), some of which have been shown to be phosphorylated by \textit{in vitro} kinase assays. Through its many binding partners including ASK1 (Chen et al., 2001, Yamaguchi et al., 2004), MST2 (O'Neill et al., 2004), Rb (Wang et al., 1998, Jamal and Ziff, 1995), and Cdc25 (Galaktionov et al., 1995), CRaf has been shown to have ERK-independent functions in processes including apoptosis and the control of the cell cycle (as discussed later).
1.7 Apoptosis

Apoptosis is an important process in many biological processes, including embryonic development, the immune system, and normal cell turnover. The process of apoptosis is mediated through two major pathways in mammalian cells; the intrinsic and the extrinsic pathways. The intrinsic pathway is activated upon DNA damage and is often mediated by the activation of a pro-apoptotic member of the Bcl-2 family. These family members dimerise at the mitochondrial membrane to regulate cytochrome c release into the cytoplasm (Liu et al., 1996, Gross et al., 1999). Cytochrome c interacts with apoptotic protease factor-1 (Apaf-1), and procaspase-9 to form a structure known as the apoptosome (Li et al., 1997a, Zou et al., 1999). In this complex, caspase-9 becomes proteolytically activated. The extrinsic apoptotic pathway is initiated at the cell surface. Members of the death receptor family such as CD95/Fas become activated upon the binding of a ligand such as CD95 ligand (CD95L). This induces trimerisation and the recruitment of an adapter protein such as Fas-associated death domain (FADD). Subsequently many procaspase-8 proteins bind to form the death-inducing signalling complex (DISC), this results in the trans-catalysis of caspase-8. The activation of either caspase-8 or 9 causes the activation of so-called effector caspases (caspase-3, 6 and 7) which in turn cause the cleavage of specific proteins resulting in the cellular alterations which typify in apoptosis.

1.7.1 ERK-dependent roles for Raf proteins in apoptosis

The activation of the MEK/ERK pathway by BRaf has been linked with the suppression of apoptosis (Erhardt et al., 1999). Additionally the ERK proteins have been shown to regulate a number of proteins involved in controlling apoptosis including Bim, p90RSK and caspase 9.

Growth factor deprivation induces apoptosis via the intrinsic pathway. The over-expression of components of the ERK cascade protects cells from such apoptosis following serum starvation (Erhardt et al., 1999). The over-expression of constitutively activated MEK, Ras,
or CRaf prevented apoptosis after growth factor deprivation. When BRaf was over-expressed, the anti-apoptotic effect was shown to be mediated through the ERK cascade, since ERK was shown to be constitutively active and treatment with either the specific MEK inhibitor PD98059 or expression of a dominant inhibitory MEK mutant blocked the anti-apoptotic activity. The activation of the ERK cascade did not interfere with the release of cytochrome c from mitochondria after growth factor deprivation. However, the addition of cytochrome c to the cytoplasm of cells over-expressing BRaf failed to induce caspase activation. Therefore the ERK cascade appeared to protect cells against apoptosis at the level of caspase activation, downstream from cytochrome c release. A study by Allan et al., (2003) showed that the ERKs suppress apoptosis via phosphorylation of caspase 9, preventing its processing, activation and subsequent effector caspase activation (Allan et al., 2003), which may account for post-mitochondrial effects.

However, the ERK cascade is also known to have pre-mitochondrial effects mediated by Bcl-2-interacting mediator of cell death (Bim). Bim is a pro-apoptotic member of the Bcl-2 protein family. Its mRNA is alternatively spliced to give rise to the short, long and extra long protein variants (BimS, BimL and BimEL). Of the various Bim isoforms, BimEL and BimL have been linked to the ERK cascade. BimEL and BimL promote apoptosis by binding to the anti-apoptotic proteins Bcl-2 or Bcl-xL, which prevent cytochrome c release by binding to the pro-apoptotic proteins Bax and Bak. The binding of BimEL or BimL to Bcl-2 or Bcl-xL releases Bax and Bak leading to apoptosis. ERK1/2 can repress Bim expression and targets BimEL by phosphorylation, for degradation via the proteasome (Ley et al., 2003, Ewings et al., 2007).

As discussed above, ERK1/2 activate p90RSK proteins, which have roles in cell survival. The p90RSK-2 isoform has been shown to inactivate the Bcl-2 pro-apoptotic protein Bad by phosphorylation of S112 (Bonni et al., 1999, Tan et al., 1999). The p90RSK proteins are also able to phosphorylate and hence activate the transcription factor CREB. This induces the
expression of the anti-apoptotic protein, Bcl-2 (Bonni et al., 1999). Therefore the activation of the p90<sub>RSK</sub> proteins by ERK can protect against apoptosis via two mechanisms.

1.7.2 ERK-independent roles for Raf proteins in apoptosis

The abolition of the <i>craf</i> gene in mice causes apoptosis and embryonic lethality, despite the normal regulation of the ERK cascade by BRaf. This suggests that CRaf protects the cell from apoptosis via an ERK-independent mechanism (Huser et al., 2001, Mikula et al., 2001). In addition, inhibition of CRaf by agonists to cAMP induces apoptosis in <i>v-abl</i>-transformed cells despite high constitutive ERK activity (Weissinger et al., 1997). CRaf has been found to interact with a number of proteins that mediate apoptosis both <i>in vitro</i> and <i>in vivo</i>. CRaf has been reported to phosphorylate the pro-apoptotic protein Bad <i>in vitro</i> (Wang et al., 1996a). However this occurs on sites distinct from the regulatory sites S112 and S136, whose phosphorylation prevents the binding of Bad to Bcl-<i>x</i><sub>L</sub>, hence preventing the release of cytochrome <i>c</i> from the mitochondria (Zha et al., 1996). CRaf has been reported to interact with Bcl-2 <i>in vitro</i>, and these proteins act to prevent apoptosis upon withdrawal of interleukin-3 (Wang et al., 1994, Wang et al., 1996a). However the interactions in the prevention of apoptosis is controversial since it has also been reported that Bcl-2 does not stably interact with CRaf and CRaf is not necessary for Bcl-2 activity (Olivier et al., 1997). Three proteins with which CRaf interacts, Rok-α, ASK1 and MST2 are more likely to be mediators of anti-apoptotic effects.

As described above CD95/Fas is involved in the extrinsic apoptotic pathway. The clustering of Fas and binding of a ligand induces internalisation of the receptor and the formation of the DISC leading to apoptosis. Fas clustering can be promoted by ezrin, when phosphorylated on T567 by Rok-α. CRaf was found to inhibit the ability of Rok-α to control Fas clustering and internalisation at the cell membrane (Piazzolla et al., 2005). In this study the authors demonstrated that in wild-type cells the formation of a CRaf/Rok-α complex restrains Rok-α activity and ezrin phosphorylation. However in the absence of CRaf, Rok-α activity and ezrin
phosphorylation generated a prolonged Fas signal leading to an increase in apoptosis. This was exemplified in craf⁻/⁻ embryonic livers and MEFs which have been shown to be hypersensitive to Fas-induced apoptosis (Mikula et al., 2001, Piazzolla et al., 2005). Piazzolla et al., (2005) observed 5-fold increased membrane expression of Fas in craf⁻/⁻ cells as determined by flow cytometry. Immunofluorescence staining demonstrated that the loss of CRaf in these cells appeared to inhibit the ability of Fas to internalise. The effects of CRaf were shown to be mediated by Rok-α in experiments where a dominant-negative Rok-α mutant was transfected into craf⁻/⁻ cells which abrogated both ezrin phosphorylation and Fas clustering. Furthermore siRNA against Rok-α abrogated the hypersensitivity of craf⁻/⁻ cells to Fas-induced apoptosis and dramatically reduced Fas clustering and ezrin phosphorylation. It has previously been demonstrated in unstimulated and migrating fibroblasts that the ability of CRaf to restrain the activation of Rok-α is independent of its kinase activity (Ehrenreiter et al., 2005). In agreement with this, Piazzolla et al., (2005) expressed a CRaf kinase dead mutant in craf⁻/⁻ cells which rescued the craf⁻/⁻ phenotype. The apoptotic craf⁻/⁻ phenotype was also rescued in cultured cells by heterozygosity at the lpr locus which has been shown to functionally inactivate Fas (Watanabe-Fukunaga et al., 1992). Furthermore this mutation also prevented fetal liver apoptosis and embryonic lethality caused by the knockout of craf (Piazzolla et al., 2005).

Apoptosis signal-regulating kinase (ASK1) is a pro-apoptotic stress activated MAPKKK, which, when active, promotes apoptosis via pathways including the JNK and p38 MAPK cascades (Ichijo et al., 1997). ASK1 is activated by a number of stress related stimuli including TNF-α, CD95L, oxidative stress and DNA damage. The over-expression of CRaf disrupts ASK1-induced apoptosis. It is clear that the mechanism of ASK1 regulation does not involve the ERK cascade from experiments using MEK inhibitors. CRaf has been shown to physically associate with ASK1 at its N-terminal, and antagonise it. The use of kinase dead CRaf mutants demonstrated that ASK1 regulation is independent of CRaf kinase activity, although the mechanism of regulation is not clear (Chen et al., 2001). One
possibility is that CRaf binds to ASK1 promoting an inactive ASK1 conformation. Yamaguchi et al., (2004) generated cardiac muscle-specific craf knockout mice in which ASK1 activity was found to be increased when compared to the wild-type and there were an increased number of apoptotic cardiomyocytes. This phenotype was rescued by the abolition of ASK1, suggesting that ASK1 mediated apoptosis in these mice, and was inhibited by CRaf (Yamaguchi et al., 2004).

The role of CRaf in the regulation of apoptosis by MST2 is controversial and further investigations are required to substantiate the data presented here. MST2 is a kinase which is activated by the pro-apoptotic agents staurosporine and CD95L (Taylor et al., 1996). In CRaf depleted cells, MST2 was active and cells were hypersensitive to apoptosis. This phenotype was rescued by increased expression of CRaf (O'Neill et al., 2004). CRaf has been observed to bind to MST2. This interaction has been mapped to residues 151 - 303 of CRaf, a region that is variable between isoforms and so may mediate isoform-specific interactions. Indeed MST2 was not found to bind to BRaf, suggesting that MST2 is part of a CRaf specific pathway (O'Neill et al., 2004). MST2 is activated by homodimerisation and trans-phosphorylation of a threonine residue at the activation loop (Praskova et al., 2004). However, CRaf has been shown to form heterodimers with MST2 and to recruit the phosphatase PP2A to dephosphorylate the protein, rendering MST2 inactive (O'Neill et al., 2004). Kinase dead mutants of CRaf are as efficient as wild-type CRaf in binding to, and antagonising, MST2, indicating that the kinase activity of CRaf is dispensable for this pro-survival function. CRaf regulates MST2 by sequestering it into an inactive complex, preventing apoptosis. Stress signals or the over-expression of MST2 lead to apoptosis by the disruption of this complex. MST2 has also been implicated in cell cycle control, as will be discussed below.
1.8 Cell cycle

The cell cycle is a ubiquitous process involved in the growth and proliferation of cells, development of organisms and the regulation of DNA damage repair. The cell cycle involves numerous regulatory proteins that direct the cell through a specific sequence of events, culminating in mitosis and the production of two daughter cells. The cycle can be divided into stages, interphase (cell growth) and mitosis (nuclear division), which is followed by cytokinesis (cell division). Interphase can be further divided into three phases, referred to as G1, S and G2. During G1, the cell prepares for DNA synthesis. S phase is a time where cells synthesise their DNA and therefore have aneuploid DNA content between 2N and 4N. The G2 phase allows cells to prepare for mitosis or the M phase, which consists of prophase, metaphase, anaphase and telophase. A further phase called G0 describes cells which are not actively cycling and may have exited the cell cycle. Such cells may later re-enter the cell cycle at G1 (Schafer, 1998).

1.8.1 Components of the cell cycle

Progression through the cell cycle is controlled mainly by complexes consisting of cyclins and cyclin dependent kinases (CDKs). Such complexes control cell cycle progression by the phosphorylation of a variety of substrates. Cyclins bind to and direct CDKs to their appropriate substrates. However due to the varying expression during the cell cycle, cyclins can only activate CDKs at specific times during the cell cycle. CDKs are serine/threonine protein kinases that can phosphorylate a variety of substrates, so catalysing the process of cell division. For example in G1 an important substrate which must be phosphorylated to allow the progression of the cell cycle is the retinoblastoma protein (Rb). Other G1 and M phase CDK substrates include nuclear lamins and microtubules that form the nuclear cytoskeleton (Kill, 1995).
The cell cycle is negatively regulated by cyclin dependent kinase inhibitors (CKIs), causing cell cycle delay/arrest. These blocks are removed by proteolysis or phosphorylation of the CKIs. CKIs are small proteins (15-57 kDa) and belong to one of two families: the INK4 family (consisting of p15, p16, p18 and p19) or the Cip/Kip family (consisting of p21\textsuperscript{CIP1}, p27\textsuperscript{KIP1} and p57\textsuperscript{KIP2}). The INK4 family inactivate only CDK4 and CDK6 by the formation of stable complexes with the CDKs before they are bound to their appropriate cyclin. Excess expression of cyclin does not dissociate them from the CDK. The Cip/Kip family can inhibit CDK 2; however inhibition can be relieved by increasing cellular concentrations of cyclins.

1.8.2 Cell cycle progression

The progression through the cell cycle is dependent upon the combined actions of cyclin/CDK complexes and CKIs as depicted in Figure 1.7. The restriction point is perhaps the most important regulatory mechanism imposed on cell cycle progression. This is the point in G1 after which cells no longer respond to withdrawal of growth factors and are committed to cell cycle progression. This checkpoint monitors whether extracellular conditions are suitable for DNA replication and cell division. If not, the cell can exit the cell cycle and enter a quiescent state (G0), where cells remain viable with minimal metabolism and can later re-enter the cell cycle (Zetterberg, 1990). Rb is known as the gatekeeper of the restriction point. Rb, in its active, hypophosphorylated state binds the transcription factor E2 promoter binding factor (E2F), making it unavailable for transcription.

Cyclin D forms complexes with CDK 4/6 and phosphorylates Rb (Kato et al., 1993). The hyperphosphorylation of Rb causes it to dissociate from E2F. This frees E2F to participate in transcription of genes necessary for the progression of the cell cycle such as cyclin E, cyclin A, CDK1 and cdc25A (Arroyo, 1992). Since mitogens induce the expression of cyclin D which mediates the phosphorylation of Rb, this checkpoint connects extracellular stimuli to the transcriptional machinery, by controlling the expression of genes required for G1/S progression. The withdrawal of extracellular mitogens causes the cessation of cyclin D
Progression through the cell cycle is regulated by various CDK/cyclin complexes and their inhibitors which belong to either the Cip/Kip or the INK4 family. Mitogens induce the synthesis of cyclin D and are required to pass the restriction point. This checkpoint is regulated by the phosphorylation status of Rb which, when phosphorylated by cyclin D and E-directed CDKs, releases E2F. Release of this transcription factor causes the production of proteins required for the continuation of the cell cycle.
synthesis, its destabilisation, and degradation. If this occurs before the restriction point, the cell exits the cycle without replicating its DNA, but if it occurs afterward, cells complete mitosis and exit from the cycle in the next G1 interval (Matsushime et al., 1994). Cyclin E/CDK2 complexes also phosphorylate Rb, albeit preferentially on sites distinct from those phosphorylated by cyclin D/CDK4/6. Rb remains hyperphosphorylated through the remainder of the cell cycle and reverts to its hypophosphorylated form at the end of mitosis.

The INK4 family of CKIs regulate early G1, before the restriction point, by inhibiting CDK4 and CDK6. The inhibition of these CDKs prevents the phosphorylation and inactivation of Rb. The regulation of the cell cycle mediated by the Cip/Kip family of CKIs is more complex. p21\(^{CIP1}\) and p27\(^{KIP1}\) are major inhibitors of CDK2, so inhibit the phosphorylation of Rb by cyclin E/CDK2 complexes. However, p21\(^{CIP1}\) can also promote G1 progression by facilitating the assembly of cyclin D/CDK4 and cyclin D/CDK6 complexes (LaBaer, 1997). Although, the role of p27\(^{KIP1}\) in binding to cyclinD/CDK4/6 is controversial, some studies have implied that it is important for the assembly of cyclin D/CDK4 and cyclin D/CDK6 complexes. For example in early G1, p27\(^{KIP1}\) assembles cyclin D1/CDK4/6 in the cytoplasm and facilitates the import of cyclin D1 complexes into the nucleus (LaBaer, 1997). Indeed mouse embryo fibroblasts (MEFs) lacking p27\(^{KIP1}\) and p21\(^{CIP1}\) do not stably express the cyclin D isoforms and show a 90% reduction in cyclin D dependent kinase activity (Cheng et al., 1999). However, these cells proliferate normally, thus introducing the concept of redundancy among components of the cell cycle (this will be explored further in the next section).

Furthermore the binding of p21\(^{CIP1}\) and p27\(^{KIP1}\) to cyclin D complexes sequesters these inhibitory proteins, so preventing their inhibition of cyclin E/CDK2 and cyclin A/CDK2 complexes at late G1 and S phase. Therefore, mitogen withdrawal not only leads to the disassembly of the cyclin D/CDK4/6 complexes but also mobilises a pool of CKIs, which blocks the activity of cyclin E/CDK2 and facilitates cell cycle exit.
1.8.3 Redundancy in components of the cell cycle

Initially, the expression of all cyclins and CDKs were thought to be necessary for progression through the cell cycle. For example microinjection of antibodies against cyclin E into cultured mammalian cells prevented the transition from G0 into S phase (Ohtsubo et al., 1995). The reduction of CDK4 activity by inhibitors of the INK4 gene family (Serrano et al., 1993), or by drugs against CDK4 (Tetsu and McCormick, 2003) causes G1-phase arrest. Also the inhibition of CDK2 activity by a variety of means including dominant-negatives (van den Heuvel, 1993), microinjection of antibodies against CDK2 (Pagano, 1993, Tsai et al., 1993), or inhibitors of the Cip/Kip family (Sherr, 1995) result in G1 arrest. However, more recent work using knockout mice challenges the view that all cyclins and CDKs are essential for cell cycle progression and implies functional redundancy between them. Mice which have mutations of one or more of these cell cycle regulators undergo an almost normal embryogenesis. Where developmental defects are present, they emerge late in development, and occur in a minority of cell lineages. For example mice which individually lack any of the cyclin D isoforms are viable, but display a subset of specific defects, confined to those tissues where only one cyclin D isoform is principally expressed. The mice seem to be able to undergo embryonic development, because the expression of the remaining cyclin D isoforms become up-regulated in most tissues (Ciemerych et al., 2002). However defects arise in tissues in which the residual cyclins are not up-regulated. Furthermore MEFs derived from E13.5 cyclin D null embryos grow in vitro. These cells show a slight defect in their ability to exit from quiescence. Surprisingly E2F target genes are still induced in the absence of all cyclin D isoforms, implying that cyclin E/CDK2 complexes may be sufficient to inhibit Rb. In fact, siRNA against CDK2, while having a negligible effect on wild-type MEFs, strongly inhibits the proliferation of cells lacking all D-type cyclins.

1.8.4 ERK-dependent roles for Raf proteins in the cell cycle

ERK1/2 can regulate both cell cycle progression and arrest by the phosphorylation of cytoplasmic and nuclear substrates which regulate the transcription of multiple target genes.
The creation of chimaeric Raf isoforms enabled the role of the ERK cascade in the cell cycle to be further investigated. The CR3 domain of each of the Raf isoforms was fused to the human hormone-binding domain of the estrogen receptor (hbER) to produce ΔARaf:ER, ΔBRaf:ER, and ΔCRaf:ER (Samuels et al., 1993, Pritchard et al., 1995). In the absence of estradiol the chimaeric proteins are inactive, due to steric hindrance upon the formation of a multi-protein complex including HSP90. Upon addition of estradiol, the steric hindrance of the complex is released and the Raf:ER proteins become oncogenically active. Addition of estradiol to quiescent 3T3 cells expressing ΔBRaf:ER, or ΔCRaf:ER inhibited cell cycle progression and response to EGF or PDGF. Furthermore ΔBRaf:ER prevented the mitogenic response of these cells to serum. Contrastingly, addition of estradiol to quiescent 3T3 cells expressing ΔARaf:ER enabled cell cycle progression and had no effect on the mitogenic response to stimulation with growth factors (Pritchard et al., 1995). It is believed that the reason for the differing responses of the cells to the activation of the chimaeric Raf proteins is due to their differing efficiencies towards activation of MEK/ERK. Indeed, ΔBRaf:ER was found to activate MEK to the greatest extent and ΔARaf:ER the least (Pritchard et al., 1995). The use of these protein constructs demonstrated that all the active Raf isoforms could induce cyclin D1 and cyclin E expression and cause a decrease in the expression of p27^KIP1. This correlates with the progression of the cell cycle and a positive influence on proliferation. However both ΔBRaf:ER and ΔCRaf:ER strongly induce the expression of p21^CIP1, which is most likely responsible for the observed cell cycle arrest (Woods et al., 1997). Furthermore the activation of ΔCRaf:ER in p21^CIP1−/− MEFs led to a robust mitogenic response. In the study by Woods et al., (1997) the activation of the chimaeric proteins was manipulated by altering the concentration of estradiol used. It was concluded that each of the Raf proteins could cause cell cycle progression or arrest depending upon the degree of their kinase activity. Low levels of kinase activity were found to cause cell cycle progression whereas higher levels elicited cell cycle arrest (Figure 1.8).
Figure 1.8 A model for Raf-induced cell cycle progression or arrest. (Adapted from Woods et al 1997)

(A) Raf-induced cell cycle progression. Low-level activation of Raf leads to cell cycle progression via the induction of cyclins D and E and reduced p27^KIP1 expression. (B) Raf-induced cell cycle arrest. Higher Raf activity also leads to the induction of cyclins D and E as well as reduced p27^KIP1 expression. However expression of p21^{CIP1} is also induced, which together with p27^KIP1, inhibits cyclin E/CDK 2 complexes causing cell cycle arrest at the G1/S transition.
Evidence demonstrates that ERK1/2 activity is responsible for the control of the transcription of genes which mediate the transition through the stages of the cell cycle, such as cyclin D1. Indeed ERK regulates cyclin D1 transcriptional induction via Fos family members. The first report that ERK activation was implicated in the induction of cyclin D1 expression was made by Lavoie et al., (1996). The authors demonstrated that the activation of the ERK pathway by stimulating the stably expressed ΔCRaf:ER chimera led to increased cyclin D1 expression; whereas blocking ERK activity by expressing a dominant negative form of MEK led to decreased cyclin D expression (Lavoie et al., 1996). Members of the fos family were later found to function as an intermediate between activated ERK and cyclin D expression since MEFs lacking fos-B and c-fos genes displayed a defect in proliferation which could be rescued by the ectopic expression of cyclin D1 (Brown et al., 1998). Upon activation, ERKs can translocate to the nucleus where they can phosphorylate pre-existing transcription factors, such as Elk-1 to induce transcription of c-fos gene as described above. Members of the c-Fos and c-Jun families can heterodimerise to form AP-1 complexes, which are able to bind to AP-1 binding sites located in the promoter region of many genes including cyclin D1 (Angel and Karin, 1991, Albanese et al., 1995). This promotes expression of cyclin D1, leading to the phosphorylation of pRb by cyclin D/CDK4/6 complexes and progression through the restriction point.

Another substrate of the ERK proteins is c-Myc (Gupta et al., 1993). ERK phosphorylates S62 of c-Myc which increases its stability (Seth et al., 1991). c-Myc functions as a transcription factor stimulating both cell cycle progression and apoptosis. c-Myc has a critical role in normal cell cycle progression, especially during the transition from G0 to S phase (Spencer CA, 1991), where it responds directly to mitogenic signals to push cells in to the G1 phase of the cell cycle. c-Myc affects the progression of the cell cycle by controlling the transcription of genes including Cdc25A, cyclin D1, cyclin D2, cyclin E, cyclin A, CDK1, CDK2, CDK4 and E2F. Another important role of c-Myc is to suppress the transcription of the cell cycle inhibitors Gadd45, p15, p21cip1, and p27kip1 (Vermeulen K., 2003). Kerkhoff et al.
al, in 1998 demonstrated that the isolated Raf/MEK/ERK signal transduction cascade was sufficient to induce the expression of the c-Myc gene (Kerkhoff et al., 1998).

Interestingly ERK activity has been shown to be required for proper nuclear translocation of CDK2 in the nucleus. Blocking ERK activation did not alter the levels of cyclin E/CDK2 complexes, only the nuclear localisation of CDK2 (Keenan et al., 2001). Furthermore ERK has been shown to regulate phosphorylation of T160 of CDK2, which is an activating site (Lents et al., 2002) although the mechanism linking ERK to the nuclear translocation and phosphorylation of CDK2 are not known.

p53 is widely recognized as a protein mediating apoptosis and functioning during the cell cycle as an inducer of cell cycle arrest in response to DNA damage. The p53 protein has a short t½ because an associated protein, Mdm2, induces its ubiquitination and degradation by the proteasome. In certain stress conditions, p53 is stabilised and can function in cell cycle arrest or apoptosis. It mediates cell cycle arrest in part due to its transcriptional activation of p21<sup>CIP1</sup> which inhibits cyclin E/CDK2 complexes, preventing the inactivation of Rb, so that the restriction point cannot be overcome. The expression of Mdm2 is enhanced by p53 itself, forming a self-regulatory negative feedback loop. p53 stability is also regulated by p19<sup>ARF</sup>. This protein prevents the interaction between Mdm2 and p53, therefore increasing p53 levels. The p19<sup>ARF</sup> protein is under the transcriptional control of E2F; thus, Rb inactivation not only allows cells to pass the restriction point, but also enables a cell to arrest at the G1/S checkpoint in response to increased p21<sup>CIP1</sup> expression mediated by p53. Activation of the MAPK pathway has been shown to lead to increased levels of p53 and also to stimulate transcription of the p53 gene (Agarwal, 2001). High intensity ERK signalling has been shown to result in a p21<sup>CIP1</sup> mediated cell cycle block. Additionally, the activation of the ERK cascade has been shown to reduce p53 levels, by the activation of Mdm2 which targets p53 for destruction in the absence of p19<sup>ARF</sup>. However, p19<sup>ARF</sup> is also activated by
the ERK cascade, therefore levels of p53 are determined by the opposing effects of the ERK-mediated activation of p19^{ARF} and Mdm2 (Ries et al., 2000).

As discussed above, other ERK substrates include the p90^{RSK} proteins. Upon activation, the p90^{RSK} isoforms phosphorylate their downstream targets which, among others described previously, include p27^{KIP1}. Phosphorylation of p27^{KIP1} allows the binding of 14-3-3, resulting in its cytoplasmic localisation (Fujita et al., 2003), hence promoting cell cycle progression. Lefevre et al in 2003 demonstrated that the inhibition of CRaf and the MEK/ERK module increases p27^{KIP1} production suggesting that this pathway may be involved in the degradation of p27^{KIP1} in an ERK-dependent manner (Lefevre et al., 2003). This is supported by an investigation that shows ERK1 phosphorylates p27^{KIP1} and targets it to the ubiquitin–proteasome pathway for degradation (Coats et al., 1996).

1.8.5 ERK-independent roles for Raf proteins in the cell cycle

The Raf isoforms clearly influence the cell cycle by signalling through the ERK cascade. However, CRaf has also been shown to interact with a number of proteins which mediate the cell cycle in an ERK-independent manner, including Rb and Cdc25. Additionally, studies have shown that CRaf becomes activated in mitosis (Laird et al., 1995) and that this activation has a unique mechanism, which is not dependent upon Raf binding or membrane localisation (Laird et al., 1999). Such mitotic CRaf does not activate ERK and is active in the cytoplasm and not localised at the plasma membrane (Ziogas et al., 1998). However no target of mitotically active CRaf has yet been identified.

Rb must be inactivated by phosphorylation to allow the cell cycle to progress from G1 into S phase. This phosphorylation is dependent upon the action of cyclin D and E dependent kinases. However, CRaf has also been identified as a Rb kinase and therefore contributes to Rb inactivation and hence to the direct control of the cell cycle (Wang et al., 1998, Jamal and Ziff, 1995). The binding of CRaf to Rb was found to occur after stimulation with
mitogens, and was only present in proliferating cells. The interacting region of CRaf was identified as between residues 1 and 28, a region which is not conserved in the other Raf isoforms. CRaf kinase activity was necessary for the interaction with Rb, but phosphorylation of Rb by CRaf has only been seen in vitro. However, the role of CRaf as a Rb kinase is at odds with results of timed microinjection studies. These experiments show that, for cell cycle progression in response to insulin, CRaf activity is required only in the first 20 minutes after stimulation (Rose et al., 1998), but Rb phosphorylation occurs later in G1. Therefore the significance of Rb association is not clear.

Cdc25 is a dual specificity phosphatase which dephosphorylates threonine and tyrosine residues in CDK1. CDK1 regulates G2/M progression of the cell cycle; it is activated by Cdc25-mediated dephosphorylation. CRaf can bind to and stimulate the activity of Cdc25 via 14-3-3, therefore indirectly regulating the cell cycle (Galaktionov et al., 1995). Humans have three Cdc25 genes, of which CRaf has been shown to bind to two; Cdc25A and B, but not to Cdc25C (Galaktionov et al., 1995). Cdc25A plays an important role at the G1/S-phase transition (Nilsson and Hoffmann, 2000). Cdc25B and Cdc25C are regulators of the G2/M transition via their activity on cyclinA/CDK2, cyclinA/CDK1, and cyclinB/CDK1 (Kristjansdottir and Rudolph, 2004).

MST2 is also involved in control of the cell cycle. CRaf is capable of binding to MST2 via residues 151-303, sequestering it into an inactive complex and recruiting phosphatases to remove activating phosphorylations. The inhibition of MST2 in this way causes downstream activation of the Lats proteins, which function to restrict the cell cycle (O'Neil, 2005). The Lats proteins are tumour suppressors which have been shown to function in cell cycle control by inhibiting G1/S or G2/M transition through interactions with cyclins E, A and B (Xia et al., 2002, Li et al., 2003).
1.9 The ubiquitin-proteasome system (UPS)

1.9.1 Ubiquitin

Ubiquitin, a 76 amino acid peptide, tags proteins which are intended for degradation by the ATP-dependent 26S proteasome. It binds covalently to the target protein by an isopeptide linkage between the C terminal glycine of ubiquitin and, usually, the ε-amino group of lysine in the target protein. Polyubiquitin chains can be formed by further isopeptide linkages between the C terminus of ubiquitin with the ε-amino group of lysine of another ubiquitin molecule (Nandi et al., 2006). The lysine residue on which polyubiquitination occurs is important; the formation of polyubiquitin chains by linkage at Lys-48 act as a signal for proteasome mediated degradation, whereas ubiquitination at Lys-63 act as signals for DNA repair or activation of transcription factors. Mono-ubiquitination of proteins has other functions such as endocytosis, histone regulation or virus budding. The UPS plays major roles in several biological processes including regulation of the cell cycle, cancer and cell survival, the inflammatory response, the immune response, protein misfolding and ER associated degradation. Here the focus is on the degradation of misfolded proteins.

1.9.2 Mechanism of ubiquitination

An activating enzyme, E1, transfers ubiquitin to a carrier E2 enzyme, which in turn tags ubiquitin to the substrate protein with the help of E3 enzymes (Figure 1.9). There are few genes encoding E1, tens of E2 encoding genes and hundreds of genes encoding E3 ligases. The diverse E3 ligases recognise substrates harbouring various degradation signals and contribute to the selectivity and specificity of the UPS. Ubiquitin needs to be removed from tagged proteins before they enter the proteolytic core of the proteasome. The classical deubiquitinating enzymes belong to ubiquitin processing (UBP) and ubiquitin carboxy-terminal hydrolases (UBH) families.
Figure 1.9 The mechanism of ubiquitination. (Adapted from Nandi et al 2006)

An E1 enzyme in the presence of ATP activates ubiquitin, forming an ubiquitin-E1 thiol ester. This is recognized by E2 enzymes, to which ubiquitin is transferred by another thiol ester linkage. E2 enzymes specifically associate with E3 enzymes, which are responsible for the final target selection and specificity. E3 enzymes belong to two distinct families: those containing the HECT domain, which form a covalent bond with the ubiquitin before transferring it to the substrate, and those that contain the RING domain. Ubiquitination via RING finger E3 ligases involves binding to E2-ubiquitin complex and facilitating direct transfer of ubiquitin to the targeted protein without the additional thiol ester formation as observed in the HECT family of E3 ligases.
1.9.3 The proteasome

Proteins which have been tagged with polyubiquitin chains are marked for degradation by the 26S proteasome. This is a cytoplasmic protease complex, consisting of the 20S proteasome, harbouring the proteolytic core, bound to a 19S cap (Nandi et al., 2006). The proteolytic active site of 20S proteasome is present within the lumen of the complex to avoid non-specific degradation of cellular proteins. 20S proteasomes consist of four heptameric rings, the outer rings contain α-type subunits whereas the inner two rings are β-type subunits. The α subunit rings form a selective barrier between the catalytic chamber and the cytoplasm. They are the sites for the binding of various regulatory particles and entry and exit of substrates. The β subunits harbour the catalytic site. The 19S cap binds to either or both ends of 20S proteasomes, in the presence of ATP, to channel ubiquitinated proteins into the central active site chamber for degradation. The 19S cap has diverse activities including ATPase, ubiquitin-binding, deubiquitinating and reverse chaperone activity (Nandi et al., 2006).

1.9.4 E3 ubiquitin ligases

As described above, E3 ubiquitin ligases are proteins which facilitate the transfer of ubiquitin from an E2 enzyme to a substrate. E3 enzymes enable the specific binding of E2 enzymes, although E2 enzymes are capable of binding to more than one E3 ligase. Importantly, E3 enzymes are responsible for the final target selection and therefore enable specificity in the coordination of ubiquitination of the selected substrate.

Three classes of E3 have been identified: the HECT (homologous to E6-AP C-terminus), the RING (really interesting new gene) finger, and U-box domain types. HECT domain proteins form a covalent (thiolester) bond with an ubiquitin moiety to a cysteine residue in the ~350-residue HECT domain. Subsequently, the HECT E3 interacts with target protein, which results in transfer of ubiquitin. By contrast ubiquitination via RING finger E3 ligases involves binding to E2-ubiquitin complex. This facilitates the direct transfer of ubiquitin to the targeted
protein (Pickart, 2001, Weissman, 2001). RING finger and U-box domains are structurally related (Hatakeyama et al., 2001). The RING finger E3s fall into one of two categories, either being based on a single component or existing as multi-component complexes such as the SCF (Skp1/Cullin/F-box/Rbx1/2) family.

### 1.9.5 CHIP

The UPS interacts with members of the heat shock family and cofactors to eliminate misfolded proteins. CHIP (carboxyl terminus of Hsc70 interacting protein), which is an E3 ligase and a Hsc70 interacting protein (McDonough and Patterson, 2003), forms a direct link between protein unfolding and degradation. CHIP is a 34.5 kDa protein containing three 34-amino acid TPR domains at its N terminus (Ballinger et al., 1999) and a U-box domain at its C terminus. CHIP interacts with the molecular chaperones HSC70, HSP70 and HSP90 through its TPR domains (Connell et al., 2001), whereas its U-box domain contains its E3 ubiquitin ligase activity. The molecular chaperones check and enable correct folding of newly synthesised proteins by binding to exposed hydrophobic regions. They are also required for the refolding of proteins which have denatured due to age, stress, disease etc. During synthesis of new proteins and refolding of denatured proteins, cooperation between the cell’s molecular chaperones and its degradation machinery must occur because some proteins cannot attain their correct tertiary conformation spontaneously. The interaction of CHIP with these molecular chaperones results in their client substrate’s ubiquitination and degradation by the proteasome if the substrate protein cannot fold correctly (Figure 1.10) (Connell et al., 2001, Demand et al., 2001, Murata et al., 2003). This occurs by the recruitment of members of the Ubc4/5 family to the chaperone complex, which are E2 ubiquitin-conjugating enzymes. CHIP then acts as an E3 ubiquitin ligase by adding ubiquitin residues to the chaperone substrate, therefore inducing its targeting to the proteasome. Furthermore CHIP inhibits the folding activity of the chaperones (Connell et al., 2001, Ballinger et al., 1999).
Figure 1.10 **Chaperone mediated folding or degradation of client proteins.**

Client proteins form immature multi-protein complexes, including chaperones HSP90 and HSP70. HSP90 dimerisation and the activation of its ATPase activity leads to the dissociation of HSP70. This allows the formation of the mature complex. In this state, the client protein is correctly folded such that it can become active. However if the client protein remains misfolded, an E3 ubiquitin ligase such as CHIP is recruited to the complex, mediating ubiquitination and degradation via the proteasome. For simplicity, a range of other chaperones are not included on this figure.
However, the role of CHIP in the degradation of proteins does not appear to end with their ubiquitination. It may also participate in the transport of ubiquitinated proteins to the proteasome. Indeed CHIP has been shown to co-localise with the proteasome in cells treated with proteasome inhibitors and interacts with the S5a proteasome subunit in yeast 2-hybrid and GST pull down assays (Connell et al., 2001).

The activity of CHIP may be mediated by its interaction with a second co-chaperone called BAG1 (Wang et al., 1996). A direct protein interaction has been established between the two proteins and they both associate with chaperone proteins. BAG1 binds to HSC/HSP70 via its carboxy-terminal BAG domain, which associates with the ATPase domain of the chaperones. It acts as a nucleotide exchange factor for HSC/HSP70, stimulating the release of protein substrates from these chaperones (Hohfeld and Jentsch, 1997, Luders et al., 1998, Takayama et al., 1998, Luders et al., 2000b, Sondermann et al., 2001). BAG1 targets ubiquitinated chaperone substrates for degradation as it possesses a ubiquitin-like domain at its amino terminus that targets it to the proteasome and binds to it in an ATP-dependent manner (Luders et al., 2000a). The ability of BAG1 to associate with the proteasome, and simultaneously with HSC/HSP70 allows the chaperone/substrate complex to be brought into close association with the proteasome. Furthermore since BAG1 stimulates the release of protein substrates from HSC/HSP70 through its nucleotide exchange activity, it facilitates the release and proteasomal degradation of ubiquitinated chaperone substrates.

### 1.9.6 Regulation of CRaf by ubiquitination

Although CRaf is ubiquitously expressed it has been shown to be ubiquitinated and targeted for degradation by the proteasome in varied settings (Schneider et al., 1996, Demand et al., 2001, Schulte et al., 1997, Manenti et al., 2002, Du et al., 2006). For example, treatment with geldanamycin, an inhibitor of the chaperone activity of HSP90, alters the CRaf/HSP90 complex by binding to the chaperone protein (Schulte et al., 1995, Schneider et al., 1996, Stancato et al., 1997). This leads to a reduction in CRaf stability that finally results in CRaf
depletion which blocks normal CRaf signalling, demonstrated by the inhibition of phorbol-12-myristate-13-acetate (PMA) stimulated CRaf signalling to MEK/ERK in NIH3T3 cells (Schulte et al., 1996). Work by Schulte et al. (1997) demonstrated that the degradation of CRaf upon geldanamycin treatment was due to the increased turnover of the protein by the proteasome. The authors demonstrated that CRaf degradation was prevented by treatment with several proteasomal inhibitors in geldanamycin-treated cells. Furthermore CRaf in cells treated with geldanamycin and proteasome inhibitors was of a higher molecular weight and showed a laddering pattern consistent with its poly-ubiquitination (Schulte et al., 1997). This laddering pattern was more pronounced when N-ethylmaleimide was added to the lysis buffer, which inhibits the de-ubiquitination of proteins. The authors found that unlike CRaf in untreated cells, the CRaf in geldanamycin- and proteasome inhibitor-treated cells was mainly insoluble in NP40 or Triton-X100 based buffers, but was soluble in SDS buffers. However this insoluble CRaf appeared to have enzymatic activity as it restored RAF/MEK/ERK signalling in geldanamycin-treated NIH3T3 cells induced by PMA. As described above, CRaf is part of a multiprotein complex containing proteins with various scaffolding, modulating and chaperoning abilities. The chaperone proteins include HSP90, p50 and HSP70. HSP90 ensures the correct folding of CRaf in an ATP driven process. Indeed the disruption of HSP90 binding by treatment with geldanamycin results in misfolding of CRaf and the proteins degradation by the proteasome (Demand et al., 2001, Schulte et al., 1997, Schulte et al., 1995). The E3 ubiquitin ligase, CHIP has been implicated in the stimulation of the ubiquitination of CRaf (Demand et al., 2001). The investigators showed that CHIP, together with the E2 UbcH5b, formed a conjugation system that mediated the attachment of ubiquitin moieties to CRaf. The requirement of CHIP for an E2 enzyme was found to be specific, as in contrast to UbcH5b, the ER-associated UbcH7 was unable to cooperate with CHIP. The use of CHIP mutants in this study demonstrated that the U box of CHIP was essential for ubiquitin conjugation. The degradation of CRaf is further facilitated by the ubiquitin binding protein BAG-1 that targets ubiquitinated proteins to the proteolytic complex (Demand et al., 2001, Song et al., 2001).
Secondly, CRaf has been shown to be down-regulated when cell anchorage is disrupted and it was found that this is due to ubiquitination and targeting of CRaf to the proteasome (Manenti et al., 2002). It is known that adhesion of cells to the extracellular matrix is a requirement for ERK activation by growth factors. Several studies identified that this requirement was at the level of Raf (Chen et al., 1996, Lin et al., 1997, Howe and Juliano, 1998). CRaf was shown to be protected against ubiquitin-dependent proteasome degradation by cell adhesion to the extracellular matrix in work by Manenti et al. (2002). When cell anchorage was disrupted, CRaf expression was down-regulated. However when cells kept in suspension were treated with proteasome inhibitors the expression of CRaf was restored, indicating that cell adhesion disruption triggered a mechanism of CRaf degradation by the proteasome. Interestingly, it was found that CRaf expression was not raised in adherent cells treated with the proteasomal inhibitor suggesting that, in adherent culture conditions, the expression of CRaf does not depend on proteasomal activity. The authors investigated whether CRaf could be ubiquitinated in cells. They did this by co-expressing ectopic CRaf and ubiquitin in HEK-293 and NIH3T3 cells. Western blot analysis with an antibody against CRaf revealed the appearance of a population of CRaf with a higher molecular weight, thus indicating that it was modified in some way. The authors confirmed the ubiquitination of CRaf by co-transfection of His-tagged ubiquitin and HA-tagged CRaf. Ubiquitinated products were purified on Nickel affinity columns and the ubiquitinated form(s) of CRaf were detected by Western blot with an α-HA antibody. In all experiments, one main population of ubiquitinated CRaf was identified with an apparent molecular weight corresponding to the addition of two ubiquitin molecules per molecule of CRaf (Manenti et al., 2002). This is not consistent with the expectation of higher molecular weight poly-ubiquitinated populations of CRaf which are produced prior to proteasomal degradation.

Finally, CRaf expression was reduced by treatment with methylglyoxal via a ubiquitin-mediated proteasomal degradation process, which was associated with the phosphorylation of CRaf (Du et al., 2006). Methylglyoxal is a physiological glucose metabolite, which possibly
contributes to a decrease in CRaf expression in some diabetic patients. Methylglyoxal modifies cellular proteins by glycation and oxidation, resulting in their dysfunction or loss. The authors observed a decrease in CRaf protein expression induced by methylglyoxal in HEK293 cells. A more substantial decrease was seen when cells were pre-treated with the CRaf activator PMA or when cells were transfected with vectors expressing active forms of CRaf and Ras, indicating that active CRaf is degraded more quickly in this system than the inactive protein. Pre-treatment with bisindolylmaleimide, a specific PKC inhibitor, prevented CRaf down-regulation induced by co-treatment with methylglyoxal and PMA, suggesting that a phosphorylation event mediated by PKC is required for the loss of CRaf in such treated cells. Treatment of cells with methylglyoxal and PMA caused ubiquitination of CRaf as analysed by western blot. The authors excluded the possibility that the treatment with methylglyoxal altered binding of CRaf to HSP90, which indicates that this mode of CRaf degradation may occur by a distinct mechanism.

It is not clear if the other Raf isoforms are regulated in a similar manner. Both BRaf and CRaf contain a PEST domain. However this sequence is lacking in ARaf. PEST domains are sequences of over 12 amino acids enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) which are uninterrupted by positively charged residues. They act as a signal peptide for protein degradation (Rechsteiner and Rogers, 1996). The CRaf PEST domain is present in the variable region between CR2 and CR3 at residues 284-309. The PEST domain in BRaf is also not conserved and occurs within residues 298-338 in the variable region between CR1 and CR2. This suggests that both BRaf and CRaf may be regulated by protein degradation.

1.10 ERK cascade mutations in cancer

The ERK cascade is hyperactivated in approximately 30% of human cancers (Hoshino et al., 1999). Various components of the ERK signalling pathway can be mutated in cancer,
including RTKs, RAS, GAPs, and BRAF. Mutations of these proteins bring about a cancerous phenotype at least in part due to the deregulation of ERK activity.

1.10.1 Oncogenic RAS

The RAS genes are mutated in 15-30% of human cancers, predominantly those of the pancreas, biliary tract, colon and lung, although the specific RAS gene mutated varies with the type of cancer (Bos, 1989), with the KRAS gene being the most frequently mutated. KRAS mutations are found in 70–90% of adenocarcinomas of the pancreas, approximately 50% of colon carcinomas and 25–50% of lung adenocarcinomas.

Oncogenic RAS proteins contain single amino acid missense mutations located at residues G12, G13 or Q61 that render them chronically active (Herrmann, 2003) by the impairment of their intrinsic and GAP-stimulated GTP hydrolysis so that they reside in a persistently GTP-bound and active state. Inappropriate activation of RAS can also be caused by mutation of RTKs, leading to their persistent activation. For example the EGF receptor is mutated in human cancers. Additionally inappropriate RAS activation may be due to a mutation of RAS GAPs such as the loss of function of the NF1 tumour suppressor. Oncogenic RAS can aid the progression towards cancer by signalling to many diverse pathways which regulate proliferation, differentiation and survival. The best characterised RAS effector pathway is the ERK cascade. However RAS can also signal to effectors other than RAF, including PI3K, phospholipase Cε and RalGEFs, although the activation of these substrates may be cell type specific. The main activity of PI3K involves conversion of phosphatidyl inositol (4,5)-bispophosphate to phosphatidyl inositol (3,4,5)-trisphosphate. This allows the activation of PKB and hence its downstream targets such as nuclear factor-κB and Bcl-2 family proteins therefore mediating cell survival. PI3K is persistently activated in some, but not all RAS-transformed cells (McFall et al., 2001, Ulku et al., 2003).
1.10.2 Oncogenic BRAF

BRAF was identified as an oncogene in 2002 in a sequencing screen of 923 cancer samples (Davies et al., 2002). Activating mutations in the BRAF gene have been identified in up to 70% of human malignant melanomas, up to 15% of human colorectal cancers and also up to 35% of thyroid cancers. BRAF mutations have been identified at lower frequencies in gliomas, lung cancers, sarcomas, ovarian carcinomas, breast cancers and liver cancers. In total BRAF is mutated in approximately 7% of human cancers. The BRAF mutations identified may not be the actual cause of the cancers. However, the acquisition of BRAF mutations may be a prerequisite for cancer development although further mutations are required for the transition to malignancy. Indeed evidence exists that BRAF mutations are a founder event for the development of cancer. For example activating mutations of BRAF have been observed in premalignant colon polyps and the early Duke’s stages (A and B) of colorectal cancer (Yuen et al., 2002, Rajagopalan et al., 2002) Furthermore such mutations have also been observed in premalignant naevi (Pollock et al., 2003, Yazdi et al., 2003), which can remain dormant for years before the development of malignant melanoma.

Over 45 oncogenic mutations have been identified in BRAF (Emuss et al., 2005), most of which are present in the kinase domain, localised either in the activation segment or the glycine-rich loop (Figure 1.11 and 1.12). Indeed 89% of the BRAF mutations identified encode amino acid substitutions in the activation segment of the protein, with 92% of these mutations having a T to A change at nucleotide 1799, producing a V600E mutation in the protein. In a separate study by Rajagopalan et al of colorectal tumours, 10% of the samples had a BRAF mutation, and of this 10% 88% of samples contained the V600E mutation (Rajagopalan et al., 2002). Other mutations were detected in the activation loop at lower frequencies, which include V600D, L597V, L597R, G596R and F595L. The glycine-rich loop GXGXXG motif was another site that harbourd a significant proportion of mutations, affecting the glycine residues 464, 466 and 469. The V600E mutation accounts for over 90% of the mutations in melanoma and thyroid cancer.
Figure 1.11 **Oncogenic BRAF mutations.** (Adapted from Wellbrock et al 2004a)

Mutations identified in BRAF are mainly found in the kinase domain, localised either in the activation segment or the glycine-rich loop. The green bars donate the frequency of each mutation. The most commonly mutated residue is V600.
Figure 1.12 **The location of oncogenic BRAF mutations in the kinase domain.**
(Taken from Garnett and Marais, 2004)

The BRAF kinase domain from figure 1.4 is shown again, highlighting the location of mutations. These are indicated and coloured red or orange (colour is used for clarity, but has no other significance). Note the majority of the mutations are confined to the glycine rich loop and activation segment, although a few occur elsewhere in the kinase.
(Fukushima et al., 2003, Kimura et al., 2003, Kumar et al., 2003, Brose et al., 2002). In the absence of the V600E substitution, \textit{BRAF} mutations in cancer would be quite rare. It is unknown why the V600E mutation predominates; certainly other mutants such as G469A have similarly elevated kinase activity and are also generated through a single nucleotide substitution, but occur much less frequently, accounting for 1% of mutations (Davies et al., 2002). The high incidence of V600E mutations in malignant melanomas may at first glance suggest that this mutation is induced by ultraviolet radiation. However, this would not seem to be the case as the T>A transversion which occurs is distinct from the C>T or CC>>TT pyrimidine dimer mutations common to UV-induced DNA damage (Daya-Grosjean et al., 1995). Furthermore such \textit{BRAF} mutations are not found in other forms of skin cancer and UV exposure cannot be responsible for the V600E mutations which are frequent in colorectal cancer for example.

\textbf{1.10.3 High and intermediate activity oncogenic \textit{BRAF} mutations}

The majority of \textit{BRAF} mutants confer elevated kinase activity, and such mutants can be split into the “high activity” group and the “intermediate activity” group. Seven mutants can be classified as having high activity, including E586K, V600E and E600D which have 130-, 500- and 700-fold the kinase activity of wild-type \textit{BRAF}, respectively. The kinase activities of these mutants exceed the level achieved by activating wild-type \textit{BRAF} with an oncogenic form of \textit{RAS}. Eleven mutants can be classified as having intermediate kinase activity of 1.3 fold (G469E) to 64 fold (L597V) greater than wild-type \textit{BRAF} (Wan et al., 2004). These mutants phosphorylated MEK above the basal level (non-oncogenic \textit{RAS} stimulated) for wild-type \textit{BRAF}, but below the level reached by oncogenic \textit{RAS} activation of wild-type \textit{BRAF}. The high and intermediate activity mutants all stimulate the hyperphosphorylation of ERK when transfected into cells. However, they only raise ERK activity by 2 – 4.6 fold, suggesting the presence of regulatory proteins which control the signal flow from RAF to MEK and to ERK.
1.10.4 How do the high and intermediate oncogenic BRAF mutants lead to higher kinase activity?

The crystal structure of the wild-type BRAF kinase domain has been solved as well as the kinase domain of the high activity mutant BRAF$^{V600E}$ (Wan et al., 2004), which is the predominant BRAF mutant in human malignant melanomas (Davies et al., 2002). These have provided a useful insight to explain how some of the high and intermediate oncogenic BRAF mutations confer an elevated kinase activity. The wild-type and mutant BRAF kinase domain were co-crystallised with a small-molecule inhibitor of RAF, BAY43-9006 in order to obtain crystals of suitable quality.

The structure of the kinase domain indicates that residues G596-V600 of the activation segment engage in hydrophobic interactions with residues G464-V471 of the glycine rich loop; this holds the kinase domain in an inactive conformation. As described earlier, phosphorylation of residues within the kinase domain regulate BRAF activity. Specifically, phosphorylation of threonine 599 and serine 602 in the activation segment are necessary to stabilise an active conformation allowing functional catalytic activity (Chong et al., 2001). Most of the oncogenic mutations of BRAF are present in the kinase domain, localised either in the activation segment or the glycine-rich loop. Mutations in these regions are likely to disrupt this interaction allowing the kinase domain to assume a constitutively active conformation (Wan et al., 2004). For example V600 is located in the activation segment. This residue makes hydrophobic contacts with F468 in the glycine-rich loop in the wild-type BRAF kinase structure, stabilising the inactive conformation of BRAF. The V600E mutation disrupts this inhibitory interaction and furthermore may introduce a negative charge that mimics the phosphorylation events that occur at threonine 599 and serine 602 during wild-type BRAF activation process. This would allow activation without the requirement for phosphorylation.
Since V600 appears to be important in stabilising the inactive BRAF kinase and its mutation to E600 leads to huge elevations in kinase activity, it would be expected that in the crystal structure of the V600E mutant, the activation segment would adopt a conformation more similar to the phosphorylated, active conformation. However the conformation of the activation segment is almost identical to that in the wild-type structure. An explanation for this may be the presence of BAY43-9906. This inhibitor has a higher affinity for the inactive conformation of the activation segment, and therefore stabilises this conformation. Hence in the V600E mutant, the inhibitor dictates the conformation of the activation segment more so than glutamic acid at residue 600.

It is more difficult to explain how other mutations lead to increased BRAF activity. For example, the presence of activating mutations in the glycine rich loop is difficult to understand, as similar mutations in PKA lead to reduced kinase activity (Hemmer et al., 1997, Grant et al., 1998). Many of the residues mutated such as the glycines (G463, G465, and G468) of the glycine-rich loop, the asparagine (N580) of the catalytic loop and the phenylalanine (F594) of the DFG motif serve key catalytic functions and are conserved within the kinase superfamily (Hanks and Hunter, 1995, Johnson et al., 1998). In spite of this, such mutations can be activating. The amino acid substitutions may disrupt the inhibitory interactions between the activation segment and the glycine rich loop. However, since some of the mutations involve important catalytic residues, the full activation potential of the BRAF kinase domain is not achieved, explaining why some mutants have only modestly increased kinase activity.

Activating mutations are also located outside of the glycine rich loop/activation segment interaction including E585, D586, and R681. These residues are located on the surface of the kinase domain in a pattern similar to one found in the tyrosine kinase Abl. In this kinase, this region is responsible for negative regulation through an intramolecular interaction with the regulatory N-terminal domains (Nagar et al., 2003), suggesting mutation of these
residues in BRAF may disrupt a negative interaction between the kinase domain and the N-terminus.

1.10.5 The impaired activity oncogenic BRAF mutants

Four of the cancer associated BRAF mutations investigated by Wan et al., (2004) confer reduced kinase activity of 30-80% compared to that of wild-type BRAF. These are termed the “impaired activity group”. They consist of G466E, G466V, D594V, and G596R (Wan et al., 2004). The loss of kinase activity in the impaired activity mutants can be explained more easily than the gain of activity in the high and intermediate activity mutants. For example two mutations lead to substitutions of G466 to valine or glutamic acid. Any amino acid other than glycine at this position would adversely affect ATP binding and hence reduce kinase activity. Surprisingly, when transfected into COS cells, three of the four impaired activity BRAF mutants (G466E, G466V, and G596R) induce endogenous ERK activation. In fact they appeared to be more efficient at stimulating ERK phosphorylation than wild-type BRAF. However, one of the impaired activity mutants, with a D594V substitution, is not capable of inducing MEK/ERK activity. The loss of function of this mutant is not surprising because D594 is a key catalytic residue, located in the DFG motif. However this residue is mutated in ~1% of cancers containing oncogenic BRAF mutants. It is not clear how a mutation of this residue could lead to a cancerous phenotype. It is possible that this mutant acts as a dominant-negative regulator of oncogenic RAS to modulate ERK signalling, as mutation of the D594 residue has been observed to occur concurrently with mutation of RAS (Yuen et al., 2002, Houben et al., 2004). Indeed ERK signalling must be carefully regulated to stimulate proliferation. If the signal is too strong, cells stop cycling and differentiate or senesce (Marshall, 1995, Kerkhoff and Rapp, 1998, Sewing et al., 1997, Woods et al., 1997). It is also possible that BRAF mutants, with a D594 substitution function in kinase independent pathways, although no such kinase-independent substrates have been identified as yet.
1.10.6 How do the impaired oncogenic B Raf mutants lead to activation of ERK?

As described previously, wild-type BRaf and wild-type CRaf have been shown to form heterodimers. Using siRNA against CRAF, the impaired BRAF mutants were shown to form complexes with CRAF, stimulating CRAF activation and signalling to ERK. The phosphorylation of ERK in cells transfected with the impaired BRAF mutants was shown to be dependent on endogenous CRAF activity (Wan et al., 2004). High and intermediate activity BRAF mutants also activate CRAF but do not rely on CRAF for ERK stimulation. For example Braf$^{V600E}$ activates CRAF (Garnett et al., 2005) but this activity is not required for ERK activation because, unlike the impaired activity mutants, Braf$^{V600E}$ directly activates MEK in cells. This is demonstrated in the siRNA experiments performed by Wan et al., (2004) in which, although CRaf expression was knocked down, ERK activity was not reduced in cells expressing Braf$^{V600E}$.

Both kinase inactive wild-type BRAF and the impaired activity BRAF$^{D594V}$ mutant, which lacks kinase activity due to the location of the mutation in the DFG motif, failed to activate CRAF. This suggests that BRAF must be both in an activated conformation and retain at least some kinase activity to activate CRaf. The impaired BRAF mutants appear to be considerably more effective than wild-type BRaf in stimulating CRaf activity. Indeed oncogenic BRaf proteins interact constitutively with CRaf in a RAS-independent manner, whereas binding between wild-type BRaf and CRaf is induced by RAS activation (Garnett et al., 2005). Using mutants, the activation of CRaf by BRaf was found to be dependent upon 14-3-3 mediated heterooligomerisation and CRAF trans-phosphorylation, specifically of the activation segment sites T491 and S494. Hence BRaf induces activation segment phosphorylation on CRaf either directly or indirectly through recruitment of another kinase. Such a process is subverted by mutant BRaf, to deregulate CRaf activation.
1.10.7 CRAF as an oncogene

The Raf proteins were identified as oncogenes in mice and chickens. However initial studies failed to link activating mutations in the human RAF genes to the development of human cancers (Storm et al., 1990a). This failure can be attributed to the techniques used which were not sensitive enough to detect single point mutations in cancer samples and also because investigators focused their efforts on the CRAF gene, rather than the more oncogenic BRAF gene. Indeed BRAF is mutated in approximately 7% of human cancers. However, CRAF has been shown to be mutated in only 0.7% of cancer cell lines. Traditionally, CRaf was considered to be the more important isoform of the Raf proteins, so the oncogenic nature of BRaf rather than CRaf was surprising. However the oncogenic nature of BRaf would seem to make sense when it is put into context. Firstly, studies using mice with targeted mutations of the Raf genes show that CRaf is dispensable for MEK/ERK activation and that BRaf appears to be the primary MEK/ERK activator in most tissues and cell types (Wojnowski et al., 2000, Huser et al., 2001, Mikula et al., 2001). Secondly, BRAF has also been shown to have a higher basal kinase activity than the other isoforms because of key differences in its primary structure at residues 446, 448 and 449. (Mason et al., 1999, Zhang and Guan, 2000). The presence of aspartic acids at positions 448 and 449 confers a negative charge which may mimic phosphorylated residues and the constitutive phosphorylation of S446 means that BRAF regulation is simpler. BRAF appears to only require RAS binding and phosphorylation of the activation loop on T599 and S602 for activation, in contrast with the more numerous phosphorylation events which occur in the activation process of ARaf or CRaf. Presumably, because of the constant charge in the N-region together with the elevated BRAF basal kinase activity, single amino acid substitutions are sufficient to stimulate its kinase activity. ARAF and CRAF require more molecular events for activation and so single point mutations are insufficient to induce activity.

However evidence does exist that implies a role for CRaf in cancer. The removal of the regulatory domain of CRaf generates an oncogenic kinase, which can transform NIH 3T3
and other cell lines in culture, causing changes in morphology, reduced requirement for growth factors, anchorage-independent growth and loss of contact inhibition. This CRaf mutant can also induce the formation of tumours in immunodeficient mice. However, the expression of this CRaf mutant in the lungs of transgenic mice resulted in adenomas, which did not invade or progress to a malignant phenotype (Zebisch and Troppmair, 2006, Kerkhoff et al., 2000, Rutters et al., 2006, Rapp et al., 2003, Kramer et al., 2004). Other mutations of CRaf including point mutations which produce a constitutively active kinase can also transform cells in culture. However such mutations have not been found to naturally occur in human cancers (Zebisch and Troppmair, 2006, Miwa et al., 1994, Emuss et al., 2005, Lee et al., 2005). Recently, several naturally occurring CRAF mutations (E478K, S427G and I448V) have been identified which demonstrate an increase in kinase activity and transforming activity (Emuss et al., 2005, Zebisch et al., 2006). Further investigation will be required to determine their role and frequency in human cancer. Interestingly, the CRafV492E mutation equivalent to the oncogenic BRafV600E mutation has not been detected in human cancers and actually has a ten fold lower kinase activity; hence CRAF is not converted to an oncogene. This mutation although not transforming in itself, requires concurrent mutation in the N-region to introduce a negative charge by the substitution of Y340 and Y341 for aspartic acid residues (Emuss et al., 2005). This is most likely due to the fact that CRaf has tighter regulation of its kinase domain than BRaf, thus, a single mutation is insufficient for substantial kinase activation.

The over-expression of wild-type CRaf has also been investigated in relation to its oncogenic potential. C-Raf has been found to be over-expressed in a variety of human cancers, including prostate, lung, liver, myeloid leukaemia, primitive neuroectodermal tumours, and head and neck squamous cell carcinoma (Rapp et al., 2003, Schmidt et al., 1994, Riva et al., 1995, Hwang et al., 2004, Mukherjee et al., 2005, Damodar Reddy et al., 2001). However, the significance of this to the progression of the disease needs further investigation. Initial studies where CRaf was over-expressed in a variety of cell culture
models failed to demonstrate its activation or transforming activity (Heidecker et al., 1990). Since then over-expression of CRaf in the lung has demonstrated its transforming potential (Kerkhoff et al., 2000, Rutters et al., 2006, Rapp et al., 2003).

Although the role of CRaf as an oncogene is somewhat uncertain, it is clear that it functions downstream of other oncogenes in a large number of human cancers and therefore is important to cancer development. The role of CRaf in mediating signals from oncogenic RAS has been demonstrated in studies which used agents to block C-Raf function or expression such as anti-sense RNA, small interfering RNA and small molecule kinase inhibitors. Such investigations found that the transformed phenotype induced by oncogenic RAS could be reversed by inhibiting CRaf (Avruch et al., 1994, Kolch et al., 1991, McFarlin et al., 2003, Repasky et al., 2004, Cuadrado et al., 1993, Shields et al., 2000, Kasid and Dritschilo, 2003, Leng and Mixson, 2005, Pal et al., 2005, Kim et al., 2004). In addition, CRaf is believed to mediate the effects of some oncogenic BRAF mutants as described above.

1.10.8 The role of BRAF mutations in cancer

The BRAF^{V600E} mutant stimulates constitutive ERK signalling, induces proliferation and transformation in NIH3T3 cells and murine melanocytes and can induce such cells to form tumours in nude mice (Davies et al., 2002, Ikenoue et al., 2003, Ikenoue et al., 2004, Houben et al., 2004, Wan et al., 2004, Wellbrock et al., 2004). These data identify BRAF^{V600E} as an oncogene. However such mutations have been observed in premalignant colon polyps and the early Duke’s stages (A and B) of colorectal cancer (Yuen et al., 2002, Rajagopalan et al., 2002) and in premalignant naevi (Pollock et al., 2003, Yazdi et al., 2003). Polyps and naevi can remain dormant for many years, indicating that their progression towards cancer requires additional mutations. In the case of premalignant naevi the mutation of the \( INK4a \) gene which encodes the cyclin dependent kinase inhibitor p16^{INK4A} and p19^{ARF} may be just such a “second hit”. Homozygous deletion of this gene has been
observed in some family cases of melanoma (Ruas and Peters, 1998). Point mutations and promoter hypermethylation of \textit{INK4a} are also detected in sporadic tumours (Flores et al., 1996, Funk et al., 1998) and many melanoma cell lines have abnormalities in p16$_{\text{INK4A}}$ (Ruas and Peters, 1998, Rizos et al., 1999). In addition, mutation of p19$_{\text{ARF}}$, the second protein expressed from the \textit{INK4a} gene has been detected in some melanoma cell lines (Kumar et al., 1998). In contrast to BRAF, some mutations of \textit{INK4a} may be caused by UV radiation, as many of the mutations identified are C–T or CC–TT transitions that can arise from pyrimidine dimer formation.

Mutants of BRaf influence all of the hallmarks of cancer as defined by Hanahan and Weinberg (Hanahan and Weinberg, 2000) via the hyperactivation of the ERK pathway. BRAF mutations most likely mediate early tumour development by their alteration of the cell cycle, allowing cell proliferation to accelerate (Halaban, 1999). For example the V600E mutation of BRAF leads to constitutive ERK activation. ERK is known in part to mediate the G1/S transition of the cell cycle by the regulation of numerous genes including cyclin D (Woods et al., 1997). Indeed its expression has been found to be increased in melanomas (Ewanowich et al., 2001). However, the V600E mutation is also associated with the senescent phenotype of naevi. This phenotype may be mediated by the ERK induced elevation of p21$_{\text{CIP1}}$ expression. p21$_{\text{CIP1}}$ is known to inhibit the cell cycle via its action on cyclin D/CDK4/6 and cyclin E/CDK2 complexes. The duration and the strength of the ERK signal from mutated BRAF and the presence of secondary signals from other mutated proteins will influence whether the signal leads to proliferation or senescence. Mutation of BRAF can also influence the progression of the tumour at later stages of the disease. In sporadic melanomas, p53 mutations are rare. However, abnormal p53 function may arise by the deregulated ERK activity caused by oncogenic BRaf (Ries et al., 2000). ERK signalling may also induce the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) allowing the tumour to develop its blood supply (Eliceiri et al., 1998). In addition, ERK signalling often leads to the suppression of apoptosis by a number of

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mechanisms as described previously. Metastasis and cell migration may also be favoured by ERK signalling, by the expression of integrins. For example, a study by Woods et al., (2001) inhibited MEK in human melanoma and pancreatic carcinoma cell lines and demonstrated the reduced cell surface expression of α6- and β3-integrin. Consistent with this, the conditional activation of the ERK cascade in NIH 3T3 cells caused up to a 20-fold induction of cell surface α6- and β3-integrin expression (Woods et al., 2001).

1.10.9 Braf\textsuperscript{V600E} mice

Mouse models have been produced to investigate tumour development caused by the Braf\textsuperscript{V600E} mutation. Mice have been generated which express a conditional knock-in of the Braf\textsuperscript{V600E} mutation, commonly found in human cancers. Activation of the transgene during embryogenesis under the control of a Cre/Lox system (described below) results in an embryonic lethal phenotype, with the embryos dying at E7.5. Hence the expression of a constitutively active Braf allele during embryogenesis is lethal (Mercer et al., 2005a). When the mice were bred to induce expression of the transgene under the control of the IFN-inducible promoter, they survived only four weeks post birth. The Braf\textsuperscript{V600E} gene was expressed in various tissues including heart, liver, lung, kidney and spleen and its expression correlated with increased Braf kinase activity. The mice displayed several hyperproliferative and developmental abnormalities. For example, the spleen was enlarged and the liver showed both hyperproliferation and increased apoptotic regions. Both these tissues displayed a significant increase in ERK phosphorylation. However, there were no developmental defects observed in the lung and no increase in ERK phosphorylation, although Braf\textsuperscript{V600E} expression and increased Braf activity were detected. Therefore it would seem there is a correlation between the level of ERK activation and the physiological effects observed. MEFs produced from the Braf\textsuperscript{V600E} Cre/Lox mice induced to express Braf\textsuperscript{V600E} showed an increase in proliferation and some characteristics of cell transformation, such as the loss of contact inhibition and the ability to form colonies in soft agar. It would seem that
the expression of BRafV600E is capable of causing hyperproliferation and induce some characteristics of transformation in certain cell lineages.

In addition a study by Dankort et al., (2007) conditionally expressed BRafV600E in the lungs of mutant mice by intranasal infection with an adenovirus expressing Cre recombinase. The expression of BRafV600E induces the formation of multiple benign lung adenomas, as visualised by histological analysis, within 7 weeks following infection. Use of the MEK inhibitor PD0325901 indicates that the ability of BRafV600E to elicit lung tumours is dependent on activation of MEK/ERK. The lung tumours only rarely progressed to adenocarcinomas. Tumours were analysed for markers of the state of the cell cycle, including proliferative markers (phospho-histone H3, Ki67, or BrdU incorporation) and senescence markers (SA-βGal, p19ARF, Dec1). The results indicated that in tumours induced at early times after BRafV600E expression, a high percentage of cells stain positively for proliferative markers. However in tumours observed at later times, the percentage of Ki67-positive cells was dramatically decreased even though the BRafV600E/MEK/ERK signalling pathway remained active and cells stained positively for expression of the senescence markers p19ARF and Dec1. This suggests that sustained activation of BRafV600E promotes an initial period of cell proliferation followed by senescence restraining further tumour progression. The reason for this switch is not clear, but may involve some form of feedback regulation. However mutation of a tumour suppressor gene (either INK4a or TP53) allowed cancer progression (Dankort et al., 2007).

1.10.10 Are oncogenic BRAF and RAS functionally equivalent?

It has been suggested that oncogenic BRAF and RAS mutations are functionally equivalent. Indeed, less than 1% of the cancer samples with a BRAF mutation also have a mutation in RAS and those that do have simultaneous mutations are mainly non-V600E (Davies et al., 2002, Rajagopalan et al., 2002). Their incidence in the same types of cancers is also equivalent. For example, in the case of melanoma, 70% contain a BRAF mutation, and 25%
have a mutation in NRAS, but their occurrence do not overlap (Mercer and Pritchard, 2003). BRAF and RAS mutations also occur independently in colorectal cancers (Rajagopalan et al., 2002), papillary thyroid carcinomas (Vos et al., 2003) and ovarian cancers (Singer et al., 2003, Sieben et al., 2004). The mutually exclusive occurrence of BRAF and RAS mutations suggests that they share equivalent roles in oncogenesis, so that the key role of oncogenic RAS involves RAF activation facilitating cancer progression through the deregulation of ERK activity.

However, it is unlikely that mutation of BRAF alone is truly equivalent to oncogenic RAS, because RAS acts upstream from BRAF and has the capability to cause deregulation of pathways other than the ERK cascade. For example the conditional expression of endogenous activated KRAS in mouse embryo fibroblasts caused proliferation, yet ERK activation was not observed (Tuveson et al., 2004), indicating this may have been mediated via another pathway. In addition, the frequent mutation of RAS, but not BRAF, in pancreatic, follicular thyroid and mucinous ovarian cancers indicates that for these cancer types activation of RAF alone might not be equivalent to activation of RAS (Sieben et al., 2004, Ishimura et al., 2003, Calhoun et al., 2003, Gemignani et al., 2003). Indeed, the activation of Raf in some epithelial cell lines (RIE-1, MCF-10A, HEK) cannot mimic the oncogenic properties of RAS, as these cells can become transformed by activated RAS but not by Raf (Oldham et al., 1996, Schulze et al., 2001, Hamad et al., 2002) and mutant BRaf has a significantly lower transforming potency in rodent fibroblasts when compared with mutant RAS (Davies et al., 2002). Furthermore some melanomas which have BRAF mutations also have mutations of the PTEN tumour suppressor, resulting in the activation of PI3K–PKB RAS effector pathway (Tsao et al., 2004).
1.11 Manipulating the mouse genome

1.11.1 Initial transgenic approaches

The development of transgenic mice allows the generation of animals carrying extremely precise genetic modifications of endogenous genes, enabling the investigation of gene function in normal development and in disease situations in a favoured mammalian model system. Mouse models for human genetic diseases have been created, which allow the mechanisms of the disease to be established and provide a medium to develop appropriate therapies.

The first deliberate genetic modification of the mouse embryo was reported in 1974 (Jaenisch and Mintz, 1974). Blastocysts were infected with the SV40 virus and the progeny of such mice showed stable integration of viral sequences in their cells. The next major technique developed was the transfer of cloned DNA into the pronucleus of a zygote by microinjection. The eggs were then transferred into pseudo-pregnant surrogate mothers. In this procedure anywhere between one and hundreds of copies of the microinjected DNA integrated randomly into the mouse genome, although multiple insertions often occurred at a single site as head-to-tail arrays of the injected DNA. The frequency of insertions is rare, however affected mice usually carry the introduced gene in both somatic cells and germ cells, hence they pass the introduced DNA to their progeny. In more recent years a technique has been developed whereby the genome of embryonic stem cells is manipulated by incorporating targeted exogenous DNA. These cells are then used to produce transgenic mice which harbour the same targeted allele.

1.11.2 Gene targeting in embryonic stem cells

Embryonic stem (ES) cells are derived from the inner cell mass of mouse blastocyst stage embryos at E3.5. These cells are pluripotent; they can differentiate into all cell types. ES cells were first isolated and cultured successfully in 1981 (Evans and Kaufman, 1981,
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Martin, 1981). The culture of ES cells in their undifferentiated state requires the addition of leukaeemia inhibitory factor (LIF) to the growth media (Smith et al., 1988) and a feeder layer of mitotically inactivated MEFs which provide secreted proteins, which in combination with LIF are necessary for the ES cells to retain their pluripotent capacity. The feeder cells also provide a matrix for the ES cells to adhere to. By culturing ES cells in this way they can be passaged many times without loosing their pluripotent capacity. Such cells can be induced to differentiate by altering their growth conditions in culture. They will also differentiate in concert with normal embryo cells following their re-introduction into host blastocysts.

Investigations conducted in the 1980s demonstrated that mammalian cells have the capacity to allow recombination between exogenous DNA and the homologous endogenous sequence. However, such events were found to be rare in comparison with the random integration of this same DNA (Smithies et al., 1985, Wong and Capecchi, 1986). This discovery led to the manipulation of cultured ES cells to incorporate exogenous DNA to create targeted mutations by knockout strategies.

A common technique to introduce DNA into ES cells is electroporation; a high voltage pulse is employed to enable DNA present in a suspension of ES cells to enter such cells. Specific genes can be targeted by double reciprocal homologous recombination between the endogenous DNA and a targeting vector (Figure 1.13). The targeting vector (Figure 1.13A) is constructed in such a way as to specifically recombine with and mutate a specific chromosomal locus, because it is designed to include DNA sequences homologous to the target gene. Homologous recombination events are very rare. Therefore the ES cell population must be enriched for cells with such an event. To this end, targeting vectors are designed to include a positive selectable marker, and a negative selectable marker, allowing positive and negative selection strategies to be employed. The neomycin resistance gene \((\text{neo}^R)\) is an example of a typical positive selection marker. The protein produced from this gene confers resistance to the drug G418 and therefore isolates cells which stably express...
Figure 1.13 Embryonic stem cell gene targeting.

The black lines represent non-homologous DNA sequences, blue lines represent homologous DNA in the targeting vector and locus. The neo\(^R\) and HSV-tk genes, encoding the selectable markers, are indicated as a green box and a red box, respectively. Coding exons are indicated as a blue box. (A) Examples of targeting vectors. In the first, the neo\(^R\) gene is located within an exon, which is homologous to the locus. In the second, neo\(^R\) is not present within an exon, but has DNA flanking it which is homologous to the introns in the locus on either side of the exon(s) to be deleted. (B) The positive selectable marker interrupts the exon in the locus. (C) the selectable marker replaces the exon. Both (B) and (C) result in a null mutation of the locus.
the targeting vector. The herpes simplex virus thymidine kinase gene (HSV-tk) is a typical negative marker; this produces a protein which converts the drug gancyclovir to a form which is toxic to the cell. Therefore if the HSV-tk gene is placed just outside the region of homology, it provides selection for targeted recombination because it is lost after homologous recombination. After the recombination event the targeted gene is equivalent to the replacement of the endogenous DNA with the components of the targeting vector, flanked on both sides by homologous sequences.

This relatively simple strategy is used to produce null mutations by a knockout strategy. The insertion of a selection cassette (such as neo<sup>R</sup>) into a coding exon serves as a mutagen by disrupting the coding exon of a gene (Figure 1.13b), or an exon (or exons) can be entirely replaced by the insertion of a selection cassette (Figure 1.13c). In 1987, the first targeted mutagenesis of ES cells was achieved obtaining ES cells carrying a null mutation in the hprt gene. Even so it should be noted that the interruption of exons and small deletions may result in the production of truncated transcripts and proteins which still posses some activity and do not always abolish the total function of the gene.

1.11.3 The Cre/LoxP system

The Cre/LoxP site-specific recombination system (Figure 1.14) widened the scope of targeted mutagenesis. This system allowed more subtle mutations to be possible such as point mutations, small deletions and insertions by a knock-in strategy. When creating mutated alleles which require functionality, it is essential to remove the selection cassettes which are present, because they may interfere with the regulation of the expression of the targeted gene or adjacent genes (Fiering et al., 1999). The Cre/LoxP system is one of several that can create “clean” mutations, i.e. a mutant allele without foreign sequences with the exception of a single LoxP site. The previous technology only enabled null mutations because such selection sequences could not be removed, and the gene was irreversibly disrupted by the mutation. In addition the Cre/LoxP system has provided the possibility of
Figure 1.14 **The Cre/LoxP system used to produce knockin mutations.**

**(A)** The sequence of a LoxP (locus of crossover (x) in P1) site. The LoxP sequence consists of two 13 bp inverted repeats which are separated by an 8 bp non-palindromic spacer sequence. This spacer determines the orientation of the LoxP site. When two LoxP sites are oriented in the same direction, Cre induces the deletion of the DNA segment placed between them **(B)** Cre is a 38 kDa protein, the product of the cre (cyclization recombination) gene from the bacteriophage P1. Cre recognises and mediates site-specific recombination between 34 bp LoxP sites, causing the deletion of a floxed gene.

A

![Diagram of the Cre/LoxP system](#)

- **5’** – ATAACCTTCGTATA\_GCATACATT\_TACGAAGTTAT – **3’**
- **3’** – TATTGAAGCATAT\_CGTATGTA\_ATATGCTTCAATA – **5’**

B

![Diagram of the Cre/LoxP interaction](#)

- Floxed gene
- +Cre
- LoxP
conditional mutagenesis, allowing a gene to be mutated in any cell type and/or at any time of development.

The Cre/LoxP system requires a targeting vector that contains the desired mutation in the gene of interest and the selectable marker in an intron, flanked by two LoxP sites in the same orientation to create a floxed sequence. Targeted ES cells can be transfected with a Cre-expressing vector, which mediates a site specific recombination event between the two LoxP sites and results in the deletion of the DNA between them (Hoess et al., 1982, Hoess and Abremski, 1984). This results in ES cells bearing the desired mutation in the gene of interest, with the presence of only one LoxP site as a foreign sequence present in an intron. Alternatively, the floxed selection cassette may be excised in vivo by crossing mice bearing the targeted allele with mice expressing Cre.

1.11.4 Generation of transgenic mice

Transgenic mice were first created from genetically modified ES cells in 1986 (Gossler et al., 1986, Robertson et al., 1986), introducing a new method of generating transgenic mice which allowed new possibilities in comparison with classical transgenics by microinjection of DNA into the zygote. In 1989 the first transgenic mice were obtained by homologous recombination in ES cells. The subsequent generation of transgenic mice from targeted ES cells allowed precise mutations of a gene, aiding the study of gene’s function in vivo.

The process of creating a transgenic mouse is depicted in Figure 1.15. A timed mating is set up and the blastocysts are removed from the female at E3.5. Normally between 6 and 12 targeted ES cells with the required genetic modification are microinjected into each host blastocyst. These blastocysts are then implanted into a surrogate pseudo-pregnant female. The pups which are produced from this manipulation are chimeras. Some of their cells are derived from the host embryo, and some are derived from the reintroduced ES cells (Bradley et al., 1984). As part of this manipulation the coat colour of the host blastocyst and the donor
Figure 1.15 *Generation of transgenic mice from targeted embryonic stem cells.*

Targeted ES cells with albino coat colour are microinjected into a black coat colour E3.5 host blastocyst, which are then transferred into a pseudo-pregnant female. Chimaeric pups are produced which contain cells derived from the host blastocyst and the injected targeted ES cells. Chimaeric mice are mated with wild-type mice and successful germline transmission can be assessed by the coat colour of their progeny. Brown coloured mice have transmitted the targeted ES cells, whereas black coloured offspring originate from the host blastocyst.
ES cells are designed to be distinguishable, meaning that the chimaeric mice produced exhibit coat colour mosaicism, which is used as a genetic marker of chimaerism. Since all tissues in a chimaeric mouse are generated by the proliferation and differentiation of any ES cells, donor ES cells may or may not be responsible for the development of the germline (sperm or ova). This can be investigated by breeding to a mouse with an appropriate coat colour. The coat colour of the offspring from such a cross indicates whether the germline transmission of the ES donor cells has been achieved. Once germline transmission has been achieved, the animals are screened for the presence of the mutation which was originally introduced into the ES cells in culture. Animals that contain the mutation are heterozygous: each of their cells contains the mutation on one allele. These mice can be used to create a breeding colony.

1.12 Studies in mice with targeted Raf mutations

ARaf, BRaf and CRaf knock-out mice have very different phenotypes, strongly suggesting that these proteins are non-redundant and serve distinct functions (Hagemann and Rapp, 1999).

1.12.1 ARaf

ARaf-deficient mice (arafr<sup>−/−</sup>) are born alive but dependent upon their genetic background, have differing phenotypes. Those with a C57BL6 genetic background display neurological and intestinal abnormalities and die between 7 and 21 days after birth (Pritchard et al., 1996), while those with an outbred 129Ola background survive to adulthood, do not display intestinal abnormalities, but do show a subset of the neurological defects. MEFs and other tissues derived from ARaf-deficient mice proliferate normally, do not show abnormalities with apoptosis and show no disruption of ERK activation (Mercer et al., 2002).
1.12.2 CRaf

The first CRaf-knockout mice (craf-/-) that were generated displayed an embryonic lethal phenotype at E10.5 to E12.5. However, they expressed a low amount of an N-terminal truncated CRaf protein that maintained some kinase activity. This complicated the interpretation of the data (Wojnowski et al., 1998). Subsequently craf-/- mice were generated where CRaf protein expression was completely abolished by Huser et al., (2001) and independently by Mikula et al., (2001).

Huser et al., (2001) constructed a targeting vector which introduced the neoR gene into the endogenous craf gene, replacing approximately 6kb of DNA including exons 10 to 13 that encodes amino acids 338 to 472. This mutation resulted in the complete knockout of CRaf; no fragments of CRaf were detected in mice homozygous for this mutation using Western blot or CRaf kinase assays. Huser et al., (2001) demonstrated that the mutation on a mixed C57BL6 inbred genetic background was embryonic lethal, with embryos surviving to E9.5. Mutant embryos observed at this stage were abnormal: they were developmentally arrested, being approximately one third smaller than their wild-type litter mates. Such embryos were anaemic and staining with an antibody to platelet endothelial cell adhesion molecule-1 (PECAM-1) indicated embryos had abnormal vascular network formation. In the embryo head region, a reduction of large and small blood vessels was observed, with those present appearing disordered, and there were no capillary sprouts into the neuroectoderm. Additionally embryos lacked small and large blood vessels in the yolk sac. There was a significant reduction in the number and density of cells throughout the mutant embryos, but cell size appeared to be increased. These mice displayed an increase in apoptosis of embryonic tissues, observed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assays. However cell proliferation did not appear to be affected as assayed by the staining of embryo sections with Ki67, a marker for cells in S phase (Huser et al., 2001)
craf⁻/⁻ mice were also generated by Mikula et al (2001). Exon 3 of the craf gene in these mice was removed resulting in the complete loss of CRaf as demonstrated by western blot analysis. Again no CRaf fragments were detected and CRaf kinase activity in these cells was nil. Mikula et al (2001) established their knockout craf mutation primarily on the inbred 129Sv background, but also investigated the phenotype on a mixed 129Sv/C57BL6 background. Similar to the results of Huser et al (2001), the craf⁻/⁻ embryos were found to be growth retarded and died at midgestation with anomalies in the placenta and in the foetal liver. The placenta was smaller and poorly vascularised and the liver was hypocellular, although cells were larger in size. Liver cells were again apoptotic, with the proliferation of cells not affected. Furthermore fibroblasts derived from these embryos were more sensitive than wild-type cells to specific apoptotic stimuli. However, these embryos did not possess yolk sac vascularisation defects and survived to a later stage of development (up to E16.5) than the embryos on the C57BL6 background.

However when the craf⁻/⁻ mutation was backcrossed to the outbred MF-1 strain, craf⁻/⁻ embryos were observed from E12.5 up to 3 weeks of age (Kamata et al., 2004). All animals were small, morphologically abnormal, anaemic, and displayed a smaller, disordered placenta. Analysis of the liver demonstrated a reduction in the number of cells, with cells being greater in size. However there was no significant difference in levels of spontaneous apoptosis found by TUNEL analysis, which is not consistent with the apoptotic phenotype seen on the C57BL6 background (Huser et al., 2001). In addition craf⁻/⁻ MEFs on the outbred MF1 background showed a potential cell cycle defect, displaying a reduced ability to grow as monitored by assessing the growth rates of craf⁻/⁻ MEFs in comparison with wild-type cells (Mercer et al., 2005). The differences in phenotypes of the craf⁻/⁻ mice on the different genetic backgrounds suggests that the phenotype observed in craf⁻/⁻ mice is subject to genetic modifiers which are dependent upon the strain of mouse under observation, the nature of which are not known.
ERK activation in $craf^{-/-}$ cells and embryos on all genetic backgrounds was found to be normal, supporting other evidence that it is most likely BRaf that mediates activation of the ERK cascade. These results indicate that the essential function of CRaf is to counteract apoptosis, having unique effectors to those of the ERK cascade.

Mice were also generated with a $craf^{Y340F/Y341F}$ mutation, by a knockin strategy (CRaf$^{FF}$) (Huser et al., 2001). Studies in COS7 cells had demonstrated a requirement for the phosphorylation of Y340 and Y341 for activation of CRaf, because their mutation to phenylalanine blocked the activation of CRaf by oncogenic Ras and v-Src, or by ligand stimulation (Marais et al., 1995, Marais et al., 1997, Barnard et al., 1998). However, during the generation of these mice, it was identified that the phosphorylation of Y341 was critical in activating CRaf rather than the phosphorylation of Y340, which was not essential (Mason et al., 1999). When CRaf was immunoprecipitated from MEFs derived from embryos which were homozygous for the $craf^{Y340F/Y341F}$ mutation, the protein was shown to have no activity towards MEK in vitro (Huser et al., 2001). Even so mutant mice on both genetic backgrounds displayed a normal phenotype; they survived to adulthood, were normal in weight and were fertile. MEFs were isolated from E14.5 embryos resulting from $craf^{+/FF}$ intercrosses on both genetic backgrounds. Analysis of these MEFs did not show any defect in the cell cycle; there was no difference in the growth rates of the MEFs homozygous for the $craf^{Y340F/Y341F}$ mutation, and no difference in their ability to undergo DNA synthesis as measured by BrdU incorporation. These cells showed no evidence of spontaneous apoptosis under normal growth conditions, and no increase of apoptosis above wild-type cells when treated with apoptotic inducers. Like $craf^{-/-}$ mice, ERK activation was also not affected. It was concluded that the MEK kinase activity of CRaf is not required for normal mouse development. However the protein itself is necessary. Hence CRaf is required to control apoptosis and the cell cycle in a MEK/ERK independent manner.
1.12.3 BRaf

BRaf-knockout embryos die at midgestation, by E12.5. Embryos show overall growth retardation and increased apoptosis in endothelial tissues, which leads to vascular defects and death from vascular haemorrhage (Wojnowski et al., 1997). These mice also suffer from a range of other defects, including severe neuronal abnormalities (Wiese et al., 2001) which may arise as a consequence of a significant disruption to ERK activation in these cells (Wojnowski et al., 2000). This indicates that BRaf serves distinct essential functions in embryonic development. Such observations support the hypothesis that the BRaf isoform is the major MEK activator. An important point of note is that in both ARaf-deficient and CRaf-deficient mice, the activity of BRaf has been found to be up-regulated, suggesting that ARaf and CRaf isotypes do have some MEK kinase activity which is compensated for in their absence (Huser et al., 2001, Mikula et al., 2001, Mercer et al., 2002). Interestingly, braf/− MEFs have altered migratory characteristics. The knockout of BRaf induces a ~1.5-fold increase in cell migration concurrent with a reduction of actin stress fibres. Furthermore ROCKII expression and phosphorylation of S3 on ADF/cofilin was reduced, but could be restored by the expression of human BRAF, active MEK and over-expression of LIM kinase (LIMK). Therefore a role for BRaf in the regulation of cell motility by the proper assembly of actin stress fibers and contractility through a ROCKII/LIMK/cofilin signalling pathway has been proposed (Pritchard et al., 2004). Further insight into BRaf function has been provided by conditional BRaf knockout mice, in which exon 11 was deleted using a Cre/LoxP system. This deletion causes a shift in the open reading frame and completely abrogated expression of the protein. The production of these mice circumvented the early embryonic lethality observed in the conventional targeting of this gene. The conditional knockout of BRaf in neuronal precursors led to severe neurological defects including dysmyelination, defective oligodendrocyte differentiation, which may be the result of reduced ERK activation in the brain (Galabova-Kovacs et al., 2008). Additionally an investigation using conditional BRaf and CRaf knockouts (Galabova-Kovacs et al., 2006) showed that BRaf is required for placenta development, possibly by its regulation of VEGF expression, and was the major
activator of the ERK cascade in the placenta. However, CRaf appeared to be necessary for the activation of ERK in the embryo and for erythrocyte differentiation. This investigation contradicts the view that BRaf is the main activator of the ERK cascade in all cell types, as the embryos lacking BRaf did not have a significant reduction in phospho-ERK levels.

**1.12.4 CRaf kinase defective mice**

Given the strong evidence for MEK-independent functions of CRaf, kinase defective CRaf mice were generated in our laboratory. This project aims to characterise these CRaf kinase defective mice. These animals have a D486A mutation in exon 14 of the *craf* gene. Hereafter this mutation will be referred to as DA. The mutation is located in the DFG motif, which is located in the C-terminal lobe of the kinase domain. This motif is known to be important for ATP coordination. By interacting with other residues, the DFG motif is believed to provide a favourable structural arrangement for the catalytic loop to accommodate ATP binding. Substitution of an amino acid within this motif alters an essential triad of amino acids that is required for coordination of the ATP substrate. As such, kinase activity is abolished.

A key finding prior to this project was that the CRaf^{DA} protein was expressed at very low levels in the cell. Furthermore primary *craf^{DA/DA}* MEFs grew slower than wild type MEFs in culture. This would suggest there is either an increase in apoptosis or a defect in the cell cycle; possibly either a delay at one or more stages of the cycle, or an overall reduction in the rate. Both the lack of a functional kinase domain and the reduced expression of CRaf may play in role in the observed phenotypes.
1.13 Aims of the project

The aim of this thesis was to investigate the phenotypes observed in kinase defective CRaf mice and cells in order to further characterise the functions of CRaf. This was accomplished by:

- Investigating how CRaf expression is controlled by CRaf kinase activity. The stage at which regulation of the protein occurs was investigated i.e. transcription, translation, or protein stability.
- Elucidating the mechanism for this regulation.
- Characterising the growth defects of C-Raf kinase deficient and knockout cells. The cell cycle of such cells was investigated in order to discover at what stage a possible defect may exist and to examine the signalling pathways involved in this.
2. MATERIALS AND METHODS

The H$_2$O used in all methods (including those requiring RNase-free H$_2$O) was always MilliQ water, dispensed in sterile containers.

2.1 Molecular Biology

All chemicals and reagents were supplied by Fisher Scientific or Sigma unless otherwise stated. All restriction endonucleases were supplied by New England Biolabs.

2.1.1 Plasmids

The plasmids used are detailed below and in Figure 2.1. All apart from pBluescriptSK' HA-Ubiquitin were gifts from Prof. Richard Marais (Cancer Research UK Centre for Cell and Molecular Biology, The Institute of Cancer Research, London, UK).

- pEF HA-CRAF$^{WT}$
- pEF myc-CRAF$^{WT}$
- pEF myc-CRAF$^{K375M}$
- pEF myc-CRAF$^{S621A}$
- pEF myc-CRAF$^{T491A/S494A}$
- pEF myc-CRAF$^{D486A}$
- pEF myc-CRAF$^{K375M/S621D}$
- pBluescriptSK' HA-Ubiquitin – A gift from Prof. Dirk Bohmann (Dept. of Biomedical Genetics, University of Rochester, New York, USA)
Figure 2.1 **Plasmid maps.**

(A) pEF myc-CRAF\(^{WT}\). The myc tag is substituted for a HA tag in the case of the pEF HA-CRAF\(^{WT}\) plasmid. Point mutants of CRAF were produced by site-directed mutagenesis. (B) pBluescriptSK\(^{-}\) HA-Ubiquitin.
2.1.2 Transformation of competent DH5α

50µl of DH5α library competent cells (Invitrogen) were thawed on ice and transferred to a polypropylene tube, 2µl of the plasmid DNA was added and gently mixed before the tube was incubated on ice for 30 min. The bacteria were then heat shocked at 42°C for 45 sec and then placed on ice for 5 min. 200µl Luria Bertani (LB) medium (1% [w/v] bactotryptone [Melford], 0.5% [w/v] bactoyeast extract [Melford], 17mM NaCl) was added to the tube and placed at 37°C in a 225 rpm shaking incubator for 1 h. 40µl and 100µl of the transformation reaction were spread onto LB agar plates (1.5% [w/v] agar bacteriological [Oxoid], 1% [w/v] bactotryptone, 0.5% [w/v] bactoyeast extract, 17mM NaCl) containing 50µg/ml ampicillin. The plate was inverted and incubated at 37°C overnight to allow the formation of discrete bacterial colonies.

2.1.3 Mini-preparation of plasmid DNA from bacteria

Bacterial colonies were picked for screening using a sterile yellow tip into 5ml LB, containing 50µg/ml ampicillin and grown at 37°C in a 225 rpm shaking incubator overnight. 1ml of the culture was then transferred to an eppendorf tube and centrifuged at 13000 rpm for 1 min. The supernatant was removed and the bacterial pellet was re-suspended by vortexing in 100µl ice cold resuspension buffer (P1: 50mM Tris-HCl [pH 8.0], 10mM EDTA, 100µg/ml ribonuclease [RNase A]). 200µl of lysis buffer (P2: 200mM NaOH, 1% [w/v] sodium dodecyl sulphate [SDS]) was added and the tube inverted several times to mix. 150µl of neutralisation buffer (P3: 3M KOAc, [pH5.5]) was added and the tube inverted five times to neutralise. The tube was centrifuged at 13000 rpm for 10 min. The supernatant was transferred to a fresh tube. To precipitate the DNA, 400µl ice-cold 100% [v/v] isopropanol was added and the tube was centrifuged at 13000 rpm for 10 min. The supernatant was discarded and the DNA pellet washed with 70% (v/v) ethanol and air-dried. The pellet was then resuspended in 50µl TE (10mM Tris [pH 8.0], 1mM EDTA [pH 8.0]) and stored at 4°C.
2.1.4 Maxi scale preparation of plasmid DNA from bacteria

1ml of bacterial culture was taken from a 5ml starter culture and inoculated into a sterile flask containing 400ml of LB media and 50µg/ml ampicillin. The bacteria was grown overnight at 37°C in a 225 rpm shaking incubator. The bacteria were then harvested and the plasmid DNA extracted using an EndoFree Plasmid Maxi Kit (Qiagen) according to the manufacturer’s manual.

2.1.5 Caesium chloride preparation of plasmid DNA from bacteria

1ml of bacterial culture was taken from a 5ml starter culture and inoculated into a sterile flask containing 400ml of LB media and 50µg/ml ampicillin. The flask was placed at 37°C in a 225 rpm shaking incubator overnight. The bacteria was harvested by centrifugation at 6000 rpm for 10 min at 4°C (Sorvall Evolution RC centrifuge using SLA-1500 rota). The pellet was resuspended in 10ml resuspension buffer. 20ml of lysis buffer was then added to lyse the cells and the tube was gently inverted several times. 15ml neutralisation buffer was added and the tube swirled until a white precipitate formed. The tube was centrifuged at 9000 rpm for 15 min at 4°C and the supernatant was filtered (Miracloth, Calbiochem) into a clean tube. 50ml 100% [v/v] ice cold isopropanol was added to precipitate the DNA and the tube was centrifuged at 9000 rpm for 15 min at 4°C. The pellet was air dried and then resuspended in 6ml TE. This was transferred into a polypropylene tube containing 6g CsCl (Invitrogen) and gently mixed until the CsCl had dissolved. 275µl of 10mg/ml ethidium bromide was then added to the tube. The tube was centrifuged at 4000 rpm for 5 min at 4°C. The resulting clear solution was transferred into two quick seal ultra centrifuge tubes (Beckman). The tubes were heat sealed and centrifuged at 100,000 rpm at 20°C for 16h (Beckman Optima™ Max-E Ultracentrifuge). The lower plasmid band was extracted from the tube using a syringe and retained. The DNA was extracted by ten or more washes with an equal volume of water-saturated isobutanol; washes included vigorous shaking and removal of the upper aqueous layer. Once the upper layer became clear, it was discarded and the lower layer retained. To precipitate the DNA, two volumes of water plus two volumes of
100% (v/v) ethanol were added. The tube was centrifuged at 11000 rpm for 15 min at 4°C (Sorvall Evolution RC; SS-34 rota) and the supernatant discarded. The pellet was washed in 70% (v/v) ethanol and the tube was centrifuged at 11000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet allowed to air dry before being resuspended in 250µl TE. The DNA was quantitated using a spectrophotometer (BioPhotometer, Eppendorf) to measure optical density at 260nm.

2.1.6 Diagnostic restriction digest of plasmid DNA

For analytical purposes, 5µl of plasmid DNA obtained from a mini-preparation (section 2.1.3) was added to 1 unit of the appropriate enzyme and 1 unit of the appropriate buffer (supplied by the manufacturer at 10x concentration) in a final volume of 15µl. The reaction was then incubated at the optimal temperature recommended by the manufacturer for 2 h before analysis on a 2% agarose gel.

2.1.7 Lysis of mouse tissue samples to extract DNA

An ear sample, removed from adult mice for identification, was used for genotyping. Likewise, the tail was removed from embryos for the same purpose. These samples were lysed by addition of 70µl of 0.05M NaOH and heated to 95°C for 15 min. The samples were neutralised with 7µl of 1M Tris (pH 7.5) and then used for PCR.

2.1.8 Lysis of cells to extract genomic DNA

Media was aspirated from confluent 10cm plates, and cells were washed in 1x PBS (137mM NaCl, 8.1mM Na₂HPO₄, 2.7mM KCl, 1.5mM KH₂PO₄). 500µl DNA lysis buffer (50mM Tris-HCl [pH 7.6], 1mM EDTA, 100mM NaCl, 0.2% [w/v] SDS, fresh 100µg/ml proteinase K) was added to plates and cells were scraped and collected into an eppendorf tube. Samples were incubated for 2h at 60°C. 1ml 100% (v/v) ethanol was added and mixed with the sample until the DNA precipitated. The tube was centrifuged at 13000 rpm for 1min at 4°C, the supernatant was discarded and 1ml of 70% (v/v) ethanol was added to wash the DNA pellet.
Ethanol was removed and DNA was allowed to air dry for 10min. DNA was then dissolved in 300µl of water at 60°C for 2h.

2.1.9 Polymerase Chain Reaction (PCR)

PCR was preformed in a final volume of 20µl using: 16µl ReddyMix PCR master mix (ABgene); 1µl of each of the relevant primers (Table 2.1.1) at a final concentration of 1 pmol/µl; and 2µl of sample DNA. Each PCR reaction was overlaid with mineral oil and the tubes were placed in a G-Storm™ PCR machine. The PCR conditions used are indicated below:

\[
\begin{align*}
94°C & \text{ for } 5 \text{ min} \\
94°C & \text{ for } 1 \text{ min,} \\
X°C & \text{ for } 1 \text{ min (Table 2.1.1)} \\
72°C & \text{ for } 1 \text{ min} \\
72°C & \text{ for } 5 \text{ min}
\end{align*}
\]

\[\text{35 x}\]

Table 2.1.1 Details of primers used for PCR and their corresponding optimal annealing temperature.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5' to 3'</th>
<th>Annealing temperature (X°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCP 166</td>
<td>CTCCTGGAATTAGCATCTTTAGAACC</td>
<td>60</td>
</tr>
<tr>
<td>OCP 167</td>
<td>GGTATTACCACCACTGGTC</td>
<td>60</td>
</tr>
<tr>
<td>OCP 173</td>
<td>ACAGTGCCAGAAACCATGT</td>
<td>57</td>
</tr>
<tr>
<td>OCP 59</td>
<td>TTACATGTCCACAAGACTCTTAC</td>
<td>57</td>
</tr>
<tr>
<td>OCP 2</td>
<td>CGTGCAAGCATGATGTCCTC</td>
<td>57</td>
</tr>
<tr>
<td>OCP 23</td>
<td>GGAGATGTGAGTAAAGATC</td>
<td>57</td>
</tr>
</tbody>
</table>
2.1.10 Agarose gel electrophoresis

Pure agarose (Roche) was heated by microwaving in 1x TAE buffer (40mM Tris, 1mM EDTA [pH 8.0]) until dissolved to produce a 2% (w/v) agarose solution. Ethidium bromide (10mg/ml) was added to a final concentration of 0.5µg/ml. The agarose was poured into a gel mould, containing a comb and left to set. The comb was removed and the gel submerged in 1x TAE buffer in a horizontal electrophoresis tank. DNA samples were loaded into the wells of the gel. PCR products did not require loading dye to be added as ReddyMix PCR master mix contains loading buffer. Other DNA samples required the addition of loading dye (0.5% (w/v) Orange G, 30% (v/v) glycerol). The DNA was separated by electrophoresis at 100 volts until the required separation was achieved. Samples were compared to a 1kb plus DNA ladder (Invitrogen) for the determination of their size. The DNA was visualised and recorded on a UV transilluminator and camera (Bio-Rad gel documentation system).

2.1.11 Lysis of cells to extract total RNA

Media was removed from a confluent 10cm dish and the cells were washed in 1x PBS. RNA was harvested from cells using the Qiagen RNeasy kit as described in the manufacturer’s manual. A DNA-free kit (Ambion) was then used as per the manufacturer’s protocol in order to remove DNA contamination. The rigorous DNase-treatment method was followed and repeated to ensure complete DNA removal. RNA samples were then quantified using a spectrophotometer and the OD$_{260}/_{280}$ value was ensured to be >1.6.

2.1.12 Production of cDNA from total RNA

Complementary DNA (cDNA) was prepared in a reaction mixture containing 1µg RNA, 1µl of 50µM oligodT primer (Promega), and water up to 13µl. This was heated to 65°C for 5 min, and then cooled on ice, allowing the primers to anneal to the RNA. The following components (Promega) were then added: 1µl RNaseIN, 5µl 5x reaction buffer, 1µl of 10mM dNTPs, 3µl reverse transcriptase (SuperscriptIII R [Invitrogen]), 2.5µl of 25mM MgCl2. The
reaction was then heated to 42°C for 1 hour to allow the synthesis of cDNA. The reverse transcriptase was then inactivated by heating to 95°C for 5 min.

2.1.13 Quantitative Real Time PCR (qPCR)

Quantitative RT-PCR was performed using a BioRad MiniOpticon Real Time PCR system in a final reaction volume of 25µl. All reagents and reaction mixes were kept on ice. All samples were performed in triplicate. For one sample, the master mix contained: 12.5µl SYBR green (Bio-Rad), 300nm of each primer (Table 2.1.2), water to a total volume of 15µl.

The master mix was divided into aliquots containing enough mix for 3.5 reactions. To each aliquot was then added 10µl of the appropriate cDNA (1ng/µl) sample, RNA (1ng/µl) control sample, or water. 25µl of each reaction was added to a PCR plate in triplicate. The reaction conditions varied according to the primers (see Table 2.1.2 for annealing temperature) but the following template was followed:

Step 1 Incubate 95°C for 5 min
Step 2 Incubate 95°C for 30 sec
Step 3 Incubate X°C for 30 sec
Step 4 Incubate at 72°C for 30 sec
Step 5 PLATE READ
Step 6 Go to step 2, 39 more times.
Step 7 Melting curve from 55°C - 95°C, read every 1.0°C, hold for 1 sec.

END
Table 2.1.2 Details of primers used for quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence 5’ to 3’</th>
<th>Annealing temperature (X°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Craf</td>
<td>OCP 183</td>
<td>AAT ACT ATC CGG GTT TTC TTG CC 60°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OCP 184</td>
<td>GCG TGC TTT CTT ACC TTT GTG T</td>
<td></td>
</tr>
<tr>
<td>p21CIP1</td>
<td>OCP 191</td>
<td>GTA CTT CCT CTG CCC TGC TG 63°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OCP 192</td>
<td>TCT GCG CTT GGA GTG ATA GA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>OCP 174</td>
<td>AGG TCG GTG TGA ACG GAT TTG 61°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OCP 175</td>
<td>TGT AGA CCA TGT AGT TGA GGT CA</td>
<td></td>
</tr>
</tbody>
</table>

Optimisation of qPCR conditions was required before the actual assays were performed. A temperature gradient was conducted using the primer pairs detailed in Table 2.1.2 to determine the optimum annealing temperature for the amplification of craf (Figure 2.2) and p21CIP1 (Figure 2.3). cDNA from wild-type MEFs was used in this temperature gradient which ranged from 55°C to 68°C. The optimum annealing temperature is the one that gives the lowest C_T value and hence allows the reaction to progress most efficiently. The graphs of the average C_T against annealing temperature for the amplification of craf (Figure 2.2B) showed that temperatures in the range of 56°C to 63°C produced similarly low C_T values. However 60.2°C gave the lowest C_T value, and therefore was selected as the optimal annealing temperature for the craf qPCR assay. The optimal annealing temperature for the p21 specific primers was identified as 63.1°C by an identical strategy (Figure 2.3B). The amplification curves (Figures 2.2C and 2.3C) provide readout of the efficiency of each qPCR reaction under their specific conditions. The specificity of the reaction products can also be monitored in the melt-curve data graph. A single peak corresponds to a single PCR product produced in the reaction and, as can be seen in Figure 2.2C and 2.3C, the melt-curves show one peak corresponding to the specific amplification of craf and p21 respectively.
Figure 2.2 *craf* qPCR temperature gradient to determine optimum annealing temperature.

(A) Microtitre plate showing loading of samples in triplicate (indicated by colour), subjected to different annealing temperatures (55 C-68 C, rows A-H). Average $C_T$ values obtained at each temperature are shown. A water control is shown in column 2. (B) Graph of the average $C_T$ against annealing temperature. The annealing temperature of 60.2 C gave the lowest $C_T$ value, and therefore was selected as the optimal annealing temperature for the qPCR assay. (C) Data graphs of the melt curve showing one peak, corresponding to a single PCR product. The amplification curves for each sample are shown with the threshold line indicated, positioned at the point where the curves begin to linearise.
Figure 2.3. *p21* qPCR temperature gradient to determine optimum annealing temperature.

(A) Microtitre plate showing loading of samples in triplicate (indicated by colour), subjected to different annealing temperatures (55 C-68 C, rows A-H). Average C_T values obtained at each temperature are shown. (B) Graph of the average C_T against annealing temperature. The annealing temperature of 63.1 °C temperature gave the lowest C_T value, and therefore was selected as the optimal annealing temperature for the qPCR assay. (C) Data graphs of the melt curve showing one peak, corresponding to a single PCR product. The amplification curves for each sample are shown with the threshold line indicated positioned at the point where the curves begin to linearise.

<table>
<thead>
<tr>
<th>Sample lanes</th>
<th>Temp</th>
<th>Average C_T</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1-A3</td>
<td>68.0 °C</td>
<td>N/A</td>
</tr>
<tr>
<td>B1-B3</td>
<td>67.1 °C</td>
<td>29.45</td>
</tr>
<tr>
<td>C1-C3</td>
<td>65.4 °C</td>
<td>21.93</td>
</tr>
<tr>
<td>D1-D3</td>
<td>63.1 °C</td>
<td>20.42</td>
</tr>
<tr>
<td>E1-E3</td>
<td>60.2 °C</td>
<td>20.46</td>
</tr>
<tr>
<td>F1-F3</td>
<td>57.7 °C</td>
<td>20.83</td>
</tr>
<tr>
<td>G1-G3</td>
<td>56.0 °C</td>
<td>21.25</td>
</tr>
<tr>
<td>H1-H3</td>
<td>55.0 °C</td>
<td>21.42</td>
</tr>
</tbody>
</table>
The second optimisation step involved running serial dilutions of the template cDNA (again cDNA from wild-type MEFs was used) at the established optimal temperatures. This allows standard curves to be constructed which can be used to determine the efficiency of the \textit{craf} and \textit{p21} primer pairs (Figures 2.4 and 2.5). The experiments were performed with a series of eight sequential dilutions of mRNA in triplicate. Standard curves were constructed by plotting the log of the dilution factor of the template against the C\textsubscript{T} value obtained during amplification at each dilution to produce a linear regression line. The equation of this line and the coefficient of determination (R\textsuperscript{2}) were used to determine whether the assays were optimised. The R\textsuperscript{2} value should be >0.98, indicating the experimental data fits the regression line well, that there is not too much variability between the triplicate samples and that the efficiency of the amplification is the same for different starting template copy numbers. The amplification efficiencies of the reactions were calculated from the equations of the lines, using the formula: $E = 10^{-1/\text{gradient}}$. The value obtained should be between 90-105%. A value of 100% would indicate that the PCR product exactly doubles during each cycle of exponential amplification. The R\textsuperscript{2} value for the amplification of \textit{craf} was 0.98. However, the calculated amplification efficiency was 84\% (Figure 2.4). Therefore the amplification of \textit{craf} is not perfectly exponential. The re-design of the primers may enable a higher amplification efficiency to be achieved, however because of time limitations this was not possible. The solution was to use the Pfaffl method (described in Chapter 3) to calculate expression levels of mRNA, as this takes into account the amplification efficiency of the primers. The R\textsuperscript{2} value for the amplification of \textit{p21} was 0.99 and the calculated amplification efficiency was 94\% (Figure 2.5). These values fall within the accepted limits to assume exponential amplification of \textit{p21}, hence the livack method (described in Chapter 5) was used to calculate expression levels of mRNA.

In order to compare the expression levels of a specific mRNA between two samples, the total levels of transcript must be equalised. The accuracy in quantitating the cDNA using a spectrophotometer and equalising the samples' concentrations is not sufficient for qPCR.
Figure 2.4 *craf* qPCR concentration gradient to determine efficiency of primers.

(A) Microtitre plate showing loading of samples in triplicate (indicated by colour), at different dilution factors (rows A-H). Average C<sub>T</sub> values obtained at each dilution are shown. (B) Data graphs of the melt curve showing one peak, corresponding to a single PCR product. The amplification curves for each sample are shown with the threshold line indicated, positioned at the point where the curves begin to linearise. (C) *craf* standard curve, with the average C<sub>T</sub> values plotted against the log of the dilution factors. The equation for the regression line and R<sup>2</sup> value of 0.98 are shown. The calculated amplification efficiency was 84%.

<table>
<thead>
<tr>
<th>Sample lanes</th>
<th>Dilution factor</th>
<th>Average C&lt;sub&gt;T&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1-A3</td>
<td>1</td>
<td>26.36</td>
</tr>
<tr>
<td>B1-B3</td>
<td>0.5</td>
<td>26.57</td>
</tr>
<tr>
<td>C1-C3</td>
<td>0.25</td>
<td>28.85</td>
</tr>
<tr>
<td>D1-D3</td>
<td>0.125</td>
<td>29.97</td>
</tr>
<tr>
<td>E1-E3</td>
<td>0.0625</td>
<td>30.77</td>
</tr>
<tr>
<td>F1-F3</td>
<td>0.03125</td>
<td>31.93</td>
</tr>
<tr>
<td>G1-G3</td>
<td>0.015625</td>
<td>32.90</td>
</tr>
<tr>
<td>H1-H3</td>
<td>0.007813</td>
<td>34.01</td>
</tr>
</tbody>
</table>
Figure 2.5. *p21* qPCR concentration gradient to determine efficiency of primers.

(A) Microtitre plate showing loading of samples in triplicate (indicated by colour), at different dilution factors (rows A-H). Column 5 is a water control, column 6 is a RNA control. Average C\(_T\) values obtained at each dilution are shown. (B) Data graphs of the melt curve showing one peak, corresponding to a single PCR product. The amplification curves for each sample are shown with the threshold line indicated positioned at the point where the curves begin to linearise. (C) *p21* standard curve, with the average C\(_T\) values plotted against the log of the dilution factors. The equation for the regression line and R\(^2\) value of 0.99 are shown. The calculated amplification efficiency was 94%.

<table>
<thead>
<tr>
<th>Sample lanes</th>
<th>Dilution factor</th>
<th>Average C(_T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1-A3</td>
<td>1</td>
<td>20.35</td>
</tr>
<tr>
<td>B1-B3</td>
<td>0.5</td>
<td>21.51</td>
</tr>
<tr>
<td>C1-C3</td>
<td>0.25</td>
<td>N/A</td>
</tr>
<tr>
<td>D1-D3</td>
<td>0.125</td>
<td>24.29</td>
</tr>
<tr>
<td>E1-E3</td>
<td>0.0625</td>
<td>24.82</td>
</tr>
<tr>
<td>F1-F3</td>
<td>0.03125</td>
<td>25.68</td>
</tr>
<tr>
<td>G1-G3</td>
<td>0.015625</td>
<td>26.74</td>
</tr>
<tr>
<td>H1-H3</td>
<td>0.007813</td>
<td>27.80</td>
</tr>
</tbody>
</table>
Therefore it is necessary to compare the amount of mRNA by using a reference gene which has equal expression in all samples. The expression of the gene of interest (the target gene) can then be normalised against the expression of the reference gene, which in this case was *GAPDH*. The *GAPDH* primers used were OCP 174 and OCP 175. These had previously been optimised for qPCR by Dr E. Debrand (Department of Biochemistry, University of Leicester). The efficiency of the *GAPDH* primers was found to be 93% and the $R^2$ value was 0.99 (Figure 2.6).

2.2 Animal Cell Culture

All reagents were from Invitrogen unless otherwise stated and all procedures were performed in a tissue culture hood unless otherwise stated.

2.2.1 Isolation of Mouse Embryonic Fibroblasts (MEFs)

Embryos at 13.5dpc were obtained and stored in ice cold PBS until all were processed. Each embryo was transferred to a clean Petri dish and the tail was removed for genotyping. Livers were dissected out and discarded. The remainder of the embryos were sliced and the tissue was collected in 5ml PBS. The PBS was aspirated and 2ml of 0.25% (w/v) trypsin-EDTA was added. The tissue was left to incubate at 4°C for 6 hours to allow dissociation of the tissues, and then was incubated at 37°C for 30 min. The tissue was pelleted by centrifugation at 1200 rpm and the trypsin was aspirated. 5ml of complete media (DMEM with L-glutamine and 4500 mg/l D-glucose, supplemented with 10% [v/v] foetal bovine serum [Sigma], 100 Units/ml penicillin, 100µg/ml streptomycin) was then added to inhibit the trypsin and cells were disaggregated by pipetting. Cells were then transferred to plates, prepared with an appropriate volume of complete media. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO$_2$. The media was changed every two days until cells reached confluence.
Figure 2.6 **GAPDH standard curve.** (Data obtained from Dr. E. Debrand)

(A) Dilutions used to calculate the *GAPDH* standard curve. The average Cₜ values for the various dilutions of template plotted to generate the standard curve are shown. (B) *GAPDH* standard curve with the average Cₜ plotted against the log of the dilution factor. The equation for the regression line and the R² value are shown. The calculated amplification efficiency was 93%.

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>Average Cₜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.36</td>
</tr>
<tr>
<td>0.2</td>
<td>19.85</td>
</tr>
<tr>
<td>0.1</td>
<td>21.19</td>
</tr>
<tr>
<td>0.05</td>
<td>21.77</td>
</tr>
</tbody>
</table>

\[ y = -3.5012x + 17.417 \]

\[ R^2 = 0.9898 \]
2.2.2 Trypsinising Cells

Media was aspirated, and cells were washed in 1x PBS. Sufficient 0.05\% (w/v) trypsin-EDTA was added to cover cells. Plates were then incubated for 5 min at 37\(^\circ\)C. Complete media was added to inactivate trypsin and cells were disaggregated by pipetting. If the cells required passaging, an appropriate number of cells in suspension were then added to fresh plates, prepared with an appropriate volume of complete media. Plates were incubated at 37\(^\circ\)C in a humidified atmosphere containing 5\% CO\(_2\).

2.2.3 Freezing and storage of cells

Cells were trypsinised and transferred to 12ml conical bottomed tubes (Falcon). The cells were pelleted by centrifugation at 1200 rpm for 5 min at 4\(^\circ\)C. Cells were resuspended in freezing media (10\% DMSO in foetal bovine serum) and 1ml transferred to labelled cryo-vials (Nunc). The cells were frozen at -80\(^\circ\)C for 48 hours, then transferred to liquid nitrogen for long term storage.

2.2.4 Thawing cells

Cells were thawed quickly, for less than 1 min, at 37\(^\circ\)C and then transferred to 5ml media. Cells were pelleted by centrifugation at 1200 rpm and then re-suspended in media. Cells were then transferred to fresh plates, prepared with an appropriate volume of complete media, which were then incubated at 37\(^\circ\)C.

2.2.5 Synchronising MEFs by serum starvation

Media was aspirated, and cells were washed twice in 1x PBS. Media, lacking foetal bovine serum, was then added to cells. Plates were then incubated for 24 hours at 37\(^\circ\)C. Such treatment synchronises cells in G0. In order to create populations of cells at different time-points throughout the cell cycle, complete media was later added back to such cells, which were later harvested after the appropriate incubation time.
2.2.6 Transfections of MEFs using Nucleofector technology

Immortalised craf\(^{-}\) or craf\(^{+/+}\) MEFs, or CHIP\(^{-}\) or CHIP\(^{+/+}\) immortalised lung fibroblasts were seeded on 15cm plates 24 hours prior to the transfection being performed so that they were ~50% confluent on the day of the experiment. The media was replaced 3h prior to the transfection, which was then performed using a Nucleofector according to the manufacturer’s instructions (Amaxa Biosystems, Germany). For immortalised craf\(^{-}\) or craf\(^{+/+}\) MEFs, the nucleofector programme used was T20 with solution MEF1. For CHIP\(^{-}\) or CHIP\(^{+/+}\) immortalised lung fibroblasts, the nucleofector programme used was A23 with solution MEF1. The MEFs were co-transfected with 5µg of test plasmid and 2µg of a vector expressing GFP, which was supplied with the kit, to allow an assessment of the percentage transfection efficiency by fluorescent microscopy. Transfected cells were then seeded onto either one 10cm plate or divided between 6cm plates for analysis over a time course. Plates were incubated at 37° C in a humidified atmosphere containing 5% CO\(_2\). Protein lysates were harvested between 24 and 72 h post transfection.

2.2.7 Addition of proteasome inhibitors to MEFs

MG132 (Calbiochem) dissolved in DMSO at a concentration of 10mM was added to cells at a final concentration of 30µM. Lactacystin (Calbiochem) was dissolved in DMSO to obtain a concentration of 25mM. It was added to cells at a final concentration of 0.5µM. Epoxomicin (Merck Biosciences), dissolved in DMSO at a concentration of 10mM was added to cells at a final concentration of 0.5µM. Treated cells were incubated at 37° C for 6h before protein lysates were harvested for analysis by western blotting.

2.2.8 Addition of A-769662 to MEFs

Immortalised craf\(^{-}\) cells were nucleofected and 24 h post nucleofection the media was changed and A-769662 (a gift from Professor Grahame Hardie, Division of Molecular Physiology, College of Life Sciences, University of Dundee, Dundee DD1 5EH), dissolved in DMSO at a concentration of 30mM, was added to cells at final concentrations of 30µM,
100µM or 300µM. Treated cells were incubated at 37°C for 1 h before protein lysates were harvested.

### 2.2.9 Addition of forskolin and IBMX to MEFs

Immortalised craf−/− cells were nucleofected and 24 h post nucleofection the media was changed and forskolin (a gift from Prof. Richard Marais), dissolved in DMSO at a concentration of 40mM, was added to cells at a final concentration of 25µM. This was added in conjunction with IBMX (also a gift from Prof. Richard Marais), dissolved in DMSO at a concentration of 100mM, and added to cells at a final concentration of 500µM. Treated cells were incubated at 37°C for 20 min before protein lysates were harvested.

### 2.2.10 Addition of sorafenib to MEFs

Immortalised craf−/− cells were nucleofected and 24 h post nucleofection the media was changed and sorafenib (a gift from Prof. Caroline Springer, ICR, Sutton, UK), dissolved in DMSO at a concentration of 10mM, was added to cells at a final concentration of 20µM. Treated cells were incubated at 37°C for 2 h before harvesting protein lysates.

### 2.2.11 siRNA knockdown of CHIP and BAG

The media used for this experiment lacked penicillin and streptomycin. Cells were seeded at an appropriate density so that cells reached 80% confluency the day of the experiment. 3-4 hours prior to the transfection the media was replaced. The siRNA 20µM stock solution (Dharmacon) (see Table 2.2.3) was diluted with Opti-MEM (Invitrogen) to a concentration of 0.4µM. This was mixed with a solution of 2% (v/v) lipofectamine 2000 (Invitrogen) in Opti-MEM to produce a siRNA solution of 0.2µM. This was incubated at room temperature for 30 min to generate transfection complexes. An appropriate amount of the siRNA solution was added drop-wise to media in order to incubate cells in 0 – 100nM siRNA. As controls, cells were mock treated; this involved the omission of siRNA to the Opti-MEM. Cells were then
cultured overnight before the media was replaced. Cell lysates were harvested between 24 h and 72 h post transfection.

### Table 2.2.3 siRNA oligos

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence 5’-3’</th>
<th>Optimal concentration</th>
<th>Time post transfect to harvest lysates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bag-1 pool</td>
<td>AAG AAG AGG UUG AGU UAA AUU GGU CCU ACC AGA ACA AUU UUU CUA ACC ACC UGC AAG AAU UUU CGA GUC AUG UUA AUU GGU GUU</td>
<td>75nM</td>
<td>72h</td>
</tr>
<tr>
<td>CHIP</td>
<td>GGG AUG AUA UUC CUA GUG CUU</td>
<td>100nM</td>
<td>24h</td>
</tr>
<tr>
<td>Control non-targeting siRNA pool 2</td>
<td>AUG AAC GUG AAU UGC UCA A UAA GGC UAU GAA GAG AUA C AUG UAU UGG CCU GUA UUA G UGG UUU ACA UGU CGA CUA A</td>
<td>Used under same conditions as the experimental siRNA</td>
<td></td>
</tr>
<tr>
<td>siGLO RISC Free siRNA</td>
<td>(Proprietary sequence)</td>
<td>100nM</td>
<td>N/A</td>
</tr>
</tbody>
</table>

### 2.3 Protein Analysis

All chemicals and reagents were supplied by Fisher Scientific or Sigma unless otherwise stated.

#### 2.3.1 Preparation of soluble and insoluble protein lysates from MEFs

Media was aspirated from plates, which were immediately put on ice. Cells were then washed in ice cold 1x PBS. Cells were lysed by the addition of Gold lysis buffer (GLB) (1% [v/v] Triton X-100, 0.5% NP-40, 50mM Tris [pH7.5], 150mM NaCl, 5mM EDTA, 5mM EGTA, 10mM NaF, 1mM sodium orthovanadate, corrected to pH 7.0 at room temperature. Immediately prior to use 10µg/ml aprotinin, 10µg/ml leupeptin and 1mM AEBSF were
added). The cells were scraped from the plate and collected in an eppendorf tube. Samples were placed on ice for 15 min with occasional 5 sec vortexing. Samples were centrifuged at 13000 rpm at 4°C for 15 min to pellet the nuclei and insoluble debris. Supernatants were removed into a separate tube on ice and were quantitated using Bradford’s reagent (Pierce). If the insoluble fraction was required, the pellet was solubilised by the addition of 2% (w/v) SDS solution. SDS-PAGE reducing loading buffer (182mM Tris [pH6.8], 30% [v/v] glycerol, 6% [w/v] SDS, 0.12% [w/v] Bromophenol blue, 4.57% [w/v] DTT) was added to protein samples, which were then stored at -20°C.

2.3.2 Preparation of protein lysates from embryos
Embryos were snap frozen in liquid nitrogen and stored at -80°C. Working on dry ice, the embryos were sliced as small as possible using a scalpel and forceps. 500µl GLB was added to the tissue to lyse cells. The tissue was further homogenised with a rotor-stator homogeniser. Samples were stored on ice for 15 min with occasional 5 sec vortexing. Samples were centrifuged at 13000 rpm at 4°C for 15 min to pellet the nuclei and insoluble debris. Soluble protein lysates were collected and quantitated using Bradford’s reagent. SDS-PAGE reducing loading buffer was added to protein lysates, which were then stored at -20°C.

2.3.3 Quantitation of protein lysates
1µl of the protein lysate sample collected in GLB was mixed with 1ml of Bradford’s reagent (Pierce) in a cuvette and the optical density at 595nm was analysed using a spectrophotometer (BioPhotometer, Eppendorf). The concentration of the sample was determined by comparing the optical density of the sample against a standard curve. The standard curve was produced by measuring the optical density at 595nm of a series of BSA solutions of known concentrations of 0, 2, 4, 8, 16 µg/µl.
2.3.4 Immunoprecipitation of proteins

Media was aspirated from plates, and cells were washed in ice cold 1x PBS. Cells were lysed by the addition of IP lysis buffer (20mM Tris [pH7.5], 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% [v/v] Triton X-100, 2.5mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM sodium orthovanadate, 1µg/ml leupeptin, 1mM ABESF), scraped and collected in an eppendorf tube. Samples were stored on ice for 15 min with occasional 5 sec vortexing. Samples were centrifuged at 13000 rpm at 4°C for 15 min to pellet the nuclei and insoluble debris. The supernatant was collected for soluble protein lysates, which were quantitated using Bradford’s reagent (Pierce). If the insoluble fraction was required, the pellet was solubilised by the addition of 2% SDS solution before adding RIPA buffer lacking SDS (50mM Tris.HCl [pH8], 150mM NaCl, 0.5% [w/v] sodium deoxycholate, 1% [v/v] NP40. Immediately prior to use 20ml was taken and 10µl leupeptin [25mg/ml], 10µl aprotinin [25mg/ml], 40µl 0.5M ABESF, 10µl 1M Benzamidine, 200µl 100mM sodium orthovanadate, 2ml 500mM sodium fluoride, 20µl β-mercaptoethanol, and 200µl1M β-glycerolphospbate were added). The protein lysate samples were then quantitated and to equal amounts of protein (between 100 and 400µg) primary antibodies were added at the appropriate dilution (Table 2.3.1) in a total volume of 100µl. Samples were incubated overnight with gentle rocking at 4°C. The next day Sepherose A beads were prepared. Stock beads in PBS were centrifuged and the PBS removed. The beads were washed with IP lysis buffer and finally re-suspended in IP lysis buffer to create a 50/50 bead slurry. 40µl of the bead slurry was then added to the cell lysates and primary antibody and incubated at 4°C for 1-3 h with gentle rocking. Bead complexes were then washed three times in IP lysis buffer before the addition of SDS-PAGE loading buffer.
Table 2.3.1. Details of antibodies used for immunoprecipitation

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Species</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Raf</td>
<td>1:20</td>
<td>Rabbit</td>
<td>Santa Cruz sc-227</td>
</tr>
<tr>
<td>9B11 (Myc tag)</td>
<td>1:200</td>
<td>Mouse</td>
<td>Cell Signaling 2276</td>
</tr>
<tr>
<td>HA</td>
<td>1:20</td>
<td>Rabbit</td>
<td>Santa Cruz sc-805</td>
</tr>
</tbody>
</table>

2.3.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were cast using a Mini-protean III cell (BioRad). The appropriate percentage separating gel was made (Table 2.3.2), depending on the size of the protein of interest, with a 5% stacking gel (Table 2.3.3). The TEMED was added immediately prior to pouring the gel. The gel was placed in a cassette, and this in turn was placed in a running tank. The gels were immersed in SDS-PAGE running buffer (192mM glycine, 25mM Tris-base, 0.1% [w/v] SDS). Protein lysates were boiled for 5 min and then equal amounts of protein were loaded into the wells. The samples were electrophoresed at 100 volts alongside a pre-stained molecular weight SDS-PAGE marker (All Blue Precision Plus Protein Standards, BioRad) until the desired separation had been achieved.

Table 2.3.2 Composition of resolving gels for SDS-PAGE.

<table>
<thead>
<tr>
<th>% gel</th>
<th>H₂O (ml)</th>
<th>30% acrylamide (ml)</th>
<th>1M Tris-HCl pH 8.8 (ml)</th>
<th>10% SDS (µl)</th>
<th>10% APS (µl)</th>
<th>TEMED (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.62</td>
<td>0.8</td>
<td>1.5</td>
<td>40</td>
<td>40</td>
<td>2.5</td>
</tr>
<tr>
<td>8</td>
<td>1.35</td>
<td>1.07</td>
<td>1.5</td>
<td>40</td>
<td>40</td>
<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>1.09</td>
<td>1.33</td>
<td>1.5</td>
<td>40</td>
<td>40</td>
<td>2.5</td>
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<tr>
<td>12</td>
<td>0.82</td>
<td>1.6</td>
<td>1.5</td>
<td>40</td>
<td>40</td>
<td>2.5</td>
</tr>
</tbody>
</table>
2.3.6 Western blot – semi dry transfer

SDS-PAGE gels were soaked in transfer buffer (192mM glycine, 25mM Tris, 0.01% [w/v] SDS) and the proteins were electroblotted onto 0.2µm nitrocellulose membrane (Schleicher & Schuell), pre-soaked in transfer buffer using a semi-dry blotter (BioRad) at 10 volts for 1 h.

2.3.7 Western blot – wet transfer

Three foam pads soaked in transfer buffer (25mM Tris base, 190mM glycine, 15% [v/v] methanol, 0.05% [w/v] SDS) were placed on the rear electrode plate (XCell II, Invitrogen). A piece of pre-soaked 3MM paper was placed on top of these. Next the SDS-PAGE gel, which had been washed in transfer buffer, was placed on top. The pre-soaked 0.2µm nitrocellulose membrane was put on top of the gel, avoiding the introduction of air bubbles. A second piece of pre-soaked 3MM paper was placed on top of this and finally three more foam pads were placed on top. The front electrode plate was then added and the electrodes with the SDS-PAGE gel and nitrocellulose membrane sandwiched in between were placed into a running tank. The electrodes were immersed in transfer buffer and the proteins electroblotted onto the nitrocellulose membrane at 30V for 1 h.

2.3.8 Western blot – treatment with antibodies

Non-specific sites were blocked with 5% (w/v) dried milk (Marvel) in TBST (10mM Tris HCl [pH 7.6], 150mM NaCl, 0.1% [v/v] Tween 20) for 1 h at room temperature with gentle shaking. After three 5 min washes with TBST, the blots were incubated overnight with an appropriate dilution of primary antibody (Table 2.3.4) at 4°C with gentle shaking. The membranes were then washed three times with TBST. Membranes were then incubated, at

---

**Table 2.3.3** Composition of stacking gels for SDS-PAGE.

<table>
<thead>
<tr>
<th>% gel</th>
<th>H₂O (ml)</th>
<th>30% acrylamide (ml)</th>
<th>1M Tris-HCl pH8.8 (µl)</th>
<th>10% SDS (µl)</th>
<th>10% APS (µl)</th>
<th>TEMED (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.03</td>
<td>0.25</td>
<td>188</td>
<td>15</td>
<td>15</td>
<td>1.5</td>
</tr>
</tbody>
</table>
room temperature for 1 h, with a 1:3500 dilution in TBST of the appropriate secondary antibody (Table 2.3.5) coupled to horseradish peroxidise. After three 10 min washes with TBST the Supersignal West Pico Chemiluminescent Substrate kit (Pierce) was used to visualise antigen-antibody complexes. The two solutions provided in the kit were mixed at an equal ratio and added to the blots which were then wrapped in Saranwrap and placed into a cassette. Membranes were exposed to photographic film (Fuji) at room temperature in the dark.

Table 2.3.4 Primary antibodies used to detect proteins by western blot

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Secondary</th>
<th>Dilution</th>
<th>Diluent</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-MEK 1/2</td>
<td>Rabbit</td>
<td>1:500</td>
<td>5% BSA</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>MEK 1/2</td>
<td>Rabbit</td>
<td>1:500</td>
<td>5% BSA</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>P-ERK 1/2</td>
<td>Rabbit</td>
<td>1:500</td>
<td>5% BSA</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>ERK 2</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% milk</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>B-Raf</td>
<td>Mouse</td>
<td>1:1000</td>
<td>5% milk</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>C-Raf</td>
<td>Mouse</td>
<td>1:200</td>
<td>5% milk</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>A-Raf</td>
<td>Rabbit</td>
<td>1:200</td>
<td>1% BSA</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>P C-Raf (ser 621)</td>
<td>Rabbit</td>
<td>1:200</td>
<td>5% milk</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>P C-Raf (ser 259)</td>
<td>Rabbit</td>
<td>1:200</td>
<td>5% BSA</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>P C-Raf (ser 338)</td>
<td>Rabbit</td>
<td>1:200</td>
<td>5% BSA</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Mouse</td>
<td>1:200</td>
<td>5% milk</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% milk</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>Mouse</td>
<td>1:500</td>
<td>5% milk</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% milk</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% milk</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% milk</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Cdc2</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% milk</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>P-cdc2 (tyr15)</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>5% BSA</td>
<td>Cell Signaling</td>
</tr>
<tr>
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Table 2.3.4b Primary antibodies used to detect proteins by western blot.

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Table 2.3.5 Secondary antibodies used in western blot.

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<td>Mouse HRP</td>
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<td>5% milk</td>
<td>Sigma A4416</td>
</tr>
</tbody>
</table>

2.3.9 Stripping western blots

Western blots were incubated with sufficient stripping buffer (0.2M glycine, 1% [w/v] SDS. pH to 2.5 with HCl at room temperature) to cover them for 45 min. They were then washed with TBST three times for 5 min each. The membrane was blocked again before incubation
with a different antibody as described in section 2.3.8 (Western blot – treatment with antibodies).

**2.3.10 $^{35}S$ met/cys labelling of proteins for determination of protein $t_{1/2}$**

Immortalised craf$^-$ cells were nucleofected and each transfection reaction was plated onto 5x 6cm plates. 24 h post transfection, the plates were washed twice in 1x PBS to remove traces of media and any dead cells from the nucleofection process. The cells were then incubated in DMEM lacking cysteine and methionine (Invitrogen). Cells were then pulsed with 1175Ci/mmol Tran$^{35}$S-Label (MP Biomedicals Inc.) for 1 h and were washed twice in 1x PBS. The media was replaced with fresh media without Tran$^{35}$S-Label. Protein lysates were harvested over a time course of between 0 and 315 min in an appropriate volume of GLB and equal amounts of protein subjected to immunoprecipitation. Samples were electrophoresed through an SDS-PAGE gel. SDS-PAGE gels were fixed by two 15 min washes in fixative solution (50% [v/v] methanol, 10% [v/v] acetic acid). The fixative was removed and SDS-PAGE gels were incubated in Amplify (GE Healthcare) for 30 min. Gels were then dried in a flat bed vacuum drier (SCIE-PLAS Gel drier) at 80°C for 3 h. Dried gels were exposed to X-Ray film at -80°C for between 1 day and 3 weeks. The optical density of bands on the X-Ray film was quantitated using Image J (National Institute of Health, USA). The percentage reduction in optical density compared to the optical density at time 0 was determined and plotted on a graph of log optical density versus time. $t_{1/2}$ was calculated as the time required for the optical density to decrease by 50%.

**2.3.11 Emetine dihydrochloride hydrate treatment for determination of protein $t_{1/2}$**

Emetine dihydrochloride hydrate (Sigma), dissolved in DMSO at a concentration of 180mM, was added to cells, plated onto multiple 6cm plates, at a final concentration of 300µM to inhibit protein synthesis. Treated cells were incubated at 37°C for between 0 and 16 h before protein lysates were harvested and the expression of proteins over the time-course were analysed by western blot. Membranes were exposed to photographic film at room
temperature, in the dark. The optical density of bands on the film was quantitated using Image J (National Institute of Health, USA). The percentage reduction in optical density compared to the optical density at time 0 was determined and plotted on a graph of log optical density versus time. \( t_{1/2} \) was calculated as the time required for the optical density to decrease by 50%.

### 2.4 Analysis of the cell cycle

#### 2.4.1 Flow Cytometry

Confluent plates of cells were trypsinised and collected into a 12ml conical bottomed tube (Falcon). Cells were pelleted by centrifugation at 1500 rpm for 5 min at 4°C. Cells were resuspended in 200µl 1x PBS and to this 2ml of ice cold 70% (v/v) ethanol/30% (v/v) PBS solution was added while vortexing. Samples were left at 4°C for 30 min, and then cells were pelleted by centrifugation at 1500 rpm for 5 min. Cells were resuspended by pipetting in 800µl PBS. 100µl of RNase (1mg/ml) and 100µl propidium iodide (400µg/ml) were then added and samples were incubated at 37°C for 30 min. Samples were then analysed using an argon laser tuned to 488nm and measuring FSc, SSc and red fluorescence (Becton Dickenson FACScan).

#### 2.4.2 Bromodeoxyuridine Analysis

Bromodeoxyuridine (BrdU) incorporation assays for proliferation were performed using the kit supplied by Roche. Cells were seeded onto cover-slips in 24 well plates. Upon reaching 50% confluency, cells were synchronised by serum starvation for 24 h. Cells were then simultaneously stimulated with serum and treated with BrdU labelling reagent (1:1000). Cells were incubated for between 0 and 18 h at 37°C. Cells were then washed three times with PBS and fixed with ethanol fixative (50mM glycine in 70% [v/v] ethanol [pH 2.0]) for at least 20 min at -20°C. Cells were washed three times with 1x PBS and then covered with a 1:100 dilution of anti-BrdU antibody and incubated at 37°C for 30 min. Cells were washed
again three times with 1x PBS and covered with a 1:100 dilution of anti-mouse-FITC and 1:1000 dilution of DAPI, diluted in 1x PBS for 30 min at 37°C. Cells were washed again in 1x PBS three times before being mounted on microscope slides, using Gel Mount (Sigma). A minimum of 100 cells per cover-slip were counted, and the percentage of BrdU positively stained cells was visualised by immunofluorescence microscopy (Nikon Eclipse TE300).
3. CRAF IS REGULATED AT THE LEVEL OF PROTEIN STABILITY

3.1 Introduction

3.1.1 Regulation of CRaf protein stability by the ubiquitin-proteasome system

CRaf is part of a multi-protein complex. Amongst other proteins, it binds to chaperones including HSP90, HSP70 and p50cdc37, which assist and maintain the correct tertiary structure of the protein in an ATP driven process (Kolch, 2000, Wartmann and Davis, 1994). These chaperone proteins recognise aberrantly folded proteins via associations with exposed hydrophobic stretches (Bukau and Horwich, 1998). They are capable of stabilising the folding of damaged proteins, as well as newly synthesised proteins including steroid hormone receptors and some protein kinases such as CRaf and PKB, which associate with the chaperones in their inactive state. If the correct active state is not attainable, the substrate protein is targeted for degradation. A major pathway for protein degradation involves the ubiquitination of the substrate protein which marks it for degradation by the proteasome, a process described in detail in Chapter 1. CRaf has been shown to be ubiquitinated and targeted for degradation by the proteasome in varied settings. For example, CRaf is down-regulated when cell anchorage is disrupted (Manenti et al., 2002) and it was suggested that this is due to ubiquitination and targeting of CRaf to the proteasome. Additionally, investigation has described the down-regulation of CRaf by methylglyoxal through a ubiquitin-mediated proteasomal degradation process, which was associated with the phosphorylation of CRaf (Du et al., 2006).

Powers and Workman (2006) described a model for how chaperone client proteins such as CRaf are able to achieve a stable tertiary structure or become ubiquitinated and degraded (Powers and Workman, 2006) (Figure 1.12). Initially, substrate proteins interact with an HSP70/HSP40/HIP complex. The binding of HOP to this complex allows the recruitment of HSP90 to form an intermediate complex. However HOP can only bind to ADP-bound-
CRAF is regulated at the level of protein stability

HSP90. When HSP90 exchanges ADP for ATP, the protein undergoes a conformational change. This allows transient dimerisation of HSP90 via its N-terminus and stimulates its intrinsic ATPase activity (Prodromou et al., 2000). This leads to the dissociation of HSP70/HSP40/HIP and HOP and allows the ATP-dependent association of other co-chaperones including p50\textsuperscript{cdc37} to form the mature complex. In this state, the client protein is correctly folded such that it can become active. However if the correct folding of the client protein fails to occur, the substrate protein is ubiquitinated. This can occur by the recruitment of the E3-ubiquitin ligase, CHIP, which can interact with HSP70 and HSP90 through multiple tetratricopeptide (TPR) repeats. CHIP, together with another co-chaperone BAG1, then facilitates the targeting of the ubiquitinated protein to the proteasome (Demand et al., 2001, Song et al., 2001). This model is believed to be important for the stability of CRaf since treatment with geldanamycin, an inhibitor of the chaperone activity of HSP90, leads to a reduction in CRaf stability associated with its ubiquitination and its increased turnover by the proteasome (Schulte et al., 1996, Schulte et al., 1997).

3.1.2 Manipulation of the craf gene in mice

The generation and analysis of \textit{craf}\textsuperscript{-/-} mice indicated that CRaf is necessary for normal mouse development. However, analysis of the phenotype of mice on different strain-backgrounds has demonstrated very different effects. \textit{craf}\textsuperscript{-/-} mice were created by Huser et al., (2001) and independently by Mikula et al., (2001). These mice, when maintained on a C57BL6 background, died in embryogenesis and showed poor development of the placenta, liver, and haematopoietic organs, as well as increased apoptosis of embryonic tissues (Huser et al., 2001, Mikula et al., 2001). Analysis of embryo sections, MEFs and hepatocytes on a mixed genetic background, showed that cells grew poorly due to an increase in apoptosis and not a defect in the cell cycle (Mikula et al., 2001). However, \textit{craf}\textsuperscript{-/-} embryos on the outbred MF1 background survived to later ages (Kamata et al., 2004) and MEFs showed a reduced ability to grow, but no apoptotic phenotype (Mercer et al., 2005).
This suggests that the phenotype observed in \textit{craf}^- mice is subject to genetic modifiers which are dependent upon the strain of mouse under observation. The apoptotic phenotype of \textit{craf}^- mice on a C57BL6 background may have shielded the cell cycle defect seen in \textit{craf}^- mice on a MF1 background. Thus, the true \textit{craf}^- phenotype may encompass an array of severities of apoptosis and cell cycle defects.

ERK activation, however, was found to be normal, or even increased, in all the strains of \textit{craf}^- mice, indicating that the essential functions of CRaf are independent of its MEK kinase activity and the ERK cascade. Arguably the most important function of CRaf is to counteract apoptosis, having unique effectors to those of the ERK cascade, for example ASK1 as discussed in Chapter 1.

Mice were also generated with a \textit{crf}^{Y340F/Y341F} mutation. This mutation encodes a version of CRaf which has been shown not to be able to phosphorylate or activate MEK (Huser et al., 2001). This confirms that the phosphorylation of tyrosines 340/341 in CRaf is vital for its MEK kinase activity. The phenotype of these mice was apparently normal, and no increase in apoptosis or changes to proliferation were observed. Like \textit{craf}^- mice, ERK activation was also not affected. The apparently normal phenotype of these mice further supports the evidence that the activation of the ERK cascade by CRaf is not necessary to suppress apoptosis or for normal placental development or vascularisation. While the MEK kinase activity of CRaf is not required for normal mouse development, comparison of the \textit{craf}^- and \textit{crf}^{FF/FF} phenotypes indicate that the full length protein itself is necessary. CRaf has a number of substrates described in Chapter 1 which may allow normal development in an MEK/ERK-independent manner. It is possible that the \textit{craf}^- phenotype may be due to the disruption of the activities of these substrates, whereas CRaf^{Y340F/Y341F} may be able to phosphorylate and activate these substrates. The mutation of Y340 and Y341 to phenylalanine generates a kinase incapable of phosphorylating and activating MEK, but may not however abolish all kinase activity. It is possible that a level of kinase activity
remains in the CRaf\textsuperscript{Y340F/Y341F} protein such that other substrates can become phosphorylated which may control apoptosis, for example, in a MEK-independent manner. However, another possibility exists, that the main function of CRaf may not be that of a kinase but may act as a scaffold protein. In order to address this, our laboratory generated kinase inactive CRaf mice.

### 3.1.3 Work leading up to this project; kinase inactive CRaf mice

CRaf kinase inactive mice were generated by Dr. K. Mercer using a gene targeting knockin approach (Figure 3.1). These mice have an A1587C mutation in the craf gene leading to a D486A substitution in exon 14. The mutation is located in the DFG motif, which is in the C-terminal lobe of the kinase domain. This motif is important for ATP coordination; by interacting with other residues the DFG motif provides a favourable structural arrangement for the catalytic loop to accommodate ATP binding. Substitution of an amino acid within this motif alters an essential triad of amino acids that is required for coordination of ATP and hence renders the kinase domain inactive. Sequencing confirmed the presence of the mutation (Figure 3.2 A) and the basal and serum stimulated activity of the CRaf\textsuperscript{DA} kinase was analysed to confirm that the mutation had produced a kinase defective CRaf protein (Figure 3.2 B). The kinase activity was measured using MEK-ERK kinase cascade assays (Marais et al., 1997). The CRaf\textsuperscript{DA} protein does appear to display some basal kinase activity in this assay. This is not unusual, as kinase dead mutation of the Raf proteins and other kinases always retain an amount of residual activity, of approximately 1% (Wan et al., 2004, Garnett et al., 2005). This is most likely not actually due to residual kinase activity, but due to co-precipitation with other kinases. In this case co-precipitation of BRaf is the most likely explanation because CRaf and BRaf are known to heterodimerise.
Figure 3.1 Generation of $craf^{D486A}$ mice.

Gene targeting was used to create the $craf^{D486A}$ allele. A knockin approach was used to insert the A1587C mutation in the murine $craf$ gene leading to a D486A substitution in exon 14, indicated by *. The mutation is located in the DFG motif of the kinase domain; mutation of this essential motif inactivates the kinase. In order to delete the neoR cassette, $craf^{D486A}$ mice were crossed to CMV-Cre mice (Schwenk et al., 1995). Black arrow heads represent LoxP sequences.
Figure 3.2 Analysis of craf^D486A mice (Data obtained by Dr. K. Mercer)

(A) Sequencing confirmed the presence of the A1587C mutation in the craf gene (B) Kinase cascade assays investigated the kinase activity of CRaf in craf^DA/DA and wild-type MEFs. craf^DA/DA and craf^+/+ MEFs were serum starved and then either treated with 10% FCS for 10 min or left untreated. Protein lysates were subjected to a Raf kinase cascade assay. Activity of CRaf is low in both unstimulated craf^DA/DA and wild-type MEFs. However, when stimulated, the activity in wild-type cells increases markedly, whereas the activity in craf^DA/DA cells remains low. This demonstrates that the kinase activity of CRaf in these cells has been rendered inactive.
On the C57BL6 background, craf\textsuperscript{DA/DA} mice and cells displayed an apoptotic phenotype similar to that of craf\textsuperscript{−/−} mice and cells on the same genetic background (Figure 3.3). The craf\textsuperscript{DA/DA} embryos are developmentally retarded dying at E10.5-E12.5. Furthermore TUNEL analysis demonstrated widespread apoptosis. MEFs derived from these embryos also showed increased levels of apoptosis in response to serum withdrawal, etoposide and α-CD95 antibody. Consistent with observations in craf\textsuperscript{−/−} cells (Huser et al., 2001; Mikula et al., 2001), ERK activation was not disrupted in craf\textsuperscript{DA/DA} cells, providing additional evidence to suggest that the MEK kinase activity of CRaf is not essential in suppressing apoptosis.

These data would suggest that CRaf kinase activity is required for the prevention of apoptosis. However preliminary data also showed that CRaf\textsuperscript{DA} is expressed at very low levels in the cell. Hence, a possible consequence of the low level of expression of kinase-inactive CRaf is that craf\textsuperscript{DA/DA} embryos have a phenotype similar to the craf\textsuperscript{−/−} phenotype.

3.2 Aims

The work described above was undertaken prior to my PhD studies. The aim of this chapter was to investigate why CRaf expression is reduced in craf\textsuperscript{DA/DA} mice and to clarify the role of CRaf kinase activity in controlling its own expression.

3.3 Results

3.3.1 Derivation of craf\textsuperscript{DA/DA} primary MEFs

A breeding colony of craf\textsuperscript{+/DA} was maintained at the University of Leicester. Primary MEFs were produced from embryos at E13.5, resulting from a timed intercross of craf\textsuperscript{+/DA} heterozygote animals. Each MEF line was generated from a single embryo and was genotyped by the removal of the tail for DNA extraction to be used in a PCR reaction using primers OCP 166 and OCP 167. These primers span a section of intron 13 in the craf gene, disrupted as a consequence of its mutation which includes the presence of a residual 34 bp LoxP site. Therefore the primers are capable of detecting the difference between the wild-
Figure 3.3 *craf<sup>DA/DA</sup>* mice and MEFs show an apoptotic phenotype. (Data obtained by Dr. K. Mercer, Dr. L. Carragher and Mrs S. Giblett)

(A) E10.5 *craf<sup>+/+</sup>* and *craf<sup>DA/DA</sup>* embryos were subjected to TUNEL analysis to detect apoptotic cells. Scale bars, 250μm (B) MEFs were either not treated (NT) or treated with the apoptosis inducers serum-free media (SFM), etoposide (E), or α-CD95 antibody and apoptosis was quantitated by annexin V staining. The mean of three independent experiments for three different MEFs of each genotype is shown, and error bars represent standard error. (C) ERK phosphorylation following FCS stimulation. MEFs were serum-starved for 24 hours and then stimulated with 10% (v/v) FCS over a time course of up to 16 hours, protein lysates harvested and analysed with the antibodies indicated.
type and mutated alleles. They amplify a 378 bp product of the wild-type allele and a 198 bp product of the targeted allele. PCR products were run on a 2% agarose gel and an example of genotyping from one litter is shown in Figure 3.4.

### 3.3.2 CRaf protein expression is reduced in CRaf\(^{DA/DA}\) MEFs

Preliminary results indicated that the expression of CRaf was reduced in craf\(^{DA/DA}\) MEFs (Dr. K. Mercer). To investigate this further, a series of western blots were preformed to examine the expression of key proteins in the ERK cascade in craf\(^{DA/DA}\) cells compared with craf\(^{+/+}\) cells, using actin as a loading control (Figure 3.5). Protein lysates were harvested from asynchronous, cycling MEFs. The results indicate that the expression of ARaf, BRaf, ERK2, MEK1/2 and phospho-MEK1/2 are unchanged in craf\(^{DA/DA}\) cells. The expression of phospho-ERK appears slightly elevated in craf\(^{DA/DA}\) MEFs, although this was not found to be statistically significant. However the expression of CRaf in craf\(^{DA/DA}\) cells was markedly reduced in comparison with wild type cells. Quantitation of the expression of the proteins, using ImageJ, demonstrated that CRaf protein expression was reduced by over 40 fold in craf\(^{DA/DA}\) MEFs. An unpaired T test, showed this to be significant, with a P value of < 0.0001. However the variations in protein expression of the other components of the Raf/MEK/ERK cascade between cell types was not shown to be statistically significant. Therefore the abolition of CRaf kinase activity results in the reduction of CRaf expression, but does not affect the expression of other components of the Raf/MEK/ERK cascade, and furthermore does not reduce the activation of MEK or ERK as monitored by their phosphorylation.

### 3.3.3 CRaf protein expression is reduced in CRaf\(^{DA}\) embryo protein lysates

To further investigate the reduced CRaf expression in craf\(^{DA/DA}\) cells, and to confirm that this phenotype was not an artefact of the cell culture process, protein lysates were made from craf\(^{+/+}\), craf\(^{+/DA}\) and craf\(^{DA/DA}\) embryos. Western blots were then conducted with a CRaf antibody, using actin as a loading control, which confirmed that CRaf protein expression was drastically reduced in craf\(^{DA/DA}\) embryos (Figure 3.6). Quantitation of CRaf expression
Figure 3.4 Production of craf\textsuperscript{DA/DA}, craf\textsuperscript{+/DA} and craf\textsuperscript{+/+} MEFs.

(A) The pertinent portion of the craf\textsuperscript{WT} and craf\textsuperscript{DA} alleles are shown. The primers used for identifying PCR reactions are indicated. The D486A mutation in exon 14 is indicated by *. (B) Primary MEFs were produced from embryos at E13.5, resulting from a timed intercross of craf\textsuperscript{+/DA} heterozygote animals. Each MEF line was genotyped by the removal of the tail for DNA extraction to be used in a PCR reaction using primers OCP 166 and OCP 167. PCR products were run on a 2% agarose gel and an example of genotyping from one litter is shown in this figure. Five craf\textsuperscript{+/+} embryos (lanes 1,3,4,5,6), one craf\textsuperscript{+/DA} embryo (lane 7) and one craf\textsuperscript{DA/DA} embryo (lane 2) were obtained on this occasion.
Figure 3.5 Expression of CRaf\textsuperscript{DA} is reduced, whereas the expression of other key ERK cascade proteins is unaltered in craf\textsuperscript{DA/DA} MEFs.

(A) Western blot analysis of RAF/MEK/ERK cascade proteins in craf\textsuperscript{+/+} and craf\textsuperscript{DA/DA} MEFs. Representative images from at least three independent experiments for two different MEF lines of each genotype are shown. Actin was used as a loading control. (B) The expression of the proteins in at least 3 independent experiments was quantitated using ImageJ. The reduction in CRaf\textsuperscript{DA} expression is statistically significant with a P <0.0001 (by unpaired T test). Error bars denote the standard error in the values shown.
Figure 3.6 Expression of CRaf is reduced in craf^{+/DA} and craf^{DA/DA} embryos.

(A) Western blot analysis of proteins isolated from whole craf^{+/+}, craf^{+/DA} and craf^{DA/DA} embryos. Levels of CRaf expression were detected using a specific antibody. Actin was used as a loading control. The figure shown is representative of four independent experiments. (B) The expression of the proteins in 4 independent experiments was quantitated using ImageJ. The reduction in CRaf expression in craf^{+/DA} and craf^{DA/DA} embryos is statistically significant with respective P values of 0.0039 and 0.0001 (by unpaired T test). Error bars denote the standard error in the values shown.
demonstrated that, similar to results obtained from $\text{craf}^{\text{DA}/\text{DA}}$ MEF lysates, the expression of CRaf in $\text{craf}^{\text{DA}/\text{DA}}$ embryo lysates was reduced by over 35-fold. An unpaired T test, showed this to be significant, with a P value of 0.0001.

The expression of CRaf in the $\text{craf}^{+/\text{DA}}$ embryos was intermediate between the expression of CRaf in $\text{craf}^{+/+}$ and $\text{craf}^{\text{DA}/\text{DA}}$ embryos. If the CRaf protein expression was reduced by ~2-fold, it could be theorised that although protein expression derived from the targeted allele is drastically reduced, the protein produced from the wild-type allele is not affected. However, quantitation of CRaf expression demonstrates that in $\text{craf}^{\text{DA}}$ embryo lysates CRaf expression is reduced by ~4-fold, which was identified as statistically significant with a P value of 0.0039 (by unpaired T test). This may suggest that the mutation of one allele affects the expression of the other. Indeed it is possible that the CRaf$^{\text{DA}}$ protein may act as a dominant negative and destabilise the CRaf$^{\text{WT}}$ protein by an unknown mechanism, hence accounting for the lower than expected CRaf expression. Further investigation would be required to confirm this theory.

### 3.3.4 craf mRNA levels are not reduced in $\text{craf}^{\text{DA}/\text{DA}}$ MEFs

The reduced protein expression of kinase inactive CRaf observed in $\text{craf}^{\text{DA}/\text{DA}}$ MEFs may be due to altered regulation of CRaf mRNA production or stability, its translation, or its protein stability. If one or more of these are affected in $\text{craf}^{\text{DA}/\text{DA}}$ MEFs, it can be said that CRaf regulates itself in a kinase dependent manner. Therefore abolition of CRaf kinase activity results in the reduced expression of the protein. Alternatively, it is possible that the targeting event itself may have disrupted mRNA production or processing. To investigate if CRaf mRNA production or stability is altered by the kinase inactivating mutation the expression of $\text{craf}$ cDNA was measured in $\text{craf}^{+/+}$ and $\text{craf}^{\text{DA}/\text{DA}}$ MEFs.
To accurately determine precise expression levels of craf mRNA, quantitative RT-PCR (qPCR) was performed. To ensure reliable results were obtained, all qPCR reactions were performed in triplicate. This technique measures the accumulation of the amplified product by the addition of a fluorescent molecule whose signal increases proportionally to the amount of DNA present. Initially in a qPCR reaction, the fluorescence is not detectable above background levels, but as the amount of PCR product increases exponentially, approximately doubling each cycle, enough product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs is called the threshold cycle (C\text{\textsubscript{T}}). As the qPCR reaction continues, the components of the reaction are used up and become limiting to the reaction resulting in a slowing of the reaction into the stationary phase.

Before the actual assay, the conditions of the qPCR reaction were optimised, as described in Chapter 2. The craf qPCR experiment was performed using cDNA derived from craf\textsuperscript{+/+} and craf\textsuperscript{DA/DA} MEFs, which was produced by reverse transcription of mRNA obtained from these cells. The data for a representative qPCR amplification of craf and gapdh are presented in Figures 3.7 and 3.8 respectively. In these experiments, mRNA controls were included. The mRNA used was obtained from craf\textsuperscript{+/+} and craf\textsuperscript{DA/DA} MEFs and was the template for the production of the cDNA used in this assay. From the data presented in the melt curves and amplification curves, it is clear that no amplification of craf or gapdh occurred from this RNA and therefore excluded the possibility of genomic DNA contamination of the RNA and of the subsequently derived cDNA. In addition the lack of amplification in all water controls demonstrated the absence of contamination of any reagents and showed the amplification of craf and gapdh was specifically from the cDNA used in each assay.

The Pfaffl method was used calculate the fold difference of cDNA and hence mRNA between the samples. This method assumes that each gene (both craf and gapdh) have the same amplification efficiency in all samples, but the amplification efficiencies of the two genes are not necessarily the same as each other and takes the actual amplification
Figure 3.7 qPCR analysis of craf expression in craf\textsuperscript{+/+} and craf\textsuperscript{DA/DA} MEFs.

(A) Microtitre plate showing loading of samples in triplicate (indicated by colour), as described in the table. (B) Data graphs of the melt curve showing one peak, corresponding to a single PCR product. The amplification curves for each sample are shown with the threshold line indicated, positioned at the point where the curves begin to linearise. Note the absence of amplification in the RNA control samples, indicating the lack of genomic DNA contamination. Representative data is shown from three independent experiments.

### Samples

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<tr>
<td>A4-A6</td>
<td>DA/DA</td>
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<tr>
<td>F1-F3</td>
<td>water</td>
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<tr>
<td>G1-G3</td>
<td>+/- RNA</td>
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<td>G4-G6</td>
<td>DA/DA RNA</td>
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Figure 3.8 qPCR analysis of *gapdh* expression in *craf*<sup>+/+</sup> and *craf*<sup>DA/DA</sup> MEFS.

(A) Microtitre plate showing loading of samples in triplicate (indicated by colour), as described in the table. (B) Data graphs of the melt curve showing one peak, corresponding to a single PCR product. The amplification curves for each sample are shown with the threshold line indicated, positioned at the point where the curves begin to linearise. Note the absence of amplification in the RNA control samples, indicating the lack of genomic DNA contamination. Representative data is shown from three independent experiments.

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<td>D4-D6</td>
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<td>G4-G6</td>
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141
efficiency of each gene into account. To calculate the difference in expression levels between multiple samples, one of them was chosen as a calibrator (a \( craf^{+/+} \) sample) and the expression of the other test genes in all other samples was expressed as an increase or decrease relative to the calibrator. The equation used to calculate the fold difference in expression levels of craf cDNA, relative to the calibrator and normalised to the expression of GAPDH was:

\[
\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CT, \text{target (calibrator-test)}}}{(E_{\text{ref}})^{\Delta CT, \text{ref (calibrator-test)}}}
\]

where the \( E_{\text{target}} \) is equal to the efficiency of the primers used to amplify the gene of interest (in this case the efficiency of the craf primers was 84.29%), and \( E_{\text{ref}} \) is equal to the efficiency of the primers used to amplify the reference gene (the efficiency of the gapdh primers was 93.03%).

The fold difference was then averaged in \( craf^{+/+} \) and \( craf^{DA/DA} \) MEFs, and the averaged \( craf^{DA/DA} \) values were normalised to the wild-type value (Figure 3.9). The expression of craf mRNA in \( craf^{DA/DA} \) MEFs was found not to be significantly different to the expression level seen in \( craf^{+/+} \) MEFs. Since the craf mRNA levels are not reduced in \( craf^{DA/DA} \) cells, it excluded the possibility that CRaf is regulated at the level of mRNA production or stability in a kinase dependent manner. Additionally the data shows that the targeting event itself did not disrupt craf mRNA production or stability.

### 3.3.5 Mutation of the craf gene does not affect its splicing

As a result of the targeting event which introduced the mutation into the craf gene, a 34 bp LoxP site was introduced in intron 13. Many other studies using knockin mutations also result in the presence of a residual LoxP site within an intron, and data from such investigations has established that the presence of such a residual LoxP site does not affect
cDNA derived from \textit{craf}\textsuperscript{+/+} and \textit{craf}\textsuperscript{DA/DA} MEFs, produced by reverse transcription of mRNA obtained from these cells was used in qPCR assays to quantitate relative levels of \textit{craf} mRNA, using \textit{gapdh} as a control. Each sample was analysed in triplicate with each primer set and the Pfaffl method was used to calculate relative levels of expression. The values for \textit{craf} were normalised to the values for \textit{gapdh} and subsequently the \textit{craf}\textsuperscript{DA/DA} value was normalised against the \textit{craf}\textsuperscript{+/+} value. There is no significant difference in \textit{craf} mRNA expression in \textit{craf}\textsuperscript{DA/DA} MEFs. Error bars denote the standard error in the values shown. Results are from 3 independent samples for each cell line.
gene function. qPCR analysis of craf<sup>DA</sup> mRNA has demonstrated that the mutation itself and the LoxP site do not affect the level of expression of craf<sup>DA</sup> transcripts (Figure 3.9).

However it is conceivable that the LoxP site may disrupt splicing in the region of intron 13, although this is unlikely because western blot analysis has demonstrated that only full length CRaf is expressed in craf<sup>DA/DA</sup> MEFs, albeit at a low level (Figure 3.5 and 3.6). Even so, in order to completely exclude the possibility of abnormal splicing events, an RT-PCR reaction was performed in the region from exon 11 to exon 17. This used cDNA prepared from a reverse transcription reaction with the template being RNA extracted from craf<sup>DA/DA</sup> and craf<sup>/+</sup> MEFs. The primers used were OCP 23 which anneals in exon 11 and OCP 2 which anneals in exon 17. Therefore the primers flank the region between exon 11 and exon 17. Hence the PCR product they amplify includes the targeted region. The primers amplify a 807 bp product, calculated based on the size of the exons in this region and the annealing sites of the primers. The RT-PCR product was run on a 2% agarose gel and a single transcript of the size expected was expressed in both cell types and there was no evidence of smaller or larger transcripts (Figure 3.10). This confirms that the targeting event and the residual LoxP site in craf<sup>DA/DA</sup> do not cause differential splicing between exons 11 and 17. The data presented in Figures 3.9 and 3.10 excludes the possibility that the targeting event has disrupted craf mRNA production or processing. Therefore the reduction in CRaf<sup>DA</sup> expression can only be explained if CRaf kinase activity is required for controlling its own expression at the protein level, by regulating its translation or protein stability.

### 3.3.6 Stability of endogenous CRaf<sup>WT</sup>

To determine whether the kinase activity of CRaf is required to stabilise the protein, the t<sub>1/2</sub> of endogenous CRaf<sup>WT</sup> and CRaf<sup>DA</sup> were attempted to be measured in <sup>35</sup>S met/cys pulse chase experiments (Figure 3.11). Cellular protein in craf<sup>/+</sup> and craf<sup>DA/DA</sup> MEFs was labelled with a <sup>35</sup>S met/cys pulse. Then cells were incubated in fresh media and lysates were harvested in the subsequent chase. CRaf was immunoprecipitated and samples were electrophoresed
Figure 3.10 craf mRNA splicing is not affected by the kinase inactivating mutation.

(A) The pertinent portion of the craf<sup>DA/DA</sup> gene is shown. The primers used for the RT-PCR reaction are OCP 23 and OCP 2 which flank the targeted region. (B) RNA prepared from craf<sup>+/+</sup> and craf<sup>DA/DA</sup> MEFs was transcribed to produce cDNA and subjected to RT-PCR with primers OCP 23 and OCP 2. The single expected product of 807 bp is produced in both samples, confirming that the presence of the mutation in exon 14, or the residual LoxP site in intron 13 does not affect splicing between exons 11-17. Results are representative of 2 independent samples from each cell line.
Figure 3.11 Assessment of the stability of endogenous CRafWT assayed by $^{35}$S met/cys pulse chase.

(A) Cellular protein in craf$^{+/+}$ MEFs was labelled with a $^{35}$S met/cys pulse and chased over a 20 hour time-course. CRafWT was immunoprecipitated and samples were electrophoresed through an SDS-PAGE gel. Gels were dried and exposed to X-Ray film at -80°C. A typical example from two independent experiments is presented (B) The optical density of bands on the X-Ray film was quantitated. The percent reduction in optical density was determined and plotted. The $t_{1/2}$ was calculated as the time required for the optical density to decrease by 50%. Data is representative of two independent experiments.
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through an SDS-PAGE gel. Gels were dried and exposed to X-Ray film at -80°C. The optical
density of bands on the X-Ray film was quantitated. The percent reduction in optical density
was determined and plotted. The $t_{1/2}$ was calculated as the time required for the optical
density to decrease by 50%. The $t_{1/2}$ of endogenous wild-type CRaf was assayed and was
found to be approximately 7 h. However, although several attempts were made to measure
the $t_{1/2}$ of endogenous CRaf$^{DA}$ in craf$^{DA/DA}$ MEFs, it proved impossible to pulse label this
protein because of its low expression. Therefore in order to compare the $t_{1/2}$ of wild-type and
mutant protein, CRAF expression vectors were used as described below.

3.3.7 Kinase inactive CRAF has reduced stability

The $t_{1/2}$ of wild-type and kinase inactive CRAF were compared by $^{35}$S met/cys pulse chase
experiments (Figure 3.12). Experiments were performed by transient transfection of myc-
tagged CRAF expression vectors into immortalised craf$^-$ MEFs. The vectors expressed
CRAF$^{WT}$ or a kinase defective CRAF mutant, either CRAF$^{K375M}$ or CRAF$^{D486A}$. The CRAF$^{D486A}$
construct is analogous to the mutation in endogenous CRaf$^{D486A}$ in craf$^{DA/DA}$ MEFs. The CRAF$^{K375M}$
is rendered kinase inactive by the mutation of the kinase domain ATP binding
site at position 375 from lysine to methionine. The constructs were transfected into craf$^-$
MEFs in order to prevent any possible effects of endogenous CRaf in cross-stabilising the
transfected proteins. Cellular protein in transfected cells was labelled with a $^{35}$S met/cys
pulse. Then cells were incubated in fresh media and lysates were harvested in the
subsequent chase. CRAF was immunoprecipitated using an antibody against the myc tag
and samples were electrophoresed through an SDS-PAGE gel. Gels were dried and
exposed to X-Ray film at -80°C. The optical density of bands on the X-Ray film was
quantitated. The percent reduction in optical density was determined and plotted. The $t_{1/2}$ was
calculated as the time required for the optical density to decrease by 50%. CRAF$^{WT}$ was
found to have an average $t_{1/2}$ of 81 min. However the kinase inactive CRAF mutants had a
reduction in their protein stability, being approximately 40-60% less stable than CRAF$^{WT}$;
CRAF$^{K375M}$ had an average $t_{1/2}$ of 46 min and CRAF$^{D486A}$ has a $t_{1/2}$ of 54 min.
Figure 3.12 Kinase inactive CRAF has reduced stability.

The $t_{1/2}$ of wild-type and kinase inactive CRAF were compared by $^{35}$S met/cys pulse chase experiments. (A) Myc-tagged CRAF$^{WT}$, CRAF$^{K375M}$ or CRAF$^{D486A}$ expression vectors were transfected into $craf^{-/-}$ cells. Cellular protein was labelled with a $^{35}$S met/cys pulse and chased over a time-course of up to 195 min. CRAF was immunoprecipitated using an α-myc antibody and equal amounts of samples were electrophoresed through an SDS-PAGE gel. Gels were dried and exposed to X-Ray film at -80°C. Data for CRAF$^{WT}$ and CRAF$^{K375M}$ is representative of four independent experiments. However only one experiment was performed by this method for CRAF$^{D486A}$. (B) The optical density of bands on the X-Ray film was quantitated. The percent reduction in optical density was determined and plotted. The $t_{1/2}$ was calculated as the time required for the optical density to decrease by 50%. The kinase dead CRAF protein has reduced stability (CRAF$^{KM}$ $t_{1/2}$ is ~57% of CRAF$^{WT}$; CRAF$^{DA}$ $t_{1/2}$ is ~66% of CRAF$^{WT}$.)
The \( t_{1/2} \) of endogenous wild-type CRaf of \( \sim 7 \) h (Figure 3.11) is considerably higher than the average \( t_{1/2} \) of 81 min for ectopically expressed \( \text{CRAF}^{\text{WT}} \) determined by \( ^{35}\text{S met/cys} \) pulse chase. An explanation for this difference may be that proteins involved in binding and stabilising CRaf, such as 14-3-3 and HSP90 are titrated out as a consequence of the over expression.

Experiments to investigate the \( t_{1/2} \) of \( \text{CRAF}^{\text{WT}} \) and \( \text{CRAF}^{\text{K375M}} \) were performed four times. However the \( \text{CRAF}^{\text{D486A}} \) construct was not available until the final stages of this investigation. Therefore only one experiment was performed to analyse the \( t_{1/2} \) of \( \text{CRAF}^{\text{D486A}} \) by this method due to time constraints. However in order to verify the results obtained from the \( ^{35}\text{S met/cys} \) pulse chase experiment, a second strategy was employed; using the protein synthesis inhibitor emetine (Figure 3.13). Results are obtained in significantly less time using this method in comparison to the \( ^{35}\text{S met/cys} \) pulse chase experiment.

Immortalised \( \text{craf}^{-} \) cells were transfected with a vector expressing \( \text{CRAF}^{\text{WT}} \) or \( \text{CRAF}^{\text{D486A}} \). 24 h later emetine was added to the cell culture media and lysates were harvested at time-points up to 16 hours. Total protein lysates were analysed by western blot with antibodies for CRaf and actin. The optical density of bands on the X-Ray film were quantitated and the percent reduction in optical density was determined and plotted, allowing the \( t_{1/2} \) to be calculated. The results obtained from this experiment support those from the \( ^{35}\text{S met/cys} \) pulse chase experiment demonstrating that the kinase inactive \( \text{CRAF}^{\text{D486A}} \) mutant was less stable, having a \( t_{1/2} \) of approximately 30\% of \( \text{CRAF}^{\text{WT}} \).

It is important to note that this experiment found the average \( t_{1/2} \) of ectopically expressed \( \text{CRAF}^{\text{WT}} \) to be 16 h 40 min, whereas the average \( t_{1/2} \) of ectopically expressed \( \text{CRAF}^{\text{WT}} \) determined by the \( ^{35}\text{S met/cys} \) pulse chase experiment was only 81 min. In analogy, the \( t_{1/2} \) of \( \text{CRAF}^{\text{D486A}} \) determined by the emetine experiment was 5 h, whereas it was 54 min by the \( ^{35}\text{S met/cys} \) pulse chase experiment. Although both methodologies show a similar decrease
Figure 3.13 **The CRAFd486A kinase inactive mutant has reduced stability.**

The $t_{1/2}$ of CRAF$^{WT}$, and CRAF$^{D486A}$ were compared by emetine time-course experiments. (A) Immortalised craf$^{-/-}$ cells were transfected with vectors expressing CRAF$^{WT}$ and CRAF$^{D486A}$. 24 h later emetine was added to the cell culture media and lysates were harvested at time-points up to 16 hours. Total protein lysates were analysed by western blot with antibodies for CRAF and actin. (B) The optical density of bands on the X-Ray film were quantitated. The percent reduction in optical density was determined and plotted. The $t_{1/2}$ was calculated as the time required for the optical density to decrease by 50%. The CRAFd486A mutant has a $t_{1/2}$ ~30% of CRAF$^{WT}$. 

![Western Blot Image](image)

![Graph Image](image)
in the $t_{1/2}$ of the kinase inactive CRAF$^{DA86A}$ mutant, the actual values for the proteins’ $t_{1/2}$ are not reconcilable. The difference between these values can only be due to the different assay conditions used.

### 3.3.8 CRaf expression in craf$^{DA/DA}$ MEFs is rescued by treatment with proteasome inhibitors

It has been demonstrated that CRaf expression in craf$^{DA/DA}$ cells is reduced and that both kinase inactive CRaf mutants (CRAF$^{DA}$ and CRAF$^{KM}$) have impaired stability. To examine the role of the proteasome in regulating CRaf protein stability, MEFs were treated with a range of proteasome inhibitors, specifically MG132, epoxomicin and lactacystin, and CRaf expression levels were investigated in Triton-X100 soluble and insoluble protein lysate fractions by western blot.

It was necessary to optimise the conditions used for each inhibitor. Initial treatments with high concentrations of lactacystin (10µM to 25µM) for long time points (18 hours) appeared to cause high levels of apoptosis, as examined by light microscopy, and did not rescue CRaf expression (data not shown). Reducing the concentration of the inhibitor to below 10µM and the time to 6 hours rescued CRaf expression (Figure 3.14 A) and did not appear to cause apoptosis of cells. The most significant rescue of expression was seen when MEFs were treated with a final concentration of 0.5µM lactacystin for 6 hours and as such this condition was used for future experiments. Identical conditions were used for the inhibitor epoxomicin. Treatment with MG132 appeared to rescue CRaf expression in a dose dependent manner (Figure 3.14 B). The most significant rescue of expression was seen when MEFs were treated with a final concentration of 40µM MG132 for 6 h. However a slight increase in apoptosis was observed in cells treated with this concentration of MG132 over those treated with 20µM. Therefore, it was decided that a compromise of a final concentration of 30µM MG132 should be added to cells in future experiments.
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Figure 3.14 **CRaf expression is rescued by proteasome inhibition.**

craf^{DA/DA} MEFs were treated with proteasome inhibitors (A) lactacystin and (B) MG132 at the concentrations indicated for 6 hours in order to optimise the conditions for these inhibitors. The most significant rescue of CRaf^{DA} expression was seen when MEFs were treated with either a final concentration of 0.5µM lactacystin or 40µM MG132 for 6 hours. (C) Cells were either treated with proteasome inhibitors lactacystin (L), epoxomicin (E) and MG132 (M) or treated with DMSO (C) as a control for 6 hours. Protein lysates were prepared from soluble and insoluble fractions and CRaf levels assessed. All the proteasome inhibitors rescued CRaf expression to some degree in both soluble and insoluble fractions. Data is representative of three independent experiments.
Using the conditions decided upon in preliminary experiments, craf\textsuperscript{DA/DA} MEFs were treated with the proteasome inhibitors lactacystin, epoxomicin and MG132. Both soluble and insoluble protein lysate fractions were analysed by western blotting with a CRaf antibody, using actin as a loading control (Figure 3.14 C). Insoluble Triton-X100 precipitates were re-solubilised in 2\% (w/v) SDS. All proteasome inhibitors rescued CRaf\textsuperscript{DA} expression considerably in both soluble and insoluble fractions. With epoxomicin and MG132 in particular, a significant proportion of CRaf\textsuperscript{DA} was located in the insoluble fraction and showed a laddering pattern typically observed in proteins that are ubiquitinated. These data indicate that increased proteasomal degradation is the cause of the reduced CRaf expression, and that CRaf may regulate its own stability by controlling its degradation by the proteasome in a kinase-dependent manner.

\textbf{3.3.9 CRaf\textsuperscript{DA} has a greater affinity with chaperone proteins}

HSP90 and HSP70 are chaperone proteins which bind with a high affinity to unfolded and misfolded proteins. It is well established that these proteins are necessary for establishing and stabilising CRaf tertiary structure. Additionally it has also been demonstrated that the E3 ubiquitin ligase CHIP stimulates the degradation of HSP90/HSP70 substrates such as CRaf by a process involving the chaperone cofactor BAG1. To examine whether this pathway is involved in the increased turnover of CRaf\textsuperscript{DA}, the binding of HSP90 and HSP70 to CRaf was investigated by Robert Hayward (Cancer Research UK Centre for Cell and Molecular Biology, The Institute of Cancer Research, London, UK). CRaf was immunoprecipitated from craf\textsuperscript{+/+} and craf\textsuperscript{DA/DA} MEFs and immunoblotted with either a HSP90 or a HSP70 antibody. CRaf\textsuperscript{DA} was shown to have an increased ability to bind to HSP90 than CRaf\textsuperscript{WT} (Figure 3.15); supporting the data that CRaf\textsuperscript{DA} is unstable in comparison with CRaf\textsuperscript{WT}. However the interaction of CRaf\textsuperscript{DA} with HSP70 did not appear to be any stronger than the wild-type protein. Even so, in the absence of its own kinase activity, CRaf is shown to be a misfolded protein which binds with a high affinity to HSP90.
Figure 3.15 **CRAF** association with HSP90 is greater than CRAF WT. (Data obtained from Dr. Robert Hayward, ICR, London)

(A) CRAF DA has an increased ability to bind to HSP90. Protein lysates were harvested from *craf*+/+ and *craf*DA/DA MEFs and CRAF (C) or B RAF (B) proteins were immunoprecipitated. Immunoprecipitated proteins as well as the total protein lysate (L) were analysed with an HSP90 antibody by western blot. The strong interaction between CRAF DA and HSP90 is marked by *. (B) A longer exposure of the X-ray film shows an interaction between CRAF WT and HSP90 indicated by **. There is no difference in the interaction between B RAF and HSP90 in *craf*DA/DA MEFs. (C) Interaction of CRAF DA with HSP70 is not altered. Protein lysates were prepared from *craf*+/+ and *craf*DA/DA cells and CRAF (C) or B RAF (B) proteins were immunoprecipitated. Immunoprecipitated proteins as well as total protein lysate (L) were analysed with an antibody for HSP70. Similar levels of HSP70 are co-immunoprecipitated with wild-type CRAF and CRAF DA.
3.3.10 CRAF is ubiquitinated

It has been demonstrated that CRaf is degraded by the proteasome and in addition, previous published work (Manenti et al., 2002, Schulte et al., 1997, Du et al., 2006) suggests that CRaf can be ubiquitinated. To investigate CRaf ubiquitination, various human myc tagged CRAF expression vectors were transfected into craf\(^{-}\)/ cells with or without a vector expressing HA tagged ubiquitin. It was necessary to use these CRAF expression vectors as opposed to analysing endogenous CRaf in craf\(^{+/+}\) and craf\(^{DA/DA}\) MEFs because the low levels of expression of CRaf\(^{DA}\) made it difficult to investigate directly. The CRAF expression vectors used were CRAF\(^{WT}\), kinase inactive CRAF\(^{KM}\), and the CRAF\(^{S621A}\) mutant. When this experiment was performed the kinase inactive CRAF\(^{DA}\) construct, homologous to the endogenous CRaf\(^{DA}\) mutation was not available. Cells were treated with the proteasome inhibitor, lactacystin to aid the detection of ubiquitinated CRAF. Both Triton-X100 soluble and insoluble protein lysates were immunoprecipitated with an antibody against the myc tag and western blots were conducted to determine if CRAF was ubiquitinated (Figure 3.16). Results consistently demonstrated that all forms of CRAF showed evidence of mono- and/or poly-ubiquitination, showing a laddering pattern above 74kDa (the molecular weight of CRAF) in the lanes co-transfected with HA-ubiquitin, which was absent in lanes not co-transfected with HA ubiquitin. Furthermore, although the loading of all samples was equal as shown by the IgG bands, the amount of CRaf present at 74kDa was lower in the samples co-transfected with ubiquitin. This may be due to the ubiquitination of CRaf in these samples, hence resulting in its degradation.

3.3.11 CHIP and BAG1 do not uniquely mediate the ubiquitination and subsequent proteasomal degradation of CRaf

The E3 ubiquitin ligase CHIP has been shown to stimulate the degradation of CRaf by ubiquitination and subsequent targeting to the proteasome, in a mechanism involving the chaperone cofactor BAG1. siRNA was used to knockdown both CHIP and BAG1 in craf\(^{DA/DA}\) MEFs, to assess their involvement in the degradation of CRaf. In order to ensure the siRNA
Figure 3.16 **CRAF is ubiquitinated.**

Expression vectors for human myc-tagged CRAF<sup>WT</sup>, CRAF<sup>K375M</sup> or CRAF<sup>S621A</sup> were transfected into craf<sup>-/-</sup> cells with or without HA-tagged ubiquitin. Cells were treated with lactacystin for 5 h and protein lysates were prepared from soluble and insoluble Triton-X100 fractions. CRAF was immunoprecipitated using the α-myc antibody. Immunoprecipitated material was analysed with antibodies for HA and CRAF. A laddering pattern is seen above 74kDa, the molecular weight of CRAF in the lanes co-transfected with HA-ubiquitin indicating that CRAF is ubiquitinated. The data shown are representative of three independent experiments.
delivery conditions used were optimal, 100nM siGLO was transfected into cells. This reagent is an indicator of transfection efficiency and hence siRNA uptake. Cells which have been transfected fluoresce red, allowing the percentage efficiency of siRNA transfection to be determined by fluorescence microscopy, which was found to be approximately 75% in crafDA/DA MEFs (data not shown).

The concentration of CHIP siRNA was optimised by transfecting crafDA/DA cells with different concentrations of siRNA ranging from 0.1 to 100 nM (Figure 3.17 A). Protein lysates were harvested 24 h post transfection and western blots were conducted to analyse the expression of CHIP. It was found that 100nM of siRNA produced the greatest knockdown of the protein. It would be undesirable to use more than 100nM of siRNA due to the accumulation of non-specific off target effects which occur with higher concentrations of siRNA. As a control, the same concentration of non-targeting siRNA did not show a knockdown of CHIP, indicating the specificity of the siRNA. The 100nM optimised concentration of siRNA was used in subsequent experiments, in which siRNA was transfected into crafDA/DA MEFs. Protein lysates were harvested 24 h post transfection and western blots were conducted to analyse the expression of CRaf in cells with reduced CHIP expression. The knockdown of CHIP did not lead to an increase in the expression of CRafDA (Figure 3.17 B), suggesting that CRaf is not uniquely ubiquitinated by CHIP.

These data are supported by the fact that the expression of the kinase inactive CRAFKM construct is not stabilised in chip−/− cells (Figure 3.18). The vector expressing the CRAFKM375M mutant was co-transfected into chip+/− or chip−/− immortalised lung fibroblasts (obtained from Dr. C. Patterson, University of North Carolina at Chapel Hill, USA) with a vector expressing GFP. Transfection efficiencies were confirmed to be ~50% for each cell line by microscopy. Protein lysates were then harvested at 24, 48 and 72 hours post transfection and Craf was immunoprecipitated with an antibody for the myc tag. Craf expression was analysed by western blot which demonstrated that, even in the complete absence of CHIP, Craf
Figure 3.17 **CRAf\textsuperscript{DA} degradation by the proteasome does not rely on CHIP.**

(A) Cells were either mock transfected (M) or transfected with varying concentrations of CHIP or control siRNA as indicated to identify optimal conditions for the knockdown of CHIP. 24h post transfection protein lysates were harvested and western blots were conducted with the antibodies indicated. Concentrations of siRNA above 10nM knocked down CHIP expression, with 100nM CHIP siRNA providing the greatest reduction in CHIP expression levels. (B) Cells were either mock transfected (M) or transfected with the optimised concentration (100nM) of CHIP siRNA, or control siRNA. 24h post transfection protein lysates were harvested and western blots were conducted with the antibodies indicated. Although CHIP was knocked down, CRaf expression was not increased. The data shown are representative of five independent experiments.
Figure 3.18 CHIP is not required for the proteasomal degradation of CRAF<sup>K375M</sup>.

A vector expressing CRAF<sup>K375M</sup> was co-transfected into chip<sup>+/+</sup> or chip<sup>−/−</sup> immortalised lung fibroblasts with a vector expressing GFP. Transfection efficiencies were confirmed to be ~50% for each cell line by microscopy (data not shown). Protein lysates were harvested at 24, 48 and 72 h post transfection and CRAF was immunoprecipitated with an antibody for the myc tag. CRAF expression was analysed by western blot. Even in the complete absence of CHIP, CRAF<sup>K375M</sup> expression was not increased compared to its expression in cells expressing CHIP. The figure shown is representative of three experiments.
expression was not increased. Indeed in chip<sup>−/−</sup> cells, rather than an increase in CRAF<sup>KM</sup> expression, the expression of CRAF<sup>KM</sup> was found to be consistently reduced at each time-point. An explanation for this may be that in chip<sup>−/−</sup> cells, there may be compensation and up-regulation of additional unidentified E3 ubiquitin ligases that target CRAF and in fact promote its degradation.

Furthermore, the knockdown of the ubiquitin binding protein BAG1 by siRNA also did not rescue CRaf<sup>DA</sup> levels (Figure 3.19). The concentration of BAG1 siRNA was optimised by transfecting varying concentrations of BAG1 siRNA into cells, harvesting protein lysates and monitoring the expression levels of BAG1 by western blot (Figure 3.19 A). In the course of this analysis it was found that the greatest degree of BAG1 knockdown occurred 72 h post transfection. Non-targeting control siRNA did not show a significant knockdown of the protein at concentrations up to 100nM, indicating the specificity of the siRNA. It was found that 100nM of BAG1 siRNA produced only a slightly greater knockdown of the protein than 50nM siRNA. Since the lower the concentration of siRNA used the fewer off target events expected, a compromise of 75nM siRNA was used in subsequent experiments, in which siRNA was transfected into craf<sup>DA/DA</sup> MEFs. Protein lysates were harvested 72 h post transfection and western blots were conducted to analyse the expression of CRaf<sup>DA</sup> in cells with reduced BAG1 expression. However BAG1 knockdown did not lead to an increase in the expression of CRaf<sup>DA</sup> (Figure 3.19 B). Thus, although kinase inactive CRaf is misfolded, ubiquitinated and degraded by the proteasome, this process is not uniquely modulated by CHIP and BAG1.

### 3.4 Conclusions

The protein expression of CRaf is reduced in craf<sup>PA/DA</sup> embryos and MEFs. Analysis of the expression of components of the ERK cascade by western blot in craf<sup>+/−</sup> and craf<sup>PA/DA</sup> MEFs
Figure 3.19 **CRAF degradation by the proteasome does not rely on BAG1.**

**(A)** Cells were either mock transfected (M) or transfected with varying concentrations of BAG1 or control siRNA as indicated to identify optimal conditions for the knockdown of BAG1. 72h post transfection protein lysates were harvested and western blots were conducted with the antibodies indicated. Concentrations above 50nM knocked down BAG1 expression.  **(B)** Cells were either mock transfected (M) or transfected with the optimised concentration (75nM) of BAG1 siRNA, or control siRNA. 72h post transfection protein lysates were harvested and western blots were conducted with the antibodies indicated. Although BAG1 was knocked down, CRAF expression was not increased. The data shown is representative of three experiments.
demonstrated that the reduction in expression is specific to CRafDA. Quantitation of the expression of CRaf indicates that CRafDA protein expression is reduced by over 40 fold compared to the wild-type protein. A similar reduction of CRafDA expression of over 35-fold is also observed in protein lysates derived from embryos expressing the mutant protein. This supports previous preliminary data obtained by Dr. K. Mercer that demonstrated CRaf expression was reduced in crafDA/DA MEFs. A consequence of the low expression levels of kinase-inactive CRaf is that crafDA/DA embryos have a similar phenotype to that of craf-/- embryos (Figure 3.3) (Huser et al., 2001, Mikula et al., 2001). Like craf-/- embryos, the crafDA/DA embryos are developmentally retarded and have widespread apoptosis of embryonic tissues. This results in embryonic lethality at E10.5–E12.5 on the C57BL6 genetic background. crafDA/DA MEFs also demonstrate increased levels of apoptosis in response to serum withdrawal, etoposide, and α-CD95 antibody. However, similar to the craf-/- phenotype, ERK activation is not disrupted in crafDA/DA cells, indicating that the MEK kinase activity of CRAF is not essential in suppressing apoptosis.

Altered regulation of CRaf mRNA production or stability, translation, or protein stability may be responsible for the observed reduction in CRafDA expression. However using qPCR, craf mRNA levels were not found to be reduced in crafDA/DA cells and crafDA mRNA was not alternatively spliced, demonstrating that CRaf does not regulate itself at the mRNA level in a kinase dependent manner, and the targeting event itself does not disrupt craf mRNA production, stability or cause differential splicing.

With the regulation of CRaf occurring at the RNA level ruled out, CRaf protein stability was investigated. Kinase inactive CRAF was found to have a t½ of 40-60% that of the wild-type protein, indicating that CRaf regulates its protein stability in a kinase dependent manner. The kinase inactive protein was shown to associate with the chaperone protein HSP90 to a greater extent than the wild-type protein, indicating that in the absence of its kinase activity, CRaf is a misfolded and unstable protein. Indeed the use of proteasome inhibitors
demonstrated that the reduction in CRaf protein expression may be due to increased degradation by the proteasome in craf<sup>DA/DA</sup> MEFs. In addition CRAF was shown to be ubiquitinated, but this does not occur exclusively via the actions of the E3 ubiquitin ligase CHIP and its co-chaperone BAG1. The data in this chapter indicate that CRaf kinase activity is required for regulating its own protein expression by preventing its own proteasomal degradation.

The average t½ of ectopically expressed CRAF<sup>WT</sup> presented here by 35S met/cys pulse chase experiments was found to be 81 min. This figure is much lower than the t½ of CRaf found in the literature of 17.5 h in CHP100 cells, 11 h in MCF7 cells (Schulte et al., 1995) and approximately 30 h in human coronary artery smooth muscle cells (Schumacher et al., 1998). These values were all obtained by 35S met/cys pulse chase experiments. However, there does seem to be a great deal of variation between the published values of CRaf t½. It is possible that the different assay conditions and the different cell types used affect the value obtained. Even so the t½ of CRAF presented here is low in comparison with these values. This difference may be explained because the experiments performed by Schulte et al (1995) and Schumacher et al (1998) focused on endogenous CRaf, whereas the value of 81 min obtained here was determined for ectopically expressed CRAF<sup>WT</sup>. In this system, the over-expression of CRAF<sup>WT</sup> may have the effect of titrating out proteins with chaperone and stabilising activities, thus rendering CRAF more unstable. Indeed when the t½ of endogenous wild-type CRaf was investigated by 35S met/cys pulse chase experiments, its t½ was approximately 7 h, a time more consistent with the published literature.

The experiments to determine CRaf t½ in the literature were all conducted using an 35S met/cys pulse chase methodology, and no other experimental strategy has been used to assess the t½ of CRaf. However, due to the time constraints of this project, it was not always possible to use this methodology. When the t½ of ectopically expressed CRAF<sup>WT</sup> was determined using an emetine bock, the average value was found to be 16 h 40 min and the
\( t_{1/2} \) of CRAF\(^{DA} \) was found to be 5 h. This value is not reconcilable with the value of 81 min for CRAF\(^{WT} \) and 54 min for CRAF\(^{DA} \) obtained by \(^{35}\)S met/cys pulse chase. The only explanation in the difference between these values can be due to the different assay conditions used. Indeed similar discrepancies have been observed in the measurement of other \( t_{1/2} \) by different strategies in the literature. Gannon et al (1998) measured the \( t_{1/2} \) of p34\(^{cdc2} \) and found it to vary from 6 h to 50 h dependent upon the cell line and the method used. The \( t_{1/2} \) for p34\(^{cdc2} \) determined by the addition of a protein synthesis inhibitor (cycloheximide) in an experiment analogous to the emetine experiments performed here was 21 h in SV3T3 cells and 20 h in 3T3 cells. However they determined that the average \( t_{1/2} \) when measured by \(^{35}\)S met/cys pulse chase experiments in SV3T3 cells was 9 h, with values ranging from 6 to 12 h. In the non transformed 3T3 cell line, the average \( t_{1/2} \) of this protein increased to 33 hours, with a range of between 18 and 50 h, after 6 independent experiments were performed (Gannon et al., 1998). This indicates that the stability of a protein varies with the exact cell type and growth conditions and furthermore that different methodologies produce different results. This raises the question as to which method can be used to assess the true \( t_{1/2} \) of a protein. Since in the experiments presented here CRAF was ectopically expressed, the determination of the actual \( t_{1/2} \) of CRaf is moot, because the over expression of this protein results in the reduction of its stability due to the relative dilution of chaperone proteins. Importantly both the \(^{35}\)S met/cys pulse chase and emetine methodologies show a similar decrease in the \( t_{1/2} \) of the kinase inactive CRAF\(^{D486A} \) mutant, and so although values cannot be directly compared between the methodologies, relative comparisons of stability are possible.

The data presented here demonstrate that CRaf is ubiquitinated. Furthermore analysis of the CRaf primary sequence indicates that the protein contains a PEST domain in the variable region between CR2 and CR3 at residues 284-309. These are sequences within proteins which serve to promote degradation (Rechsteiner and Rogers, 1996). Furthermore, a lysine at residue 309 may serve as a ubiquitin attachment site, although this remains to be
proven. The PEST domain found in CRAF is not conserved in ARaf or BRaf, although BRaf does contain a PEST sequence within residues 298-338 in the variable region between CR1 and CR2, suggesting that it may also be regulated by protein degradation. Previous reports have also demonstrated that CRAF is ubiquitinated and targeted for degradation by the proteasome (Schneider et al., 1996, Demand et al., 2001, Schulte et al., 1997, Manenti et al., 2002, Du et al., 2006).

Schulte et al (1997) treated cells with geldanamycin, an inhibitor of the chaperone activity of HSP90, and demonstrated a rapid degradation of CRAF, thus supporting data that HSP90 stabilises the CRAF protein. Furthermore, it was shown that only treatment with proteasome inhibitors prevented such CRAF degradation whereas treatment with other protease inhibitors did not, highlighting the role of the proteasome in the degradation of CRAF (Schulte et al., 1997). Interestingly, in this study CRAF in geldanamycin- and proteasome inhibitor-treated cells was mainly insoluble in NP40 or Triton-X100 bases buffers, but was soluble in SDS buffers. This was in contrast to CRAF in untreated cells which was found to be distributed in the soluble and insoluble fractions equally. Schulte et al (1997) excluded the possibility that the addition of the proteasome inhibitors caused CRAF to become insoluble, but the possibility remains that CRAF solubility may be altered by its instability or the loss of HSP90 binding. Furthermore the authors claimed that CRAF appeared to be unique in its accumulation in the insoluble fraction when its degradation was prevented, in comparison with other geldanamycin targets such as the IGF-1 receptor, glucocorticoid receptor, p185^{erbB2}, and mutant p53, which are also degraded by the proteasome in a drug-dependent manner. The results presented in this study led to the investigation of Triton-X100 insoluble CRAF in this project. Analogous with the results of Schulte et al (1997), untreated wild-type CRAF appeared to be distributed equally between the soluble and insoluble fractions (Figure 3.14C). This distribution may be representative of active and inactive populations of CRAF; the inactive protein is thought to be cytoplasmic and upon RAS activation translocates to the plasma membrane. Cytoplasmic proteins are known to be soluble in detergents including
Triton-X100, whereas the majority of the cytoskeleton and specific membrane micro-domains are insoluble. Stokoe et al (1994) observed that the active fraction of CRaf resides in a detergent-insoluble compartment, and furthermore the insoluble CRaf identified by Schulte et al (1997) had enzymatic activity as it restored RAF/MEK/ERK signalling in geldanamycin treated NIH3T3 cells induced by PMA.

Furthermore, although the expression of CRafDA is reduced, the distribution between the soluble and insoluble fractions does not appear to be affected; the protein was present in approximately equal amounts in both fractions. However when degradation of CRafDA was prevented by the addition of proteasome inhibitors, the rescue of Triton-X100 insoluble CRaf was greater than that of soluble CRaf. This effect is most clearly demonstrated when cells were treated with MG132. This suggests that unstable CRaf moves to the insoluble fraction and this unstable CRaf is degraded by the proteasome preferentially; meaning that such protein may not be able to accumulate as seen in the distribution of untreated CRafDA. However the inhibition of the proteasome allows insoluble CRaf accumulation. The location of CRafDA (and CRaf in cells treated with geldanamycin) in the insoluble fraction is not thought to be due to the activation of the protein at the plasma membrane, because such activation is not possible in the case of CRafDA. However it may be due to abnormal protein binding events which occur to the instable protein connecting the protein to insoluble membrane components or the cytoskeleton. It is possible that the association with ubiquitin moieties attached to instable CRaf causes such a redistribution of its location, although Figure 3.17 clearly demonstrates ubiquitinated CRaf present in the soluble fraction.

CRaf has also been shown to be ubiquitinated and degraded by the proteasome when cell anchorage is disrupted (Manenti et al., 2002). However, the authors did not demonstrate an increase in the expression of CRaf when adherent cells were treated with proteasomal inhibitors, suggesting that in adherent culture conditions the expression of CRaf does not depend on the proteasomal activity. This contradicts the results presented here, although
the authors did show that CRaf was ubiquitinated in both adherent and non-adherent populations of cells. It is unknown why the authors of this paper did not see an increase of CRaf expression in adherent cells when treated with proteasomal inhibitors. In Figure 3.14 B the expression of wild-type CRaf was raised upon treatment with MG132. However no increase in the expression of wild-type CRaf was observed in cells treated with lactacystin. This demonstrates the efficacy of different inhibitors against the proteasome. Thus the results obtained with one inhibitor may not exactly mirror the results obtained by another. It was for this reason a panel of inhibitors were used here to assess the contribution of the proteasome in the degradation of CRaf. It is also possible that in the system the authors used, the turnover of CRaf was lower than occurred in the MEFs investigated here and so any increase in CRaf expression upon treatment with an inhibitor was small.

The molecular weight of ubiquitinated forms of CRaf identified by Manenti et al (2002) corresponded to the addition of two ubiquitin molecules per molecule of CRaf, which is not consistent with the expectation of higher molecular weight poly-ubiquitinated populations of CRaf produced prior to proteasomal degradation. The authors postulated that this absence is due to the instability of the modification. In Figure 3.17, a laddering effect is seen in all samples co-expressed with ubiquitin, which indicates the presence of multiple ubiquitinated forms of CRaf. However, distinct bands are present at approximately 80kDa and 250kDa. The bands at ~80kDa probably correlate with mono-ubiquitinated CRaf. However the bands present at 250kDa may be due to the attachment of around 20 ubiquitin molecules.

It is known that poly-ubiquitination marks proteins for proteasome mediated degradation if the linkage of the polyubiquitin chains occurs at Lys-48, whereas poly-ubiquitination at Lys-63 signals for other functions including DNA repair or activation of transcription factors. Mono-ubiquitination of proteins mediates processes such as endocytosis and histone regulation. From the data presented here, it is impossible to distinguish between multiple
events of mono-ubiquitination at many sites on CRaf or the formation of polyubiquitin chains, and it is also impossible to identify the site for ubiquitin linkage in such poly-ubiquitin chains.

CRaf expression has also been shown to be reduced by treatment with methylglyoxal, via a ubiquitin-mediated proteasomal degradation process (Du et al., 2006). This study indicated that methylglyoxal, a physiological glucose metabolite caused the degradation of CRaf by a pathway distinct to the others described in the literature and to the degradation observed here. The authors described the down-regulation of CRaf upon its activation, whereas the data presented here indicates that active CRaf is more stable than CRaf which has an inactive kinase domain. They show that this form of proteasomal degradation is dependent upon phosphorylation by PKC. Furthermore this form of regulation did not involve altered binding of CRaf to HSP90. These data would suggest that more than one mechanism of CRaf proteasomal degradation exists and these mechanisms are regulated in distinct ways.

It is known that CRaf binds to chaperones including HSP90, HSP70 and p50cdc37, which assist and maintain the correct tertiary structure of the protein (Kolch, 2000, Wartmann and Davis, 1994). Furthermore the dissociation of the CRaf/HSP90 complex, caused for example by treatment with geldanamycin, leads to the ubiquitination and degradation of CRaf (Schulte et al., 1995, Schulte et al., 1997). A model has been proposed for how these HSP90 client proteins are able to achieve a stable tertiary structure, or become ubiquitinated and degraded (Powers and Workman, 2006). The client protein forms an initial immature complex with chaperones including HSP70 and later HSP90. Activation of the ATPase activity of HSP90 results in a conformational change in the protein which allows transient dimerisation. This leads to HSP70, and associated chaperone dissociation and enables the ATP-dependent association of p50cdc37 among other chaperones, forming a mature complex. In this state, the client protein is correctly folded such that it can become active. If the correct folding of the client protein fails to occur, HSP70 does not dissociate and mediates the targeting of the client protein to the proteasome for degradation. The E3 ubiquitin ligase
CHIP has been shown to interact with HSP70 and HSP90 and stimulate the degradation of chaperone substrates (Connell et al., 2001, Demand et al., 2001, Murata et al., 2003). This occurs by the recruitment of the Ubc4/5 family to the chaperone complex, which are E2 ubiquitin-conjugating enzymes. CHIP then acts as an E3 ubiquitin ligase by adding ubiquitin residues to the chaperone substrate, therefore inducing its targeting to the proteasome. BAG1, a ubiquitin domain binding protein and co-chaperone of CHIP facilitates the targeting of the ubiquitinated protein to the proteasome (Demand et al., 2001, Song et al., 2001).

This chapter investigated if this pathway was involved in the increased turnover of CRafDA. It was found that the kinase inactive protein associated with HSP90 to a greater extent than the wild-type protein. However no noticeable increase in the association with HSP70 was observed. Even so, the proteasome did appear to mediate the increased degradation of CRafDA. The data presented here support data in the literature showing that CRaf can be ubiquitinated (Manenti et al., 2002, Schulte et al., 1997, Du et al., 2006) and since CRaf is a HSP90 client protein, and is known to bind BAG1, the best candidate for the CRaf E3 ubiquitin ligase was CHIP. Indeed previous reports demonstrate that CHIP can ubiquitinate CRaf (Demand et al., 2001). However the data presented here demonstrates that CHIP and BAG1 knockdown by siRNA did not restore the expression of CRafDA. This would seem to contradict a more recent investigation which demonstrated that knockdown of CHIP by siRNA stabilised CRaf in HeLa cells (Dogan et al., 2008). Indeed the authors observed a ~2 fold increase of CRaf expression. However it has been shown here that CHIP does not uniquely mediate the ubiquitination and degradation of CRaf, suggesting that CRaf can be targeted for degradation by alternative mechanisms. It is likely that other proteins can also ubiquitinate CRaf. Indeed the reduction of expression of CRAFKM in chip−/− cells indicates that other E3 ubiquitin ligases can compensate for the lack of CHIP activity. However the nature of other pathways leading to CRaf degradation by the proteasome, and particularly the E3 ubiquitin ligases involved, are currently unknown.
4. CRAF IS REGULATED BY AUTOPHOSPHORYLATION OF SERINE 621

4.1 Introduction

4.1.1 CRAf phosphorylation sites

CRAf activity is believed to be regulated by its phosphorylation status in a reversible manner, a concept introduced in Chapter 1. A number of phosphorylation sites have been identified which positively or negatively regulate kinase activity. Sites that are phosphorylated to mediate activation of CRAf include S338 and Y341, which are located in the N-region. It has been proposed that phosphorylation of these residues introduces a negative charge which is necessary to overcome the inhibitory function of the N terminus on the kinase domain (Tran and Frost, 2003). Additional sites which are phosphorylated upon CRAf activation include T491 and S494 located within the activation loop of the kinase domain. Several sites are phosphorylated to suppress CRAf activity including S43, S233 and S259 and the dephosphorylation of these negatively regulating residues is required for CRAf activation.

In the analysis of CRAf by western blot, CRAfDA has been consistently observed to migrate faster in SDS-PAGE than CRAfWT (Figures 3.5A, 3.6, 3.14C). An explanation for this may be that CRAfDA lacks one or more phosphorylation events that occur on the wild-type protein. Data in the literature have already demonstrated a role for phosphorylation events in the direct regulation of CRAf kinase activity. However, it may be that such phosphorylation events may also contribute by regulating the stability of the protein itself. In addition the results presented in Figure 3.5 demonstrated that the best characterised effector pathway of CRAf, the MEK/ERK pathway, is not altered in crafDA/DA cells and therefore is likely not to be responsible for the stabilisation of CRAf. Therefore the phosphorylation status of kinase inactive CRAf was investigated to determine if an abnormal phosphorylation status was
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responsible for its reduced stability. Furthermore data presented in Chapter 3 showed that kinase activity of CRaf was implicated in the protein’s stability. Therefore, analysis of the status of autophosphorylation sites in CRaf became a priority. At the time of this investigation, the best characterised site at which this occurs is S621, a binding site for 14-3-3.

4.1.2 Phosphorylation of S621

Morrison et al., (1993) identified S621 as a site in CRaf that was phosphorylated in mammalian cells and in Sf9 insect cells infected with a recombinant baculovirus encoding human CRAF by phospho-peptide mapping. They found that S621 was constitutively phosphorylated in resting cells and cells treated with the growth factor, PDGF. This study demonstrated that the phosphorylation of S621 was uncompromised in the kinase dead CRAF mutant (CRAF<sup>K375M</sup>) expressed in insect cells (Morrison et al., 1993), therefore implying S621 is not auto-phosphorylated and requires the presence of a second kinase to phosphorylate this residue.

Subsequent studies proposed protein kinase A (PKA) and AMP-activated protein kinase (AMPK) as S621 kinases (Mischak et al., 1996, Sprenkle et al., 1997). Sprenkle et al., (1997) used the synthetic peptide SLPKINRSAS<sup>S621</sup>EPSLHRR to screen for S621 kinases in extracts from NIH 3T3 cells. The authors identified two peaks of S621 peptide kinase activity in fractions from MonoQ chromatography of cytoplasmic supernatants prepared from these cells. Interestingly the minor peak of S621 peptide kinase activity was inhibited by Walsh PKI peptide (an inhibitor of PKA) demonstrating that S621 is also phosphorylated by PKA in vitro to some degree. However the major peak was identified to correspond to AMPK by the immunodepletion of AMPK in the MonoQ fractions and subsequent depletion of the major peak of S621 peptide kinase activity. It was also shown that AMPK phosphorylates GST-22W CRaf (consisting of residues 306–648) on S621 in bacteria. Furthermore using phosphopeptide mapping, the partially purified kinase-defective CRaf<sup>K375M</sup> from Sf9 cells
was observed to be phosphorylated on S621 by AMPK. Indeed the sequence surrounding S621 conforms to the AMPK consensus (Dale et al., 1995). However, as is the case for PKA, the phosphorylation of S621 by AMPK has not been demonstrated in vivo.

Reports in the literature have established that an elevation of cAMP levels correlates with the inhibition of CRaf. One way in which this effect can be mediated is through the activation of PKA which is implicated in the phosphorylation of CRaf on S43 (Morrison et al., 1993, Wu et al., 1993), S259 (Dhillon et al., 2002) and S621 (Hafner et al., 1994, Mischak et al., 1996). Mischak et al (1996) co-expressed CRaf with the constitutively active catalytic subunit of PKA. They identified that PKA-phosphorylated CRaf had a reduced affinity for RAS.GTP as well as impaired catalytic activity. However, using CRaf mutants that cannot bind RAS because of a point mutation of arginine 89 or deletion of the regulatory domain, the authors demonstrated that the downregulation of CRaf kinase activity by PKA occurs independently of RAS. Using mass spectroscopy and point mutants, the authors mapped the PKA phosphorylation site to S621. However the phosphorylation of S621 by PKA was only demonstrated in vitro and evidence for the forskolin (a PKA agonist) or cAMP-stimulated phosphorylation of S621 in vivo was not presented in this investigation. Conversely, phospho-peptide mapping of CRaf in forskolin-stimulated cells showed no increase in the phosphorylation of S621 (Sidovar et al., 2000). Furthermore this study observed that the region containing the S621 phosphorylation site lacks the optimal consensus sequence of two basic residues, rendering it a poor substrate for PKA. Indeed Sidovar et al (2000) observed the PKA mediated phosphorylation of a typsin-digested peptide containing S621 occurred at a rate approximately 100 times lower than that observed with the S43 peptide in vitro.

The data presented by Sidovar et al., (2000) agues against S621 being a valid PKA phosphorylation site in vivo and is consistent with this amino acid being a major autophosphorylation site in vivo. Moreover, even the study by Mischak et al (1996)
demonstrated that the purified kinase domain displayed autophosphorylation of S621, although the authors observed that this mode of phosphorylation was not found to be as efficient as PKA in targeting S621. Indeed studies using phospho-antibodies support a view that phosphorylation of S621 is mediated by autophosphorylation and that S621 is not phosphorylated in insect, HeLa, or Sf9 cells expressing kinase dead CRaf (Mischak et al., 1996, Hekman et al., 2004, Thorson et al., 1998). Furthermore preliminary experiments by Thorson and Shaw cited by Thorson et al., (1998) demonstrate that the phosphorylation of S621 may occur in cis, since S621 phosphorylation of CRaf<sup>K375M</sup> could not be detected even when co-expressed with wild-type CRaf. It is unclear if phosphorylation of this site is subject to regulation. Initial studies reported that phosphorylation of S621 does not change due to activation of CRaf (Fabian et al., 1993), yet more recent work has indicated that this site is subject to rapid and transient regulation (Hekman et al., 2004). In this latter study Hekman et al., (2004) used immunohistochemical analysis with a phospho-specific antibody directed against S621 to demonstrate that S621 autophosphorylation occurs in the cytoplasm of PC12 cells within 30 seconds after stimulation with growth factors. Hence phosphorylation of S621 occurs before the translocation of CRaf to the plasma membrane. However the study by Mischak et al (1996) found autophosphorylation of S621 to only become detectable after 20 min upon incubation with ATP, although this analysis was performed in vitro.

**4.1.3 The role of S621 phosphorylation**

The role of S621 phosphorylation has been a subject of controversy. Claims that it acts as an activating site are supported by the observation that its mutation leads to loss of kinase activity (Thorson et al., 1998, Morrison et al., 1993). It is possible that phosphorylation at this site may be important in the structural conformation of the catalytic domain, or that binding of 14-3-3 to this residue is required for the kinase activity of CRaf. Morrison et al., (1993) expressed a CRaf<sup>S621A</sup> mutant in Sf9 cells, which was inactive under basal conditions, and was not stimulated by co-expression with RAS and Src<sup>Y527F</sup> as assayed by in vitro kinase assays (Morrison et al., 1993). Thorson et al., (1998) also expressed a CRaf<sup>S621A</sup> mutant by
infecting Sf9 cells with recombinant baculoviruses encoding the CRaf construct and MEK1. The CRaf\(^{S621A}\) mutant showed significantly reduced kinase activity by *in vitro* kinase assays which monitored MEK1 activation by CRaf, using kinase dead ERK as a substrate.

However phosphorylation of S621 has also been correlated with the ability of PKA to inhibit the kinase domain of CRaf (Mischak et al., 1996), leading to counter claims that it has an inhibitory role. Mischak et al., (1996) expressed the CRaf kinase domain as a GST fusion protein in COS-1 and Sf9 cells. Although this study also found that mutation of S621 to alanine or cysteine resulted in loss of CRaf activity, the authors argued that S621 also is a site of negative regulation. They observed that CRaf autophosphorylates on S621, hence they incubated the CRaf-kinase-domain-GST-fusion construct in kinase buffer in the presence or absence of ATP. This reaction mixture was tested for the ability to phosphorylate MEK2 by kinase assay. Consistent with the hypothesis that S621 is a site of inhibitory phosphorylation (therefore an increase in S621 phosphorylation, mediated by the autophosphorylation, should be accompanied by a decrease in catalytic activity) the authors observed that CRaf auto-inactivated upon prolonged incubation with ATP. CRaf incubated without ATP stably retained the ability to phosphorylate substrate whereas incubation with ATP progressively reduced its activity. Concurrently two-dimensional phosphopeptide mapping showed a time-dependent increase in the phosphorylation of S621. Furthermore CRaf which had been allowed to autophosphorylate when treated with serine/threonine-specific phosphatases led to a recovery of kinase activity which was accompanied with the dephosphorylation of S621. Mischak et al., (1996) suggested a dual role for S621; they concluded that S621 is essential for CRaf activity but that its phosphorylation mediates the inhibition of CRaf kinase activity by PKA.

S621 is the C terminal 14-3-3 binding site. The association between CRaf and 14-3-3 was identified by several groups (Fantl et al., 1994, Freed et al., 1994, Fu et al., 1994, Irie et al., 1994). The role of 14-3-3 binding is not fully understood. Many studies suggest that 14-3-3
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binding is essential for kinase activity (Fantl et al., 1994, Freed et al., 1994, Irie et al., 1994, Li et al., 1995, McPherson et al., 1999, Roy et al., 1998, Thorson et al., 1998, Yip-Schneider et al., 2000), however others suggest that it is not (Fu et al., 1994, Michaud et al., 1995, Suen et al., 1995). The discrepancy between these studies may in part be due to the presence of two main 14-3-3 binding sites in CRaf; S259 and S621, which appear to have opposing roles. Phosphorylation of S259 allows 14-3-3 binding to the CR2 domain; this appeared to suppress CRaf activity. Indeed when 14-3-3 binding to CR2 was disrupted the basal kinase activity of CRaf was increased (Light et al., 2002). However, phosphorylation of S621 allowed 14-3-3 binding to the CR3 domain and appeared to be essential for kinase activity (McPherson et al., 1999, Thorson et al., 1998, Tzivion et al., 1998, Yip-Schneider et al., 2000). Peptide displacement studies have demonstrated that active CRaf can be inactivated by phosphopeptides that displace 14-3-3 and the addition of recombinant 14-3-3 reactivates CRaf (McPherson et al., 1999, Thorson et al., 1998, Tzivion et al., 1998). Furthermore similar results were obtained using the isolated kinase domain, indicating that binding to CR3 is essential for activity (Yip-Schneider et al., 2000).

Interestingly Thorson et al., (1998) showed that in cells expressing wild-type 14-3-3, S621 was phosphorylated. However in cells expressing dominant negative forms of 14-3-3, very little S621-phosphorylated CRaf was detected. This may imply that 14-3-3 binding may protect such sites from phosphatases. The authors mutated the CRaf 14-3-3 binding site, and found that forms of CRaf that cannot bind 14-3-3 did not maintain phosphorylation on S621. Furthermore they found that the kinase activity correlated with S621 phosphorylation. The authors therefore proposed that their findings support the idea that 14-3-3 binding is required for CRaf kinase activity, at least in part, because it stabilises the S621 phosphorylation. Indeed, they found that CRaf expressed in HeLa cells was much less active than when it was expressed in Sf9 cells and immunoblotting confirmed that S621 phosphorylation of CRaf was much lower in HeLa cells than in Sf9 cells. Further experiments which co-expressed 14-3-3β with CRaf enhanced both the S621
phosphorylation of CRaf and its kinase activity. This suggests that the phosphorylation on S621 can be directly modulated by 14-3-3 expression levels. Furthermore the displacement of 14-3-3 using a zwitterionic detergent, Empigen-BB, reduced CRaf kinase activity, demonstrating the requirement of 14-3-3 for activity. Thorson et al., (1998) proposed that the inability to detect S621 phosphorylation when 14-3-3 function is inhibited suggests that CRaf is in a dynamic equilibrium between phosphorylated and unphosphorylated forms. S621 is phosphorylated by autophosphorylation but in the absence of 14-3-3, this phosphorylation is rapidly lost in the cell, presumably via the action of a phosphatase. This dephosphorylation is prevented by the binding of 14-3-3 to this site and allows the kinase to become competent for activation.

The binding of 14-3-3 to CRaf via phospho-S621 may also be important for CRaf to make contacts with other proteins including BCR, PKC and the cell cycle regulator Cdc25 (Conklin et al., 1995). Moreover the dimerisation of the Raf proteins has been shown to be dependent upon S621 phosphorylation implicating a role for 14-3-3. It has been demonstrated that the association of CRaf and BRaf was reduced when S621 in CRaf was substituted for alanine. However no change was observed using CRafS259A mutants. Hence 14-3-3 binding to phospho-S621 was suggested to be important for formation of the heterodimers. (Weber et al., 2001, Garnett et al., 2005, Rushworth et al., 2006). This heterodimerisation is important as both wild-type and oncogenic BRaf have been shown to activate CRaf, thus CRaf may mediate signals downstream of BRaf through this route.

These data pertaining to the S621 phosphorylation site are divergent. While some studies conclude that S621 phosphorylation requires a second kinase and that its phosphorylation has an inhibitory role, most evidence supports S621 as a site of autophosphorylation which is required for 14-3-3 binding and kinase activity. Therefore it seems unlikely that a phosphorylation site that is essential for CRaf activity would also be required for inhibition.
Further investigation is clearly required into the *in vivo* S621 kinase and its role in regulating CRaf activity.

### 4.2 Aims

The aim of this Chapter is to identify the mechanism of CRaf kinase-dependent regulation of protein expression.

### 4.3 Results

#### 4.3.1 Phosphorylation of S621 is absent in kinase inactive CRaf

The phosphorylation of S621 was first investigated in the CRaf\(^{DA}\) protein expressed in *craf\(^{DA/DA}\)* cells (Figure 4.1A). Endogenous CRaf in *craf\(^{+/+}\)* and *craf\(^{DA/DA}\)* MEFs was immunoprecipitated and the phosphorylation status of S621 was investigated in Triton-X soluble and insoluble fractions using an antibody specific for phosphorylated S621. Phosphorylation of S621 was found to be virtually absent in the CRaf\(^{DA}\) protein expressed in *craf\(^{DA/DA}\)* cells. Additionally, although the majority of CRaf\(^{WT}\) was detected in the soluble fraction and was phosphorylated on S621, a significant portion of CRaf\(^{WT}\) was in the insoluble fraction, but this was not phosphorylated on S621. This indicates that phosphorylation of S621 primarily occurs on protein in the soluble fraction. Equal amounts of total protein were immunoprecipitated, thus the loading for this experiment reflects the difference in expression of CRaf in *craf\(^{+/+}\)* and *craf\(^{DA/DA}\)* MEFs as seen previously and the difference in expression of soluble and insoluble CRaf.

The phosphorylation of S621 was also investigated in ectopically expressed wild-type and kinase inactive CRAF, using the CRAF\(^{S621A}\) mutant as a control (Figure 4.1B). Myc-tagged vectors expressing CRAF\(^{WT}\), kinase defective CRAF mutants, CRAF\(^{K375M}\) or CRAF\(^{D486A}\), or the CRAF\(^{S621A}\) mutant were transfected into immortalised *craf\(^{-/-}\)* MEFs. The constructs were transfected into *craf\(^{-/-}\)* MEFs in order to prevent any possible effects of endogenous CRaf in
Figure 4.1 Kinase inactive CRaf is not phosphorylated on S621.

(A) Soluble (S) and insoluble (I) protein lysates were produced from craf<sup>+/+</sup> and craf<sup>DA/DA</sup> MEFs which had been pre-treated with 0.5μM lactacystin for 5 h. CRaf was immunoprecipitated with a specific antibody and samples were analysed with P-S621 and CRaf antibodies by western blotting. The IgG light chain confirms equal loading of the samples. (B) CRAF<sup>WT</sup>, CRAF<sup>K375M</sup>, CRAF<sup>D486A</sup>, or CRAF<sup>S621A</sup> myc-tagged expression vectors were transfected into craf<sup>−/−</sup> immortalised MEFs. 24 h post transfection, lysates were harvested and immunoprecipitated for CRAF with an α-myc antibody. Loading was equalised for CRAF and phosphorylation of S621 was investigated by western blotting.
phosphorylating the transfected proteins. Protein lysates were immunoprecipitated with an antibody for the myc tag and western blots were conducted. In this experiment it was necessary to adjust the protein loading in order to obtain equivalent levels of CRaf in all samples because of the reduced stability of the CRAF mutants. The phosphorylation of S621 was seen to be virtually absent in both of the kinase inactive CRAF mutants. These data indicate that the kinase activity of CRaf is required for autophosphorylation of S621.

4.3.2 The phosphorylation of S259 and S338 are not affected in kinase inactive CRaf

The phosphorylation status of S259 and S338 were also investigated in ectopically expressed wild-type and kinase inactive CRAF (Figure 4.2A). The myc-tagged vectors expressing CRAFWT, CRAFK375M or CRADF486A, or the CRAFS621A mutant were transfected into immortalised craf-/- MEFs. Protein lysates were immunoprecipitated with an antibody against the myc tag and western blots were conducted with antibodies specific for the phosphorylated residues. It was again necessary to adjust the protein loading in order to obtain equivalent levels of CRaf in all samples. The phosphorylation of S259, a second 14-3-3 binding site, and S338 were not affected by the kinase inactivating mutations. Additionally, their phosphorylation was also not affected in the CRAFS621A mutant.

4.3.3 CRaf expression is not reduced in CRafT491A/S494A MEFs

It was not possible to examine the phosphorylation of the activation segment phosphorylation sites, T491 or S494, due to the lack of good phospho-antibodies for these sites. However it was possible to investigate whether mutation of these sites affected the stability of the protein. Expression vectors for the activation segment mutant CRAF T491A/S494A or CRAFWT were transfected into immortalised craf-/- cells in equal ratios. A vector expressing GFP was co-transfected into these cells, allowing the percentage efficiency of transfection to be determined by fluorescence microscopy. The percentage of cells transfected with each expression vector was approximately equal: the CRAFWT transfection efficiency was ~58% and the CRAF T491A/S494A transfection efficiency was ~60% (data not
Figure 4.2 The stability of CRAf is not dependent on the phosphorylation of S259, S338, T491 or S494.

(A) CRAF<sup>WT</sup>, CRAF<sup>K375M</sup>, CRAF<sup>D486A</sup>, or CRAF<sup>S621A</sup> myc-tagged expression vectors were transfected into <i>craf</i><sup>−/−</sup> immortalised MEFs. 24 h post transfection, lysates were harvested and CRAF was immunoprecipitated using an antibody for the myc tag. Loading was equalised for CRAF and phosphorylation of S259 and S338 was investigated by western blotting. (B) CRAF<sup>WT</sup>, or CRAF<sup>T491A/S494A</sup> expression vectors were transfected into <i>craf</i><sup>−/−</sup> immortalised MEFs. The transfection efficiencies were approximately equal: The CRAF<sup>WT</sup> transfection efficiency was ~58% and the CRAF<sup>T491A/S494A</sup> transfection efficiency was ~60%. 24 h post transfection, lysates were harvested and the expression of CRAF was analysed by western blotting. (C) CRAF<sup>WT</sup>, or CRAF<sup>T491A/S494A</sup> expression vectors were transfected into <i>craf</i><sup>−/−</sup>-immortalised MEFs. 24 h post transfection, lysates were harvested and CRAF was immunoprecipitated. The phosphorylation of S621 was analysed by western blotting.
shown). Total protein lysates were analysed by western blot, showing that the activation segment mutant CRAF\textsuperscript{T491A/S494A} was expressed at similar levels as CRAF\textsuperscript{WT} (Figure 4.2B). If the expression of CRAF\textsuperscript{T491A/S494A} was reduced in comparison with CRAF\textsuperscript{WT}, it could be said that these phosphorylation sites are required to stabilise the protein. However since the results demonstrate that the expression of CRAF\textsuperscript{T491A/S494A} is equal to CRAF\textsuperscript{WT}, it implies that these sites are not important for maintaining the protein’s stability.

In addition the phosphorylation status of S621 in CRAF\textsuperscript{T491A/S494A} and CRAF\textsuperscript{WT} was also compared (Figure 4.2C), in order to ensure that the CRAF\textsuperscript{T491A/S494A} mutant was still capable of phosphorylation of this residue. The human myc tagged expression vectors were transfected into craf\textsuperscript{−} cells. Protein lysates were immunoprecipitated with an antibody for the myc tag and western blots were conducted. The results show that even though the activation segment phosphorylation sites T491 and S494 have been mutated, CRAF\textsuperscript{T491A/S494A} is still able to phosphorylate S621 to similar levels as CRAF\textsuperscript{WT}.

4.3.4 CRaf expression is not reduced in CRaf\textsuperscript{FF/FF} MEFs

It was also impossible to study the phosphorylation of Y341 due to the lack of a good phospho-antibody for this site. However as with T491 or S494, it was possible to examine whether mutation of Y341 affected the stability of the protein through the use of MEFs with the Y340F/Y341F knockin CRaf mutation (CRaf\textsuperscript{FF}) (Huser et al., 2001).

A breeding colony of craf\textsuperscript{−/−} mice is maintained at the University of Leicester. Primary MEFs were produced from embryos at E13.5, resulting from a timed intercross of craf\textsuperscript{−/−} heterozygote animals. Each MEF line was generated from a single embryo and was genotyped by the removal of the tail for DNA extraction to be used in a PCR reaction using primers OCP 59 and OCP 173. The PCR product was run on a 2% agarose gel (Figure 4.3 B). The MEFs produced from these embryos were cultured and expanded.
Figure 4.3 Production and analysis of $craf^{Y340F/Y341F}$ mice and MEFs.

(A) A knockin approach was used to mutate the murine $craf$ gene leading to a Y340F/Y341F substitution in exon 11, indicated by *. In order to delete the neo$^R$ cassette, $craf^{+/FF}$ mice were crossed to CMV-Cre mice (Schwenk et al., 1995). Black arrow heads represent LoxP sequences. The primers used for identifying PCR reactions are indicated. (B) Primary MEFs were produced from embryos at E13.5, resulting from an intercross of $craf^{+/FF}$ heterozygote animals. Each MEF line was genotyped in a PCR reaction using primers OCP 59 and OCP 173. PCR products were run on a 2% agarose gel and an example of genotyping from one litter is shown. Three $craf^{+/+}$ embryos (lanes 1,3,8), five $craf^{+/FF}$ embryos (lanes 4,7,11,12,13) and five $craf^{FF/FF}$ embryos (lanes 2,5,6,9,10) were obtained. (C) Protein lysates were harvested from $craf^{+/+}$, $craf^{+/FF}$ and $craf^{FF/FF}$ MEFs and analysed for CRaf expression by western blotting. Mutation of Y340 and Y341 does not reduce CRaf stability.
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Protein lysates were produced from craf+/+, craf+/FF and crafFF/FF MEFs, and the expression of CRaf was analysed by western blot using actin as a loading control (Figure 4.3C). The expression of CRaf was not reduced in craf+/FF and crafFF/FF MEFs. In fact the expression of CRaf actually appears to be increased in craf+/FF and crafFF/FF MEFs. However this experiment was only performed once and therefore it is difficult to draw strong conclusions. However, these data suggest that Y340 and Y341, like T491 and S494, do not play a role in stabilising CRaf. Put together these data identify a role for S621, and not any of the other sites investigated so far, in the stabilisation of CRaf.

4.3.5 Sorafenib disrupts S621 phosphorylation and destabilises CRafWT

It is possible that the lack of S621 phosphorylation in kinase inactive CRaf is due to misfolding of the mutant protein in such a way as to sterically restrict the phosphorylation site, rather than the lack of CRaf kinase activity preventing this phosphorylation. To investigate this, the status of S621 phosphorylation and CRaf stability was assessed in cells treated with the Raf kinase inhibitor sorafenib. The use of sorafenib provides an alternative to using mutants, which may be misfolded. Sorafenib (also known as BAY 43-9006) is a multi-kinase inhibitor with activity against CRaf, BRaf, vascular endothelial growth factor receptor 2 (VEGFR2), platelet-derived growth factor receptor (PDGFR), FLT3, and c-Kit. However sorafenib is an extremely potent inhibitor of CRaf, having an IC50 of 2nmol/L.

In order to optimise the conditions for this experiment, immortalised craf−/− MEFs were transfected with a vector expressing myc tagged CRAFWT and 24 hours later cells were treated with sorafenib at concentrations of 0-20μM for 2 hours. Lysates were harvested and immunoprecipitated with a CRaf antibody. The loading was equalised for CRaf and western blots were then conducted to examine the phosphorylation status of S621 (Figure 4.4A). Visualisation of the cells by light microscopy did not indicate an increase in apoptosis as a result of treatment with up to 20μM of sorafenib (data not shown). Although the amount of total protein loaded was equalised between samples, the loading for this experiment was not
Figure 4.4 Sorafenib disrupts S621 phosphorylation and destabilises CRAf.

(A) Optimisation of conditions. Immortalised craf<sup>−/−</sup> cells were transfected with a vector expressing CRAFWT and 24 h later the cells were treated with 0-20μm sorafenib for 2 hours. Protein lysates were harvested and CRAF was immunoprecipitated. Loading was equalised for CRAF and the lysates were analysed by western blot with antibodies for P-S621 and CRAF. Concentrations of over 10μm sorafenib showed a decrease in the phosphorylation of S621. (B) Immortalised craf<sup>−/−</sup> cells transfected with CRAFWT were treated with 20μm sorafenib for 2 hours. In the panel on the left, CRAF was immunoprecipitated. Loading was equalised for CRAF and the lysates were analysed by western blot with antibodies for P-S621 and CRAF. Treatment with sorafenib resulted in a decrease in the phosphorylation of S621. The right hand panel shows total protein lysates which were analysed with antibodies for CRAF and actin. This shows a reduction in CRAF expression when cells were treated with sorafenib.
as equal as desired. The loading of the sample treated with 5μM sorafenib was higher than the other samples and this corresponds with a higher observed level of S621 phosphorylation. For the interpretation of these data, it is perhaps sensible to discount this sample and compare the phosphorylation of S621 between the other samples. When cells were treated with 10-20μM of sorafenib, the phosphorylation of S621 on CRAF\textsuperscript{WT} was consistently reduced. It was decided that a concentration of 20μM sorafenib would be used in future experiments in order to maximise the inhibition of CRaf kinase activity.

This experiment was repeated, treating cells with the optimised concentration of 20μM sorafenib (Figure 4.4B left hand panel). Again this demonstrated that sorafenib treatment reduced the phosphorylation of S621 to levels seen in kinase inactive CRaf. This indicates that the kinase activity of CRaf is responsible for phosphorylating this residue. Furthermore when total protein lysates from such sorafenib treated and untreated cells were examined by western blot, CRaf expression was found to be reduced in cells treated with sorafenib (Figure 4.4B right hand panel). This indicates a reduction in CRaf stability in sorafenib-treated cells which would be expected due to the decrease in S621 phosphorylation. This supports the hypothesis that the kinase activity of CRaf is required for autophosphorylation of S621, and this phosphorylation event is required for CRaf stability.

The reduction in stability of CRaf when treated with sorafenib was further investigated by determination of its t\textsubscript{1/2} (Figure 4.5). Due to time constraints this experiment was not performed using a \textsuperscript{35}S met/cys pulse chase experiment. Instead, the t\textsubscript{1/2} was determined using the protein synthesis inhibitor emetine. Immortalised \textit{craf\textsuperscript{-/-}} cells were transfected with a vector expressing CRAF\textsuperscript{WT} and 24 h later the cells treated with 20μm sorafenib for 2 hours. Emetine was added to the cell culture media and lysates were harvested at time-points up to 16 hours. Total protein lysates were analysed by western blot with antibodies for CRaf and actin. The optical density of bands on the X-Ray film were quantitated and the
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Figure 4.5 **Sorafenib destabilises CRAF.**

The stability, measured by $t_{1/2}$, of CRAF in cells treated with or without sorafenib were compared by emetine time-course experiments. *(A)* Immortalised $craf^{-}$ cells were transfected with a vector expressing $CRAF^{WT}$ and 24 h later the cells treated with 20$\mu$m sorafenib for 2 hours. Emetine was added to the cell culture media and lysates were harvested at time-points up to 16 hours. Total protein lysates were analysed by western blot with antibodies for CRAF and actin. A typical example from three independent experiments is presented. *(B)* The optical density of bands on the X-Ray film were quantitated. The percent reduction in optical density was determined and plotted. The $t_{1/2}$ was calculated as the time required for the optical density to decrease by 50%. Treatment with sorafenib reduced the stability of CRAF, the $t_{1/2}$ of CRAF in cells treated with sorafenib was ~34% of the $t_{1/2}$ of CRAF in untreated cells.

**A**

<table>
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<tr>
<th>control</th>
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Emetine + time (h)

- CRAF
- actin

**B**

Average $t_{1/2}$
- CRAF Control: 16h 40
- CRAF + Sorafenib: 5h 40

Optical density (% of 0 time)

- control
- sorafenib

Time (h)
percent reduction in optical density was determined and plotted. The \( t_{1/2} \) was calculated as the time required for the optical density to decrease by 50%. Treatment with sorafenib significantly reduced the stability of CRaf; the \( t_{1/2} \) of CRaf in treated cells was \(~34\%\) of the \( t_{1/2} \) of CRaf in untreated cells.

4.3.6 The stability of CRAF\(^{S621A}\) is reduced compared to CRAF\(^{WT}\)

To confirm a role of S621 phosphorylation in stabilising CRaf, the stability of the nonphosphorylatable CRAF\(^{S621A}\) mutant was examined by \(^{35}\)S met/cys pulse-chase labelling (Figure 4.6). Experiments were performed in an analogous way to the determination of the \( t_{1/2} \) of ectopically expressed CRAF\(^{K375M}\) and CRAF\(^{D486A}\) in Chapter 3. Vectors expressing myc-tagged CRAF\(^{WT}\) or CRAF\(^{S621A}\) were transfected into immortalised \( ^{craf^{-}} \) MEFs. Cellular protein in transfected cells was labelled with a \(^{35}\)S met/cys pulse, then cells were incubated in fresh media and lysates were harvested in the subsequent chase. CRAF was immunoprecipitated using an antibody for the myc tag and samples were electrophoresed through an SDS-PAGE gel. Gels were dried and exposed to X-Ray film at \(-80^\circ\)C. The optical density of bands on the X-Ray film was quantitated. The percent reduction in optical density was determined and plotted. The \( t_{1/2} \) was calculated as the time required for the optical density to decrease by 50%. In a similar way to kinase inactive CRaf, the CRAF\(^{S621A}\) mutant was found to be less stable than CRAF\(^{WT}\) with an average \( t_{1/2} \) of 24 min. Put together, these results indicate that the kinase activity of CRaf is required for S621 phosphorylation and, in its absence, an unstable protein is formed.

4.3.7 Neither ARaf nor BRaf act as an intermediate kinase for S621 phosphorylation

Although it has been shown that the phosphorylation of S621 requires its own kinase activity, it is conceivable than an intermediate kinase is involved whose activity is dependent upon the kinase activity of CRaf. Given the known heterodimerisation of the Raf kinases it was investigated whether ARaf or BRaf may play a role in phosphorylating and stabilising
Figure 4.6 **CRAF<sup>S621A</sup> has reduced stability.**

The stability, measured by t<sub>½</sub>, of wild-type and CRAF<sup>S621A</sup> were compared by <sup>35</sup>S met/cys pulse chase experiments. (A) Myc-tagged CRAF<sup>WT</sup> or CRAF<sup>S621A</sup> expression vectors were transfected into craf<sup>−/−</sup> cells. Cellular protein was labelled with a <sup>35</sup>S met/cys pulse and chased over a time-course of up to 195 min. CRAF was immunoprecipitated using the α-myc antibody and equal amounts of samples were electrophoresed through an SDS-PAGE gel. Gels were dried and exposed to X-Ray film at -80°C. A typical example from four independent experiments is presented. (B) The optical density of bands on the X-Ray film was quantitated. The percent reduction in optical density was determined and plotted. The t<sub>½</sub> was calculated as the time required for the optical density to decrease by 50%. The CRAF<sup>S621A</sup> mutant protein has reduced stability; CRAF<sup>S621A</sup> t<sub>½</sub> is ~30% of CRAF<sup>WT</sup>.

![Graph showing optical density over time for CRAF<sup>WT</sup> and CRAF<sup>S621A</sup>](image)
CRaf (Figure 4.7). Lysates were harvested from *araf*+/Y, *araf*−/Y, *braf*+/+ and *braf*−/− MEFs, kindly supplied by Prof. C Pritchard, and immunoblotted with antibodies against ARaf, BRaf and CRaf to investigate these proteins’ expression. Additionally, CRaf was immunoprecipitated and these samples were immunoblotted with an antibody specific for phosphorylated S621 to investigate the phosphorylation status of this site in cells lacking ARaf or BRaf. It was found that neither expression of CRaf nor phosphorylation of S621 was altered in MEFs with knockout mutations of ARaf or BRaf, suggesting that neither ARaf nor BRaf act as an intermediate kinase for the phosphorylation of S621.

4.3.8 AMPK is not a S621 kinase

AMPK has been previously suggested to be a S621 kinase. AMPK is a heterotrimeric αβγ enzyme consisting of catalytic α-subunit and non-catalytic β- and γ-subunits (Woods et al., 1996, Davies et al., 1994, Dyck et al., 1996). Its multi-subunit nature made over-expression of this kinase prohibitive. Therefore to investigate if AMPK was acting as a S621 kinase, *craf*DA/DA or *craf*+/+ cells were treated with 0-300μm of the AMPK agonist A-769662 for 1 hour. The phosphorylation of acetyl-CoA carboxylase provided a read-out of AMPK activation, and confirmed activation of AMPK following treatment with 100 and 300μm A-769662. However, CRafDA expression was not increased under these conditions as would be expected if S621 was phosphorylated (Figure 4.8A). The phosphorylation status of S621 was investigated when *craf*DA/DA cells were treated with 300μm A-769662, by immunoprecipitating CRaf from treated cells and analysing lysates with an antibody specific for phospho-S621. As expected by the unchanged expression of CRaf, treatment with A-769662 did not increase phosphorylation of S621 (Figure 4.8B).

4.3.9 PKA is not a S621 kinase

PKA has also been previously suggested to be S621 kinase. PKA is a tetramer which consists of two catalytic subunits and two regulatory subunits that bind cAMP. As a consequence, the regulatory subunits dissociate and release the active catalytic subunits
Figure 4.7 CRaf expression and S621 phosphorylation are not dependent on ARaf or BRaf.

(A) Protein lysates were prepared from *araf*+/Y and *araf*−/Y MEFs (provided by Prof. C Pritchard) and immunoblotted with antibodies against ARaf, BRaf and CRaf, using actin as a loading control (left hand panel). Additionally, CRaf was immunoprecipitated from such protein lysates and these samples were immunoblotted with an antibody specific for phosphorylated S621 to investigate the phosphorylation status of this site in cells lacking ARaf (right hand panel). (B) Lysates were harvested from *braf*+/+ and *braf*−/− MEFs (provided by Prof. C Pritchard) and western blots were performed with the same antibodies as before (left hand panel). CRaf was immunoprecipitated and samples were immunoblotted with an antibody specific for phosphorylated S621 to investigate the phosphorylation status of this site in cells lacking BRaf (right hand panel). Neither expression of CRaf nor phosphorylation of S621 was altered in MEFs with knockout mutations of ARaf or BRaf.
Figure 4.8 CRaf expression and S621 phosphorylation are not dependent on AMPK.

(A) craf$^{DA/DA}$ or craf$^{+/+}$ cells were treated with 0-300μm A-769662 for 1 h. Protein lysates were harvested and analysed with the antibodies indicated. Phosphorylation of acetyl-CoA carboxylase (P-ACC) provides a read-out of AMPK activation, confirming activation of AMPK following treatment with 100 and 300μm A-769662. However, CRaf$^{DA}$ expression is not increased under these conditions. (B) craf$^{DA/DA}$ or craf$^{+/+}$ MEFs were treated with 0 or 300μm A-769662 for 1 hour. Protein lysates were harvested and CRaf was immunoprecipitated. Lysates were analysed with antibodies for CRaf or phospho-S621. Treatment with A-769662 did not increase phosphorylation of S621. Due to the reduced stability of CRaf$^{DA}$, protein loading was adjusted to obtain equivalent levels of immunoprecipitated CRaf between craf$^{+/+}$ and craf$^{DA/DA}$ samples.
(McKnight, 1991). Again, because this kinase is a multi-subunit protein, it was decided that activation of endogenous protein using an agonist would be preferential to over-expression of PKA. In order to investigate if PKA is a S621 kinase, \textit{craf}^{DA/DA} or \textit{craf}^{+/+} cells were serum starved for 24 h and were then treated with the PKA agonist forskolin/IMBX for 20 min. Forskolin binds to and activates adenylate cyclases, whereas cAMP hydrolysis is prevented by IBMX (3-isobutyl-1-methylxanthine) leading to the activation of PKA. In order to confirm that PKA had been stimulated by the treatment with forskolin/IMBX, the phosphorylation of CRaf on S43 was assessed (Figure 4.9A) since PKA is known to phosphorylate CRaf on this residue. After serum starvation and treatment of \textit{craf}^{DA/DA} or \textit{craf}^{+/+} cells with forskolin/IMBX for 20 min protein lysates were harvested and CRaf was immunoprecipitated. Lysates were analysed by western blot. The increase in S43 phosphorylation by forskolin/IMBX shown here provides a read-out of PKA activation and confirms activation of PKA in both cell types. However when the phosphorylation of S621 was investigated, this was not increased by the treatment (Figure 4.9A). The results shown here indicate that the levels of S621 phosphorylation were unusually low in the \textit{craf}^{+/+} control sample in comparison with previous results. It is important to note that these cells had been serum starved for 24 h prior to harvesting lysates. The phosphorylation of S621 is known to be growth factor dependent. Therefore, in serum starved cells it can be expected that levels of phosphorylation would be low. Total protein lysates were also harvested and analysed for the expression of CRaf. Treatment with forskolin/IMBX did not raise the expression of CRaf^{DA} (Figure 4.9B). Thus PKA does not influence S621 phosphorylation or CRaf stability.

\textbf{4.3.10 Phosphorylation of S621 does not occur in trans}

CRaf is known to autophosphorylate on residue S621 and the results presented above have confirmed this. To further this investigation the possibility of S621 phosphorylation occurring \textit{in cis} or \textit{in trans} was assessed. If phosphorylation of S621 was found to occur \textit{in cis} it would exclude the possibility of the involvement of an intermediate kinase.
Figure 4.9 **CRaf expression and S621 phosphorylation are not dependent on PKA.**

craf+/+ and crafDA/DA cells were serum-starved for 24 hours and were then treated with the PKA agonist forskolin/IMBX (F/I) for 20 min or left untreated. (A) Protein lysates were immunoprecipitated for CRaf and analysed with antibodies for phospho-S621 or S43. The increase in S43 phosphorylation by F/I shown here provides a read-out of PKA activation and confirms activation of PKA in both cell types. The phosphorylation of S621 is not increased upon treatment with F/I. Levels of S621 phosphorylation are also low in craf+/+ cells, due to serum starvation. Again, protein loading of the immunoprecipitates was adjusted to obtain equivalent levels of immunoprecipitated CRaf between cell lines. (B) Total protein lysates were harvested and analysed for expression of CRaf. Treatment with F/I does not raise the expression of CRafDA.
Protein lysates were made from \textit{craf}^{+/+}, \textit{craf}^{+/DA} and \textit{craf}^{DA/DA} MEFs. Western blots were then conducted with a CRaf antibody to investigate the expression of CRaf (Figure 4.10 A). In addition lysates were also immunoprecipitated with a CRaf antibody and phosphorylation of S621 was investigated by western blot (Figure 4.10 C). Equal amounts of total protein were immunoprecipitated, thus the loading for this experiment reflects the difference in expression of CRaf in \textit{craf}^{+/+} and \textit{craf}^{DA/DA} MEFs as seen previously. If the phosphorylation occurred \textit{in trans}, it would be expected that in the \textit{craf}^{+/DA} MEFs the presence of CRaf\textsuperscript{WT} would be sufficient to phosphorylate both CRaf\textsuperscript{WT} and CRaf\textsuperscript{DA} on S621, so stabilising the protein. Thus the levels of CRaf in \textit{craf}^{+/DA} MEFs should resemble that seen in \textit{craf}^{+/+} MEFs. Conversely, if the phosphorylation occurred \textit{in cis}, only the CRaf\textsuperscript{WT} protein would be capable of phosphorylating itself, stabilising the protein. Hence, in \textit{craf}^{+/DA} MEFs the CRaf\textsuperscript{DA} protein would be incapable of S621 phosphorylation and would be rendered instable. Subsequently the expression of CRaf and phosphorylation of S621 should be intermediate between \textit{craf}^{+/+} and \textit{craf}^{DA/DA} MEFs. This latter scenario was found to occur and tends to suggest that the phosphorylation event occurs \textit{in cis}. The reduction in CRaf expression in \textit{craf}^{+/DA} and \textit{craf}^{DA/DA} MEFs was found to be significant by unpaired T tests, with P values of 0.0230 and 0.0414 respectively (Figure 4.10 B). Furthermore the data presented in Figure 3.6 in which western blots were conducted on protein lysates produced from whole \textit{craf}^{+/+}, \textit{craf}^{+/DA} and \textit{craf}^{DA/DA} embryos also appears to support the conclusion that the phosphorylation event occurs \textit{in cis}. These data also showed the expression of CRaf to be significantly reduced in \textit{craf}^{DA/DA} embryo lysates and intermediate in \textit{craf}^{+/DA} embryo lysates. However it is important to note that in both Figures 3.6 and 4.10 the expression of CRaf in \textit{craf}^{+/DA} cells was found to be less than half that observed in wild-type cells. This may imply that the CRaf\textsuperscript{DA} protein acts as a dominant negative and destabilises the CRaf\textsuperscript{WT} protein by an unknown mechanism, as discussed in Chapter 3.
Figure 4.10 Analysis of CRaf expression and S621 phosphorylation in \textit{craf}\textsuperscript{+/+}, \textit{craf}\textsuperscript{+/DA} and \textit{craf}\textsuperscript{DA/DA} MEFs.

(A) Protein lysates were harvested from \textit{craf}\textsuperscript{+/+}, \textit{craf}\textsuperscript{+/DA} and \textit{craf}\textsuperscript{DA/DA} MEFs. Total protein lysates were analysed by western blot with the antibodies indicated. The expression of CRaf was significantly reduced in \textit{craf}\textsuperscript{DA/DA} MEFs and intermediate in \textit{craf}\textsuperscript{+/DA} MEFs. (B) The expression of the proteins from 3 cell lines were quantitated using ImageJ. The reduction in CRaf expression in \textit{craf}\textsuperscript{+/DA} and \textit{craf}\textsuperscript{DA/DA} embryos is statistically significant with respective P values of 0.0230 and 0.0414 (by unpaired T test). Error bars denote the standard error in the values shown. (C) CRaf was immunoprecipitated from lysates harvested from \textit{craf}\textsuperscript{+/+}, \textit{craf}\textsuperscript{+/DA} and \textit{craf}\textsuperscript{DA/DA} MEFs using an antibody specific for the protein. Samples were analysed with antibodies specific for phospho-S621 and CRaf. The IgG light chain confirms equal loading of the samples. The phosphorylation of S621 is absent in \textit{craf}\textsuperscript{DA/DA} MEFs and intermediate in \textit{craf}\textsuperscript{+/DA} MEFs.
Further evidence for the cis phosphorylation of S621 was provided by investigating the phosphorylation of S621 in ectopically expressed kinase inactive CRAF. Myc-tagged CRAFWT or the myc-tagged kinase inactive CRAF mutants (either CRAFKM or CRAFDA) were transfected into immortalised craf−/− MEFs. Only the ectopically expressed CRAF and not the endogenous CRaf present in the cells was immunoprecipitated with an antibody specific to the myc tag. Lysates were immunoblotted with antibodies against CRaf, or phosphorylated S621. The kinase inactive CRAF mutants were phosphorylated at very low levels when compared to the phosphorylation of S621 observed in CRAFWT (Figure 4.11). Again this is the expected result if the phosphorylation occurred in cis. If the event did occur in trans, the endogenous CRafWT protein would be capable of phosphorylating the kinase inactive CRAF mutants, thus stabilising them.

Another experiment was performed in which kinase inactive CRAF (either CRAFKM or CRAFDA) was co-transfected with HA-tagged CRAFWT in equal ratios into immortalised craf−/− MEFs. The proteins were immunoprecipitated with their corresponding tags and immunoblotted with antibodies against HA, myc, CRaf, or phosphorylated S621. In contrast to CRAFWT, both the kinase inactive mutants were phosphorylated at very low levels even in the presence of equal amounts of CRAFWT (Figure 4.12A). This is expected if phosphorylation occurred in cis, as the kinase inactive mutants would be unable to phosphorylate themselves. Put together these data obtained by three different methodologies strongly exclude the possibility of there being an intermediate kinase and furthermore suggest that the phosphorylation of S621 occurs in cis.

However some phosphorylation of S621 can still be observed in the kinase inactive mutants in Figure 4.11. Furthermore when analysing longer exposures (Figure 4.12B) of results described in Figure 4.12 it can be seen that there is a small amount of HA-CRAFWT present in the myc immunoprecipitate and a small amount of myc-CRAFKM present in the HA immunoprecipitate. An explanation for this may be due to the formation of CRAF
Figure 4.11 **S621 phosphorylation does not occur in trans.**

**(A)** Vectors expressing either myc-tagged CRAF$^{WT}$, CRAF$^{K375M}$ or CRAF$^{D486A}$ were transfected into $craf^{+/+}$ cells. CRAF was immunoprecipitated and analysed with the antibodies indicated. S621 phosphorylation of kinase inactive CRAF is compromised when expressed in $craf^{+/+}$ cells. Protein loading of the immunoprecipitates was adjusted to obtain equivalent levels of immunoprecipitated CRAF so that levels of serine 621 phosphorylation between wild-type and mutant CRAF proteins could be directly compared.
Figure 4.12 **S621 phosphorylation does not occur in trans.**

**(A)** A vector expressing HA tagged **CRAF**\(^{WT}\) was co-transfected with either myc-tagged **CRAF\(^{K375M}\)** or **CRAF\(^{D486A}\)** into **craf**\(^{-/-}\) cells. Protein lysates were harvested and either **CRAF\(^{K375M}\)/CRAF\(^{D486A}\)** were immunoprecipitated with an antibody for the myc tag or **CRAF**\(^{WT}\) was immunoprecipitated with an antibody for HA. Immunoprecipitated material was analysed with the antibodies indicated. S621 phosphorylation of kinase inactive **CRAF** is still compromised when co-expressed with **CRAF**\(^{WT}\). **(B)** A longer exposure of the X-ray film of the experiment described in **(A)** shows phosphorylation of S621 in the kinase inactive mutant and also a small amount of HA-CRAF\(^{WT}\) present in the myc immunoprecipitate and a small amount of myc-CRAF\(^{KM}\) present in the HA immunoprecipitate. This is possibly due to the formation of CRAF homodimers between **CRAF**\(^{WT}\) and the kinase inactive CRAF mutant. In both **(A)** and **(B)** protein loading of the immunoprecipitates was adjusted to obtain equivalent levels of immunoprecipitated **CRAF** so that levels of serine 621 phosphorylation between wild-type and mutant **CRAF** proteins could be directly compared.
homodimers between CRAF\textsuperscript{WT} and kinase inactive CRAF. Therefore the low amount of phosphorylated CRAF seen in the kinase inactive mutants in Figure 4.11 and in longer exposures of results in Figure 4.12 may actually be due to phosphorylation of CRAF\textsuperscript{WT} present at small amounts in this sample.

### 4.3.11 The CRAF\textsuperscript{K375M/S621D} mutant does not act as a phosphomimetic and does not rescue the phenotype

It has been established that the CRAF\textsuperscript{K375M} mutant has a lower stability than CRAF\textsuperscript{WT} assayed by \(^{35}\text{S}\) met/cys pulse chase experiments. Since data presented in this Chapter indicate that phosphorylation of S621 stabilises CRaf, it was investigated whether the mutation of S621 to negatively charged aspartic acid acts as a phosphomimetic for kinase inactive CRAF. A CRAF\textsuperscript{K375M/S621D} construct was produced by Robert Hayward (Cancer Research UK Centre for Cell and Molecular Biology, The Institute of Cancer Research, London, UK). The t\(_{1/2}\) of this mutant was compared to the t\(_{1/2}\) of CRAF\textsuperscript{K375M} and CRAF\textsuperscript{WT} by emetine time-course experiments. Immortalised craf\textsuperscript{−/−} cells were transfected with vectors expressing CRAF\textsuperscript{WT}, CRAF\textsuperscript{KM} or CRAF\textsuperscript{KM/SD}, 24 h later emetine was added to the cell culture media and lysates were harvested at time-points up to 16 hours. Total protein lysates were analysed by western blot with antibodies against CRaf and actin. The optical density of bands on the X-Ray film were quantitated. The percent reduction in optical density was determined and plotted, allowing the t\(_{1/2}\) to be calculated (Figure 4.13). The CRAF\textsuperscript{KM} mutant had a t\(_{1/2}\) approximately 17\% of CRAF\textsuperscript{WT}, as expected from previous experiments. However the CRAF\textsuperscript{KM/SD} mutant did not have a greater t\(_{1/2}\). Therefore the S621D mutation does not appear to act as a phosphomimetic.

### 4.3.12 BRaf is not regulated in a similar way to CRaf by the auto-phosphorylation of the S621 homologue.

Kinase inactive BRaf MEFs are available and were kindly supplied by Dr. T Kamata and Prof. C Pritchard; these cells encode a version of BRaf which is kinase defective due to a
Figure 4.13 The CRAF<sup>K375M/S621D</sup> mutant does not act as a phosphomimetic.

The stability, measured by t<sub>1/2</sub>, of CRAF<sup>WT</sup>, CRAF<sup>KM</sup> and CRAF<sup>KM/SD</sup> were compared by emetine time-course experiments. (A) Immortalised <i>raf</i><sup>-/-</sup> cells were transfected with vectors expressing CRAF<sup>WT</sup>, CRAF<sup>KM</sup> or CRAF<sup>KM/SD</sup>. 24 h later emetine was added to the cell culture media and lysates were harvested at time-points up to 16 h. Total protein lysates were analysed by western blot with antibodies for CRAF and actin. (B) The optical density of bands on the X-Ray film were quantitated. The percent reduction in optical density was determined and plotted. The t<sub>1/2</sub> was calculated as the time required for the optical density to decrease by 50%. The CRAF<sup>KM</sup> mutant had a t<sub>1/2</sub> ~17% of CRAF<sup>WT</sup>. However the CRAF<sup>KM/SD</sup> mutant did not have a greater t<sub>1/2</sub>. Hence the S621D mutation does not appear to act as a phosphomimetic.
CRaf expression has been shown to be reduced in CRaf kinase inactive MEFs, due to the lack of auto-phosphorylation of S621. To investigate if BRaf shares this mechanism of regulation the expression of BRaf was investigated in BRaf kinase inactive MEFs by western blot (Figure 4.14A). The expression of BRaf was found to be unchanged in \( \text{braf}^{\text{DA/DA}} \) cells. Therefore, unlike CRaf, the kinase activity of BRaf is not necessary for protein stability. Furthermore the phosphorylation of BRaf at the S621 homologue, S729 was also investigated in \( \text{braf}^{\text{DA/DA}} \) MEFs (Figure 4.14B) but was found to be unchanged when compared to the wild-type. This indicates that the S621 homologue is not involved in auto-phosphorylation-mediated regulation as occurs with CRaf and further supports the evidence that BRaf is not subjected to this form of regulation.

Furthermore, the Raf proteins are known to heterodimerise (Weber et al., 2001), and may act to stabilise each other by phosphorylation. Hence, mutation of BRaf may affect CRaf and ARaf stability. Therefore the expression of CRaf and ARaf was investigated in \( \text{braf}^{\text{DA/DA}} \) cells to see if mutation of BRaf affected their expression. The expression of the Raf proteins was found to be unchanged in \( \text{braf}^{\text{DA/DA}} \) cells when compared to wild type. Thus, the lack of BRaf kinase activity does not affect CRaf or ARaf stability. This is in agreement with previous results (Figure 4.7B) that showed the expression of ARaf and CRaf was unchanged in \( \text{braf}^{-/-} \) cells.
Figure 4.14 Neither the kinase activity of BRaf nor the phosphorylation of S729 is necessary for the protein's stability.

(A) Generation of \( braf^{D594A} \) allele. The right arm of the targeting vector contains exon 15 harbouring the mutation (*). In addition the targeting vector contains a LSL cassette, consisting of three LoxP sequences (black arrows), a minigene encoding exons 15 to 18 of wild-type \( braf \) with a splice acceptor (SA) sequence at the 5' end. Two stop sequences, represented by polyadenylation (PA) sequences, are located at the 3' end of the minigene and at the 3' end of the \( neo^R \) cassette. Homologous recombination between the targeting vector and the wild-type \( braf \) gene in embryonic stem cells generated the \( braf^{LSL-D594A} \) allele. Expression of Cre recombinase allows deletion of the LSL cassette and generation of the \( braf^{D594A} \) allele. (B) Protein lysates were harvested from \( braf^{+/+} \) and \( braf^{DA/DA} \) MEFs. Total protein lysates were analysed with the antibodies indicated. The expression of BRaf\(^{DA} \) was not reduced in comparison with BRaf\(^{WT} \). (C) BRaf was immunoprecipitated from protein lysates harvested from \( braf^{+/+} \) and \( braf^{DA/DA} \) MEFs. Samples were analysed with an antibody specific for S729, the homolog of S621 in CRaf. The phosphorylation of S729 is not reduced in \( braf^{DA/DA} \) MEFs.
4.4 Conclusions

It has been established in Chapter 3 that CRaf kinase activity is required for regulating CRaf protein expression by preventing its own proteasomal degradation. In this Chapter, data are presented to show that the principle function of CRaf kinase activity is to autophosphorylate S621.

Here it is shown that S621 phosphorylation is severely abrogated in kinase inactive CRaf in both endogenous CRaf\(^{DA}\) and the ectopically expressed CRAF\(^{K375M}\) and CRAF\(^{D486A}\) mutants, indicating that the phosphorylation of S621 is dependent upon CRaf kinase activity. Further evidence for this was obtained by treatment with the Raf kinase inhibitor sorafenib, which was found to abrogate the phosphorylation of S621. Furthermore the loss of this phosphorylation event caused a reduction in CRaf expression due to decreased stability of the protein as monitored by the protein’s t\(_{1/2}\) demonstrating the importance of phospho-S621 in stabilising CRaf. The reduced t\(_{1/2}\), of the CRAF\(^{S621A}\) mutant supports the conclusion that phosphorylation of S621 is necessary to stabilise the protein. The data presented indicates that S259, S338, T491, S494, Y340 and Y341 are not involved in stabilising CRaf: This is indicated by the fact that the phosphorylation status of sites S259 and S338 are not altered in kinase inactive CRaf and by the fact that mutation of T491, S494 and Y340/1 do not reduce CRAF expression. Put together these data identify a role for S621, and not any of the other sites investigated, in the stabilisation of CRaf. The possibility of an intermediate S621 kinase, whose activity was dependent upon CRaf kinase activity was investigated, but the phosphorylation of S621 was shown not to depend on ARaf, BRaf, AMPK, or PKA. Furthermore results from several experimental approaches indicate that the phosphorylation of S621 occurs in cis. BRaf however, does not share this mode of regulation.

The possibility existed that the lack of S621 phosphorylation in kinase inactive CRaf is due to misfolding of the mutant protein in such as way as to sterically restrict the phosphorylation of this site rather than the lack of CRaf kinase activity per se. This was investigated and
refuted by the treatment of wild-type cells with the Raf kinase inhibitor sorafenib. The phosphorylation of S621 was abolished by the inhibitor, indicating that the kinase activity of CRaf is responsible for phosphorylating this residue and furthermore, the absence of S621 phosphorylation in kinase inactive CRaf is not due to the production of a misfolded protein. Indeed treatment with sorafenib has the same effect on destabilising CRaf as the kinase inactivating mutations. The t½ of the kinase inactive CRAF mutants were ~40-60% of CRAFWT and the t½ of CRaf in cells treated with sorafenib was ~34% of the t½ of CRaf in untreated cells. Thus, this confirms the conclusion that CRaf kinase activity is required for CRaf protein stability.

This investigation has shown that phosphorylation of S621 is required for CRaf stability. Indeed reports in the literature also demonstrate that the S621A mutant construct is difficult to express in cells. When transfected into COS-1 cells, the S621A construct is expressed to a similar reduced level as kinase dead constructs (Rushworth et al., 2006). This would be expected if phosphorylation of S621 is required for the stability of the protein. Therefore the first essential role of CRaf kinase activity is to mediate the phosphorylation of this residue. However, the effect of this mutant on stability has not been correlated before.

The mode of S621 phosphorylation and its kinase has been a point of controversy. The phosphorylation of S621 was initially shown to be uncompromised in the kinase dead CRAF mutant CRAFK375M, expressed in insect cells using phospho-peptide mapping (Morrison et al., 1993). This implied that S621 is not an auto-phosphorylation site and requires the presence of a second kinase to phosphorylate this residue. Subsequent studies proposed PKA and AMPK as S621 kinases (Mischak et al., 1996, Sprenkle et al., 1997), although the phosphorylation of S621 by PKA and AMPK has only been demonstrated in vitro. Furthermore, phospho-peptide mapping of CRaf in forskolin (a PKA agonist) stimulated cells showed no increase in the phosphorylation of S621 (Sidovar et al., 2000). Therefore, although it is shown here that the phosphorylation of S621 is CRaf kinase-dependent; the
possibility of an intermediate kinase whose activity is dependent upon CRaf kinase activity was investigated. However AMPK and PKA, which have already been proposed as S621 kinases, did not induce the phosphorylation of S621 or rescue kinase inactive CRaf stability. Given the known heterodimerisation of the Raf kinases (Weber et al., 2001) it was investigated whether ARaf or BRaf played a role in phosphorylating and stabilising CRaf. It was shown here that neither ARaf nor BRaf acted as such an intermediate kinase.

Indeed studies using phospho-antibodies have accumulated evidence in favour of S621 being a site of autophosphorylation, as S621 is not phosphorylated in insect, HeLa, or Sf9 cells expressing kinase dead CRaf (Mischak et al., 1996, Hekman et al., 2004, Thorson et al., 1998) and this view is corroborated by the findings shown here. Indeed, the study by Hekman et al., (2004) demonstrated that S621 autophosphorylation occurs within seconds after growth factor stimulation, confirming that it occurs prior to CRaf plasma membrane translocation. In this regard, CRaf shows similarities to DYRKs and GSK3β which have been shown to autophosphorylate tyrosine residues within their activation loops during translation and folding (Lochhead et al., 2005; Lochhead et al., 2006).

This autokinase activity of CRaf does not involve homo- or hetero-dimerisation and transphosphorylation, but has been shown to occur in cis, further refuting the possibility of an intermediate kinase. Indeed preliminary experiments by Thorson et al., (1998) suggested that the phosphorylation of S621 may occur in cis, since S621 phosphorylation in CRafK375M could not be detected even when co-expressed with wild-type CRaf. This must invoke a model in which a C-terminal fragment of CRaf spanning S621 folds into the CRaf catalytic cleft to allow phosphorylation to occur. None of the complete Raf proteins have been crystallised but the crystal structure of the inactive BRaf kinase domain bound to sorafenib was solved in 2004 (Wan et al., 2004). In this study only residues 432–725 of BRaf were crystallised; the S621 homologue, S729, was omitted. Therefore it is not known if the BRaf structure would be conducive to allowing the positioning of this residue in the catalytic cleft.
However the data presented here and present in the literature (Hekman et al., 2004) shows that S729 of BRaf is not autophosphorylated like S621 of CRaf. Therefore the kinase domains of BRaf and CRaf must fold quite differently to allow autophosphorylation in one isoform but not the other. To understand the mechanism of cis autophosphorylation of CRaf on S621 it will be important to crystallise the molecule without deleting the C terminal fragment containing S621 and to compare it to that established for BRaf.

The role of S621 phosphorylation has been a subject of controversy. Claims that it acts as an activating site are supported by the observation that its mutation leads to loss of kinase activity (Thorson et al., 1998, Morrison et al., 1993). However its phosphorylation correlates with the ability of PKA to inhibit the kinase domain of CRaf (Mischak et al., 1996), leading to counter claims that it has an inhibitory role. S621 is a 14-3-3 binding site, and the role of 14-3-3 binding is also controversial. Some studies suggest that 14-3-3 binding is necessary for activity (Fantl et al., 1994, Freed et al., 1994, Irie et al., 1994, Li et al., 1995, McPherson et al., 1999, Roy et al., 1998, Thorson et al., 1998, Yip-Schneider et al., 2000). However others suggest that it is not (Fu et al., 1994, Michaud et al., 1995, Suen et al., 1995). The data presented here and in Chapter 3 provide clarification on this issue. S621 phosphorylation is required to enable the correct tertiary structure of CRaf preventing its degradation by the proteasome. Therefore, these data support the view that that 14-3-3 binding is essential for CRaf activity.

The data presented here allow the extension of the model described in Chapter 3 (Figure 4.15). CRaf forms an initial immature complex with chaperones including HSP70 and later HSP90. Activation of the ATPase activity of HSP90 results in a conformational change in the protein which allows transient dimerisation. This leads to HSP70, and associated chaperone dissociation and enables the ATP-dependent association of p50cdc37 among other chaperones, forming a mature complex. In this state, CRaf becomes correctly folded such that it can become active; autophosphorylation of S621 is also a key step in this process. If
Figure 4.15 **A comprehensive model of CRaf auto-regulation.**

CRaf forms an immature multi-protein complex, including HSP90 and HSP70. HSP90 dimerisation and the activation of its ATPase activity leads to the dissociation of HSP70. In conjunction with S621 autophosphorylation CRaf acquires its correct tertiary structure and becomes competent to either activate MEK or inactivate ASK1/MST2. Alternatively, if CRaf remains misfolded, an E3 ubiquitin ligase is recruited to the complex, mediating CRaf ubiquitination and its degradation via the proteasome. The autophosphorylation of S621 is a key modulator of this binary switch. For simplicity, a range of other chaperones are not included in this figure.
the correct folding fails to occur, HSP70 does not dissociate and mediates the targeting of CRaf to the proteasome for degradation. However this is not uniquely caused by the actions of CHIP and BAG1. Therefore CRaf can be targeted for degradation by alternative unknown mechanisms. Hence CRaf expression levels are rendered prohibitively low and the protein is unable to participate in normal signalling.

This model neatly combines the two previous modes for the regulation of CRaf. First, CRaf is a client of chaperone proteins including HSP90 and HSP70 which are responsible for either establishing the correct tertiary structure of the protein or for targeting CRaf molecules that remain misfolded for degradation by the proteasome. Second, in a well established model of CRaf regulation, inactive CRaf is cytoplasmic and phosphorylated on S259 and S621 allowing the binding of 14-3-3. Activation of CRaf is caused by RAS mediated translocation to the plasma membrane, where CRaf is converted to its active state by a succession of critical regulatory steps, including phosphorylation of key residues and 14-3-3 is displaced from S259.

Evidence is accumulating for a link between phosphorylation, ubiquitination and proteasomal degradation. Many kinases are known to be involved in regulatory steps that generally promote proteasome mediated degradation of their target proteins (Hoeller et al., 2006). For example the activation of cytoplasmic IκB kinase leads to the phosphorylation and subsequent poly-ubiquitination of IκB on lysine residues 21 and 22, which results in the proteasomal degradation of IκB. There are also numerous examples of phosphorylation preventing proteasome-mediated degradation. For example p53 levels are kept low in unstressed cells mainly by its proteasomal degradation in part mediated by the p53 target MDM2 which is a RING-type E3 ligase. However upon DNA damage, the N-terminal region of p53 becomes phosphorylated by several kinases, including ataxia telangiectasia mutated (ATM), which leads to dissociation from MDM2 and the accumulation of transcriptionally
active p53 in the nucleus. However, it is believed that CRaf is the first case of autophosphorylation preventing degradation of a kinase.

CRaf regulation at the plasma membrane tightly controls the kinase activity of the protein. However the role of this kinase activity is unclear. It has been demonstrated that BRaf, rather than CRaf is the major MEK activator (Wojnowski et al., 2000, Huser et al., 2001, Mikula et al., 2001, Catling et al., 1994, Reuter et al., 1995, Jaiswal et al., 1994, Eychene et al., 1995, Jaiswal et al., 1996, Traverse and Cohen, 1994). The functions that have been determined for CRaf include the suppression of apoptosis, which occurs in a kinase-independent manner. Since the kinase activity of CRaf does not appear to be required, it is interesting that this activity is so tightly regulated. The data presented here provides an explanation: the kinase activity is required to autophosphorylate S621 and hence control CRaf expression. Therefore the kinase domain regulates the extent to which CRaf functions in signalling processes, including the control of apoptosis.

It was investigated if BRaf is regulated by a similar mechanism to CRaf by the autophosphorylation of the S621 homologue. For this investigation BRaf$^{DA}$ cells were used which contain the mutation D594A. Mutation of this residue in human cancer cells (D594V) produces an impaired activity mutant which was unable to activate either ERK or CRAF in vivo (Wan et al., 2004). BRaf expression is not decreased in BRaf$^{DA}$ cells, therefore unlike CRaf, the kinase activity of BRaf is not necessary for protein stability. In addition the phosphorylation of BRaf on the S621 homologue, S729, was found to be unchanged when compared to the wild-type, analogous to the results presented by Hekman et al (2004). This indicates that the S621 homologue is not involved in auto-phosphorylation mediated regulation, as occurs with CRaf, and further supports the evidence that BRaf is not subject to this form of regulation. This is consistent with current evidence that suggests that although BRaf is also a HSP90 client protein, it is regulated differently to CRaf as it is far less sensitive to HSP90 inhibition (da Rocha Dias et al., 2005).
Chapter 5  
CRAF is required for the kinetics of G1/S phase progression

5. CRAF is required for the kinetics of G1-S phase progression

5.1 Introduction

5.1.1 RAS/Raf/MEK/ERK and the cell cycle

The cell cycle, as described in Chapter 1, is a complex process that directs cells through a specific ordered sequence of events and ultimately results in the production of two daughter cells. The stages of the cell cycle are referred to as G1-, S-, G2- and M-phase. Progression through the cell cycle is driven by complexes of cyclins and cyclin dependent kinases (CDKs) and is restrained by the action of cyclin dependent kinase inhibitors (CKIs).

The Raf isoforms are among a number of signalling proteins that regulate the cell cycle; they are generally thought to exert their effects by the activation of ERK. The best characterised example of the Raf/MEK/ERK cascade driving cell cycle progression is via the induction of cyclin D1 expression. Active ERK can translocate to the nucleus where it induces transcription of genes including c-fos. Fos family members act as immediate early genes, allowing control of cyclin D1 transcription by ERK (Brown et al., 1998). This occurs by heterodimerisation of c-Fos and c-Jun family members to form AP-1 complexes, which bind to AP-1 binding sites located in the promoter region of cyclin D1 (Angel and Karin, 1991, Albanese et al., 1995). Thus, this induces expression of cyclin D1, leading to the phosphorylation of pRb by cyclin D/CDK4/6 complexes and progression through the restriction point.

The ERK cascade is also thought to have a role in the regulation of p21\textsuperscript{CIP1}. For instance oncogenic RAS provokes G1 arrest in primary fibroblasts, accompanied by accumulation of p53, p21\textsuperscript{CIP1}, and p16\textsuperscript{INK4A} (Serrano et al., 1997). The introduction of dominant negative p53 or antisense p21\textsuperscript{CIP1} prevented p21\textsuperscript{CIP1} induction and cell cycle arrest. The expression of an inducible ΔCRaf:ER construct in rat Schwann cells led to p53-dependent activation of
p21\textsuperscript{CIP1} resulting in G1 arrest (Lloyd et al., 1997). However the expression of high levels of activated ΔBRaf:ER in NIH3T3 cells resulted in p53-independent induction of p21\textsuperscript{CIP1} and also led to cell cycle arrest (Sewing et al., 1997, Woods et al., 1997), although lower levels of ΔBRaf:ER activity induced proliferation rather than arrest (Woods et al., 1997). Therefore the level of Raf activity and associated ERK activity, certainly in the murine fibroblasts studied here, would seem to be important in determining whether a cell would arrest or proliferate. In addition, stimulation of NIH3T3 cells expressing the NGF receptor, TrkA, with NGF caused activation of the ERK cascade which led to p21\textsuperscript{CIP1} induction and cell cycle arrest (Decker, 1995). Furthermore, expression of ΔCRaf:ER in these cells resulted in a prolonged increase in ERK activity and growth arrest of these cells concomitantly with induction of p21\textsuperscript{CIP1}. This effect was reversed by treatment of cells with the MEK inhibitor PD98059 (Pumiglia and Decker, 1997).

Whilst activation of the ERK cascade appears to induce the expression of cyclin D1 and p21\textsuperscript{CIP1}, the expression of p27\textsuperscript{KIP1} is repressed by RAS/Raf/MEK/ERK signalling. For example the activation of an inducible constitutively active CRaf protein expressed in murine fibroblasts reduced the expression of p27\textsuperscript{KIP1} and enabled proliferation in previously arrested cells (Kerkhoff and Rapp, 1997). Furthermore the activation of ΔRaf:ER protein constructs demonstrated that all the active Raf isoforms could cause a decrease in the expression of p27\textsuperscript{KIP1} (Woods et al., 1997). Proteasomal degradation of p27\textsuperscript{KIP1} has been documented as being a major regulator of the protein’s expression (Pagano et al., 1995). It can be speculated that phosphorylation of p27\textsuperscript{KIP1} observed to be mediated by ERK \textit{in vitro} (Kawada et al., 1997, Alessandrini et al., 1997) may mark the protein for degradation by the proteasome. Hence this offers a possible explanation for the mechanism of RAS/Raf/MEK/ERK downregulation of p27\textsuperscript{KIP1} expression.

As described above most of the RAS/Raf effects on the cell cycle are mediated through ERK. However CRaf may also regulate the cell cycle in an ERK-independent manner. For
example CRaf can form a direct interaction with Rb and in addition to cyclin D and cyclin E-CDK complexes, CRaf has been identified as a Rb kinase. (Wang et al., 1998, Jamal and Ziff, 1995). The disruption of the Rb/CRaf interaction by a nine amino acid peptide prevented Rb phosphorylation and cell proliferation, suggesting that the binding of CRaf is necessary for inactivation of Rb (Dasgupta et al., 2004). In addition CRaf can bind to Cdc25, stimulating its activity. Cdc25 is a dual specificity phosphatase which dephosphorylates threonine and tyrosine residues in CDK1. Cdc25-mediated dephosphorylation activates CDK1, which regulates G2/M progression (Galaktionov et al., 1995). Humans have three Cdc25 genes, of which CRaf has been shown to bind to two; Cdc25A and B, but not to Cdc25C (Galaktionov et al., 1995). Finally CRaf is capable of binding to MST2 and inactivating it. This causes downstream activation of the Lats proteins, which function to restrict the cell cycle by inhibiting G1/S or G2/M transition through interactions with cyclins E, A and B (O'Neil, 2005, Xia et al., 2002, Li et al., 2003).

5.1.2 Phenotypes of mice with targeted craf mutations

craf<sup>−/−</sup> mice, when maintained on a C57BL6 background, died in embryogenesis and displayed poor development of the placenta, liver, and haematopoietic organs, as well as increased apoptosis of embryonic tissues (Huser et al., 2001, Mikula et al., 2001). On a mixed 129Sv/C57BL6 genetic background craf<sup>−/−</sup> cells grew poorly due to an increase in apoptosis; and no cell cycle defect was identified. However, on the outbred MF1 background craf<sup>−/−</sup> embryos survived to later ages (Kamata et al., 2004) and did not display an apoptotic phenotype, but showed a reduced ability to grow as monitored by assessing the growth rates of craf<sup>−/−</sup> MEFs in comparison with wild-type cells (Mercer et al., 2005). Hence the phenotype observed in craf<sup>−/−</sup> mice is subject to genetic modifiers which are dependent upon the strain of mouse. craf<sup>Y340FY341F</sup> mice have also been generated; such mice are incapable of CRaf mediated phosphorylation or activation of MEK (Huser et al., 2001). These mice
have an apparently normal phenotype and no increase in apoptosis or changes in proliferation were observed.

In both \(\text{craf}^{\text{Y340FY341F}}\) cells and \(\text{craf}^{-/}\) cells on either genetic background ERK activation was not found to be reduced. Hence the phenotypes described above are believed to be ERK independent. It was because of the strong evidence for MEK/ERK independent functions of CRaf that kinase defective CRaf mice were generated in our laboratory, although the reduced CRaf expression observed in these cells (described in Chapters 3 and 4) has subsequently complicated the interpretation of the kinase-dependent functions of CRaf.

5.1.3 Preliminary data indicates a cell cycle defect in \(\text{craf}^{\text{DA/DA}}\) MEFs

Preliminary data obtained prior to this study by Dr. K. Mercer (Department of Biochemistry, University of Leicester; Figure 5.1A) indicated that \(\text{craf}^{\text{DA/DA}}\) mice on the 129Sv/C57BL6 mixed background survived postnatally but grew more slowly than wild-type mice, being smaller than wild-type mice of the same age. This was assessed by monitoring the weights of cohorts of \(\text{craf}^{\text{DA/DA}}\), \(\text{craf}^{\text{DA/+}}\) and \(\text{craf}^{+/+}\) mice over time. \(\text{craf}^{\text{DA/DA}}\) mice put on less weight than wild-type mice and the weight gain of \(\text{craf}^{\text{DA/+}}\) was intermediate. Furthermore cell counting assays indicated that primary \(\text{craf}^{\text{DA/DA}}\) MEFs grew more slowly than wild-type cells (Figure 5.1B). However on the pure C57BL6 background \(\text{craf}^{\text{DA/DA}}\) mice showed a similar apoptotic phenotype to \(\text{craf}^{-/}\) mice (Noble et al., 2008). The slower growth on the 129Sv/C57BL6 mixed background cannot be accounted for by the low level of increased spontaneous apoptosis. Hence this might indicate a defect in the cell cycle as well - possibly either a delay at one or more stages of the cycle, or an overall reduction in the rate. Both the lack of a functional kinase domain and the reduced expression of CRaf may play in role in the observed phenotypes.
Figure 5.1 Initial observations of the $craf^{DA/DA}$ phenotype. (Data obtained from Dr. K. Mercer)

(A) The weights of $craf^{DA/DA}$, $craf^{+/DA}$ and $craf^{+/+}$ mice on the mixed 129Sv/C57BL6 background were compared with increasing age. $craf^{DA/DA}$ mice gained less weight than wild-type mice, with $craf^{+/DA}$ mice being intermediate between the two. (B) The growth curves of $craf^{DA/DA}$ and $craf^{+/+}$ MEFs show that $craf^{DA/DA}$ MEFs grew more slowly than wild-type MEFs.
5.2 Aims

The aim of this Chapter was to characterise the growth defects of \( craf^{DA/DA} \) MEFs. Preliminary data indicated a possible cell cycle defect in these cells. If this was confirmed, it would be necessary to identify at what stage the cell cycle was affected and furthermore to investigate the signalling pathway(s) involved in this.

5.3 Results

5.3.1 Flow cytometry analysis of the cell cycle

\( craf^{DA/DA} \) MEFs have been observed to grow at a reduced rate. Although it is known that \( craf^{DA/DA} \) cells have increased apoptosis, the cell cycle is investigated here. To investigate any potential changes in the cell cycle, the DNA content in unsynchronised \( craf^{DA/DA} \) MEFs was compared to wild-type cells by flow cytometry (Figure 5.2). Due to effects of spontaneous immortalisation on the cell cycle, all cells were analysed before passage 7. Cells were harvested and fixed in 70% ethanol before being treated with RNase and the DNA stained with propidium iodide which intercalates in the DNA helix and is excited by 488nm light. Such cells were then analysed by flow cytometry which allows measurement of the DNA content of cells. The resulting profile of a population of cells consists of distinct peaks which relate to the stages of the cell cycle by their DNA content. Since G1 cells are 2n, G2/M cells are 4n and S phase cells possess an intermediate amount of DNA, this method allows the determination of the percentage of cells in each phase of the cell cycle.

The data indicate that the proportion of cells in G1, S and G2/M varies between \( craf^{DA/DA} \) MEFs and wild-type cells. \( craf^{DA/DA} \) MEFs cells have a lower proportion of cells in the G1 stage of the cell cycle and a greater proportion of cells in S and G2/M. These data would suggest that there is a defect in the \( craf^{DA/DA} \) cell cycle but the exact point at which the defect occurs is difficult to pinpoint from these data alone.
Figure 5.2 *craf*<sup>DA/DA</sup> and *craf*<sup>+/+</sup> MEF DNA content distribution.

(A) Two unsynchronised *craf*<sup>+/+</sup> and *craf*<sup>DA/DA</sup> MEF cell lines were fixed in 70% ethanol before being treated with RNase and stained with propidium iodide. Such cells were then analysed by flow cytometry. The percentage of cells in G1, S and G2/M was determined allowing comparison of proportions of cells in each cell cycle phase between populations of *craf*<sup>+/+</sup> and *craf*<sup>DA/DA</sup> MEFs. (B) The percentage of cells in G1, S and G2/M from 12 independent experiments was averaged and graphs produced. Error bars denote the standard error in the values shown. *craf*<sup>DA/DA</sup> cells have a lower proportion of cells in the G1 stage of the cell cycle and a greater proportion of cells in S and G2/M.
5.3.1 BrdU analysis of the cell cycle in craf<sup>−/−</sup>, craf<sup>DA/DA</sup> and craf<sup>FF/FF</sup> MEFs

While flow cytometry analysis had identified a potential defect in the craf<sup>DA/DA</sup> cell cycle, G0-G1-S-phase progression was investigated by BrdU assays. The BrdU labelling reagent labels the DNA of cells in S phase, so allows the progression of cells in S phase to be monitored over a time-course. Cells were counterstained with DAPI and visualised by immunofluorescence.

Initially a series of control conditions were analysed to assess the specificity of the BrdU staining. Asynchronous populations of craf<sup>+/−</sup> MEFs were subjected to the BrdU assay. However certain omissions were made to the protocol; cells which were not treated with the BrdU labelling reagent, the primary antibody or the secondary antibody all showed no BrdU positive cells. This is in contrast to asynchronous craf<sup>+/−</sup> MEFs subjected to the complete protocol which demonstrated a percentage of BrdU positive staining. Representative immunofluorescence images of the above control conditions are shown in Figure 5.3.

Subsequently, BrdU proliferation assays were conducted on craf<sup>−/−</sup>, craf<sup>DA/DA</sup> and craf<sup>FF/FF</sup> MEFs and their matched wild-type counterparts over a time-course of up to 18 hours. All three cell lines were analysed in order to investigate the role of the whole protein in the cell cycle as compared to the roles of its total kinase activity and specific MEK kinase activity. MEFs were serum starved for 24 h to synchronise cells in G0. MEFs were then simultaneously treated with BrdU labelling reagent and stimulated with serum for the time-points indicated. The percentage of BrdU positive cells for each cell line at each time-point was determined and plotted (Figure 5.4). The results indicate that both craf<sup>−/−</sup> and craf<sup>DA/DA</sup> MEFs exhibit a general delay in cell cycle progression. However at the point where the greatest rate of wild-type S-phase entry is observed (16-18 h for craf<sup>DA/DA</sup> assay and 14-16 h for craf<sup>−/−</sup> assay) both craf<sup>−/−</sup> and craf<sup>DA/DA</sup> MEFs display an approximate 2 h delay in comparison to their wild-type counterparts. This would support a G1/S delay in the cell cycle of MEFs lacking CRaf kinase activity and/or CRaf expression. craf<sup>FF/FF</sup> MEFs have...
Figure 5.3. **Representative Immunofluorescence images of BrdU control cran+/+ MEFs**

Concurrent with the BrdU assays, a series of control conditions were analysed to assess the specificity of the BrdU staining. Asynchronous populations of cran+/+ MEFs were subjected to the BrdU assay. However omissions were made to the protocol. Cells which were not treated with the BrdU labelling reagent, the primary antibody or the secondary antibody all showed no BrdU positive cells. Images are x40 magnification, scale bar = 15μm.
craf\textsuperscript{DA/DA} (A), craf\textsuperscript{-/-} (B) and craf\textsuperscript{FF/FF} (C) primary MEFs and their matched wild-type counterparts were seeded on coverslips and serum starved for 24 h to synchronise cells in G0. Cells were then simultaneously treated with BrdU labelling reagent and stimulated with serum for the time-points indicated, up to 18 h. Cells were counterstained with DAPI and visualised by immunofluorescence. The percentage of BrdU positive cells for each cell line at each time-point was determined and plotted. The data presented are representative of two independent experiments for each cell line. craf\textsuperscript{DA/DA} and craf\textsuperscript{-/-} MEFs enter into S phase up to 2 h later than their wild-type counterparts. craf\textsuperscript{FF/FF} MEFs enter S phase at the same rate as wild-type cells.
previously been shown not to have an observable defect in their cell cycle or growth (Huser et al., 2001). In agreement with this, the data obtained indicates that the progress into S phase is not altered in craf<sup>FF/FF</sup> MEFs. It is worth noting that the three wild type strains used in these experiments exhibit slightly different profiles. For example the wild-type MEFs in Figure 5.4B show the greatest rate at S-phase entry by 14 h, whereas those used in Figure 5.4C demonstrate a high level of S-phase entry by 10 h. This difference is due to the inherent variability between different MEF lines that is in part due to the age and the conditions in which the embryo and subsequent MEFs were isolated. However this variability can be reduced by only directly comparing matched MEF lines obtained from the same litter.

Representative images of craf<sup>DA/DA</sup>, craf<sup>−/−</sup> and craf<sup>FF/FF</sup> MEFs and their matched wild-type counterparts at 0h, 10h and 18h post serum stimulation/BrdU addition are shown in Appendix A in Figures A.1 - A.3 respectively.

### 5.3.2 Western blot analysis of cell cycle proteins in craf<sup>DA/DA</sup> and craf<sup>−/−</sup> MEFs

A series of western blots were performed to investigate the expression of key cell cycle proteins involved in G0-G1-S-phase progression in craf<sup>DA/DA</sup> cells compared with wild-type cells. Protein lysates were harvested from asynchronous populations of craf<sup>−/−</sup> and craf<sup>DA/DA</sup> MEFs.

In the first instance such samples were analysed by western blotting to assess the expression of the G0 and early G1 cell cycle proteins cyclin D1, cyclin D2, cyclin D3, CDK 4, CDK 6 and p16<sup>INK4A</sup> (Figure 5.5). Initially cyclin D1 expression was not detectable by western blot in asynchronous cell lysates. Therefore, in order to detect the expression of cyclin D1, MEFs were serum starved for 24 h and serum stimulated for 20 h to synchronise cells in G1, when expression of cyclin D1 would be expected to peak. The blots shown for each protein investigated represent the expression of that protein in two different cell lines for both craf<sup>DA/DA</sup> and wild-type cells. The expression level of at least 3 independent replicates for each protein of interest was quantitated and graphs were produced. The
**Figure 5.5. Analysis of the expression of G0 and Early G1 cell cycle proteins by western blot in craf^{DA/DA} and craf^{+/+} MEFs**

*(A)* Protein lysates were harvested from asynchronous populations of craf^{+/+} and craf^{DA/DA} MEFs. Samples were analysed by western blotting to assess the expression of cyclin D2, cyclin D3, CDK4, CDK6 and p16^{INK4A}. To detect cyclin D1 expression, MEFs were serum starved for 24 h then serum stimulated for 20 h to synchronise cells in G1, when expression of cyclin D1 would be expected to peak. Protein lysates were then prepared and expression levels analysed by western blotting. *(B)* The expression level of at least 3 independent replicates for each protein of interest was quantitated and graphs produced. Error bars denote the standard error in the values shown. The expression of proteins investigated was not significantly changed in craf^{DA/DA} MEFs, with the exception of cyclin D3 which had slightly reduced expression in craf^{DA/DA} MEFs. This difference was shown to be statistically significant with a P value of 0.001 by unpaired T test.

![Western blot images](image)

### A

<table>
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<th></th>
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<tr>
<td></td>
<td>0h</td>
<td>20h</td>
</tr>
<tr>
<td></td>
<td>0h</td>
<td>20h</td>
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<td></td>
<td>+FCS</td>
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- cyclin D2
- cyclin D3
- CDK4
- CDK 6
- p16^{INK4A}
- actin

### B

![Graph](image)

**Relative expression**

- cyclin D1
- cyclin D2
- cyclin D3
- CDK4
- CDK6
- p16^{INK4A}

*P = 0.001*
expression of G0 and early G1 cell cycle proteins, with the exception of cyclin D3, was unchanged in craf^{DA/DA} MEFs. The expression of cyclin D3, however, was slightly reduced in craf^{DA/DA} MEFs. An unpaired T test, showed this to be significant, with a P value of 0.001. However the variation in the expression of the other proteins investigated was not shown to be statistically significant.

Protein lysates from craf^{DA/DA} and craf^{+/+} MEFs were also analysed for the expression of the late G1 and S phase cell cycle proteins cyclin A, cyclin E, CDK2, p27^{KIP1}, p21^{CIP1} and also p53 (Figure 5.6). The expression of these proteins differed markedly between craf^{DA/DA} and craf^{+/+} samples. While the expression of cyclin A and p53 was consistent and any variations observed were not deemed to be statistically significant, the expression of cyclin E, CDK2 and p27^{KIP1} was reduced in craf^{DA/DA} MEFs and the expression of p21^{CIP1} was increased in craf^{DA/DA} MEFs. Unpaired T tests, showed these differences to be significant, with P values of 0.0065, 0.0003, 0.0415 and 0.0141, respectively. Since p53 levels did not appear to be altered in craf^{DA/DA} MEFs, the altered expression of p21^{CIP1} may not be p53 dependent. However p53 activity was not examined, so p53 dependent p21^{CIP1} regulation cannot be ruled out.

Finally, the expression of the G2 cell cycle proteins cyclin B and CDK1 was investigated. Additionally, the activity of CDK1 was monitored by investigating the phosphorylation of Y15 and T161 (Figure 5.7). The expression of the proteins in at least 3 independent experiments was quantitated using ImageJ and graphs were plotted. The expression of the G2 cell cycle proteins was unchanged in craf^{DA/DA} MEFs and any observed variations in expression were not statistically significant by unpaired T test. Furthermore the activation of CDK1 appeared to be unchanged in craf^{DA/DA} MEFs.
Chapter 5  
CRAF is required for the kinetics of G1/S phase progression

Figure 5.6. Analysis of the expression of late G1 and S phase cell cycle proteins by western blot in craf^{DA/DA} and craf^{+/+} MEFs

(A) Protein lysates were harvested from asynchronous populations of craf^{+/+} and craf^{DA/DA} MEFs. Samples were analysed by western blotting to assess the expression of the cell cycle proteins cyclin E, cyclin A, p21^{CIP1}, CDK2, p27^{KIP1} and p53. (B) The expression of the proteins in at least 3 independent experiments was quantitated using ImageJ. The expression of cyclin A and p53 was unchanged in craf^{DA/DA} MEFs. However the expression of cyclin E, CDK2 and p27^{KIP1} was reduced in craf^{DA/DA} MEFs and the expression of p21^{CIP1} was increased in craf^{DA/DA} MEFs. Unpaired T tests, showed these differences to be significant, with P values of 0.0065, 0.0003, 0.0415 and 0.0141, respectively. Error bars denote the standard error in the values shown.
Figure 5.7. Analysis of the expression of G2 cell cycle proteins by western blot in \( \text{craf}^{DA/DA} \) and \( \text{craf}^{+/-} \) MEFs

(A) Protein lysates were harvested from asynchronous populations of \( \text{craf}^{+/-} \) and \( \text{craf}^{DA/DA} \) MEFs. Samples were analysed by western blotting to assess the expression of the cell cycle proteins cyclin B and CDK1. The phosphorylation of CDK1 at T161 and Y15 was also investigated using specific phospho-antibodies.

(B) The expression of the proteins in at least 3 independent experiments was quantitated using ImageJ. Error bars denote the standard error in the values shown. The expression of the G2 cell cycle proteins was unchanged in \( \text{craf}^{DA/DA} \) MEFs, and any variations observed were shown not to be statistically significant by unpaired T test. Additionally the activation of CDK1 appeared to be unchanged in \( \text{craf}^{DA/DA} \) MEFs.
These results highlight abnormalities in the proteins regulating the cell cycle of \( \text{craf}^{\text{DA/DA}} \) MEFs, particularly at late G1, corresponding with the prior results of BrdU analysis which indicated a \( \sim 2 \) h delay in this phase of the cell cycle.

### 5.3.3 Western blot analysis of cell cycle proteins in \( \text{craf}^{-/-} \) and \( \text{craf}^{+/+} \) MEFs

The expression of cyclin E, \( \text{p21}^{\text{CIP1}} \), \( \text{p27}^{\text{KIP1}} \), CDK2 and cyclin D3 have been found to be altered in \( \text{craf}^{\text{DA/DA}} \) MEFs. It is not clear if this altered expression profile is due to the reduced expression of CRaf or the lack of CRaf kinase activity. Therefore the expression of these proteins was investigated in \( \text{craf}^{-/-} \) MEFs. Due to the time restraints of this project, the expression of these proteins was not investigated in \( \text{craf}^{\text{FF/FF}} \) MEFs. Using protein lysates from asynchronous \( \text{craf}^{-/-} \) and \( \text{craf}^{+/+} \) MEFs, consistent results were not achievable, possibly due to the degree of heterogeneity which exists in MEF populations. Therefore \( \text{craf}^{-/-} \) and \( \text{craf}^{-/-} \) MEFs were serum starved for \( 20 \) h to synchronise cells in G0. Cells were then stimulated with serum for 16 h, 18 h and 20 h. Protein lysates were harvested from these synchronised populations of cells and were analysed by western blotting to assess the expression of the cell cycle proteins cyclin E, \( \text{p21}^{\text{CIP1}} \), \( \text{p27}^{\text{KIP1}} \), CDK2 and cyclin D3 (Figure 5.8).

Cyclin D3 and CDK2 expression had previously been found to be reduced in \( \text{craf}^{\text{DA/DA}} \) MEFs. However under the experimental conditions described above, their expression was not altered in \( \text{craf}^{-/-} \) MEFs. Interestingly while \( \text{p27}^{\text{KIP1}} \) expression was reduced in \( \text{craf}^{\text{DA/DA}} \) MEFs, the expression of this particular protein was increased slightly in \( \text{craf}^{-/-} \) MEFs. However, importantly, cyclin E and \( \text{p21}^{\text{CIP1}} \) expression appeared to be altered consistently in both \( \text{craf}^{\text{DA/DA}} \) and \( \text{craf}^{-/-} \) MEFs. The expression of \( \text{p21}^{\text{CIP1}} \) was increased in \( \text{craf}^{\text{DA/DA}} \) and \( \text{craf}^{-/-} \) MEFs whereas the expression of cyclin E was reduced. The abnormal expression of either protein may in some degree account for the observed cell cycle defect in \( \text{craf}^{\text{DA/DA}} \) and \( \text{craf}^{-/-} \) MEFs. It was decided that further analysis would focus on the altered regulation of \( \text{p21}^{\text{CIP1}} \)
Figure 5.8. **Analysis of the expression of cell cycle proteins in** \( \textit{craf}^{+/+} \) **and** \( \textit{craf}^{-/-} \) **MEFs which are found to be altered in** \( \textit{craf}^{DA/DA} \) **MEFs**

**(A)** \( \textit{craf}^{+/+} \) **and** \( \textit{craf}^{-/-} \) **MEFs were serum starved for 20 h to synchronise cells in G0. Cells were then stimulated with serum for the time-points indicated. Protein lysates were harvested and samples were analysed by western blotting to assess the expression of the cell cycle proteins cyclin E, p21\(^{\text{CIP1}}\), p27\(^{\text{KIP1}}\), CDK2 and cyclin D3. The data shown are representative of 2 independent experiments. (B) The expression of the proteins in (A) was quantitated using ImageJ. Due to the lack of a third replicate, statistical analysis was not performed on these data.
due to the availability of a reliable antibody and the degree of information relating to its regulation.

5.3.4 p21\textsuperscript{CIP1} regulation by the proteasome is not altered in \textit{craf\textsuperscript{DA/DA}} or \textit{craf\textsuperscript{−/−}} MEFs

p21\textsuperscript{CIP1} expression is known to be regulated at the protein level by the proteasome. Therefore this mode of regulation was first investigated in \textit{craf\textsuperscript{DA/DA}} and \textit{craf\textsuperscript{−/−}} MEFs. \textit{craf\textsuperscript{−/−}}, \textit{craf\textsuperscript{DA/DA}} MEFs and their matched wild-type counterparts were either treated with 30μM of the proteasome inhibitor MG132 or treated with DMSO as a control for 6 hours. Protein lysates were prepared and p21\textsuperscript{CIP1} expression assessed by western blot (Figure 5.9). Consistent with previous results, p21\textsuperscript{CIP1} expression is greater in the \textit{craf\textsuperscript{−/−}} and \textit{craf\textsuperscript{DA/DA}} MEFs than in wild-type cells. However p21\textsuperscript{CIP1} levels in \textit{craf\textsuperscript{−/−}} and \textit{craf\textsuperscript{DA/DA}} cells are raised further by proteasome inhibition suggesting that regulation at the level of the proteasome is not a key factor here.

5.3.5 p21\textsuperscript{CIP1} mRNA levels are increased in \textit{craf\textsuperscript{DA/DA}} MEFs

The increased p21\textsuperscript{CIP1} protein expression in \textit{craf\textsuperscript{−/−}} and \textit{craf\textsuperscript{DA/DA}} MEFs may be a result of increased p21\textsuperscript{CIP1} mRNA production or stability. Hence the expression of p21\textsuperscript{CIP1} cDNA was measured in \textit{craf\textsuperscript{−/−}} and \textit{craf\textsuperscript{DA/DA}} MEFs by qPCR. cDNA used in this assay was derived from \textit{craf\textsuperscript{−/−}} and \textit{craf\textsuperscript{DA/DA}} MEFs, produced by reverse transcription of mRNA obtained from these cells. Before the actual assay, the conditions of the qPCR reaction needed to be optimised, as described in Chapter 2. The data for representative qPCR amplification of p21\textsuperscript{CIP1} and GAPDH are presented in Figures 5.10 and 5.11 respectively. To ensure reliable results were obtained, all qPCR reactions were performed in triplicate. Template mRNA used for the production of the cDNA was included as a control. From the data presented in the melt and amplification curves (Figure 5.10 B), it is clear that no amplification of p21\textsuperscript{CIP1} occurred from this RNA. Hence this excluded the possibility of genomic DNA contamination of the RNA and of the subsequently derived cDNA. Additionally the lack of amplification in all water
Figure 5.9. p21CIP1 regulation by the proteasome is not altered in \( \text{cr}\)af\(^{DA/DA}\) or \( \text{cr}\)af\(^{-/-}\) MEFs

craf\(^{-/-}\) MEFs and their matched wild-type counterparts (A) and \( \text{cr}\)af\(^{DA/DA}\) MEFs and their matched wild-type counterparts (B) were either treated with the proteasome inhibitor MG132 (M) or treated with DMSO (C) as a control for 6 h. Protein lysates were prepared and p21CIP1 expression assessed. Although the expression of p21CIP1 is greater in the \( \text{cr}\)af\(^{-/-}\) and \( \text{cr}\)af\(^{DA/DA}\) MEFs as shown previously, p21CIP1 levels in these cells were raised further by proteasome inhibition suggesting that regulation at the level of the proteasome is not a key factor here.

\[
\begin{array}{c|c|c|c|c}
 & +/+ & -/- &  \\
\hline
C & M & C & M \\
\hline
\text{p21CIP1} &  &  &  \\
\text{actin} &  &  &  \\
\end{array}
\]

\[
\begin{array}{c|c|c|c|c}
 & +/+ & DA/DA &  \\
\hline
C & M & C & M \\
\hline
\text{p21CIP1} &  &  &  \\
\text{actin} &  &  &  \\
\end{array}
\]
Figure 5.10 qPCR analysis of p21$^{CIP1}$ expression in craf$^{+/+}$ and craf$^{DA/DA}$ MEFs.

(A) Microtitre plate showing loading of samples in triplicate (indicated by colour), as described in the table. (B) Data graphs of the melt curve showing one peak, corresponding to a single PCR product. The amplification curves for each sample are shown with the threshold line positioned at the point where the curves begin to linearise. Note the absence of amplification in the RNA control samples, indicating the lack of genomic DNA contamination.

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Figure 5.11 qPCR analysis of gapdh expression in craf+/+ and crafDA/DA MEFs.

(A) Microtitre plate showing loading of samples in triplicate (indicated by colour), as described in the table. (B) Data graphs of the melt curve showing one peak, corresponding to a single PCR product. The amplification curves for each sample are shown with the threshold line positioned at the point where the curves begin to linearise.

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<tr>
<td>B4-B6</td>
<td>8.10 DA/DA cDNA</td>
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<tr>
<td>C1-C3</td>
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</tr>
<tr>
<td>D1-D3</td>
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<td>E1-E3</td>
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controls demonstrated the absence of contamination of any reagents and showed the amplification of \( p21^{CIP1} \) and \( GAPDH \) was specifically from the cDNA used in each assay.

The Livack method was used to calculate the fold difference of cDNA and hence mRNA between the samples. This method assumes that both \( p21^{CIP1} \) and \( GAPDH \) are amplified with efficiencies close to 100% and within 5% of each other. Preliminary experiments depicted in Figures 2.5 and 2.6 verify these assumptions; the amplification efficiency was 94% for \( p21^{CIP1} \) and was 93% for \( GAPDH \). Analogous with the Pfaffl method outlined in Chapter 3, the relative difference in the expression level of the target gene - i.e. \( p21^{CIP1} \) - in multiple samples can be determined by choosing one sample as a calibrator and representing the expression of the test genes in all other samples as an increase or decrease relative to the calibrator and normalised to the expression of a reference gene – i.e. \( GAPDH \).

First the \( C_T \) of the target gene (\( p21^{CIP1} \)) must be normalised to that of the reference gene (\( GAPDH \)) for both calibrator and test samples:

\[
\Delta C_T^{(test)} = C_T(p21^{CIP1}, test) - C_T(GAPDH, test)
\]

\[
\Delta C_T^{(calibrator)} = C_T(p21^{CIP1}, calibrator) - C_T(GAPDH, calibrator)
\]

Secondly the \( \Delta C_T \) of the test sample must be normalised to the \( \Delta C_T \) of the calibrator:

\[
\Delta \Delta C_T = \Delta C_T^{(test)} - \Delta C_T^{(calibrator)}
\]

Finally the formula to calculate the expression ratio is:

\[
2^{\Delta \Delta C_T} = \text{Normalized expression ratio}
\]

The fold difference was then averaged in \( craf^{+/+} \) and \( craf^{DA/DA} \) MEFs and the averaged \( craf^{DA/DA} \) values were normalised to the wild-type value (Figure 5.12). The expression of
Figure 5.12 Quantitation of \( p21^{CIP1} \) mRNA expression levels in \( craf^{+/+} \) and \( craf^{DA/DA} \) MEFs.

cDNA derived from \( craf^{+/+} \) and \( craf^{DA/DA} \) MEFs, produced by reverse transcription of mRNA obtained from these cells was used in qPCR assays to quantitate relative levels of \( p21^{CIP1} \) mRNA, using \( gapdh \) as a control. Each sample was analysed in triplicate with each primer set and the Livack method was used to calculate relative levels of expression. The values for \( p21^{CIP1} \) were normalised to the values for \( gapdh \) and subsequently the \( craf^{DA/DA} \) value was normalised against the \( craf^{+/+} \) value. There is an average 2.7 fold increase in \( p21^{CIP1} \) mRNA expression in \( craf^{DA/DA} \) MEFs. This difference was shown to be statistically significant with a P value of 0.0009 by unpaired T test. Error bars denote the standard error in the values shown. Results are from 4 independent experiments.

\[ * \text{ P} = 0.0009 \]
Chapter 5  CRAF is required for the kinetics of G1/S phase progression

p21<sup>CIP1</sup> mRNA in <em>craf<sup>DA/DA</sup></em> MEFs was 2.7 fold greater than the expression level seen in <em>craf<sup>+/+</sup></em> MEFs. An unpaired T test indicated that this difference was statistically significant, with a P value of 0.0009. This indicates that p21<sup>CIP1</sup> is regulated at the level of mRNA production or stability in a CRaf dependent manner. Due to the time constraints of this investigation and the variability of protein expression observed in the <em>craf<sup>-/-</sup></em> MEFs, p21<sup>CIP1</sup> mRNA levels were not investigated in these cells.

5.4 Conclusions

Preliminary observations made by Dr. K Mercer prior to this investigation pointed to a potential cell cycle defect in <em>craf<sup>DA/DA</sup></em> cells. The work presented here is consistent with this. Flow cytometry analysis has demonstrated that the proportion of cells at various stages of the cell cycle differs in <em>craf<sup>DA/DA</sup></em> MEFs. Unsynchronised populations of these MEFs have a greater percentage of cells in G2/M and S phase and fewer cells in G1. Such differing profiles may be indicative of a defect of the <em>craf<sup>DA/DA</sup></em> cell cycle, with the potential of a block/delay in the cell cycle at any phase. However BrdU assays have demonstrated that <em>craf<sup>DA/DA</sup></em> MEFs progress more slowly through the cell cycle and reach S phase up to 2 hours later than their wild-type counterparts, providing evidence of a G1/S defect. Subsequent analysis of the expression of cell cycle proteins provides further support for a delay in the G1/S-phase transition. However, this does not rule out the possibility of additional defects at other cell cycle phases.

Although a number of studies have analysed the effects of reduced CRaf expression caused by knockout mutations or by RNAi, none have investigated alterations in the expression of cell cycle proteins. However a reduction in cell proliferation and growth has been documented in the absence of CRaf (Schumacher et al., 1998, Mercer et al., 2005). Schumacher et al (1998) used an antisense oligodeoxynucleotides directed against CRaf in human coronary artery smooth muscle cells and demonstrated down-regulation of both
gene and protein expression. Concurrent with this, BrdU assays showed that there was a 31% reduction in the proportion of cells in S phase when CRaf expression was reduced. The investigation conducted by Mercer et al (2005) analysed the growth rates of craf\textsuperscript{−/−} MEFs in comparison with wild-type cells and determined that craf\textsuperscript{−/−} MEFs on the outbred MF1 background demonstrated a reduced ability to grow. Hence it is proposed that the cell cycle defect observed in this investigation is due to the reduced CRaf expression and not just due to the absence of a functional CRaf kinase domain per se. This theory is supported by the BrdU data from craf\textsuperscript{−/−} MEFs, which also progress into S phase at a similar delayed rate to craf\textsuperscript{DA/DA} MEFs.

Cell cycle proteins associated with G1 and the G1/S transition, such as cyclin D3, cyclin E, CDK2, p21\textsuperscript{CIP1} and p27\textsuperscript{KIP1} have altered expression profiles in asynchronous craf\textsuperscript{DA/DA} MEFs, whereas the expression of cell cycle proteins associated with G2/M are unchanged. It is clear that the proteins with altered expression levels must function together to create a phenotype which does not cause cell cycle arrest in asynchronous cells, but delays the progression of the cell cycle at G1/S. The expression profiles of these proteins in craf\textsuperscript{DA/DA} and craf\textsuperscript{−/−} MEFs is detailed in Table 5.1

Table 5.1 Comparison of the expression of cyclin E, p21\textsuperscript{CIP1}, p27\textsuperscript{KIP1}, CDK2 and cyclin D3 in craf\textsuperscript{DA/DA} and craf\textsuperscript{−/−} MEFs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expression level in craf\textsuperscript{DA/DA} MEFs compared to wild-type</th>
<th>Expression level in craf\textsuperscript{−/−} MEFs compared to wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclin E</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>p21\textsuperscript{CIP1}</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>p27\textsuperscript{KIP1}</td>
<td>Reduced</td>
<td>Increased</td>
</tr>
<tr>
<td>CDK2</td>
<td>Reduced</td>
<td>No change</td>
</tr>
<tr>
<td>cyclin D3</td>
<td>Reduced</td>
<td>No change</td>
</tr>
</tbody>
</table>
Since CRaf kinase activity is absent in the small amount of protein expressed in craf$^{DA/DA}$ MEFs and the CRaf protein is completely absent in the craf$^{-/-}$ MEFs, it is difficult to reconcile why p27$^{KIP1}$, CDK2 and cyclin D3 show different expression profiles in craf$^{DA/DA}$ and craf$^{-/-}$ cells. An explanation is not currently available but this may be due to the variability that exists between the MEF lines and/or due to the different experimental conditions used to monitor the expression of the proteins in the craf$^{DA/DA}$ and craf$^{-/-}$ MEFs. One potential way to resolve this discrepancy is by the use of siRNA against CRaf in a wild-type cell line such as NIH3T3 fibroblasts and subsequent analysis of these proteins’ expression levels. However, since the expression of cyclin E and p21$^{CIP1}$ appears to be altered consistently in both craf$^{DA/DA}$ and craf$^{-/-}$ MEFs, the regulatory mechanisms of these two proteins are further discussed below. Although either protein would be a good candidate to scrutinise further, due to the time restraints of this investigation, the altered regulation of p21$^{CIP1}$ was focused on in the later parts of this Chapter.

The expression of cyclin E is reduced in both craf$^{DA/DA}$ and craf$^{-/-}$ MEFs. Since cyclin E and CDK2 form a complex which promotes cell cycle progression at late G1, the reduction of cyclin E expression is likely to delay the cell cycle at this point. Down-regulation of cyclin E, by microinjection of antibodies against the protein into cultured mammalian cells has been shown to prevent the transition from G0 to S phase (Ohtsubo, 1995). Therefore, it may seem unusual that craf$^{DA/DA}$ and craf$^{-/-}$ MEFs are still capable of progression through the cell cycle. However evidence for a degree of redundancy in the regulation of this stage of the cell cycle is accumulating, which implies compensation by other cyclin/CDK complexes. In one approach cyclin E1/2 knockout mice were produced (Geng, 2003). The phenotypes of such mice and cells demonstrated that E type cyclins were not strictly required for embryonic development or continuous cell cycle progression. However, when cell cycle re-entry was examined, defects were observed in the cyclin E knockout MEFs, which failed to re-enter the cell cycle.
Although cyclin E is involved in the regulation of the phosphorylation of Rb and subsequent release of the transcription factor E2F, it is itself an E2F target gene. Indeed the cyclin E promoter contains defined E2F binding sites (Ohtani et al., 1995, Geng et al., 1996, Le Cam et al., 1999). E2F-independent cyclin E transcription has also been described. For example LRH-1 induces cyclin E transcription in pancreatic and hepatic cells (Botrugno et al., 2004). The ERK cascade has been implicated in the E2F-dependent and -independent transcriptional regulation of cyclin E. For example ERK stimulates the induction of cyclin D1 expression via Fos family members. Thus the increased expression of cyclin D1 enables phosphorylation of pRb and release of E2F which induces cyclin E expression. In addition to the transcriptional control of cyclin E, the expression of the protein is also regulated by proteolysis. Two pathways exist to degrade the protein via the proteasome. The degradation of cyclin E bound to CDK2 is dependent on the Skp1/Cullin/F box protein-related complex, SCF^{Fbw7}, ubiquitin ligase. Fbw7 is an F-box protein which binds to cyclin E and promotes its ubiquitination (Koepp et al., 2001, Strohmaier et al., 2001). Free cyclin E is ubiquitinated via the ubiquitin ligase Cul-3 which binds to cyclin E in the absence of CDK2 (Clurman et al., 1996, Singer et al., 1999).

Due to the time restraints of this investigation it was decided that the mechanism of the increased expression of p21^{CIP1} in craf^{DA/DA} and craf^{-/} MEFs would be investigated further. p21^{CIP1} is a cyclin dependent kinase inhibitor (CKI) belonging to the CIP/KIP family. CKIs bind to CDKs inhibiting their activity; p21^{CIP1} specifically inhibits the activity of cyclin/CDK2 complexes to negatively modulate cell cycle progression at the G1/S transition (Brugarolas et al., 1999). Therefore the increase in its expression in both craf^{DA/DA} and craf^{-/} MEFs would be expected to restrict the cell cycle at G1/S, as is observed in these cells.

However the overall role of p21^{CIP1} in the cell cycle is confusing. It has been shown that over-expression of p21^{CIP1} in many situations results in complete cell cycle arrest, rather than the delay that is observed here (Niculescu et al., 1998, Ogryzko et al., 1997,
Radhakrishnan et al., 2004). Therefore it is surprising that the increase of p21\textsuperscript{CIP1} in \textit{craf}\textsuperscript{DA/DA} and \textit{craf}\textsuperscript{-/-} cells does not induce complete arrest. One possible explanation for this is that the protein may not be functional or that its subcellular localisation is altered. However the role of p21\textsuperscript{CIP1} is complicated further since some reports have implicated p21\textsuperscript{CIP1} as being a positive regulator of proliferation. For example p21\textsuperscript{CIP1} is over-expressed in some advanced human tumours (Seoane et al., 2002) and the proliferation of myeloid progenitor cells is compromised in mice lacking p21\textsuperscript{CIP1}, suggesting a positive role for p21\textsuperscript{CIP1} in the proliferation of these cells (Mantel et al., 1996). Indeed p21\textsuperscript{CIP1} is known to stabilise the interaction between D-type-cyclins and CDK4/6, thereby acting as a positive regulator of cell cycle (LaBaer, 1997). p21\textsuperscript{CIP1} increases the affinity of CDK4 and cyclin D1 by approximately 35-fold and targets CDK4/cyclin complexes to the nucleus. Furthermore, the binding of cyclin D complexes to p21\textsuperscript{CIP1} sequesters the protein, restricting its availability for p21\textsuperscript{CIP1}-mediated inhibition of cyclin E/CDK2 and cyclin A/CDK2 complexes at late G1 and S phase.

In addition evidence is emerging that the sub-cellular localisation of p21\textsuperscript{CIP1} can be regulated by phosphorylation on specific serine and threonine residues. For example phosphorylation of T145 by PKB induces the re-localisation of p21\textsuperscript{CIP1} from the nucleus to the cytoplasm (Zhou et al., 2001, Xia et al., 2004, Perez-Tenorio et al., 2006). Thus this cytoplasmic p21\textsuperscript{CIP1} would be unable to participate in its inhibitory role in the cell cycle.

As mentioned above, the over-expression of p21\textsuperscript{CIP1} has been show to cause cell cycle arrest at G1, G2 or S phase (Niculescu et al., 1998, Ogryzko et al., 1997, Radhakrishnan et al., 2004). p21\textsuperscript{CIP1} may induce arrest by different mechanisms including inhibiting CDK activity or by disrupting the activity of proliferating cell nuclear antigen (PCNA), an accessory protein for DNA polymerase δ, required for processive DNA synthesis. Thus, p21\textsuperscript{CIP1} has two distinct inhibitory effects on S-phase entry (Flores-Rozas et al., 1994, Waga et al., 1994). These inhibitory effects are mediated through different domains of p21\textsuperscript{CIP1}. The N-terminal domain of p21\textsuperscript{CIP1} contains the CDK inhibitory activity and the C-terminal domain binds PCNA (Chen et al., 1995, Goubin and Ducommun, 1995, Harper et al., 1995, Luo et al., 1995).
p21\textsuperscript{CIP1} expression is controlled at the transcriptional level by both p53-dependent and – independent mechanisms. However p21\textsuperscript{CIP1} expression can also be regulated by additional mechanisms including epigenetic silencing, mRNA stability, and ubiquitin-dependent and - independent degradation of the protein (Gartel, 2005). p21\textsuperscript{CIP1} has been demonstrated to be subject to proteasome-dependent degradation (Blagosklonny et al., 1996, Rousseau et al., 1999, Sheaff et al., 2000), although the ubiquitination of p21\textsuperscript{CIP1} has been a point of controversy since the mutation of all lysine residues did not stabilise the protein. However a study in 2003 demonstrated that ubiquitination occurs at the N-terminus of p21\textsuperscript{CIP1} by a unique mechanism (Bloom et al., 2003). The SCF ubiquitin ligase containing the F-box protein Skp2 has been implicated in p21\textsuperscript{CIP1} degradation since p21\textsuperscript{CIP1} expression increases in cells treated with antisense oligonucleotides to Cul1, Skp1 or Skp2 (Yu et al., 1998). Furthermore Cul1 and Skp2 can be found in complex with p21\textsuperscript{CIP1}. More recently p21\textsuperscript{CIP1} was shown to be a substrate of SCF\textsuperscript{Skp2} \textit{in vitro} and this E3 ubiquitin ligase was shown to be important for the degradation of p21\textsuperscript{CIP1} specifically during S-phase (Bornstein et al., 2003). Further study is required to identify p21\textsuperscript{CIP1} ubiquitin ligases during different cell cycle phases and under different physiological conditions. This is important as \textit{in vitro} studies have identified that SCF\textsuperscript{Skp2} only recognises p21\textsuperscript{CIP1} when in complex with cyclin E-CDK2 (Bornstein et al., 2003). However p21\textsuperscript{CIP1} is found in other complexes (e.g. with cyclin D-CDK4/6) and in different subcellular locations. Furthermore p21\textsuperscript{CIP1} is still unstable in cells which do not express Skp2. Therefore, ubiquitin ligases in addition to SCF\textsuperscript{Skp2} may be important in the degradation of p21\textsuperscript{CIP1}. Indeed the ring finger ubiquitin ligases Mdm2 (Jin et al., 2003) and p53RFP (Ng et al., 2003) have also been suggested to regulate p21\textsuperscript{CIP1} stability.
However, the data presented in Figure 5.9 demonstrate that mis-regulation of $p21^{\text{CIP1}}$ expression by the proteasome may not be a key factor in its increased expression since inhibition of the proteasome can raise the levels of $p21^{\text{CIP1}}$ still further in $\text{craf}^{-/}$ and $\text{craf}^{\text{DA/DA}}$ MEFs. Furthermore, qPCR analysis (Figure 5.12) indicated that the increased $p21^{\text{CIP1}}$ protein expression in CRaf kinase defective MEFs was most likely due to increased $p21^{\text{CIP1}}$ mRNA production or stability. Indeed the expression of $p21^{\text{CIP1}}$ mRNA in $\text{craf}^{\text{DA/DA}}$ MEFs was 2.7 fold greater than the expression level seen in $\text{craf}^{-/}$ MEFs. Hence $p21^{\text{CIP1}}$ is regulated at the level of mRNA production or stability in a CRaf dependent manner.

$\text{p21}^{\text{CIP1}}$ is a transcriptional target of p53; sequence analysis of rat, mouse, and human $\text{p21}^{\text{CIP1}}$ promoters revealed the conservation of two p53-responsive elements (el-Deiry et al., 1995). $\text{p21}^{\text{CIP1}}$ appears to be expressed normally in murine embryos and most tissues lacking a functional p53 gene (Macleod et al., 1995), indicating that p53 is not required for $\text{p21}^{\text{CIP1}}$ transcription during development or in unstimulated adult tissues. However γ-irradiation of p53-deficient mice suggested that p53-dependent regulation of $\text{p21}^{\text{CIP1}}$ is critical for the response to DNA damage (Macleod et al., 1995). Indeed $\text{p21}^{\text{CIP1}}$ is capable of mediating growth arrest when cells are exposed to DNA damaging agents in response to p53 activation (el-Deiry et al., 1993, el-Deiry et al., 1994). Furthermore $\text{p21}^{\text{CIP1}}$-deficient cells fail to arrest in response to p53 activation after DNA damage (Waldman et al., 1995). However the data presented in Figure 5.6 indicated that p53 expression is not altered in $\text{craf}^{\text{DA/DA}}$ MEFs. Therefore the altered regulation of $\text{p21}^{\text{CIP1}}$ transcription is believed to be p53-independent, although p53 activity was not examined in this study, so p53 dependent $\text{p21}^{\text{CIP1}}$ regulation cannot be ruled out completely.

A variety of additional transcription factors can also induce $\text{p21}^{\text{CIP1}}$ transcription, independently of p53. These are capable of specifically binding to a number of cis-acting elements contained within the $\text{p21}^{\text{CIP1}}$ promoter (Figure 5.13). For example the $\text{p21}^{\text{CIP1}}$ promoter contains six Sp1 binding sites. Sp1 is a member of a multi-gene family which also
The p21<sup>CIP1</sup> promoter contains a number of positive cis-acting elements, including six Sp1 sites (numbered 1–6) to which a variety of transcription factors bind, thus regulating transcription.
includes Sp2, Sp3, and Sp4. In U937 human leukemic cells Sp1 mediates \( p21^{\text{CIP1}} \) transcription via Sp1-1 and Sp1-2 sites in response to PMA and the phosphatase inhibitor okadaic acid (Biggs et al., 1996). The tumour-suppressor protein BRCA1 induces transcription of \( p21^{\text{CIP1}} \) via the region from 2143 to 293 bp which contains the Sp1-1 and Sp1-2 sites. DNA synthesis and S-phase progression was shown to be inhibited in human cancer cell lines which contain a functional \( p21^{\text{CIP1}} \) gene when BRCA1 was over-expressed (Somasundaram et al., 1997). In addition, the Sp1-3 site has been demonstrated to be required for \( p21^{\text{CIP1}} \) transcription in response to TGF-\( \beta \) (Datto et al., 1995), calcium (Prowse et al., 1997), butyrate (Nakano et al., 1997), the HMG–CoA reductase inhibitor lovastatin (Lee et al., 1998), the histone deacetylase inhibitor, trichostatin A (Sowa et al., 1997) and NGF (Gartel and Tyner, 1999). Indeed both TGF-\( \beta \) and butyrate inhibit proliferation and induce G1 cell cycle arrest in various cell types (Datto et al., 1995, Nakano et al., 1997) and lovastatin induces cell cycle arrest in p53 deficient human prostate carcinoma cells (Lee et al., 1998). Furthermore NGF may induce \( p21^{\text{CIP1}} \) transcription via regulation of the activity of the transcriptional coactivator p300 which subsequently cooperates with Sp1 to induce \( p21^{\text{CIP1}} \) (Gartel and Tyner, 1999).

Additional transcription factors which can induce \( p21^{\text{CIP1}} \) transcription include AP2, E2Fs, STATs, C/EBP\( \alpha \) and C/EBP\( \beta \). Activator protein 2 (AP2) has been shown to induce \( p21^{\text{CIP1}} \) transcription via an AP2 consensus binding site located between 2103 and 295 bp in the \( p21^{\text{CIP1}} \) promoter. AP2 mediated transcription of \( p21^{\text{CIP1}} \) has been shown to cause growth arrest in K562 human leukaemia cells treated with phorbol ester or the phosphatase inhibitor okadaic acid (Zeng et al., 1997). Interestingly treatment with okadaic acid activated the \( p21^{\text{CIP1}} \) promoter in a different leukaemia cell line, U937, although this occurred through an Sp1 binding site. E2F1 and E2F3 activate the \( p21^{\text{CIP1}} \) promoter via cis-acting elements between 2119 to 116 bp (Gartel et al., 1998, Hiyama et al., 1998). The activation of STAT proteins induced by the binding of ligands to their receptors has been correlated with \( p21^{\text{CIP1}} \) transcription and growth arrest. For example EGF and IFN-\( \gamma \) cause the activation of STAT1,
which results in cell growth arrest and the up-regulation of the $p21^{CIP1}$ promoter via STAT binding sites located at 2690, 22590, and 24233 bp in the $p21^{CIP1}$ promoter (Chin et al., 1996). In addition the haematopoietic growth factor thrombopoietin induces transcriptional activation of $p21^{CIP1}$ via STAT5 (Matsumura et al., 1997). The CCAAT/enhancer binding protein-β (C/EBPβ) induces $p21^{CIP1}$ transcription via an NF-IL6 consensus site in the $p21^{CIP1}$ promoter centred at 21924 bp (Chinery et al., 1997). C/EBPα has been shown to activate the $p21^{CIP1}$ promoter and also induces post-translational stabilisation of the p21 protein in adipocytes (Timchenko et al., 1996).

In addition to the induction of $p21^{CIP1}$ by the various factors described above, $p21^{CIP1}$ transcription may also be negatively regulated by c-myc (Coller et al., 2000). This activity requires the proximal promoter region of $p21^{CIP1}$. However DNA binding by c-myc is not essential for repression and it is controversial as to what $p21^{CIP1}$ transcription factors c-myc binds to (Claassen and Hann, 2000, Gartel et al., 2001). In addition several viruses including human papilloma virus and hepatitis C down-regulate $p21^{CIP1}$ by decreasing p53 protein levels (Hassan et al., 2004, Munger et al., 2004).

A further possibility is that the cell cycle defect identified in this Chapter is related to aberrant ERK activity. Prior investigations of MEFs with CRaf mutations indicated that ERK activity was not altered (Huser et al., 2001). However the data presented in Figure 3.5 indicates that, rather than a reduction in phospho-ERK levels in $craf^{DA/DA}$ MEFs, there is actually a slight increase in phospho-ERK. ERK kinase activity was quantitated in wild-type and $craf^{DA/DA}$ MEFs in Noble et al (2008) demonstrating that 5 min post serum stimulation the ERK kinase activity in $craf^{DA/DA}$ MEFs was increased by ~1.3 fold. This increase may be due to a compensatory up-regulation of BRaf kinase activity in cells lacking CRaf activity/expression. Indeed Huser et al (2001) identified a slight elevation in the induction of BRaf activity in $craf^{-/-}$ MEFs compared to $craf^{+/+}$ cells. For example, at 2 min post EGF stimulation there was a ~1.5 fold increase in BRaf activity in $craf^{-/-}$ MEFs. There is growing
evidence for the formation of CRaf/BRaf heterodimers, and it has been demonstrated that
BRaf is an activator of CRaf, although CRaf was found not to activate BRaf (Garnett et al.,
2005). However the data presented here implies that CRaf is a negative regulator of BRaf,
although this remains to be proven.

Increased BRaf/ERK activity in cells lacking functional CRaf could easily account for the
increased p21\textsuperscript{CIP1} expression as previously shown by Woods et al (1997). In this
investigation ΔRaf:ER constructs were activated in NIH3T3 cells. High levels of Raf signalling
were observed to cause up-regulation of p21\textsuperscript{CIP1} and down regulation of p27\textsuperscript{KIP1} as
has been observed in craf\textsuperscript{DA/DA} MEFs. However Woods et al (1997) also saw an up-
regulation of cyclin D1 and cyclin E expression which is not observed here. It is possible that
this disparity may be due to differential levels of ERK activation in the two experimental
systems. Other investigations have also demonstrated that the ERK cascade has a role in
the regulation of p21\textsuperscript{CIP1} transcription by both p53-dependent and –independent
mechanisms. For example high Raf activity, resulting from the expression of inducible
ΔRaf:ER constructs or oncogenic RAS have caused cell cycle arrest mediated by the
induction of p21\textsuperscript{CIP1} by either p53-dependent (Lloyd et al., 1997, Serrano et al., 1997) or –
independent mechanisms (Sewing et al., 1997, Woods et al., 1997).

However several data contradict the model that increased p21\textsuperscript{CIP1} expression is mediated by
increased BRaf/ERK activity and may imply that the phenotype is actually ERK-
independent. Although Huser et al (2001) also identified a slight elevation in the induction of
BRaf activity in craf\textsuperscript{FF/FF} MEFs compared to craf\textsuperscript{+/+} cells, no cell cycle defect has been
reported in craf\textsuperscript{FF/FF} MEFs. Furthermore BrdU analysis of craf\textsuperscript{FF/FF} MEFs shown in Figure 5.4
did not detect the G1/S delay observed in craf\textsuperscript{+/+} and craf\textsuperscript{DA/DA} cells. Due to the time restraints
of this project the expression of cell cycle proteins was not investigated in these cells,
however the investigation by Woods et al (1997) observed the same alterations in cyclin D1,
cyclin E, p27KIP1 and p21CIP1 expression upon activation of a ΔCRafY340FY341F:ER construct. At the present time the contradictory CRafY340FY341F data is difficult to reconcile.

The data presented in this Chapter indicates that CRaf regulates the expression of several cell cycle proteins including p21CIP1. Although the mechanism for the altered expression of p21CIP1 is not fully understood, the data suggests three possible alternatives. CRaf may be required for the regulation of p21CIP1 transcription factors, so that in cells which lack functional CRaf activity, the expression/activity of p21CIP1 transcription factors are up-regulated. The inverse of this possibility is the requirement for CRaf in the activity and/or expression of p21CIP1 transcriptional inhibitors, thus in cells which lack functional CRaf, the expression/activity of p21CIP1 transcriptional inhibitors are down-regulated. The third possibility is that in the absence of CRaf, BRaf kinase activity is up-regulated, resulting in increased ERK activation which mediates increased p21CIP1 transcription. Hence inhibition of CRaf kinase activity and/or reduction of its expression cause a delay in S-phase progression as summarised in Figure 5.14.
Figure 5.14. CRaf mediated regulation of G1-S-phase progression via p21CIP1

The loss of CRaf kinase activity and/or reduction in its expression results in increased expression of p21CIP1. Hence this causes a delay in S-phase progression. The mechanism for CRaf mediated p21CIP1 regulation has not been elucidated. However three of the most likely possibilities are: 1) CRaf may inhibit the expression of p21CIP1 by inhibiting the expression/activity of its known transcription inducers such as Sp1, Ap2, E2F, p53 etc. 2) CRaf may induce the activity/expression of p21CIP1 transcriptional repressors including c-myc. 3) CRaf may be a negative regulator of BRaf, although this remains to be formally proven. In the absence of CRaf, BRaf kinase activity is deregulated (Huser et al (2001) resulting in increased ERK activation (Noble et al (2008) which mediates increased p21CIP1 transcription, as previously demonstrated by Woods et al (1997).
6. SUMMARY AND DISCUSSION

6.1 Project background: Phenotypes of mice with CRaf mutations

The analysis of craf\(^{-}\) mice prior to this PhD project indicated that CRaf is necessary for normal mouse development. It was found that craf\(^{-}\) mice maintained on a C57BL6 background, did not survive embryogenesis and showed poor development of many organs, as well as increased apoptosis of MEFs and embryonic tissues (Huser et al., 2001, Mikula et al., 2001). However, on the outbred MF1 background craf\(^{-}\) embryos survived to a later age (Kamata et al., 2004) and MEFs displayed a reduced ability to grow, but no apoptotic phenotype (Mercer et al., 2005). This suggested that the craf\(^{-}\) phenotype is subject to genetic modifiers which are dependent upon the strain of mouse.

ERK activation was found to be normal, or even slightly increased, in all craf\(^{-}\) mouse strains, indicating that the essential functions of CRaf are independent of its MEK/ERK kinase activity. The apparently normal phenotype of craf\(^{Y340F/Y341F}\) mice, which are incapable of CRaf mediated activation of MEK (Huser et al., 2001), supported evidence that the MEK kinase activity of CRaf is not necessary to suppress apoptosis or for embryonic development.

However, analysis of craf\(^{Y340F/Y341F}\) mice did not determine if the MEK-independent kinase activity of CRaf was necessary for the prevention of apoptosis and embryonic development, or if simply the presence of the protein was required in the absence of any kinase activity, as CRaf is known to interact with many other proteins. Therefore in order to address this, kinase inactive CRaf mice were generated in our laboratory by Dr. K. Mercer prior to my joining the laboratory. An A1587C mutation was introduced into the craf gene leading to a D486A substitution in exon 14, specifically located in the DFG motif of the kinase domain. Disruption of the DFG motif prevents ATP coordination and hence renders the kinase domain inactive.
On the C57BL6 background, craf<sup>DA/DA</sup> mice and cells displayed an apoptotic phenotype similar to craf<sup>-/-</sup> mice and cells on the same genetic background. ERK activation was not reduced in craf<sup>DA/DA</sup> cells, providing additional evidence that CRaf MEK kinase activity is not essential in suppressing apoptosis. Nonetheless these observations suggested that CRaf kinase activity is required for the prevention of apoptosis. However preliminary data demonstrated that CRaf<sup>DA</sup> is expressed at very low levels in the cell. This would imply that the low level of kinase-inactive CRaf expression results in a phenotype similar to the craf<sup>-/-</sup> phenotype, hence precluding assessment of whether CRaf kinase activity is required for the phenotype. This project aimed to characterise the role of CRaf in regulating its own expression using the craf<sup>DA/DA</sup> mice.

6.2 Autophosphorylation of serine 621 regulates CRaf protein stability

Preliminary work unexpectedly demonstrated that the CRaf<sup>DA</sup> protein was expressed at very low levels in cells. Therefore the possibility that CRaf expression is controlled by CRaf kinase activity was investigated during the course of this project.

6.2.1 Kinase inactive CRaf is unstable

In Chapter 3 it was confirmed that CRaf expression, specifically, was reduced in craf<sup>DA/DA</sup> embryos and MEFs. It was investigated at what stage in the production of the protein this aberrant regulation occurred. qPCR assays ruled out the possibility of CRaf regulation occurring at the RNA level. Furthermore it was revealed that craf<sup>DA</sup> mRNA was not subject to alternative splicing between exons 11-17 as a result of the targeting event. However, when the stability of the CRaf protein was investigated, it became clear that instability of the CRaf<sup>DA</sup> protein accounted for its reduced expression. The t<sub>1/2</sub> of kinase inactive CRAF was 40-60% that of the wild-type protein. Furthermore the kinase inactive protein associated with the chaperone protein HSP90 more so than the wild-type protein, indicating that in the
absence of its kinase activity, CRaf is a misfolded and unstable protein. Previous investigations have used kinase inactive CRaf mutants as dominant interfering proteins in studies downstream from oncogenic RAS (Schaap et al., 1993, Chao et al., 1994, Troppmair et al., 1994). However the instability of kinase inactive CRaf should be taken into consideration in the interpretation of such experiments.

6.2.2 Kinase inactive CRaf is ubiquitininated and degraded by the proteasome

Previous investigations have shown that CRaf can be ubiquitininated and degraded by the proteasome (Schulte et al., 1997, Manenti et al., 2002, Du et al., 2006), hence this possibility was investigated in Chapter 3. Analysis of CRafDA expression in crafDA/DA MEFs treated with proteasome inhibitors demonstrated that the reduction in CRafDA protein expression was due to increased degradation by the proteasome. Additionally CRAF was shown to be ubiquitinated. Thus, the data indicated that the kinase activity of CRaf is required for regulating its protein expression by preventing its own proteasomal degradation. Since CRaf is a HSP90 client protein, and is known to bind the ubiquitin binding protein BAG1, the best candidate for the CRaf E3 ubiquitin ligase was CHIP. Indeed previous reports have demonstrated that CHIP can ubiquitinate CRaf and the protein’s proteasomal degradation could be facilitated by BAG1 (Demand et al., 2001). However contrary to this, the data presented here revealed that although CRAF was ubiquitinated, this does not occur exclusively via the actions of the E3 ubiquitin ligase CHIP and its co-chaperone BAG1.

6.2.3 Inactivation of CRaf kinase activity abrogates phosphorylation of serine 621

It has been demonstrated that CRaf kinase activity is required for regulating CRaf protein expression by the prevention of its own proteasomal degradation. Subsequently the mechanism for this mode of kinase-dependent regulation was elucidated. In Chapter 4 it was shown that the phosphorylation of S621 in endogenous CRafDA and ectopically expressed CRAFK375M and CRAF486A kinase inactive mutants was considerably reduced. This suggested that the phosphorylation of S621 is dependent upon CRaf kinase activity.
Indeed previous reports in the literature corroborate the requirement of CRaf kinase activity for S621 phosphorylation, since this residue is not phosphorylated in insect, HeLa, or Sf9 cells expressing kinase inactive CRaf (Mischak et al., 1996, Hekman et al., 2004, Thorson et al., 1998). Further evidence was obtained by treatment of wild-type cells with the Raf kinase inhibitor sorafenib. This was found to reduce the phosphorylation of S621 and confirmed that loss of this phosphorylation event was not due to misfolding of the CRafDA mutant protein in such a way as to sterically restrict the phosphorylation event but due to the lack of CRaf kinase activity per se.

6.2.4 Phosphorylation of serine 621 is required to stabilise CRaf

Subsequently the loss of S621 phosphorylation was found to be responsible for a reduction in CRaf stability which results in decreased CRafDA expression. Assessment of CRaf stability was determined by measurement of the t½ of the protein. Loss of S621 phosphorylation, either in the kinase dead mutants or the CRAF\textsuperscript{S621A} mutant, was associated with a reduction of the protein’s t½ by ~30-60% of CRAF\textsuperscript{WT} indicating a reduction in CRaf stability. The use of the Raf kinase inhibitor sorafenib produced data consistent with this finding. Treatment both abrogated S621 phosphorylation and reduced CRaf stability by ~34% of the t½ of CRaf in untreated cells. Thus, CRaf kinase activity is required for S621 phosphorylation, which is necessary for CRaf protein stability. Therefore the first essential role of CRaf kinase activity is to mediate the phosphorylation of this residue.

6.2.5 The phosphorylation of S621 occurs in cis

Early publications suggested that S621 phosphorylation was not a result of auto-phosphorylation but required a second kinase (Morrison et al., 1993). S621 kinases were proposed, notably PKA and AMPK (Mischak et al., 1996, Sprenkle et al., 1997). However more recent investigations supported S621 being a site of autophosphorylation (Mischak et al., 1996, Hekman et al, 2004, Thorson et al., 1998) and this view was corroborated by the findings of this investigation which demonstrated that S621 phosphorylation is CRaf kinase-
dependent. However the possibility existed of an intermediate S621 kinase, whose activity was dependent upon CRaf kinase activity. The roles of PKA and AMPK were addressed in Chapter 4 by chemical stimulation of these kinases. However, neither induced S621 phosphorylation, nor rescued kinase inactive CRaf stability. Given the known heterodimerisation of the Raf isoforms (Weber et al., 2001) it was investigated whether ARaf or BRaf were involved in the phosphorylation and stabilisation of CRaf. However it was demonstrated that neither ARaf nor BRaf acted as such an intermediate kinase.

The mode of S621 auto-phosphorylation was further investigated by several experimental approaches which indicated that the phosphorylation of S621 does not require dimerisation and trans-phosphorylation but occurs in cis. This further refutes the possibility of an intermediate kinase. This finding is supported by experiments in which S621 phosphorylation in CRaf\(^{K375M}\) could not be detected even when co-expressed with wild-type CRaf (Thorson et al., 1998). In theory the cis-phosphorylation of S621 requires the folding of the C-terminal fragment of CRaf spanning this residue to fold into the catalytic cleft. The crystal structure of CRaf has not yet been identified, but the crystal structure of the inactive BRaf kinase domain bound to sorafenib was solved by Wan et al (2004). This study omitted the S621 homologue, S729, hence it is unknown if this residue is able to be positioned inside the catalytic cleft. Data presented by Hekman et al (2004) and here in Chapter 4 demonstrated that S729 of BRaf is not autophosphorylated in the same way as S621 of CRaf, hence BRaf and CRaf must fold quite differently to allow autophosphorylation in one isoform but not the other. Therefore the determination of the complete crystal structure of CRaf is necessary to allow full understanding of mechanism of cis autophosphorylation of CRaf on S621.
6.2.6 BRaf does not share this mode of kinase-dependent regulation

It was postulated that BRaf stability could be regulated by a mechanism analogous to CRaf, involving autophosphorylation of S729. However as mentioned above, both an investigation by Hekman et al (2004) and the work presented in Chapter 4 demonstrated that S729 is not a site of auto-phosphorylation. Furthermore investigation of cells containing kinase inactive BRaf showed that BRaf expression was not decreased. Therefore, unlike CRaf, the BRaf kinase activity is not necessary for protein stability. Hence BRaf is not believed to share this form of kinase-dependent regulation with CRaf.

6.2.7 Model for CRaf auto-regulation

It is known that CRaf forms a multiprotein complex with chaperone proteins including HSP90 that ensure the protein’s correct folding in an ATP driven process (Kolch, 2000, Wartmann and Davis, 1994). Indeed dissociation of the CRaf/HSP90 complex, resulting from geldanamycin treatment, leads to the ubiquitination and degradation of CRaf (Schulte et al., 1995, Schulte et al., 1997).

Powers and Workman, (2006) proposed a model whereby HSP90 client proteins either achieve a stable tertiary structure, or become ubiquitinated and degraded by the proteasome, which can be applied to CRaf (Figure 4.15). Initially CRaf forms an immature complex with chaperones including HSP70 and later HSP90. Dimerisation of HSP90 and the activation of its ATPase activity results in the loss of HSP70 from the complex. Phosphorylation of S621 is associated with the acquisition of the correct CRaf tertiary structure and acts as a molecular switch between protein stability and degradation. Thus CRaf becomes competent to participate in signalling. Alternatively if correct folding fails to occur, HSP70 does not dissociate from the complex and recruitment of an E3 ubiquitin ligase occurs which mediates CRaf ubiquitination and degradation via the proteasome. Although data from previous reports highlighted the E3 ubiquitin ligase CHIP and its co-chaperone BAG1 (Connell et al., 2001, Demand et al., 2001, Murata et al., 2003, Song et
al., 2001), the data presented here indicates that neither are involved in the degradation of CRaf in the system investigated. At the present time, the E3 ligase and mechanism by which it targets CRaf for degradation are unknown. The work described above has been published in Noble et al (2008), which is presented in Appendix B.

**6.2.8 Future work**

It will be important to identify the ubiquitin ligase for CRaf. Work by Demand et al (2001) identified that the protein CHIP, a co-chaperone of HSP70 and HSP90 and an E3 ubiquitin ligase, stimulated the ubiquitination of CRaf. In addition the protein BAG1 has been shown to interact with CHIP and aid the targeting of ubiquitinated proteins to the proteasome. A more recent investigation by Dogan et al (2008) found that knockdown of CHIP by siRNA stabilised CRaf. However it has been demonstrated here that CHIP and BAG1 do not uniquely mediate the ubiquitination and degradation of CRaf, because in their absence the expression of kinase inactive CRaf is not increased. Further work is needed to investigate other candidate ubiquitin ligases. It is probable that more than one ubiquitin ligase is involved in the degradation of CRaf since in CHIP−/− cells the expression of kinase inactive CRAF was decreased suggesting the possibility of compensation for CHIP by other ubiquitin ligases.

It would be interesting to investigate this mode of CRaf regulation in different physiological settings. For example to inspect the stability of CRaf, its phosphorylation status and its possible degradation by the proteasome when cell anchorage is disrupted, as Manenti et al (2002) demonstrated that CRaf expression was reduced when cell anchorage was disrupted and suggested that this was due to ubiquitination and proteasomal degradation. The status of CRaf could also be further investigated in cells with BRaf mutations such as BRafV600E MEFs and in cancer cells with BRaf and RAS mutations that utilise CRaf as an effector.
6.3 CRAF is required for the kinetics of G1/S phase progression

Preliminary data found that craf$^{DA/DA}$ MEFs grew slower than wild-type MEFs in culture. This could be indicative of either an increase in apoptosis or a defect in the cell cycle; possibly either a delay at one or more stages of the cycle, or an overall reduction in the rate. Analysis of this phenotype is complicated since it could result from the reduced expression of CRaf and/or the lack of a functional kinase domain in the small amount of protein which is expressed. Indeed craf$^{-/-}$ MEFs on the outbred MF1 background also grew more slowly than expected in culture (Mercer et al., 2005). Therefore, during the course of this investigation the growth defects of CRaf kinase deficient and knockout cells were characterised to discover at what stage a potential cell cycle defect may exist and to examine the signalling pathways involved in this.

6.3.1 G0-G1-S-phase progression is delayed in cells lacking functional CRaf

Flow cytometry analysis of unsynchronised populations of craf$^{DA/DA}$ and wild-type MEFs in Chapter 5 demonstrated that the proportion of cells at various stages of the cell cycle differed in craf$^{DA/DA}$ MEFs. Such data may be indicative of a craf$^{DA/DA}$ cell cycle defect, with the potential of a block/delay in the cell cycle at any phase. Subsequent BrdU assays identified a ~2 h delay in the progression from G0 into S-phase, although this did not rule out the possibility of additional cell cycle defects. Previous investigations have observed a reduction in cell proliferation/growth in the absence of CRaf (Schumacher et al., 1998, Mercer et al., 2005). Therefore it was postulated that the cell cycle defect observed here is due to the reduction of CRaf protein expressed and not just to the absence of kinase activity per se. This hypothesis was supported by craf$^{-/-}$ BrdU data which progress into S phase at a similar delayed rate to craf$^{DA/DA}$ MEFs and craf$^{FF/FF}$ cell data which showed no cell cycle defect at this stage.
6.3.2 The expression of several late G1 and S phase cell cycle proteins is altered in cells lacking functional CRaf

Analysis of the expression of cell cycle proteins provided further support for a delay in S-phase transition in both crafDA/DA and craf−/− cells. Cell cycle proteins associated with G1 and the G1/S transition, specifically cyclin D3, cyclin E, CDK2, p21CIP1 and p27KIP1, were found to have altered expression profiles in asynchronous crafDA/DA MEFs. While the expression levels of these proteins were not mirrored exactly in craf−/− MEFs, in both cell lines the expression of p21CIP1 was increased and the expression of cyclin E decreased. Such alteration of cyclin E and p21CIP1 expression have been observed to restrict the cell cycle in a variety of experimental systems (Ohtsubo, 1995, Geng, 2003, Niculescu et al., 1998, Ogryzko et al., 1997, Radhakrishnan et al., 2004). The altered regulation of p21CIP1 was focused on as a result of time limitations, the availability of literary data and the presence of good p21CIP1 antibodies.

6.3.3 The increase in p21CIP1 protein expression in cells lacking functional CRaf is coincident with an increase in its mRNA expression

It is well established that the expression of p21CIP1 is controlled by p53-dependent and – independent mechanisms of transcriptional regulation. However additional regulatory mechanisms exist which include epigenetic silencing, mRNA stability, and ubiquitin-dependent and -independent degradation of the protein.

The possibility of altered proteasomal degradation of p21CIP1 was first investigated. In line with published data (Blagosklonny et al., 1996, Rousseau et al., 1999, Sheaff et al., 2000) it was clear that p21CIP1 expression was regulated by the proteasome since protein levels increased upon treatment with a proteasomal inhibitor. However, the data indicated that misregulation of p21CIP1 expression by the proteasome may not be the principal explanation for its increased expression since inhibition of the proteasome raised the levels of p21CIP1 still further in craf−/− and crafDA/DA MEFs.
Subsequently qPCR analysis revealed that the increased \( p21^{\text{CIP1}} \) protein expression in CRaf kinase defective cells was most likely due to the increased production or stability of \( p21^{\text{CIP1}} \) mRNA. Indeed the expression of \( p21^{\text{CIP1}} \) mRNA in \( \text{craf}^{\text{DA/DA}} \) MEFs was found to be \( \sim 2.7 \) fold greater than the level seen in wild-type cells. Hence, the data presented in Chapter 5 showed that \( p21^{\text{CIP1}} \) is regulated at the level of mRNA production or stability in a CRaf dependent manner, although the mechanism for this mode of regulation still requires elucidation.

**6.3.4 Future work**

Many studies have documented that over-expression of \( p21^{\text{CIP1}} \) results in cell cycle arrest rather than the delay that is observed in this investigation (Niculescu et al., 1998, Ogryzko et al., 1997, Radhakrishnan et al., 2004). Therefore it is surprising that the increase of \( p21^{\text{CIP1}} \) in cells lacking functional CRaf does not induce complete arrest. One possible explanation for this is that the protein may not be functional or that its subcellular localisation is altered. It is becoming evident that phosphorylation of \( p21^{\text{CIP1}} \) on specific residues may regulate the localisation of the protein. Indeed PKB mediated phosphorylation of T145 induces the re-localisation of \( p21^{\text{CIP1}} \) from the nucleus to the cytoplasm, preventing it from participating in its role in the cell cycle (Zhou et al., 2001, Xia et al., 2004, Perez-Tenorio et al., 2006). Therefore it would be interesting to examine the phosphorylation status of \( p21^{\text{CIP1}} \) expressed in CRaf kinase dead and knockout cells by western blotting using phospho-specific antibodies and to examine its localisation by immunofluorescence.

\( p21^{\text{CIP1}} \) is regulated by a number of mechanisms which mediate either mRNA or protein stability or operate at the transcriptional level. qPCR data presented in Chapter 5 demonstrated that the increased \( p21^{\text{CIP1}} \) protein expression in CRaf kinase defective MEFs was most likely due to increased \( p21^{\text{CIP1}} \) mRNA production or stability. Many mechanisms exist to regulate \( p21^{\text{CIP1}} \) transcription; hence any one of these could be deregulated by the loss of CRaf, enabling increased \( p21^{\text{CIP1}} \) expression. For example \( p21^{\text{CIP1}} \) is a transcriptional
target of p53, thus it may be interesting to investigate if p53 expression/activity is up-regulated in cells lacking functional CRaf, although data presented in Chapter 5 indicated that p53 expression was not altered in craf^{DA/DA} MEFs. Additional p21^{CIP1} transcription factors which would be possible candidates to investigate include Sp1, AP2, E2Fs, STATs, C/EBPα and C/EBPβ. The transcription of p21^{CIP1} can also be negatively regulated by proteins including c-myc (Coller et al., 2000), hence the expression/activity of this protein could also be examined in cells lacking functional CRaf.

Work conducted by Woods et al (1997) indicated that high levels of Raf/MEK/ERK signalling caused up-regulation of p21^{CIP1} and down-regulation of p27^{KIP1}, changes that have been observed in Chapter 5 in craf^{DA/DA} MEFs. However Woods et al (1997) also saw an up-regulation of cyclin D1 and cyclin E expression which was not observed here. Even so it is theorised that a possible explanation for the identified cell cycle defect in cells lacking functional CRaf could be related to the aberrant ERK activity observed in these cells. Hence it would be desirable to assess whether the delay in G0-G1-S transition and the increased p21^{CIP1} expression in cells lacking functional CRaf is ERK-dependent. Prior investigations of MEFs with CRaf mutations indicated that ERK activity was not altered (Huser et al., 2001). However during the course of this project, phospho-ERK levels in craf^{DA/DA} MEFs were found to be consistently elevated. ERK kinase activity was quantitated in wild-type and craf^{DA/DA} MEFs in Noble et al (2008) and the results presented indicated that 5 min post serum stimulation the ERK kinase activity in craf^{DA/DA} MEFs was increased by ~1.3 fold. It can be theorised that this increase may be due to a compensatory up-regulation of BRaf kinase activity in cells lacking CRaf activity/expression. Indeed Huser et al (2001) identified a slight elevation in the induction of BRaf activity in craf^{−/−} MEFs in comparison with craf^{+/+} cells. For example, at 2 min post EGF stimulation there was a ~1.5 fold increase in BRaf activity in craf^{−/−} MEFs. This would suggest that CRaf is a negative regulator of BRaf. It has been shown that CRaf and BRaf form a heterodimer and that BRaf is an activator of CRaf, although it has been demonstrated that CRaf is not an activator of BRaf (Garnett et al.,
2005). However, that CRaf is a negative regulator of BRaf is a novel possibility, but is currently only implicated from our studies of cells lacking functional CRaf and remains to be formally proven. Increased BRaf/ERK activity could easily account for the increased p21\textsuperscript{CIP1} expression as previously shown by Woods et al (1997).

An initial experiment to determine if this phenotype is ERK-dependent would be to treat cells lacking functional CRaf with MEK inhibitors in order to rescue the phenotype. Subsequently the role of BRaf in the activation of ERK could be assessed by treatment with siRNA. Indeed this work is ongoing and preliminary results from our laboratory are encouraging.

### 6.4 Conclusions

The RAS/RAF/MEK/ERK pathway is hyperactivated in human cancers, mainly due to oncogenic RTK, RAS and BRAF mutations. The CRaf protein kinase plays a key role in relaying signals from activated RAS or BRaf proteins to downstream effectors. Because of its essential role in signaling, CRaf kinase activity must be tightly regulated in the cell. The accepted model involves translocation of CRaf to the plasma membrane upon RAS activation. Subsequently CRaf is activated by a series of events including phosphorylation. Through the generation of a kinase defective CRaf\textsuperscript{D486A} mutant in mice, or by the use of the Raf inhibitor sorafenib a new mechanism of regulation has been identified in which autophosphorylation of S621 is required to stabilise the protein, preventing its degradation by the proteasome. This novel mechanism of regulation has significant implications for the control of CRaf activity in normal and cancer cells. The reduction of CRaf expression, caused by either by knockout or kinase dead mutations, renders CRaf unable to participate in normal signalling. Thus, this leads to an increase in apoptosis and to cell cycle defects by mechanisms which have yet to be fully elucidated. The proposed model of CRaf mediated signalling is shown in Figure 6.1.
Figure 6.1. CRaf auto-regulates via autophosphorylation of S621, thus preventing its proteasomal degradation. Subsequently CRaf is required for the prevention of apoptosis and G0-G1-S phase cell cycle progression.

CRaf forms an immature multi-protein complex with HSP90 and HSP70. For simplicity, additional chaperones are not included. HSP90 dimerisation and ATPase activation leads to the dissociation of HSP70. In conjunction with S621 autophosphorylation, CRaf acquires its correct tertiary structure and becomes competent to inactivate ASK1/MST2 preventing apoptosis, activate MEK and inhibit p21CIP1 expression, allowing cell cycle progression. Alternatively, if CRaf remains misfolded, an E3 ubiquitin ligase is recruited to the complex, mediating CRaf ubiquitination and proteasomal degradation. This model proposes that CRaf is a negative regulator of BRaf, although this remains to be formally proven. Deregulation of BRaf could give rise to higher levels of p21CIP1 expression as previously demonstrated by Woods et al (1997).
Appendix A

BrdU analysis of the cell cycle in $craf^-$, $craf^{DA/DA}$ and $craf^{FF/FF}$ MEFs

Representative Immunofluorescence images of BrdU positively stained $craf^{DA/DA}$, $craf^-$ and $craf^{FF/FF}$ primary MEFs and their matched wild-type counterparts at 0h, 10h and 18h post addition of BrdU labelling reagent/serum stimulation are shown in Figures A.1 - A.3 respectively.
Figure A.1. **Representative Immunofluorescence images of BrdU positively stained craf^{DA/DA} and craf^{+/+} MEFs**

craf^{DA/DA} and craf^{+/+} MEFs were serum starved for 24 h to synchronise cells in G0. Cells were then simultaneously treated with BrdU labelling reagent and stimulated with serum for time-points up to 18h. Cells were counterstained with DAPI and visualised by immunofluorescence. Images are x40 magnification, scale bar = 15μm. **(A)** Representative images are shown of craf^{+/+} cells at 0h, 10h and 18h. **(B)** Representative images are shown of craf^{DA/DA} cells at 0h, 10h and 18h.
Figure A.2. **Representative Immunofluorescence images of BrdU positively stained craf−/− and craf+/+ MEFs**

craf−/− and craf+/+ MEFs were serum starved for 24 h to synchronise cells in G0. Cells were then simultaneously treated with BrdU labelling reagent and stimulated with serum for time-points up to 18h. Cells were counterstained with DAPI and visualised by immunofluorescence. Images are x40 magnification, scale bar = 15μm. (A) Representative images are shown of craf+/+ cells at 0h, 10h and 18h. (B) Representative images are shown of craf−/− cells at 0h, 10h and 18h.
**Figure A.3. Representative Immunofluorescence images of BrdU positively stained craf\textsuperscript{FF/FF} and craf\textsuperscript{+/+} MEFs**

craf\textsuperscript{FF/FF} and craf\textsuperscript{+/+} MEFs were serum starved for 24 h to synchronise cells in G0. Cells were then simultaneously treated with BrdU labelling reagent and stimulated with serum for time-points up to 18h. Cells were counterstained with DAPI and visualised by immunofluorescence. Images are x40 magnification, scale bar = 15μm. (A) Representative images are shown of craf\textsuperscript{+/+} cells at 0h, 10h and 18h. (B) Representative images are shown of craf\textsuperscript{FF/FF} cells at 0h, 10h and 18h.
Appendix B

Publications
CRAF Autophosphorylation of Serine 621 Is Required to Prevent Its Proteasome-Mediated Degradation

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SUMMARY

The CRAF protein kinase regulates proliferative, differentiation, and survival signals from activated RAS proteins to downstream effectors, most often by inducing MEK/ERK activation. A well-established model of CRAF regulation involves RAS-mediated translocation of CRAF to the plasma membrane, where it is activated by a series of events including phosphorylation. Here we have discovered a new mode of regulation that occurs prior to this step. By creating a kinase-defective version of CRAF in mice or by use of the RAF inhibitor sorafenib, we show that CRAF must first undergo autophosphorylation of serine 621 (S621). Autophosphorylation occurs in cis, does not involve MEK/ERK activation, and is essential to ensure the correct folding and stability of the protein. In the absence of S621 phosphorylation, CRAF is degraded by the proteasome by mechanisms that do not uniquely rely on the E3 ubiquitin ligase CHIP.

INTRODUCTION

The RAS/RAF/MEK/ERK cascade is a conserved intracellular signaling pathway that plays a crucial role in controlling the ability of cells to respond to their environment (Avruch et al., 2001; Marais and Marshall, 1996). ERKs are the ultimate effectors of the pathway that can phosphorylate and activate numerous nuclear and cytoplasmic substrates involved in mediating the appropriate cellular response (Yoon and Seger, 2006). A major point of control of ERK occurs at the level of RAF, of which there are three family members in mammals: ARAF, BRAF, and CRAF (Mercer and Pritchard, 2003; Wellbrock et al., 2004). Of these three, BRAF has by far the strongest ability to activate ERK (Huser et al., 2001; Pritchard et al., 1995) and is a key mediator of ERK activation in several physiological settings (Rushworth et al., 2006). The predominant role of BRAF is underpinned by the discovery of somatic mutations of BRAF in human cancer samples but the rarity of CRAF mutations and the absence of ARAF mutations (Davies et al., 2002; Garnett and Marais, 2004). The most common BRAF mutation, a valine to glutamic acid change at residue 600 (V600E BRAF), is activated by more than 500-fold and promotes tumor progression by inducing constitutive ERK activation (Garnett and Marais, 2004; Mercer and Pritchard, 2003; Wan et al., 2004).

Although recent emphasis has been placed on BRAF, much of our understanding of the control of RAF activity is based on CRAF (Wellbrock et al., 2004). CRAF normally resides in the cytoplasm as an inactive kinase bound to 14-3-3 adaptor/scaffold proteins (Kolch, 2000). Conversion of RAS.GDP to RAS.GTP leads to the displacement of 14-3-3, dephosphorylation of serine 259, and the recruitment of CRAF to the plasma membrane (Leevers et al., 1994; Marais et al., 1997; Stokoe et al., 1994). Here, CRAF undergoes regulatory phosphorylation events and can bind to lipids and other proteins that together convert CRAF to an active kinase. Five phosphorylation sites within or flanking the kinase domain are involved in this. Binding of 14-3-3 to the C terminus of CRAF is mediated by phosphorylation of serine 621 (S621) (Muslin et al., 1996; Wellbrock et al., 2004). Phosphorylation of serine 338 (S338) and tyrosine 341 (Y341) within the N region (Marais et al., 1995; Mason et al., 1999) and threonine 491 (T491)/serine 494 (S494) in the activation segment of the kinase domain (Chong et al., 2001) occur following CRAF membrane localization. The kinases involved in these essential steps have not all been elucidated, although S621 is known to be a site of CRAF autophosphorylation (Hekman et al., 2004; Mischak et al., 1996; Thorson et al., 1998), and it has been shown that casein kinase 2 (CK2) can phosphorylate S338 (Ritt et al., 2007).

Apart from its ability to activate MEK/ERK, CRAF is known to have other targets in the cell. Ablation of craf in mice causes widespread apoptosis and embryonic lethality without alterations in MEK/ERK activity, and the creation of a MEK kinase-inactive version of CRAF with the YY340/341FF mutations (CrafFF) confirmed that this phenotype is MEK/ERK independent (Huser et al., 2001; Mikula et al., 2001). Biochemical evidence has since...
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shown that CRAF suppresses apoptosis by binding to and inhibiting the activity of two proapoptotic kinases: apoptosis-signal-regulating kinase 1 (ASK1) (Chen et al., 2001) and mammalian sterile 20-like kinase 2 (MST2) (O’Neill et al., 2004). It can also inhibit the ability of Rokα to control Fas clustering and internalization at the cell membrane in a MEK kinase-independent manner (Piazzolla et al., 2005). The fact that knockout mutation of ask1 rescues cardiomyocyte apoptosis induced by ablation of craf is supportive of a role of ASK1 as an apoptotic effector of CRAF (Yamaguchi et al., 2004).

CRAF is also part of a multiprotein complex containing a number of chaperone proteins including heat shock protein 90 (HSP90), p50/cdc37, and HSP70 (Kolch, 2000; Wartmann and Davis, 1994). Through an ATP-driven process, HSP90 is able to ensure the correct folding of the protein and disruption of the CRAF-HSP90 interaction with geldanamycin leads to CRAF misfolding and its consequent degradation via the proteasome (Demand et al., 2001; Schulte et al., 1997; Schulte et al., 1995). The degradation of misfolded CRAF is thought to occur by the recruitment of the E3 ubiquitin ligase CHIP (C terminus of Hsp70-interacting protein) to the complex. CHIP interacts with HSP70 via tandem tetra tricopeptide (TPR) repeats and also has a RING-finger-like domain that facilitates the ubiquitylation of chaperone client proteins like CRAF (Connell et al., 2001; Demand et al., 2001). The degradation of CRAF is facilitated by the ubiquitin-binding protein BAG-1, which targets ubiquitylated proteins to the proteolytic complex (Demand et al., 2001; Song et al., 2001).

In this study, we have further investigated CRAF with the aim of clarifying its kinase-dependent and -independent functions. This involved the creation of mice expressing a kinase-inactive version of CRAF in which aspartic acid residue 486 within the DFG motif of the activation segment was converted to alanine (D486ACRAF). Homozygous D486ACRAF mice have increased levels of apoptosis in a similar way to mice with a craf knockout mutation. Here we show that this phenotype can be attributed to the fact that CRAF kinase activity is required for stabilizing its own protein expression by a mechanism involving autophosphorylation of S621 via an intramolecular reaction. In the absence of this phosphorylation event, CRAF is misfolded and targeted to the proteasome, although we show that this process does not uniquely rely on CHIP or BAG-1. Thus we have identified a new mode of regulation of CRAF that is absolutely essential to fulfill its role in signaling.

RESULTS

Generation and Analysis of Kinase-Dead CRAF in Mice

We generated homozygous mice expressing the D486A-craf mutation by using a gene-targeting knockin approach (Huser et al., 2001; Figure 1A). The mutation creates a kinase-inactive protein as measured using the immunoprecipitation MEK-ERK cascade assay (Marais et al., 1997) (Figure 1B). Unexpectedly, we found that CRAF protein levels were reduced by >50-fold in crafDA/DA embryos and mouse embryonic fibroblasts (MEFs) compared to craf+/+ embryos and MEFs (Figures 1C and 1D). The expression of other components of the RAF/MEK/ERK pathway was not affected (Figure 1D), and total protein synthesis was not reduced in crafDA/DA cells (Figure 1E), suggesting that the effect was specific for the CRAF protein. Craf mRNA levels (Figure 1F) and splicing (see Figure S1 available online) were not altered, excluding the possibility that the targeting event disrupted craf mRNA production or processing. Thus, CRAF kinase activity is required for controlling its own expression at the protein level.

A consequence of the low level of expression of kinase-dead CRAF is that crafDA/DA embryos have a phenotype similar to the craf+/− null phenotype (Figure 2) (Huser et al., 2001; Mikula et al., 2001). Like the craf+/− embryos, the crafDA/DA embryos are developmentally retarded and have widespread apoptosis (Figure 2A), dying at E10.5–E12.5. crafDA/DA MEFs have a reduced capacity to grow (Figure S2) with increased levels of apoptosis in response to serum withdrawal, etoposide, and α-CD95 antibody (Figure 2B). Consistent with observations in craf+/− cells (Huser et al., 2001; Mikula et al., 2001), ERK phosphorylation and activation is not disrupted in crafDA/DA cells in response to stimulation of cells with exogenous growth factors or apoptosis inducers (Figures 2C–2E and Figure S2). Indeed, we have consistently observed increased levels of active ERK in both crafDA/DA and craf+/− cells in response to a variety of extracellular stimuli. These results confirm that the MEK kinase activity of CRAF is not essential in suppressing apoptosis and also suggest that the MEK/ERK pathway is not involved in mediating the effect of CRAF in regulating its own expression.

Kinase-Inactive CRAF Is Unstable and Degraded by the Proteasome

To determine whether the kinase activity of CRAF is required for stabilizing the protein, the half-lives of wild-type CRAF and kinase-inactive CRAF were compared. In initial experiments, the t1/2 of endogenous CRAF in elaborated cells was examined by pulse labeling and found to be ~7 hr (Figure S3). Although several attempts were made to measure the t1/2 of endogenous D486A-CRAF in crafDA/DA cells, it proved impossible to pulse label this protein due to its low expression level. Therefore, experiments were performed by transfection of myc-tagged vectors expressing WT-CRAF and two different kinase inactive versions of CRAF: K375M-CRAF and D486C-CRAF. Transfections were performed into craf+/− cells in order to prevent any effects of endogenous CRAF in cross-stabilizing the transfected proteins. The t1/2 values were as follows: WT-CRAF, ~80 min; K375M-CRAF, ~45 min; and D486C-CRAF, ~50 min, showing that kinase-inactive CRAF is ~40–60% less stable than WT-CRAF and that both mutants are alike in this respect (Figure 3A). Transfected WT-CRAF is also considerably less stable than endogenous WT-CRAF, possibly because proteins involved in binding and stabilizing CRAF are titrated out as a consequence of the overexpression.

To examine the role of the proteasome in regulating CRAF stability, crafDA/DA MEFs were treated with a range of proteasomal inhibitors and CRAF expression levels analyzed in Triton X-100 soluble and insoluble fractions. All treatments rescued the expression of CRAF to levels similar to that in craf+/+ cells, suggesting that proteasomal degradation is the cause of the reduced CRAF expression (Figure 3B). With epoxomicin and MG132, in particular, a significant proportion of D486A-CRAF was in the insoluble fraction and showed a laddering pattern typically observed in proteins that are ubiquitylated.

CRAF is a HSP90 client protein, and this interaction is necessary for establishing and stabilizing CRAF tertiary structure (Kolch,
It has also been demonstrated that the CHIP E3-ubiquitin ligase stimulates the degradation of chaperone substrates such as CRAF by a process involving the chaperone cofactor BAG1 (Demand et al., 2001). To examine whether this pathway is involved in the increased turnover of kinase-inactive CRaf, we first examined the interaction of D486ACRaf with chaperone proteins.

2000; Wartmann and Davis, 1994). It has also been demonstrated that the CHIP E3-ubiquitin ligase stimulates the degradation of chaperone substrates such as CRAF by a process involving the chaperone cofactor BAG1 (Demand et al., 2001). To examine whether this pathway is involved in the increased turnover of kinase-inactive CRaf, we first examined the interaction of D486ACRaf with chaperone proteins, D486ACRaf had a significantly increased ability to bind HSP90 compared to the wild-type protein.
CRAF Autophosphorylation Prevents Its Degradation

(Figure 3C), indicating that the D486A craf mutation generates a misfolded protein, although binding to HSP70 was not noticeably altered (Figure S4B). To examine CRaf ubiquitylation, vectors expressing myc-tagged kinase-inactive K375M CRAF and WT CRAF as well as the S621A CRaf mutant were cotransfected into cells with HA-tagged ubiquitin. Following treatment with lactacystin, CRAF immunoprecipitates were analyzed with an antibody for HA. All forms of CRaf showed evidence of mono- and/or polyubiquitylation (Figure 3D). siRNA was used to knock down CHIP in craf DA/DA cells, but this did not lead to alterations in the level of expression of D486A CRaf (Figure 3E), suggesting that CRAF is not uniquely ubiquitylated by CHIP. These data were supported by the fact that the expression of K375M CRAf is not stabilized in CHIP−/− cells (Figure S5). In addition, knockdown of the ubiquitin-binding protein BAG1 also does not resurrect D486A CRaf levels (Figure 3F). Thus, although kinase-inactive CRaf is
misfolded and degraded by the proteasome, this process is not uniquely modulated by the CHIP E3 ubiquitin ligase/chaperone.

Phosphorylation of S621 Is Required to Stabilize CRAf

Data provided above (Figure 2) rule out a role of the best-characterized effector pathway of CRAf—the MEK/ERK pathway—in mediating the effect of CRAf in stabilizing itself. However, in our analysis, we consistently observed that D486ACRAf migrated faster in SDS-PAGE than WT CRAf (Figures 1C, 1D, and 3B). Since phosphorylation of CRAf is known to play a role in this, we analyzed phosphorylation of kinase-inactive CRAf. Phosphorylation of S621 was found to be virtually absent in the D486ACRAf protein expressed in crafDA/DA cells (Figure 4A) as well as in K375M CRAf and D486A CRAf ectopically expressed in craf−/− cells (Figure 4B). Interestingly, although the majority of WT CRAf was detected in the soluble fraction and was phosphorylated on S621, a significant portion of WT CRAf was in the insoluble fraction, but this was not phosphorylated on S621 (Figure 4A). Other CRAf phosphorylation sites including S259 and S338 were not affected by the kinase-inactivating mutations (Figures 4B). Although it was not possible to examine the phosphorylation of Y341, T491, or S494 due to the lack of good phosphoantibodies for these sites, we examined whether mutation of these sites affected the stability of the protein. Following transfection into craf−/− cells, the activation segment mutant T491A/S494ACRAf was expressed at similar levels as WT CRAf (Figure 4C). In addition, CRAf expression level was not altered in MEFs expressing CRAff (Figure 4D). These results suggest that phosphorylation of Y341, T491, and S494 does not play a role in stabilizing CRAf.

To confirm that disruption of RAF activity per se is responsible for this effect, treatment of cells with the RAF kinase inhibitor sorafenib was assessed and led to a significant disruption of S621 phosphorylation and decreased stability of the protein (Figure 4E). To confirm a role of S621 phosphorylation in stabilization of CRAf, the stability of the nonphosphorylatable S621ACRAf mutant was examined by pulse-chase labeling following transfection into craf−/− cells. In a similar way to kinase-inactive CRAf, this mutant was found to be ~50% less stable than WT CRAf, with a t1/2 of ~40 min (Figure 4F). These results suggest that the kinase activity of CRAf is required for S621 phosphorylation and that its absence an unstable protein is formed.

CRAf Autophosphorylation Occurs In cis

Although we have shown that the phosphorylation of S621 of CRAf requires its own kinase activity, it is conceivable that an
intermediary kinase is involved whose activity is dependent on CRAF kinase activity. Given the known heterodimerization of RAF kinases (Rushworth et al., 2006; Wan et al., 2004), we investigated whether ARaf or BRaf may play a role in phosphorylating and stabilizing CRAF. However, we found that neither the expression level of CRaf nor S621 phosphorylation was altered in MEFs with knockout mutations of araf or braf (Figure 5A). In addition, AMP-activated protein kinase (AMPK) and cAMP-dependent protein kinase (PKA) have both been previously suggested to be S621 kinases (Mischak et al., 1996; Sprenkle et al., 1997). However, we found that neither the expression level of CRaf nor S621 phosphorylation was altered following treatment of CRaf with S621 phosphorylation was altered following treatment of CRaf with sorafenib (Figure 5E). These results strongly suggest that phosphorylation of CRaf on S621 by itself occurs in cis.

**DISCUSSION**

In a well-established model of CRAF regulation, inactive CRAF is held in the cytoplasm and is phosphorylated on S259 and S621 that allow binding to 14-3-3 scaffold/adaptor molecules (Kolch, 2000; Mercer and Pritchard, 2003). Induction of the MEK kinase activity of CRAF by extracellular signals is achieved by RAS.GTP-mediated displacement of 14-3-3, resulting in recruitment of CRAF to the plasma membrane, where it is converted to its active state by a succession of critical regulatory steps, including phosphorylation of key residues. It is also known that CRAF is an HSP90 client protein and that the chaperone activity of HSP90, in conjunction with other chaperones such as HSP70 and p50cdc37, is responsible for either establishing the correct tertiary structure of the protein or for targeting CRAF molecules that remain misfolded for degradation by the proteasome (Kolch, 2000; Powers and Workman, 2006). The data presented here neatly combine these two modes of CRAF regulation to provide a comprehensive model (Figure 6). CRAF is a misfolded protein unless it undergoes autophosphorylation in cis of S621, thus preventing it from being degraded by the proteasome. Without this stabilization, CRAF levels are prohibited low and it is unable to participate in normal signaling.

**Autophosphorylation Is a Key First Step in CRAF Regulation**

We show here that the first essential role of CRAF kinase activity is to autophosphorylate S621. In an initial examination of CRAF phosphorylation sites using phosphopeptide mapping, Morrison et al. (1993) provided evidence that S621 phosphorylation was not compromised following the expression of the K375M/CRAF mutant in insect cells. However, using phosphoantibodies, there...
has been growing evidence in favor of S621 being a site of autophosphorylation (Hekman et al., 2004; Mischak et al., 1996; Thorson et al., 1998), and this view is corroborated by our findings here. Indeed, it has been shown that S621 autophosphorylation occurs within seconds after growth factor stimulation in the cytoplasm of the cell (Hekman et al., 2004), which confirms...
DYRKs and GSK3

Figure 6. A Comprehensive Model of CRAF Autoregulation

CRAF is part of an immature multiprotein complex that includes the chaperones HSP90 and HSP70. CRAF then has one of two fates: activation of the ATPase activity of HSP90 and HSP90 dimerization leads to the dissociation of HSP70, and, in conjunction with S621 autophosphorylation, CRAF acquires its correct tertiary structure and becomes competent to either activate MEK or inactivate MEK. Alternatively, if CRAF remains misfolded, an E3 ubiquitin ligase is recruited to the complex, leading to ubiquitylation of CRAF and its degradation via the proteasome. A key modulator of this binary switch is autophosphorylation of S621. For simplicity, a range of other chaperones thought to be involved in this process such as p50cdc37 and HSP40 are not included on this figure.

The autokinase activity of CRAF does not involve homo-or heterodimerization and transphosphorylation but occurs in cis (Figure 5). Teleologically, this must invoke a model in which a C-terminal fragment of CRAF spanning S621 folds into the CRAF catalytic cleft where it can be phosphorylated. The current crystal structure of the RAF kinase domain was established for an inactive version of BRAF bound to the RAF inhibitor sorafenib and does not accommodate this possibility, since a C-terminal fragment spanning the equivalent serine in BRAF, S729, was deleted in order to allow the protein to be crystallized (Wan et al., 2004). It is known that S729 of BRAF is autophosphorylated like S621 of CRAF (C.N. and C.A.P., unpublished data; Hekman et al., 2004), and so the BRAF and CRAF kinase domains must fold quite differently to allow autophosphorylation in one isoform but not the other. This critical difference in the modulation of the two RAFs can only be further clarified by solving the crystal structure of CRAF without deleting the C-terminal fragment containing S621 and comparing it to that established for BRAF.

CRAF Suppresses Apoptosis Independently of Its MEK Kinase Activity

A wealth of biochemical data have established CRAF as a MEK kinase that is induced by RAS.GTP (Kolch, 2000), but its relative contribution to ERK activation is low, and it is known that BRAF has considerably stronger activity as a MEK kinase in many physiological settings. A recent study showed that CRAF:BRAF heterodimers have considerably higher MEK kinase activity than either BRAF or CRAF homodimers, even though they are present at very low concentrations in the cell (Rushworth et al., 2006). Therefore, a key role of CRAF in MEK/ERK activation may be as a cofactor for BRAF. The importance of such crossregulation is highlighted by the fact that many cancer samples with oncogenic BRAF mutations possess deregulated CRAF activity that can contribute to tumor induction via ERK activation in some situations (Wan et al., 2004). Our results presented here show that the overriding role of CRAF during embryonic development is to suppress apoptosis, and this is mediated through the deregulation of other CRAF effectors, particularly ASK1, a proapoptotic kinase whose activity is negatively regulated by CRAF (Figure 6) (Chen et al., 2001; Yamaguchi et al., 2004). The MEK kinase activity of CRAF does not seem to be involved here, since ERK activation is not disrupted under these circumstances (Figure 2) (Huser et al., 2001; Mikula et al., 2001).

The Role of S621 Phosphorylation and 14-3-3 Binding Is to Stabilize CRAF

In the past, the role of S621 phosphorylation and 14-3-3 binding to the C terminus of CRAF has been controversial, with some studies suggesting that 14-3-3 binding is necessary for activity, whereas others show that dissociation of 14-3-3 from CRAF does not alter its kinase activity (Kolch, 2000; Wellbrock et al., 2004). The work presented here provides important clarification on this issue by showing that S621 phosphorylation is absolutely critical to ensure the correct tertiary structure of CRAF so that it is not targeted for degradation. As such, therefore, our results support the view that that 14-3-3 binding is essential for CRAF activity. The fact that WT-CRAF moves to the insoluble fraction if it is not phosphorylated on S621 (Figure 4A) provides evidence that, if this regulatory step does not occur, CRAF is sequestered from its normal cytoplasmic and plasma membrane-associated substrates.

CRAF Is Degraded by the Proteasome

Several previous reports have demonstrated CRAF ubiquitylation and degradation by the proteasome (Du et al., 2006; Manenti et al., 2002; Schulte et al., 1997), and, consistent with the data presented here, disruption of the CRAF-HSP90 interaction with geldanamycin leads to destabilization of CRAF by the proteasome (Schulte et al., 1997). PEST domains within proteins
frequently serve to promote degradation (Rechsteiner and Rogers, 1996), and, indeed, CRAF contains a PEST sequence in the variable hinge region between CR2 and CR3 at residues 284–309, and a lysine at residue 309 may serve as a ubiquitin attachment site. This PEST domain in CRAF is not conserved in either ARAF or BRAF, although BRAF contains a PEST sequence at a different position within residues 298–338 in the variable region between CR1 and CR2, suggesting that it may also be regulated by protein degradation.

It is well known that CRAF is an HSP90 client protein and can also bind various other chaperones including HSP70 and p50cdc37 (Kolch, 2000; Wartmann and Davis, 1994). Based on analysis of other HSP90 client proteins, a model for how these various chaperones ensure the correct folding of their substrates has been proposed (Figure 6) (Powers and Workman, 2006). Initially, client proteins interact with HSP70 and HSP90 in a complex with other chaperones to form an immature complex. When the ATPase activity of HSP90 is activated, it undergoes a conformational change that includes transient dimerization. This leads to the dissociation of HSP70 and its associated chaperones and allows the ATP-dependent association of other chaperones such as p50cdc37 (Powers and Workman, 2006). It is while in this mature state that the client protein is folded such that it can become an active protein, and, in the case of CRAF, we have shown that autophosphorylation of S621 is also a key step in this process. If the client protein does not fold, then HSP70 mediates targeting to the proteasome for degradation (Connell et al., 2001; Demand et al., 2001).

The CHIP protein has been shown to interact with HSP70 and stimulates the degradation of chaperone substrates. It is thought to do this by recruiting E2 ubiquitin-conjugating enzymes of the Ubc4/5 family to the chaperone complex and acting as a E3 ubiquitin ligase to add ubiquitin residues to the chaperone substrate, thus inducing its targeting to the proteasome. The targeting process is also facilitated by its ubiquitin domain-binding protein BAG1 (Demand et al., 2001; Song et al., 2001). Since CRAF is a HSP90 client protein and is known to bind BAG1, CHIP was considered to be the best-candidate E3 ubiquitin ligase that mediates the increased degradation of kinase-inactive CRAF described here. However, our data showing that CHIP and BAG1 knockdown do not restore the expression level of D486ACRAF suggest that CRAF can be targeted for degradation by alternative mechanisms. The nature of these other pathways, and particularly the E3 ubiquitin ligases involved, is currently unknown.

There is a growing link between phosphorylation, ubiquitylation, and proteasomal degradation, and many kinases are now known to be involved in regulatory steps that generally promote proteasome-mediated degradation of their target proteins (Hoeller et al., 2006). There are also numerous examples of phosphorylation preventing proteasome-mediated degradation such as p53 phosphorylation by ATR and ATM damage response kinases. However, CRAF is the first case of autophosphorylation preventing degradation of a kinase. Although BRAF is also an HSP90 client protein, current evidence suggests that it is regulated differently to CRAF, as it is far less sensitive to HSP90 inhibition (da Rocha Dias et al., 2005). In addition, autoregulation is not involved in stabilizing BRAF, as by creating mice expressing the analogous kinase inactive mutant D594ABraf we have found that its kinase activity does not affect either S729 phosphorylation or the expression level of the protein (C.N. and C.A.P., unpublished data).

In summary, our data have identified an important mode of regulation of CRAF and have elucidated that autophosphorylation is a key step in stabilizing the protein. These results have important implications in normal growth factor signaling as well as in cancer and suggest that S621 autophosphorylation is critical for this protein to perform its function within these contexts. Our data should also be taken into consideration in experiments using kinase-inactive CRAF as a dominant interfering protein.

**EXPERIMENTAL PROCEDURES**

**Generation of craf<sup>DA/DA</sup> Mice and Embryo Analysis.**

craf<sup>DA/DA</sup> mice with the D486ACRAF knockin mutation were generated by standard procedures. Inheritance of the targeted allele was assessed by PCR genotyping using primers A (5′-CTCTTGAATTCAGCTTTGAC-3′) and B (5′-GGTTTACCACCAACTGTGC-3′). To delete the neo cassette, craf<sup>DA/neo</sup> mice were crossed to CMV-Cre mice (Schwenk et al., 1995), and a breeding colony of craf<sup>DA/neo</sup> mice was established on the C57Bl6 background. Embryos were harvested at E10.5–14.5 and used for MEFs, protein lysates, or histology as described (Huser et al., 2001).

**Cell Treatments.**

MEFs were transfected with expression vectors using a nucleofector under the conditions recommended by the manufacturer (Amansa Biosystems, Germany). siRNA was transfected using oligofectamine according to the manufacturer’s instructions (Invitrogen). CHIP-specific siRNA (5′-GGGAUGAUJAUUC CUAUGUGCUU-3′; Drhmacron) or a Bag-1 siRNA pool (Dharmacon) was used. A siCONTROL nontargeting siRNA pool (Dharmacon) was used as a control. For protease inhibition, cells were treated with either 0.5 μM epoxomicin, 0.5 μM lactacystin, or 30 μM MG132 for 6 hr to prepare of preparation of protein lysates. Apoptosis was induced by treating MEFs with either 50 ng/ml s-CD95 antibody with 0.5 μM cycloheximide for 20 hr, 75 μM etoposide for 20 hr, or serum-free medium (SFM) for 48 hr and assessed by annexin V staining. Cells were treated with 0–20 μM sorafenib with 0–300 μM A-769662 in DMSO or with 25 μM fonskolin/500 μM IBMX in DMSO.

**Protein Analysis.**

Triton X-100 soluble proteins were prepared by taking the supernatants following 13,000 rpm centrifugation of total protein lysates as described (Lucett et al., 2000), and insoluble proteins were obtained by treating the pellets with 2% (v/v) SDS. The primary antibodies used were the following: ARaf (Santa Cruz Biotechnology, Inc., SC-408), BRAf (BD Biosciences, 610152), actin (Santa Cruz Biotechnology, Inc., SC-805), myc tag (Cell Signaling Technology, Inc., 9421), phosphoser338 (Serotec, MCA 1852), GAPDH (Chemicon International, MAB374), HA (Santa Cruz Biotechnology, Inc., SC-805), myc tag (Cell Signaling Technology, 2726), HSP90 (Stressgen, SPA-830), HSP70 (Stressgen, SPA-820), CHIP (Abcam, AB2917), BAG1 (Abcam, AB7976), phospho-ACC (Cell Signaling Technology, 3661), ACC (Cell Signaling Technology, 3662), and phosphoserine 43 (Dumaz et al., 2002). The ERK2 antibody was a kind gift from Prof. Chris Marshall (ICR, London). The Santa Cruz Biotechnology, Inc., SC-227 CRAF antibody was used for immunoprecipitation, and kinase assays were performed using the kinase cascade assay (Marais et al., 1997). ERK2 kinase assays were performed and quantitated as previously described (Wan et al., 2004).
Quantitative RT-PCR
Primers for craf were 5’-ATACATCTCGGTGTTTCTTGCC-3’ (forward) and 5’-GCCTCCTTACCTTTGTTGTG-3’ (reverse). Primers for gapdh were 5’-AGGTCGGTGTGAACGGATTG-3’ (forward) and 5’-TGTAGACCATGTAGTTGAGGCTCA (reverse). cDNA was PCR amplified using 300 nM of each primer and SYBR Green (Bio-Rad) using a Bio-Rad MiniOpticon Real Time PCR system. Each sample was amplified in triplicate with each primer set, and mean C_v values were obtained. ΔC_v for each sample was calculated by normalizing the C_v value for craf to the C_v value for gapdh. ΔΔC_v was calculated by normalizing the ΔC_v value for the craf^DA/DA sample to the ΔC_v value for the craf^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^


References


References


DA ROCHA DIAS, S., FRIEDLOS, F., LIGHT, Y., SPRINGER, C., WORKMAN, P. & MARAIS, R. (2005) Activated B-RAF is an Hsp90 client protein that is targeted by

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References


References


MATSUMURA, I., ISHIKAWA, J., NAKAJIMA, K., ORITANI, K., TOMIYAMA, Y., MIYAGAWA, J., KATO, T., MIYAZAKI, H., MATSUZAWA, Y. & KANAKURA, Y.


REFERENCES


References


REFERENCES


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References


