Mechanisms of $^{V600E}$BRAF-induced tumour development

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

By

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March 2011
ABSTRACT

Mechanisms of V600E BRAF-induced tumour development

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BRAF is a serine/threonine protein kinase that functions as a component of the RAS/RAF/MEK/ERK signalling pathway. Gain-of-function BRAF mutations have been found in ~50%-70% of malignant melanomas and in a variety of other cancer types, including thyroid, colon, and lung cancers. The activating V600E BRAF mutation is the most frequent mutation comprising a single amino acid substitution (V600E) that results in constitutive activation of BRAF kinase activity. Here, the CreERT\textsuperscript{T} system is used to induce endogenous V600E BRaf expression from a single knockin allele after 4-hydroxytamoxifen treatment in MEFs. V600E BRaf induced a faster G\textsubscript{1}/S phase progression by a significant upregulation of Cyclin D1 at the transcriptional level and was reversed by MEK inhibition. V600E BRaf also suppressed apoptosis at the pre-mitochondrial level following serum withdrawal by a drastic downregulation in Bim\textsubscript{EL} expression at the post-transcriptional level which was rescued by MEK inhibition and proteasomal inhibition. In vivo, expression of endogenous V600E BRaf in mice induced senescence and autophagy in lung adenomas and this prevented lung tumour progression. Senescence was primarily mediated by the p53/p21\textsuperscript{Cip1} and p16\textsuperscript{INK4a} pathways while autophagy was regulated by inhibition of the mTOR pathway. Mass spectrometry was used to screen for components of the senescence-messaging secretome induced by V600E BRaf in the lung. The cholesterol binding protein Npc2 was identified as being significantly induced by V600E BRaf expression. The role of Npc2 in tumour suppression was investigated and it was found that this protein induced upregulation of the p53/p21\textsuperscript{Cip1} pathway and triggered autophagy in a paracrine/autocrine manner. Knockout of Npc2 abrogated senescence and promoted growth of lung tumours by downregulation of the p53/p21\textsuperscript{Cip1} pathway in vivo, but did not prevent the induction of autophagy.
ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor Prof. Catrin Pritchard for her excellent guidance, invaluable suggestions and continued encouragement throughout my PhD. I would like to thank my committee members, Dr. L. Miguel Martins and Raj Patel for their generous help and suggestions on this project. I am grateful to both past and present members of lab 3/43. Particular thanks to Susan Giblet, Dr. Linda Carragher, Dr. Tamihiro Kamata, Dr. Katerina Andreadi, Dr. Catherine Noble, Dr. Neil Bate, Dr Kees Straatman, Dr. Emma Stringer, Bipin Patel, Kimberley Snell, Maria Aguilar and Maggie Cheung for their support, advice and friendship.

I would specially like to thank Prof. Peter Lobel for his generous help in providing NPC2 reagents and knockout animal. I am grateful to Dr. Andrew Bottrill and his colleagues for the excellent proteomic work. I would like to thank Mr Stefan Hyman and Miss Natalie Allcock for all the help and advice in ultrastructural observation. I am thankful to Jenny Edwards for the histological assistance. My thanks also go to all those involved within the Biomedical Services for their excellent technical expertise. I would also like to thank Drs Xiaoming Sun and Hailan Chen for their help and friendship.

I would also like to especially thank my parents-in-law for their support, understanding and encouragement.

I am deeply indebted to my beloved wife for her constant support, love, patience during my study.

Lastly, I wish to thank my parents. They bore me, raised me, supported me, taught me and loved me. To them I dedicate this thesis.
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<th>Description</th>
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<tbody>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
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<td>4E-BP1</td>
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<tr>
<td>ATP</td>
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<td>ATR</td>
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<td>BCL-2-interacting mediator of cell death</td>
</tr>
<tr>
<td>bNPC2</td>
<td>bovine NPC2</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAK</td>
<td>CDK-activating kinase</td>
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<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
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<tr>
<td>Caspase</td>
<td>Cysteine aspartate specific protease</td>
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<tr>
<td>CDC</td>
<td>Cell division cycle</td>
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<td>Cyclin-dependent kinase</td>
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<td>CHK</td>
<td>Checkpoint kinase</td>
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<tr>
<td>CIP</td>
<td>CDK-interacting protein</td>
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<tr>
<td>CRD</td>
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</tr>
<tr>
<td>C_T</td>
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<tr>
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<td>4’,6-diamidino-2-phenylindole</td>
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<tr>
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<td>Deleted in esophageal cancer 1</td>
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<tr>
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<tr>
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<td>Fas-associated death domain protein</td>
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<tr>
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<td>G_0</td>
<td>Gap 0 phase</td>
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<td>G₁</td>
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<td>Gap2 phase</td>
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<td>GAPDH</td>
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<td>Inhibitor of apoptosis proteins</td>
</tr>
<tr>
<td>IEG</td>
<td>Immediate early gene</td>
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<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
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<td>MKKK/MEKK</td>
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<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilisation</td>
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<tr>
<td>MPR</td>
<td>Mannose-6 phosphate receptor</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MST2</td>
<td>Mammalian sterile 20-like kinase</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>MTT</td>
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<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RBD</td>
<td>Ras binding domain</td>
</tr>
<tr>
<td>rhNPC2</td>
<td>recombinant human NPC2</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>S</td>
<td>Synthesis</td>
</tr>
<tr>
<td>S6K</td>
<td>p70 S6 kinase</td>
</tr>
<tr>
<td>SAHF</td>
<td>Senescence-associated heterochromatin foci</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SA-β-gal</td>
<td>Senescence-associated β-galactosidase</td>
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<tr>
<td>SMS</td>
<td>Senescence-messaging secretome</td>
</tr>
<tr>
<td>Stc1</td>
<td>stanniocalcin 1</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS/Tween-20</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TMRE</td>
<td>Tetramethylrhodamine ethyl ester</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR-associated death domain protein</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis</td>
</tr>
<tr>
<td>ULK</td>
<td>Unc-51-like kinase</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>v/v</td>
<td>volume to volume</td>
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<td>w/v</td>
<td>weight to volume</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
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1.1 Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) are the most extensively studied protein kinases involved in intracellular signal transduction, regulating a variety of cellular responses, including cell proliferation, differentiation and apoptosis (Avruch, 2007; Chang and Karin, 2001; Davis, 1993). There are three MAPK groups: extracellular signal-regulated protein kinases (ERK1/2), c-Jun N-terminal kinase (JNK), and p38^{MAPK} (Davis et al., 2000; Johnson and Lapadat, 2002). MAPKs can be activated by diverse stimuli, including growth factors and cellular stresses. In response to stimuli, MAPKs are activated through phosphorylation of both threonine and tyrosine residues of the TXY (Thr-X-Tyr) motif in the catalytic domain by upstream dual-specificity kinases (Ray and Sturgill, 1988). These upstream kinases named M KKs (MAPK kinases) or MEKs, include MEK1/2, MEK3/6 and MEK4/7, which in turn are activated by MAPK kinase kinases (MKKK or MEKK) (Pearson et al., 2001; Chang and Karin, 2001). Once activated, MAPKs phosphorylate a number of substrates in the cytoplasm and nucleus, and subsequently activate a set of signalling pathways controlling cell proliferation, differentiation and apoptosis (Chang and Karin, 2001). ERK1/2 generally controls cell growth and differentiation, whereas JNK and p38^{MAPK} control stress responses (Figure 1.1) (Robinson and Cobb, 1997).

1.1.1 The RAS/RAF/MEK/ERK pathway

The RAS/RAF/MEK/ERK signalling pathway transmits extracellular signals to the transcription factors in the nucleus to regulate gene expression (Robinson and Cobb, 1997). Growth factors bind to and activate receptor tyrosine kinases (RTKs) at the cell surface, which stimulate RAS activation through a series of adaptor proteins and exchange factors. RAS proteins are attached to the inner surface of the plasma membrane. In their active form they bind to and recruit RAF proteins from the cytosol to the plasma membrane, where RAF is activated. RAF then phosphorylates and activates
### Figure 1.1 Schematic overview of MAPK pathways

There are three major mammalian MAPKKK–MAPKK–MAPK protein kinase cascades. Whereas the ERK pathway is commonly activated by growth factors, the JNK and p38$_{\text{MAPK}}$ pathways are activated by environmental stress. Many of the substrates for MAPKs are nuclear transcription factors involved in growth, development, differentiation and inflammation.

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**Stimulus**
- Growth factors
- Mitogens
- Growth factors
- Cytokines
- Stress

**MAPKKK**
- RAF
- MEKK
- TAK

**MAPKK**
- MEK1/2
- MEK4/7
- MEK3/6

**MAPK**
- ERK1/2
- JNK
- p38$_{\text{MAPK}}$

**Biological response**
- Growth
- Differentiation
- Development
- Inflammation
- Apoptosis/Growth
- Differentiation
the MEK kinases (MEK1 and MEK2), which in turn phosphorylate and activate the ERK kinases (ERK1 and ERK2). ERK1/2 phosphorylates substrates in the cytosol, and it also translocates to the nucleus where it phosphorylates numerous substrates including transcription factors such as Elk1 (Marais et al., 1993; Janknecht et al., 1993; Kortenjann et al., 1994), c-Ets-1 and c-Ets-2 (Figure 1.2) (Wasylyk et al., 1993; Coffer et al., 1994).

1.1.2 RAS-upstream activator of the RAF/MEK/ERK pathway

RAS is a small GTP-binding protein, which acts as an upstream regulator of several characterised signalling pathway including RAF/MEK/ERK, PI3K/AKT/mTOR and RalEGF/Ral (Peyssonnaux et al., 2000). There are four RAS proteins identified in mammals: HRAS, NRAS, KRAS4A and KRAS4B. The two KRAS proteins are produced from the same gene by alternative splicing. RAS is a 21-kDa membrane associated guanine nucleotide binding protein, cycling between a GDP-bound and a GTP-bound state. RAS activation is initiated by binding of growth factors, cytokines and mitogens to their receptors. Upon stimulation by Shc/Grb2/SOS, the inactive RAS exchanges GDP for GTP and undergoes a conformational change; thus RAS becomes active. The active RAS can recruit RAF to the plasma membrane for its activation and subsequent stimulation of the MEK/ERK signalling pathway (Figure 1.2). It has been shown that ~30% of human cancers harbour activating RAS mutations, one of the most common genetic alterations in human tumourigenesis (Schubbert et al., 2007). Constitutive activation of the RAS signalling leads to malignant transformation by promoting proliferation, enhanced mobility, survival, and loss of anchorage dependence (Downward, 2003). In the RAS family, KRAS mutations are the most frequent mutations and occur in 30% of non-small cell lung cancers (NSCLC), 50% of colorectal adenocarcinomas and 90% of pancreatic adenocarcinomas. 15% percent of melanomas and 30% of acute myelogenous leukemias have activating NRAS mutations. Activating mutations in HRAS are fairly rare but contribute to squamous cell carcinomas, bladder carcinomas and renal cancers (Downward, 2003).
Figure 1.2 Schematic representation of the RAS/RAF/MEK/ERK pathway

Growth factors bind to and activate receptor tyrosine kinases (RTKs) at the cell surface, which stimulate RAS activation. Active RAS binds to and recruits RAF proteins to the plasma membrane for activation. RAF then phosphorylates and activates the MEK kinases, which in turn phosphorylate and activate the ERK kinases. ERKs phosphorylate proteins in the cytosol, and also translocate to the nucleus, where they phosphorylate numerous substrates to regulate proliferation, differentiation, senescence, and apoptosis.
1.2 RAF protein family

1.2.1 History of RAF proteins

RAF kinases are a family of serine/threonine kinases at the central position in the evolutionary conserved RAS/RAF/MEK/ERK signalling pathway (Robinson and Cobb, 1997). In mammalian cells, there are three RAF isoforms: ARAF, BRAF and CRAF (Marias et al., 1997; Hagemann and Rapp, 1999). The first Raf gene to be identified was v-raf, the transforming gene of the mouse sarcoma virus 3611, which induces fibrosarcomas and erythroleukaemias in newborn mice. The cellular homologue of v-raf is Craf (Rapp et al., 1983). Subsequently, the avian homologue of v-raf, the v-mil oncogene of the avian retrovirus MH2 was identified (Sut rave et al., 1984). Araf was found through the screening of a mouse spleen DNA library with a v-raf probe (Huleihel et al., 1986). Braf was also identified by homology to the avian homologue v-Rmil (Marx et al., 1988) and isolated from NIH 3T3 cells transformed with human Ewing sarcoma DNA (Ikawa et al., 1988). The three RAF isoforms differ in their, structure, regulation and ability to activate downstream effectors in the ERK kinase cascade (see below).

1.2.2 Structure of RAF proteins

All RAF proteins share three conserved regions (CR): CR1, CR2 and CR3. CR1 contains a RAS binding domain (RBD) (Scheffler et al., 1994; Vojtek et al., 1993) and a cysteine-rich domain (CRD) (Mott et al., 1996). Both interact with activated RAS-GTPases in order for recruitment of RAF to the plasma membrane. A putative zinc-binding domain is also found in the CR1 (Beck et al., 1987; Ghosh et al., 1994). In CRAF, CR1 encompasses residues 61-194. CR2 of CRAF encompassing residues 254-269 is a serine/threonine-rich domain containing a 14-3-3 binding site at S259. CR3 is the catalytic kinase domain, which in turn contains two regions important for RAF activation: the activation segment and the glycine-rich loop. The activation segment spans the conserved sequences DFG and APE, which is involved in ATP binding. CR3 of CRAF spans residues 335-627 (Daum et al., 1994; Morrison and Cutler, 1997). CR1 and CR2 in the N terminus are largely regulatory, whereas CR3 in the C-terminus is catalytic (Figure 1.3) (Wellbrock et al., 2004a).
Figure 1.3 Schematic representation of RAF proteins

Three RAF isoforms ARAF, BRAF and CRAF share three conserved regions: CR1 (blue), CR2 (green) and CR3 (purple). CR1 contains the RAS-binding domain (RBD) and the cysteine-rich domain (CRD); both are required for membrane recruitment of RAF. CR2 contains a 14-3-3 binding site at S259. CR3 contains the kinase domain. The amino acids that are highlighted in red above individual isoforms refer to known phosphorylation sites required for RAF protein regulation and activation.
1.2.3 Regulation of RAF

Both phosphorylation and dephosphorylation events play important roles in RAF regulation. RAF activation is initiated by RAS bound-GTP binding to the RBD and CRD in the N-terminus of RAF. In unstimulated cells, CRAF is located in the cytoplasm and is phosphorylated at two serine residues: S259 and S621 (S214 and S582 in ARAF, S365 and S729 in BRAF, respectively) that bind 14-3-3 proteins retaining CRAF in an inactive and closed conformation. When stimulated by growth factor signalling, active RAS displaces 14-3-3 from S259 and CRAF is translocated to the plasma membrane where it is dephosphorylated at S259 by phosphatases such as PP2A (Abraham et al., 2000; Jaumot and Hancock, 2001; Ory et al., 2003). At the plasma membrane, distinct serine and tyrosine phosphorylation events take place for full CRAF activation (Dhillon et al., 2002). Similarly, BRAF is located in the cytoplasm through binding to 14-3-3 proteins at phosphorylated S365 and S729 (Brummer et al., 2006). BRAF appears to have a more open conformation compared to CRAF. Following activation of RAS, BRAF is translocated to the plasma membrane, where S365 is dephosphorylated by PP2A and additional phosphorylation events in the activation segment are required for full BRAF activation (Figure 1.4) (Mercer and Pritchard, 2003).

RAS-triggered phosphorylation in the activation segment is an essential event in the activation of RAF after recruitment to the plasma membrane (Zhang and Guan, 2000). Within the kinase domain there is an activation segment and, in BRAF, phosphorylation of T599 and S602 within this region is a critical event for activation (Zhang and Guan, 2000). The crystal structure of the kinase domain of BRAF provides a molecular explanation of how phosphorylation of this region stimulates its kinase activity (Figure 1.5) (Wan et al., 2004). The activation segment is kept in an inactive conformation by binding directly to the glycine-rich loop in the resting state. Importantly, T599 is at the interface of the interaction surface between the activation segment and the glycine-rich loop. T599 phosphorylation is predicted to disrupt the binding between these two
Figure 1.4 Model for RAF activation (Taken from Mercer and Pritchard, 2003)

**A** BRAF is maintained in the cytoplasm in a complex with 14-3-3 by binding to S365 and S729 (S259 and S621 in CRAF). BRAF in the cytoplasm has a more open conformation than CRAF due to the presence of phospho-S446 and D449. Upon activation of RAS, BRAF binds to RAS, resulting in its translocation to the plasma membrane. PP2A may remove the phosphate group from S365 (S259 in CRAF). Activating kinases are then able to phosphorylate T599 and S602 (T491 and S494 in CRAF) resulting in full BRAF activation. **B** Additional tyrosine and serine kinases are required to phosphorylate Y341 and S338, leading to full CRAF activation.
regions, freeing the activation segment and allowing the kinase to fold into an active conformation (Wan et al., 2004). It is unclear why S602 phosphorylation is required for activation, but it appears to be less important than T599 and may form additional interactions within the kinase domain to stabilise the active conformation or perhaps it is a priming site that allows more efficient phosphorylation of T599 (Gray-Schopfer et al., 2005). The corresponding sites of T599 and S602 in CRAF, T491 and S494, must also be phosphorylated for activation and these sites are conserved in ARAF (T452 and T455), suggesting these isoforms are regulated by similar mechanisms (Figure. 1.4) (Chong et al., 2001).

Another motif that is important for RAF activity is the N-region (negative-charge regulatory region) localised upstream of CR3 (Figure 1.3). Phosphorylation in this region has important consequences for the differential regulation of RAF isoforms. In CRAF, S338 and Y341 residues (S299 and Y302 in ARAF, respectively) in the N-region are unphosphorylated in resting cells, but become phosphorylated under activating conditions (King et al., 1998; Fabian et al., 1993). Phosphorylation of these sites is essential for full CRAF kinase activity (Marais et al., 1997; Mason et al., 1999). In contrast, S446 in the N-region of BRAF (S338 in CRAF), is constitutively phosphorylated, and instead of a tyrosine residue, D449 (Y341 in CRAF) contains a permanent negative charge, mimicking phosphorylated tyrosine. Thus, unlike ARAF and CRAF, the N-region of BRAF carries a constitutive negative charge. As a result, activation of BRAF requires fewer phosphorylation steps and BRAF is primed for activation by RAS alone which mediates membrane recruitment and stimulates phosphorylation in the activation segment. Thus, compared with BRAF, regulation of ARAF and CRAF is more complex because they require both RAS and SRC for full activation (Marais et al., 1997; King et al., 1998). The N-region negative charge in BRAF appears to explain why BRAF has elevated basal kinase activity compared to ARAF and CRAF in the resting state (Marais et al., 1997). This suggests that BRAF will be activated under a greater variety of conditions and may be the isoform that is primarily responsible for signalling between RAS and MEK in the majority of cells. Importantly, it
may also make BRAF more susceptible to mutagenesis in cancer (Gray-Schopfer et al., 2005).

1.2.4 RAS/RAF mutations in human cancer

Constitutive activation of the RAS/RAF/MEK/ERK signalling pathway is commonly observed in human cancer (Hoshino et al., 1999). About 30% of human cancers harbour activating RAS mutations (Schubbert et al., 2007). High kinase activity RAS and BRAF mutations are mutually exclusive and rarely co-exist in the same cancers, but cancer types with BRAF mutations are similar to those with RAS mutations (Sensi et al., 2006). BRAF is the only RAF protein to be frequently mutated in cancer, probably due to the fact that its constitutive activation requires fewer mutation events as indicated above. No ARAF mutations have been identified so far and CRAF mutations are rarely found (Emuss et al., 2005; Lee et al., 2005; Zebisch et al., 2006). However, CRAF is overexpressed in some ovarian and pulmonary carcinomas, raising the possibility that CRAF may also act as an oncogene (Rapp et al., 1988; McPhillips et al., 2006).

1.2.4.1 BRAF mutations

BRAF mutations have been found in ~7% of human cancers overall, and BRAF activating mutations are detected in ~50-70% of malignant melanomas, ~30% of papillary thyroid and serous ovarian carcinomas, ~15% of colorectal cancers, and at a lower frequency in a wide range of other human cancers. There are over 45 missense mutations involving 24 different codons in the \textit{BRAF} gene in human cancers (Davies et al., 2002; Garnett and Marais, 2004). Most of the BRAF mutations are within or close to the glycine-rich loop or the activation segment of the kinase domain of BRAF (Figure 1.5 and 1.6). Importantly, in normal conditions, these regions interact to maintain BRAF in an inactive conformation, and the mutations in these regions are thought to disrupt this interaction and induce the active conformation (Wan et al., 2004). According to their different \textit{in vitro} kinase activities, Wan et al., tested 22 BRAF mutations and segregated them into three groups, seven of the mutants (G469A, E586K, V600D, V600E, V600K, V600R and K601E) had basal kinase activities that exceeded \textit{G12V} RAS-stimulated
WT\textsubscript{BRAF} activity, being ∼130 to 700 fold more active than basal WT\textsubscript{BRAF}. These mutants were referred to as the high activity group. A further eleven mutants (R462I, I463S, G464E, G464V, G466A, G469E, N581S, F595L, L597V, T599I and A728V) had basal kinase activities between ∼1.3 to 64 fold higher than WT\textsubscript{BRAF}. Because their activities were between basal and G12V\textsubscript{RAS}-stimulated WT\textsubscript{BRAF}, they were referred to as the intermediate activity group. There were four cancer-associated BRAF mutants (G466E, G466V, D594A, and G596R) whose kinase activities were reduced to between 30% and 80% of the activities of WT\textsubscript{BRAF}. These mutants were termed the impaired activity group. Despite their reduced \textit{in vitro} kinase activities, three of the mutants still activated ERK in COS cells by transactivating CRAF (Wan et al., 2004).

1.2.4.2 V600E\textsubscript{BRAF} mutation
Among all the BRAF mutations, the most frequent one is a single base transversion, a thymidine to adenosine at nucleotide 1799 in the kinase domain resulting in a valine to glutamic acid conversion at codon 600 (V600E) in exon 15 (Davies et al., 2002). Initially this mutation was identified at codon 599 instead of codon 600; therefore, the older publications referred to it as V599E. V600E\textsubscript{BRAF} mutation accounts for ∼83% of BRAF mutations in primary melanoma and ∼95% in melanoma cell lines (Davies et al., 2002). V600E\textsubscript{BRAF} is a conventional oncogene as V600E\textsubscript{BRAF} displays elevated kinase activity \textit{in vitro} (∼500 fold greater than that of WT\textsubscript{BRAF}), constitutively activating the MEK/ERK signalling pathway, as well as enhanced transformation activity in fibroblasts and melanocytes (Wan et al., 2004; Davies et al., 2002; Satyamoorthy et al., 2003; Ikenoue et al., 2003; Wellbrock et al., 2004b). The conserved phosphorylation sites within the activation segment of BRAF, T599 and S602, require to be phosphorylated to gain maximum kinase activity after recruitment of BRAF to the plasma membrane by activated RAS. V600E\textsubscript{BRAF} mutation probably mimics the T599/S602 phosphorylation in the activation segment by inserting a negative charged residue to the nearby regulatory phosphorylation site, resulting in the conformational change which is induced by phosphorylation of T599 and S602. This renders V600E\textsubscript{BRAF} constitutively active (Figure 1.5 and 1.6) (Davies et al., 2002; Garnett and Marais, 2004; Wan et al., 2004).
Figure 1.5  Crystal structure of BRAF kinase domain (Taken from Wan et al., 2004b)

Diagram of BRAF kinase domain in complex with BAY43-9006. The positions of Asp593 and Phe594 of the DFG motif, Asn580 of the catalytic loop, and Glu585 are shown, DFG and APE motif in yellow, rest of activation segment and the N-region are in red. N lobe is in magenta, C lobe in marine, and P-loop in orange. Residues 600-611 of the activation loop are disordered (dashed lines).
Figure 1.6 BRAF mutations in human cancer

Most of the BRAF mutations are localised within or close to the glycine-rich loop or the activation segment of the kinase domain of BRAF. The glycine-rich loop and the activation segment are highlighted in green and pink, respectively. The positions of the mutations and the most common amino acid substitutions that occur in human cancer are indicated. The length of the line provides an indication of the relative frequency with which the mutations occur. V600 is the most frequent BRAF mutation in human cancer.
\textbf{V600E-BRAF} activity is also independent of the presence of a negative charge in the N-region, which is normally required for \textit{WT}BRAF and CRAF activation (Emuss et al., 2005; Brummer et al., 2006). Furthermore, \textbf{V600E-BRAF} is insensitive to the SPRY2-mediated negative feedback loop that inhibits MEK/ERK signalling in \textit{WT}BRAF-expressing cells (Tsavachidou et al., 2004). Regarding its protein stability, \textbf{V600E-BRAF} needs Hsp90 (Grbovic et al., 2006).

\subsection*{1.2.4.3 BRAF signalling in cancer}

Most of the mutants of BRAF are all activating displaying elevated kinase activity compared to \textit{WT}BRAF and can directly phosphorylate MEK to activate ERK. There are four impaired kinase activity mutants of BRAF, including G466E, G466V, G596R, and D594V, which have reduced kinase activity (Houben et al., 2004; Ikenoue et al., 2004, 2003; Wan et al., 2004). Three of these mutants G466E, G466V, and G596R fail to directly activate MEK, but can phosphorylate MEK through heterodimerisation with \textit{WT}CRAF (Wan et al., 2004). The fourth mutant D594V is a kinase dead mutant and was initially thought not to activate the MEK/ERK pathway. However, recent data has shown that this mutation cooperates with oncogenic RAS to drive tumour progression through CRAF (Figure. 1.7) (Heidorn et al., 2010; Luo et al., 1996; Wan et al., 2004).

All BRAF cancer mutants are capable of dimerising with CRAF, and the impaired kinase activity mutants totally rely on CRAF for ERK activation (Wan et al., 2004). BRAF can activate CRAF in the cytosol in a RAS-independent manner through 14-3-3-mediated heterooligomerisation and transphosphorylation. N-region phosphorylation in CRAF is not necessary for this, but activation segment phosphorylation and binding to 14-3-3 to the C-terminus are essential. Both BRAF mutants and \textit{WT}BRAF can bind to CRAF to form a complex in which \textit{WT}BRAF binding to CRAF is RAS-inducible while BRAF mutants are constitutive (Garnett et al., 2005). A recent study showed endogenous BRAF and CRAF form heterodimers which are regulated by mitogens and enhanced by 14-3-3 proteins. This formation of heterodimers is ERK-independent and is stabilised by MEK inhibition. ERK-induced phosphorylation of T753 in BRAF disrupts the RAF
Figure 1.7 Oncogenic signalling by the RAS/RAF/MEK/ERK pathway

In response to mitogenic stimuli, wild-type RAS can signal to all three RAF proteins. Oncogenic RAS and BRAF mutants activate MEK/ERK independently of mitogenic signals. In melanoma cell lines, G12V RAS signals exclusively via CRAF. BRAF mutants associated with high kinase activity (for example, V600E) or intermediate activity (for example, G466A) stimulate MEK directly, whereas impaired activity mutants (for example, G466E) require CRAF for MEK activation.
heterodimers. Mutation of T573 results in a longer persistence of CRAF/BRAF heterodimers and the BRAF/CRAF heterodimers have significantly elevated kinase activity compared to the respective monomers or homodimers (Rushworth et al., 2006).

1.3 Cell cycle
The cell cycle is a complex process that 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 cell cycle can be morphologically divided into interphase and mitosis (M) phase. Interphase includes G\textsubscript{1} (Gap1), S (synthesis), and G\textsubscript{2} (Gap2) phases. M phase consists of prophase, metaphase, anaphase and telophase (Figure 1.8) (Norbury and Nurse, 1992). In the G\textsubscript{1} phase, the cells grow and prepare for DNA synthesis. In the S phase, the cells synthesise DNA, and in the G\textsubscript{2} phase cells prepare for M phase. In the M phase, the chromosomes and cytoplasm are divided between two identical daughter cells. At the end of the G\textsubscript{1}, G\textsubscript{2} and in the middle of M phase, checkpoints are designed to ensure that cells are ready to proceed to the next stage of cell division. When cells temporarily or permanently stop dividing, they quit the cycle and enter a state of quiescence, called G\textsubscript{0} phase (Schafer, 1998).

1.3.1 Components of cell cycle
Mammalian cell cycle progression is driven by the sequential activation of the Cyclin-dependent kinases (CDKs). CDKs are a large family of serine/threonine protein kinases whose activities require binding to specific Cyclins. CDKs form complexes with Cyclins known as heterodimeric kinases, where CDKs function as catalytic subunits and Cyclins serve as regulatory subunits (Malumbres and Barbacid, 2005). Levels of CDKs remain constant through the cell cycle, while Cyclins are synthesised and degraded cyclically during the cell cycle, thus regulating CDK activities in a timely manner. The activation of CDKs is also regulated through phosphorylation at a central threonine residue by the CDK-activating kinase (CAK) and dephosphorylation at inhibitory phosphorylation sites in their N-terminus by CDC25 phosphatases. Each Cyclin/CDK complex is believed to act at a specific stage of the cell cycle. During the cell cycle, CDK4
Figure 1.8 Cell cycle and its regulation by Cyclins, CDKs and CKIs

Cell cycle is divided into four distinct phases: G₁, S, G₂, and M. The progression of a cell through the cell cycle is promoted by CDKs, which are positively and negatively regulated by Cyclins and CKIs, respectively. Cyclin D interacts with CDK4 and CDK6 to drive the progression of a cell through G₁. The association of Cyclin E with CDK2 is active at the G₁/S transition and directs entry into S phase. S phase progression is directed by the Cyclin A/CDK2 complex, and the complex of Cyclin A/CDK1 is important in G₂. Cyclin B/CDK1 is necessary for the entry into and progression through mitosis.
and CDK6 bind to and are activated by D-type Cyclins (Cyclin D1, Cyclin D2, and Cyclin D3) in early G1 phase. CDK2 sequentially binds to and is activated by E-type Cyclins in the later G1 phase. CDK2 also binds to A-type Cyclins for activity during S phase. CDK1 is controlled by its interaction with A-type Cyclins and B-type Cyclins during G2 and M phases (Sherr and Roberts, 1999).

On the other hand, the activity of CDKs is exquisitely regulated by a group of negative regulators of CDKs, known as CDK inhibitors (CKIs) that limit cell cycle progression in response to cellular stress (Sherr and Roberts, 1999). CKIs fall into two families: the INK4 family and the CIP/KIP family. The INK4 family is composed of four members: p16\textsuperscript{INK4A}, p15\textsuperscript{INK4B}, p18\textsuperscript{INK4C}, and p19\textsuperscript{INK4D/ARF} (Ruas and Peters, 1998). The INK4 family members specifically bind and inhibit the CDK4 or CDK6 kinases from formation of active Cyclin D-CDK4/CDK6 complexes. The CIP/KIP family consists of p21\textsuperscript{CIP1}, p27\textsuperscript{KIP1}, and p57\textsuperscript{KIP2}, which associate and inhibit all Cyclin-CDK complexes including Cyclin D-CDK4/CDK6, Cyclin E/CDK2, Cyclin A/CDK2, Cyclin A/CDK1 and Cyclin B/CDK1 complexes (Sherr and Roberts, 1999; Sugimoto et al., 2002). Conversely, the CIP/KIP family is also required for efficient assembly of the CyclinD-CDK4/CDK6 kinases by facilitating and stabilising the association between the CDK4/CDK6 kinases and Cyclin D (LaBaer et al., 1997; Cheng et al., 1999).

1.3.2 Progression of cell cycle

Regulation of G1/S phase transition is tightly controlled by interactions between D-type Cyclins and E-type Cyclins with specific CDKs and CKIs as well. When quiescent cells enter the cell cycle, D-type Cyclins act as sensors of mitogenic signals, whose expression is stimulated. D-type Cyclins preferentially bind and activate CDK4 and CDK6 during G1 phase. The active Cyclins D-CDK4/CDK6 complexes phosphorylate the retinoblastoma protein (pRb), which mediates G1 arrest through sequestration of transcriptional factors of E2F family. Phosphorylation of pRb disrupts its association with E2F transcription factor and leads to release of E2F transcription factors from pRb repression and initiation of transcription of the Cyclin E gene required for G1/S phase
transition and other genes required for DNA replication (Weinberg, 1995; Harbour and Dean, 2000; Bracken et al., 2004). Subsequent expression of Cyclin E and assembly of Cyclin E/CDK2 complexes promotes the further phosphorylation and inactivation of pRb. The activity of Cyclin E/CDK2 complexes is also regulated by Cyclin D-CDK4/CDK6 complexes through sequestration of the CKI p21\textsuperscript{CIP1} and p27\textsuperscript{KIP1} and release of their binding to CDK2 (Sherr and Roberts, 1999; Polyak et al., 1994). Cyclin E/CDK2 complexes are essential to drive G\textsubscript{1}/S phase transition and in turn trigger DNA replication. Suppression of pRb by Cyclin E/CDK2 also causes induction of E2F-regulated enzymes required for DNA replication and nucleotide synthesis including Cyclin A expression (Dyson, 1998; Nevins, 1998; Trimarchi and Lees, 2002). Cyclin A subsequently binds and activates CDK2 to drive the cell cycle through S phase by phosphorylating substrates that initiate DNA replication. CDK1 is activated by Cyclin A at the end of G\textsubscript{2} phase to facilitate the onset of M phase. The decision to enter mitosis is mediated by Cyclin B/CDK1 complexes. Following nuclear envelope breakdown, degradation of Cyclin A facilitates the formation of Cyclin B/CDK1 complexes responsible for driving cells through mitosis (Sherr, 1996; Pines, 1999). Finally, degradation of Cyclin B is critical for exit from mitosis.

1.3.3 The RAF/MEK/ERK cascade and cell cycle

The RAF/MEK/ERK signalling pathway plays a crucial role in the regulation of cell proliferation by growth factors. ERK activation is an obligatory event for growth factor-induced cell cycle progression (Melonche et al., 1992; Cook and McCormick, 1996; Tamemoto et al., 1992; Vouret-Craviari et al., 1993; Roovers and Assoian, 2000). In response to mitogenic signals, both the duration and strength of ERK signalling has a significant impact on whether cells enter cell cycle or not (Marshall, 1995). In PC12 cells, it has been shown that transient ERK signalling induced by EGF stimulates cell proliferation, whereas a sustained ERK activation stimulated by NGF results in cell differentiation (Dikic et al., 1994; Traverse et al., 1994; Sasagawa et al., 2005). In fibroblasts, it has been shown that transient ERK signalling stimulates rapid transcriptional induction of immediate early genes (IEGs) including Fos and Jun family
members. It has been shown that c-fos is induced upon transient ERK activation, but the c-Fos protein is unstable and degraded (Murphy et al., 2002). When ERK signalling is sustained, c-Fos induced during the immediate early phase is phosphorylated and stabilised by active ERK and p90RSK (90K-ribosomal S6 kinase). These immediate early genes function as sensors for ERK signalling (Murphy et al., 2002, 2004) and their products are able to regulate delayed response genes including Cyclins and CDKs that are required for cell cycle progression. It has been shown that IEGs are required for induction and maintenance of the increased expression of Cyclin D1 (Figure 1.9) (Pages et al., 1993; Lavoie et al., 1996; Weber et al., 1997; Balmanno and Cook, 1999). Thus, Cyclin D1 expression could represent a critical link between ERK signalling, IEG sensors and cell cycle progression. Sustained ERK signalling can also repress the expression of genes that inhibit proliferation (Yamamoto et al., 2006). Therefore, sustained, not transient ERK activation is required for sequential induction of gene expression for the successful G1 phase progression to S phase (Weber et al. 1997; Meloche et al., 1992; Cook and McCormick, 1996; Roovers et al., 1999; Balmanno and Cook, 1999). In addition to temporal factors, ERK signalling also influences cellular processes by varying its signalling strength. It has been shown that low levels of ERK activity induced in △Braf:ER cells elicit cell cycle progression by inducing expression of Cyclin D1 and Cyclin E and suppression of p27Kip1 (Woods et al., 1997). However, high levels of persistent ERK signalling can lead to cell cycle arrest by inducing high p21Cip1 that inhibits both Cyclin D/CDK4 and Cyclin E/CDK2 complexes (Sewing et al., 1997; Woods et al., 1997; Mirza et al., 2004). Therefore, the RAF/MEK/ERK cascade must be finely tuned to properly regulate gene expression that is a rate-limiting event for S phase entry, during G1 progression of the cell cycle.

1.3.4 Regulation of Cyclin D1 in the G1 phase

A key event for cell cycle re-entry and progression through the G1 phase is expression of D-type Cyclins and consequent activation of Cyclin D-CDK4/CDK6 complex leading to inactivation of pRB and release of E2F transcription factor (Sherr and Roberts, 1999; Stacey, 2003). It has been shown that Cyclin D1 expression can be tightly regulated at
Figure 1.9 Role of the RAF/MEK/ERK pathway in G₁/S phase progression

The mitogen-activated RAF/MEK/ERK pathway promotes G₁ to S phase cell cycle progression by induction of *Cyclin D1* transcription through AP-1 transcription factors that are formed by heterodimerisation of c-Jun and c-Fos transcription factors. Cyclin D-CDK4/CDK6 and Cyclin E/CDK2 complexes phosphorylate Rb, leading to release of E2F transcription factors which are indispensable for production of proteins required for the G₁/S phase progression of the cell cycle.
the transcription, protein stability and subcellular localisation levels during the G1 phase (Marshall, 1999). It is known that growth factor-dependent activation of the RAF/MEK/ERK signalling drives cell cycle progression through stimulation of Cyclin D1 transcription. This is achieved by the ability of activated ERK to translocate to the nucleus and phosphorylate a number of pre-existing transcription factors, such as Fos and Jun family members and Ets. Members of the Jun family (c-Jun, Jun B, and Jun D) and the Fos family (c-Fos, Fos B, Fra-1 and Fra-2) can form heterodimers referred to as activator protein 1 (AP-1) complex that binds to AP-1 binding sites in the Cyclin D1 promoter and directly transactivates it (Figure 1.9) (Albanese et al. 1995; Aktas et al. 1997; Lavoie et al. 1996; Winston et al. 1996; Kerkhoff and Rapp 1997; Weber et al. 1997; Balmanno and Cook, 1999; Shaulian and Karin, 2001). ERKs also regulate Cyclin D1 transcription via c-Myc. ERK1/2 phosphorylates c-Myc at Ser62 leading to its stabilisation (Seth et al., 1991), then c-Myc directly transactivates Cyclin D1 (Daksis et al., 1994). Cyclin D1 transcription can be negatively regulated by p38MAPK, and this can be reversed by inhibition of p38MAPK (Lavoie et al., 1996).

The protein stability and subcellular localisation of Cyclin D1 has been shown to regulated by Glycogen Synthase Kinase 3β (GSK-3β) (Diehl et al., 1998). It has been demonstrated that Cyclin D1 accumulates in the nucleus throughout G1 phase, but it is localised to the cytoplasm during the remainder of interphase (Baldin et al. 1993; Lukas et al., 1995; Diehl et al. 1998; Hitomi and Stacey, 1999; Stacey, 2003). Failure to remove Cyclin D1 from the nucleus in the S phase results in G1 arrest (Baldin et al., 1993; Diehl et al., 1998; Guo et al., 2005; Alt et al., 2000). The cell cycle-specific redistribution of Cyclin D1 correlated with its phosphorylation at Thr-286 by GSK-3β, which promotes the nuclear export of Cyclin D1 and its subsequent degradation via the proteasome pathway (Diehl et al., 1998). However, this process can be inhibited by growth factor-dependent RAS-activated AKT phosphorylation and inactivation of GSK-3β during the G1 phase (Diehl et al., 1998; Rodriguez-Viciana et al. 1994, 1997; Franke et al., 1997; Klinghoffer et al. 1996; Kauffmann-Zeh et al. 1997; Vanhaesebroeck et al. 1997). Likewise, a mutant Cyclin D1 protein containing an alanine for threonine
substitution at residue 286 (D1-T286A) remained in the nucleus throughout the cell cycle and was not subject to GSK-3β-dependent redistribution (Diehl et al. 1998). Further study has demonstrated that phosphorylation of Cyclin D1 at Thr286 facilitates its binding to the nuclear exportin CRM1, which transports Cyclin D1 to the cytoplasm where Cyclin D1 is rapidly degraded (Alt et al., 2000). p21CIP1 and p27KIP1 have been shown to bind to Thr286-phosphorylated Cyclin D1, thereby preventing Cyclin D1 association with CRM1, resulting in abolition of CRM1-dependent nuclear export and maintenance of CyclinD1-CDK4/CDK6 complexes within the nucleus during the G1 phase (Alt et al., 2002).

1.4 Apoptosis

Apoptosis, one of the best known forms of programmed cell death, is a physiological process that serves as a major mechanism for the precise regulation of cell numbers and as a defence mechanism to remove unwanted and potentially dangerous cells. Apoptosis is characterised by biochemical and morphological changes such as DNA fragmentation, cytoplasm shrinkage and chromatin condensation. When misregulated, apoptosis can contribute to various diseases including cancer, autoimmune, and neurodegenerative diseases (Jacobson et al., 1997). Apoptosis progress can be divided into three different phases. First of all is the activation phase, in which various death-triggering signals initiate the cell death machinery and activate it; the second stage is the execution phase, in which activated machinery (activation of caspases) targets the multiple cellular substrates; finally, the destruction phase, in which the dead or dying cells are broken down (Fleury et al., 2002).

1.4.1 Apoptosis includes mitochondria pathway and death receptor pathway

Apoptosis can be initiated by the engagement of the plasma membrane death receptors (extrinsic pathway), or by changes in the mitochondrial integrity following either a broad range of physical and chemical stimuli or growth factor withdrawal (intrinsic pathway) (Figure. 1.10) (Budihardjo et al., 1999).
1.4.1.1 Mitochondria-dependent pathway (intrinsic pathway)

The intrinsic pathway is characterized by mitochondrial outer membrane permeabilisation (MOMP) and the release of mitochondrial cytochrome c, which results in assembly of a caspase-activating complex between caspase-9 and APAF1, the apoptosome. The BCL-2 family of proteins plays an important role in inducing mitochondria-dependent apoptosis. Pro- and anti-apoptotic members of the BCL-2 family of proteins integrate intrinsic signals, leading either to cell survival by maintaining mitochondrial homeostasis or to cell death by release of pro-apoptotic proteins stored in the mitochondria into the cytosol (Gross et al., 1999). Upon apoptotic stimulation, BH3-only proteins of the BCL-2 family bind to BCL-2 proteins resulting in activation of BAX/BAK. Activated BAX/BAK undergoes a conformational change and insertion into the outer mitochondrial membrane, allowing release of cytochrome c. Subsequently, cytochrome c assembles pro-caspase-9 and APAF-1 to form a caspase-activating complex, the apoptosome (Li et al., 1997). Through the interaction between the caspase recruitment domain (CARD) of APAF-1 and the prodomain of pro-caspase-9 (Zhou et al., 1999; Shiozaki et al., 2002), pro-caspase-9 is processed by an intrinsic autocatalytic activity of pro-caspase-9 itself. APAF-1 triggers this activity by oligomerising pro-caspase-9 (Srinivasula et al., 1998). Active caspase-9 initiates a caspase cascade involving activation of caspase-3, 6, and 7, leading to apoptosis.

1.4.1.2 Death receptor pathway (extrinsic pathway)

The extrinsic pathway involves stimulation of members of the tumour necrosis factor receptor (TNFR) superfamily, such as CD95/Fas, TNFR or TRAIL (death receptors). Stimulated by ligation of death receptors to Fas ligand or tumor necrosis factor (TNF), pro-caspase-8 is rapidly recruited by Fas-associated death domain protein (FADD)/TNFR1-associated death domain protein (TRADD) to form a death-inducing signalling complex (DISC) (Kischkel et al., 1995; Sedger et al., 1999). In this complex, pro-caspase-8 is activated by dimerisation and converted to the processed heterotetrameric mature form of caspase-8, which is released into the cytosol (Boatright et al., 2003; Donepudi et al., 2003). Active caspase-8 cleaves effector caspases such as
Figure 1.10 Mitochondria and death receptor control of apoptosis

Apoptosis can be initiated through the mitochondria-dependent and death receptor pathways. In response to cellular stress, BH3-only proteins such as BIM bind to BCL-2 proteins resulting in disruption of association of BCL-2 with BAX/BAK. Then BAX/BAK initiates cytochrome c release from mitochondria by interrupting the mitochondrial outer membrane. Subsequently, released cytochrome c binds APAF-1 and pro-caspase-9 to form the apoptosome resulting the processing of pro-caspase-9 to produce active capase-9. Caspase-9 then activates downstream effector caspases to initiate apoptosis. Death receptors bind to death ligands, resulting in activation of caspase-8. Active caspase-8 either directly activate effector caspases to induce apoptosis or cleave BID that connects the death receptor pathway to the mitochondria-dependent pathway.
caspase-3, 6, 7 to induce apoptosis. Active caspase-8 can also cleave the BH3-only protein BID. The cleavage of BID produces a truncated protein (tBID), which is translocated to the mitochondria where it promotes cytochrome c release and formation of the apoptosome (Li et al., 1998). Thus, caspase-8 provides a direct link between the death receptor pathway and mitochondria-dependent pathway.

1.4.2 Regulation of apoptosis

1.4.2.1 BCL-2

BCL-2 family proteins play key roles in apoptosis by regulating the mitochondrial outer membrane permeabilisation (MOMP). BCL-2 family members share one to four BCL-2 homology domains (BH1-4) and are classified into anti-apoptotic and pro-apoptotic proteins in terms of their ability to induce or prevent apoptosis (Cory and Adams, 2002). The pro-apoptotic members are further divided into two groups: BH3-only family members such as BID, PUMA, BIM, and multidomain (BH1-3) family members including BAX and BAK; they collaborate together to induce apoptosis. Mitochondrial outer membrane permeabilisation (MOMP) can be achieved by modulating the balance between pro- and anti-apoptotic members of the BCL-2 family. Activation of BH3-only proteins by apoptotic stimuli can either directly activate BAX/BAK to induce apoptosis (Eskes et al., 2000; Wei et al., 2001a). Alternatively, BH3-only proteins bind to and inhibit BCL-2 proteins subsequently releasing BAX/BAK from BCL-2 sequestration to induce apoptosis (Huang and Strasser, 2000; Bouillet et al., 1999; Chen et al., 2005a; Puthalakath and Strasser, 2002).

BCL-2-interacting mediator of cell death (BIM) is a BH3-only protein of the BCL-2 family with a dual function in apoptosis (Chen et al., 2005a). BIM is able to bind the anti-apoptotic proteins such as BCL-2, BCL-xL, BCL-W and MCL-1 through heterodimerisation, antagonising their anti-apoptotic activity (O’Connor et al., 1998; Chen et al., 2005a). On the other hand, BIM also interacts with pro-apoptotic multidomain proteins BAX and BAK, whose oligomerisation and insertion into the mitochondrial membrane results in release of cytochrome c from the mitochondrial
intermembrane space to the cytosol (Kuwana et al., 2005; Letai et al., 2002; Marani et al., 2002). There are three major Bim isoforms: Bim_{EL}, Bim_{L} and Bim_{S}, (where EL, L and S denote extra-long, long and short splice variants, respectively), which are generated by alternative splicing (Puthalakath et al., 1999; Bouillet et al., 2001; O’Connor et al., 1998). An extensive study of their expression pattern in a wide variety of cell lines and mouse tissues has revealed that the long splice variants Bim_{EL} and Bim_{L}, though usually expressed at low levels in the adult, are the dominant isoforms (O’Reilly et al., 2000). At physiological conditions, BIM is negatively regulated and segregated to microtubules by interacting with the LC8 (dynein light chain 1), which prevents its binding to the anti-apoptotic BCL-2 family members. Upon certain apoptotic stimuli, including UV irradiation, cytokine withdrawal, and disruption of the cytoskeleton integrity, BIM and LC8 complexes are released from microtubules and translocated to mitochondria (Puthalakath et al., 1999; Lei and Davis, 2003).

BIM expression can be upregulated at the transcriptional level through inactivation of the PI3K/AKT signalling pathway resulting in activation of the forkhead rhabdomyosarcoma-like transcription factor-1 (FKHRL1; FOXO3A) (Dijkers et al., 2000, 2002; Linseman et al., 2002; Stahl et al., 2002; Urbich et al., 2005), or by inactivation of the ERK1/2 pathway (Weston et al, 2003), or due to activation of the JNK pathway (Whitfield et al, 2001). Most importantly, at the post-translational level, ERK1/2 is the major pathway that promotes rapid phosphorylation of Bim_{EL} at Ser65 (Ser69 in humans) and subsequent degradation via the proteasome in fibroblasts and epithelial cells (Ley et al., 2003, 2004; Luciano et al., 2003; Marani et al., 2004; Wickenden et al., 2008). However, JNK-dependent phosphorylation of BIM at Ser65 enhanced its pro-apoptotic activity in neurons (Putcha et al., 2003; Becker et al., 2004). In addition, phosphorylation of BIM at Thr56 by JNK results in inhibition of the binding of BIM to dynein light chain 1 and increased pro-apoptotic activity (Lei and Davis, 2003).
1.4.2.2 Caspases

Caspases are a family of cysteine proteases. Upon activation, they cause biochemical and morphological changes by cleaving a series of target substrate proteins at specific aspartate residues. Activation of caspases is a central event during apoptosis, and the key components of this machinery are highly conserved throughout eukaryotes. Pro-caspase is composed of three distinct functional modules: a prodomain and two catalytic subunits known as the large and small subunits. Caspases are produced in an inactive form, named pro-caspases, which are proteolytically activated to form active tetramers during apoptosis in a cascade involved in the cleaving initiator caspases and effector caspases (Earnshaw et al., 1999).

Caspase activities are regulated by a variety of proteins. X-linked IAP (XIAP), the Inhibitor of apoptosis proteins (IAPs), directly interacts with the apoptosome by associating and inhibiting caspase-9 and caspase-3 (Riedl et al., 2001; Bratton et al., 2001). Smac/Diablo, a protein released from mitochondria, promotes apoptosis by binding to IAPs and removal of their inhibitory activity on caspases (Verhagen et al., 2000). Omi/HtrA2 (a proapoptotic mitochondrial factor) can also induce apoptosis in a caspase-dependent manner via its ability to disrupt caspase-IAPs interaction and in a caspase-independent manner through its serine protease activity (Verhagen et al., 2002).

1.4.3 RAF in the suppression of apoptosis

Growth factor-dependent kinases prevent apoptosis by blocking activation of the apoptotic machinery. Thus, upon stimulation of the RAS/RAF/MEK/ERK cascade, apoptosis is suppressed in a variety of different cell systems (Cleveland, 1994; Kauffmann-Zeh, 1997). Activating mutations in RAS and RAF also constitutively activate the MEK/ERK cascade resulting in suppression of apoptosis. There are many different ways to achieve the inhibition of apoptosis by RAF, which can be divided into MAP kinase-dependent and MAP kinase-independent mechanisms.
1.4.3.1 MAP kinase-dependent pathways

It has been shown that the RAF/MEK/ERK pathway can phosphorylate BAD on S112 which contributes to its inactivation and subsequent sequestration by 14-3-3 proteins. This allows BCL-2 to form homodimers and an anti-apoptotic response is generated (Zha et al., 1996). In mammalian cell extracts, it has been shown that caspase-9 is directly phosphorylated by RAF/MEK/ERK at Thr125 resulting in inhibition of caspase-9 activity and promoting cell survival (Allan et al., 2003). Caspase-9 also can be phosphorylated by AKT (Cardone et al., 1998), protein kinase A (Martin et al., 2005) and protein kinase C zeta (Brady et al., 2005). Cross-talk among these pathways results in prevention of apoptosis. The RAF/MEK/ERK cascade can also target the BCL-2 family protein BIM to inhibit apoptosis. ERK1/2 promotes rapid phosphorylation of BimEL at Ser65 (Ser69 in humans) and subsequent degradation via the proteasome in the presence of growth factors. Conversely, removal of growth factor inhibits ERK activity, restores BIM expression and leads to apoptosis induction (Ley et al., 2003, 2004; Luciano et al., 2003; Marani et al., 2004).

BRAF is the most active isoform in the RAF family due to its high ability to activate downstream effectors. Overexpression of BRAF has been shown to confer resistance to apoptosis as a result of constitutive activation of the RAF/MEK/ERK signalling pathway. This anti-apoptotic activity of BRAF blocked caspase activation but did not interfere with the release of cytochrome c from mitochondria, which suggests that inhibitor of apoptosis proteins (IAPs) may be the relevant target (Erhardt et al., 1999). Consistent with this, following the expression of BRAF in motoneurons, XIAP is upregulated to prevent apoptosis (Wiese et al., 2001). V600E BRAF-overexpressing cells also show an increase in the DNA-binding activity of NF-κB, resulting in upregulation of anti-apoptotic c-IAP-1, c-IAP-2 and XIAP (Palona et al., 2006). V600E BRAF prevention of cancer cells from apoptosis has been reported in melanoma cells, colon cancer cells, thyroid carcinomas, and ovarian cancer cells. Through specific knockdown of BRAF with siRNA, or by using RAF kinase inhibitors or MEK inhibitors, it has been shown that apoptosis can be induced in V600E BRAF mutant cancer cells (Boisvert-Adamo and Aplin, 2006;
1.4.3.2 MAP kinase-independent pathways

Alternative mechanisms have been proposed to account for the anti-apoptotic activity of RAFs that do not involve the MEK/ERK pathway, particularly for CRAF. It has been shown that CRAF targeting to mitochondria by BCL-2 leads to cell survival without ERK activation, and is associated with phosphorylation of BAD, resulting in its translocation to the cytosol and ultimately the inhibition of cytochrome c release (Wang et al., 1996; Majewski et al., 1999; Salomoni et al., 1998; Peruzzi 2001). In addition to BAD, CRAF can regulate the anti-apoptotic transcription factor NF-κB (Foo and Nolan, 1999; Li and Sedivy, 1993; Troppmair et al., 1998), which has been shown to participate in the transcriptional regulation of IAPs (Wang et al., 1998a; Stehlik et al., 1998) as well as of c-FLIP, an inhibitor of caspase-8 (Micheau et al., 2001). CRAF apparently induces NF-κB activation by a mechanism that does not involve MEK/ERK but rather another MAPKKK, MEKK-1, upstream of the IκB kinase complex (Baumann et al., 2000). The MEK/ERK-independent pro-survival function of CRAF has also been reported to be mediated by antagonising the activity of apoptotic signal-regulated kinase (ASK1), which phosphorylates and activates mitogen-activated protein kinase kinase 4/7 (MKK4/7) and MKK3/6, upstream of the JNK and p38 MAP kinase pathways (Chen et al., 2001a). ASK-1 plays an essential role in TNF-α, H₂O₂, oxidative stress, and DNA damage induced apoptosis (Ichijo et al., 1997; Chang et al., 1998; Gotoh and Cooper, 1998; Saitoh et al., 1998; Chen et al., 1999; Chen et al., 2001a; Tobiume et al., 2001). Dominant negative mutants of ASK1 can inhibit TNF-α and Fas ligand-induced cell death (Ichijo et al., 1997; Chang et al., 1998) and overexpression of ASK1 is sufficient to induce apoptosis in a number of cell lines through a mitochondria-dependent caspase activation pathway (Hatai et al., 2000). It has been shown that CRAF directly binds to the N-terminus of ASK1 independently of its kinase activity (Chen et al., 2001a). Further studies showed that CRAF specifically phosphorylates apoptosis-linked gene-2 (ALG-2) to cause its conformational change and may mediate its anti-apoptotic function by
interrupting ASK1-dependent phosphorylation of ALG-2 (Chen et al., 2005b). In mammalian cells, the pro-apoptotic kinase, mammalian sterile 20-like kinase (MST2) (O’Neill et al., 2004), is activated by stress and causes apoptosis when overexpressed. Finally, Craf has been shown to inhibit MST2 by preventing its dimerisation and phosphorylation of the activation loop on MST2. Both functions required Craf binding to MST2, but were independent of Craf kinase activity and the ERK pathway (O’Neill and Kolch, 2005). Depletion of MST2 from Craf−/− mouse or human cells abrogated sensitivity to apoptosis, whereas overexpression of MST2 induced apoptosis. Conversely, depletion of Craf from Craf+/+ mouse or human cells led to MST2 activation and apoptosis. The concomitant depletion of both Craf and MST2 prevented apoptosis (O’Neill et al., 2004).

1.5 Senescence

1.5.1 Replicative senescence

Senescence is a signal transduction program leading to irreversible cell cycle arrest. Replicative senescence was first described in normal human diploid fibroblasts as a state of irreversible cell cycle arrest resulting from limited proliferative lifespan (Hayflick and Moorhead 1961; Hayflick, 1965). Senescent cells display specific features including changes in morphology and gene expression patterns compared to proliferative cells (Hayflick and Moorhead 1961). Morphologically, senescent cells appear as flattened and have an enlarged cell phenotype (Berube et al., 1998). These cells are permanently growth arrested in the G1 phase of the cell cycle, are incapable of synthesising DNA and are unresponsive to growth factor stimulation. However, these cells remain metabolically alive (DiLeonardo et al., 1994; Herbig et al., 2004; Ogryzko et al., 1996; Serrano et al., 1997). The first biomarker characterising the senescence phenotype was senescence-associated β-galactosidase (SA−β-gal) activity at pH 6, and distinguished senescent cells from pre-senescent, immortal, quiescent or terminally differentiated cells (Dimri et al., 1995). Gene expression patterns involved in cell cycle progression and inhibition are dramatically altered during senescence. Two CDK inhibitors p21CIP1 and p16INK4A are often expressed in senescent cells and they are the components of
tumour suppressor pathways controlled by p53 and pRb, respectively. Senescent cells also repress genes that govern cell cycle progression such as c-Fos, Cyclin A, Cyclin B and PCNA. Some of these genes are suppressed due to the inactivation of E2F transcriptional factors by pRb sequestration (Stein et al., 1991). Therefore, replicative senescence has been proposed as an intrinsic limitation of proliferative capacity for protecting long-lived multicellular organisms from the developing of cancer (Lloyd, 2002).

1.5.2 The DNA damage response in senescence

The major cause of replicative senescence was found to be telomere attrition as human fibroblasts cultured towards the end of their replicative lifespan have gradually decreased telomere length (Harley et al., 1990). The telomeres are located at the end of linear chromosomes and protect and stabilise chromosome ends. Due to an end replication problem, which is represented by incomplete DNA replication on either DNA end, telomeres are gradually eroded with each cell doubling. Once telomeres reach a critically short length, their protective structures collapse leading to telomere uncapping (Campisi 2001; Smogorzewska and de Lange, 2002; Ben-Porath and Weinberg, 2004). An uncapped telomere can be recognised as a site of DNA damage represented by DNA double-strand breaks (DSBs) associated with a number of molecular markers, which prove indistinguishable from those induced by DNA damage detected in senescent human fibroblasts (d'Adda di Fagagna et al., 2003; Herbig et al., 2004). Thus, a correlation between telomere attrition and DNA damage response (DDR) in replicative senescence has been established (d'Adda di Fagagna et al., 2003; Reaper et al., 2004).

Following the generation of DNA double-strand breaks (DSBs) at the uncapped telomeres, the DNA damage response machinery is activated. DNA double-strand breaks are sensed by ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3-related (ATR) kinases at the site of DNA damage (Shiloh, 2006; Zou, 2007). Phosphorylation of histone H2AX (γ-H2AX) by ATM or ATR is a robust marker for double-strand breaks and facilitates the formation of DNA damage foci around uncapped telomeres (Celeste et al., 2002). Subsequently checkpoint kinases (CHK1
and CHK2) are activated by ATM or ATR at the DNA damage foci. Finally, activation of the checkpoint kinases results in the induction and stabilisation of p53 by phosphorylation leading to induction of p21CIP1 and a stable cell cycle arrest (d’Adda di Fagagna, 2008). Collectively, telomere attrition-induced activation of the ATM/ATR-CHK1/CHK2-p53 pathway plays a critical role in irreversible growth arrest and maintenance of a replicative senescence phenotype.

1.5.3 Stress-induced premature senescence

In addition to replicative senescence that is related to telomere attrition, senescence can also be triggered in normal and cancer cells by a variety of intracellular and extracellular stresses including disruption of heterochromatin or functional telomere structures (Takai et al., 2003; Meier et al., 2007), oncogene activation (Serrano et al., 1997; Zhu et al., 1998; Ferbeyre et al., 2000, 2002; Pearson et al., 2000), inadequate culture conditions (Sherr and DePinho, 2000; Ramirez et al., 2001; Parrinello et al., 2003), subcytotoxic doses of hydrogen peroxide (Chen and Ames, 1994; Chen et al., 1995), DNA damaging agents used in cancer therapies (Di Leonardo et al., 1994; Robles and Adami, 1998; Shay and Roninson 2004; Gewirtz et al., 2008; Roninson et al., 2001), ultraviolet radiation (UV) (Chainiaux et al., 2002) and ionizing radiation (IR) (Oh et al., 2001a). These forms of stress-induced senescence exhibit morphological and biochemical features indistinguishable from those of senescent cells undergo replicative senescence activated by telomere attrition (Suzuki and Boothman, 2008; Oh et al., 2001a; Chainiaux et al., 2002; Chen and Ames 1994; Robles et al., 1999; Wang et al., 1998b; Toussaint et al., 2002), leading to a suggestion that senescence is a common response to cellular stress with characteristics of the activation of a DNA damage response (Ben-Porath and Weinberg, 2005). However, stress-induced senescence is induced in a relatively short period of time before reaching the Hayflick limit and is not associated with telomere shortening. Therefore, stress-induced senescence is collectively referred to as premature senescence (Ben-Porath and Weinberg, 2004; Chen et al., 2001b; Drayton and Peters, 2002) or stress-induced premature senescence (SIPS) (Toussaint et al., 2002). However, genome-wide gene
expression analysis has found that there is very limited overlap among the gene expression profiles in cells induced to senescence by telomere attrition and various cellular stresses, indicating fundamental differences in gene regulation during senescence activated by various signals (Shelton et al., 1999; Schwarze et al., 2002; de Magalhaes et al., 2004; Collado et al., 2005; Darbro et al., 2005; Franco et al., 2005).

1.5.4 Oncogene-induced senescence

Oncogene activation is a hallmark of cancer and activated oncogenes are required for tumour growth in animal models (Hanahan and Weinberg, 2000). However, oncogene activation in normal cells does not always induce unscheduled proliferation, but cellular senescence, called oncogene-induced senescence (OIS) (Collado and Serrano, 2006; Mooi and Peeper, 2006). OIS was first observed by overexpression of oncogenic HRAS in normal human and mouse cells leading to an initial proliferative burst followed by induction of premature senescence with features of cell cycle arrest in late G1 phase and expression of p53, p16\(^{INK4A}\), p19\(^{ARF}\), p21\(^{CIP1}\), and SA-\(\beta\)-gal \textit{in vitro} (Serrano et al., 1997). Subsequently, activation of oncogenic Raf-1, MEK, and \(V_{600E}\)BRAF were also shown to induce senescence in human fibroblasts and melanocytes (Lin et al., 1998; Zhu et al., 1998; Michaloglou et al., 2005; Denoyelle et al., 2006). OIS shares many features and markers indistinguishable from replicative senescence, but is independent of any telomere attrition and dysfunction (Jones et al., 2000). Evidence to illustrate the mechanisms for OIS is emerging. Similar to telomere-dependent replicative senescence, OIS causes a DNA damage response. Activation of oncogenic RAS, CDC6, Cyclin E, and STAT5 induce hyperproliferation and DNA hyper-replication resulting in termination of replicon prematurely, which generates DNA breaks that initiate an ATR/ATM-mediated DNA damage response and phosphorylation of p53 by DNA-damage response kinases (Di Micco et al., 2006; Bartkova et al., 2006; Mallette et al., 2007). Inhibition of the ATM kinase was shown to suppress the induction of senescence and in a mouse model led to increased tumour size and invasiveness (Bartkova et al., 2006). These findings also establish the link between DNA damage response and OIS (Bartek et al., 2007; Hemann and Narita, 2007). Thus, OIS operates through known mechanisms that
converge on p53. It has also been shown that p19ARF is the critical sensor that activates p53 during tumorigenesis in response to oncogenic signals in mice (Christophorou et al., 2006; Efeyan et al., 2006). Apart from a p53-dependent pathway, activation of oncogenic RAS or RAF has been shown to induce high levels of p16INK4A expression in a MEK/ERK dependent manner (Lin et al., 1998; Zhu et al., 1998; Ohtani et al., 2001) via Ets transcription factors (Ohtani et al., 2001). Oncogenic RAF also induces senescence independently of p53 and p16INK4A (Olsen et al., 2002; Michaloglou et al., 2005). The involvement of two tumour suppressor pathways, p53 and p16INK4A, leads to the hypothesis that OIS may represent a tumour suppressive mechanism, by which cells are prevented from uncontrolled proliferation in response to the aberrant activation of proliferation-driving oncogenes.

1.5.5 Oncogene-induced senescence suppresses tumourigenesis in vivo

Cellular senescence has been shown to be an important mechanism of tumour suppression to prevent the proliferation of damaged or stressed cells at risk of neoplastic transformation (Campisi, 2005). The p53/p21CIP1 and p16INK4A/pRb pathways are recognised as master regulators of senescence and are activated upon entry into senescence. Inactivation of these two pathways prevents the induction of senescence in mouse and human cells (Campisi, 2005). However, these two pathways are frequently disabled in cancer cells owing to mutations or deletions, contributing to neoplastic transformation (Sherr and McCormick, 2002; Ben-Porath and Weinberg, 2004). Thus, senescence appears to involve an intricate program that actively suppresses tumourigenesis.

The role of OIS acting as a tumour suppressor mechanism in vivo has been established in preventing tumourigenesis by restraining the proliferative cells that bear mutations in oncogenes and tumour suppressor genes (Campisi, 2005). OIS occurs in vivo during the early stages of tumour development both in mouse models and in humans via the expression of distinct oncogenes including RAS (Braig et al., 2005; Collado et al., 2005), BRAF (Michaloglou et al., 2005; Gray-Schopfer et al., 2006; Dankort et al., 2007, 2009;
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Dhomen et al., 2009; Carragher et al., 2010), E2F (Lazzerini Denchi et al., 2005) or the loss of the tumour suppressor PTEN (Chen et al., 2005c) and NF1 (Courtois-Cox et al., 2006). It has been shown that oncogenic G^{12V}Kras induces senescence in benign lung adenomas with several senescence markers such as p15^{ink4b}, p16^{ink4a}, Dec1, DcR2, and HP1-γ being expressed (Collado et al., 2005). NRAS-induced senescence occurred in lymphomas and depends on the presence of the Rb-bound H3K9 histone methyltransferases (HMT) Suv39h1. Loss of Suv39h1 promoted aggressive lymphomas in the presence of oncogenic RAS (Braig et al., 2005). Senescence has also been observed in mouse pituitary glands by expression of inducible E2F (Lazzerini Denchi et al., 2005). Interestingly, inactivation of PTEN led to senescence in a prostate mouse model. When combined with loss of PTEN and p53, invasive prostate cancer was induced (Chen et al., 2005c). Analysis of benign neurofibromas from patients revealed that loss of NF1 induced senescence with high levels of p16^{INK4A} and SA-β-gal. This is due to deficiency in NF1 inducing a negative feedback response that suppresses RAS and its effector pathways (Courtois-Cox et al., 2006). On the contrary, restoration of p53 in the mouse models of sarcoma and liver cancer, enabled senescence re-establishment and tumour regression (Ventura et al., 2007; Xue et al., 2007).

In terms of oncogenic BRAF, overexpression of V^{600E}BRAF in human melanocytes has been shown to induce benign nevi that remain in a growth-arrested state expressing markers of senescence including p16^{INK4A} and SA-β-gal activity but without telomere shortening (Michaloglou et al, 2005). SA-β-gal was also positive in the human congenital nevi indicating senescence occurs in neoplastic lesions (Michaloglou et al, 2005). Similarly, Gray-Schopfer et al., (2006) showed that growth inhibition accompanied by high levels of p16^{INK4A} occurred in V^{600E}BRAF transfected human melanocytes. Benign nevi showed a p16^{INK4A}-dependent senescent phenotype and dysplastic nevi displayed low levels of p16^{INK4A}, p21^{CIP1} and p53. However, advanced melanomas totally lacked both p16^{INK4A} and p21^{CIP1} (Gray-Schopfer et al., 2006). Dhomen et al., (2009) showed that expression of V^{600E}BRAf at physiological levels in mouse melanocytes stimulated skin hyperpigmentation due to increased melanocyte
proliferation. $V_{600E}$BRaf also induced nevi harbouring senescent melanocytes that were positively stained with SA-β-gal. Approximately 70% of $V_{600E}$BRaf-expressing mice developed melanomas. It has been shown that $p16^{\text{Ink4a}}$ was not required to induce melanocyte senescence and its loss was not required for tumour development (Dhomen et al., 2009). Similarly, Dankort et al. (2009) showed that, after the induction of $V_{600E}$BRaf expression, mice developed benign melanocytic hyperplasias that did not progress to melanoma. By contrast, the expression of $V_{600E}$BRaf in combination with the silencing of the PTEN tumour suppressor, promoted the development of a metastatic melanoma with 100% penetrance. After a short latency period, melanomas metastasised to the lymph nodes and lungs (Dankort et al., 2009). In a separate study, it was shown that expression of physiological levels of $V_{600E}$BRaf led to development of multiple pulmonary tumours with adenoma morphology dependent on the MEK/ERK cascade (Dankort et al., 2007). These benign adenomas accumulated senescence markers including $p19^{\text{Arf}}$ and Dec1, but not SA-β-gal. $V_{600E}$BRaf-induced benign lung adenomas rarely progressed to adenocarcinomas, unless either $p53$ or $\text{Ink4a}/\text{Arf}$ was inactivated (Dankort et al., 2007). Most recently, expression of $V_{600E}$BRaf in the intestine induced a MEK-dependent burst of proliferation, leading to the formation of hyperplastic crypts (Carragher et al., 2010). However, crypt hyperplasia was not sustained due to the induction of crypt senescence accompanied by upregulation of SA-β-gal and $p16^{\text{Ink4a}}$. Tumour progression occurred only when $\text{Cdkn2a}$ was knocked out or $p16^{\text{Ink4a}}$ was downregulated through CpG methylation (Carragher et al., 2010). These observations strongly support the hypothesis that OIS functions as an important in vivo physiological response to prevent tumour development driven by the high activity $V_{600E}$BRaf oncogene.

1.5.6 Molecular pathways in senescence

Cellular senescence can be induced by a variety of stimuli and regulated by different signalling pathways and the execution and maintenance of the senescence phenotype converges on the p53/p21$^{\text{CIP1}}$ and $p16^{\text{INK4A}}$/pRb pathways in most cases resulting in irreversible cell cycle arrest. The p53/p21$^{\text{CIP1}}$ and $p16^{\text{INK4A}}$/pRb pathways are activated
upon entry into senescence and function as central integration points for the various signalling pathways leading to senescent growth arrest (Campisi, 2005; Campisi and d’Adda di Fagagna, 2007). These two pathways are well known tumour suppressor mechanisms and are often mutated in tumours, illustrating the importance of disrupting growth arrest to the tumourigenic process (Ruas and Peters, 1998; Sharpless and DePinho, 2004; Gil and Peters, 2006; Kim and Sharpless, 2006). These two pathways can operate either independently or cooperatively to permanently block cell cycle progression depending on cell types, culture conditions and the intensity of the stress (Figure 1.11) (Campisi and d’Adda di Fagagna, 2007).

1.5.6.1 The p53/p21\textsuperscript{CIP1} pathway
Senescence triggered by various stimuli that commonly generate DNA damage response is thought to be induced primarily through p53 since it is a pivotal effector of these DNA damage signals (Wahl and Carr, 2001). p53 is a tumour suppressor and inactivated in ~50% of human cancers (Hollstein et al., 1991; Levine et al., 1991). Under physiological conditions, the activity of p53 is low due to MDM2 (HDM2 in human) binding and subsequent targeting of p53 for proteasome-dependent degradation. MDM2 is also a transcription target of p53. Therefore, p53-induced MDM2 transcription results in its own degradation (Levine et al., 1997; Kruse and Gu, 2009). Following telomere shortening in replicative senescence or DNA damage response up stress, p53 is stabilised and activated by phosphorylation through ATM/ATR-CHK1/CHK2 pathways, resulting in disruption of its binding with MDM2 (d’Adda di Fagagna et al., 2003; Herbig et al., 2004). p53 is also activated by ARF (p14 in human and p19 in mouse, respectively) that binds and sequesters MDM2, thereby stabilising and activating p53 in response to oncogene activation (Sherr and McCormick, 2002; Lowe and Sherr, 2003). Activated p53 transactivates its downstream target gene p21\textsuperscript{CIP1} to cause cell cycle arrest (El-Deiry et al., 1993). As explained above, p21\textsuperscript{CIP1} is a CDK inhibitor and acts to inhibit functional Cyclin E/CDK2 and Cyclin D-CDK4/CDK6 complexes, leading to cell cycle arrest by hypophosphorylation of Rb (Sherr and Roberts, 1999).
Figure 1.11 Senescence controlled by the p53/p21\textsuperscript{CIP1} and p16\textsuperscript{INK4A}/pRb pathways

Senescence-inducing signals usually engage either the p53/p21\textsuperscript{CIP1} or the p16\textsuperscript{INK4A}/pRb tumour suppressor pathways. p53 is negatively regulated by MDM2, which facilitates its degradation, and MDM2 is negatively regulated by the p19\textsuperscript{ARF}. Active p53 establishes senescence growth arrest in part by inducing the expression of p21\textsuperscript{CIP1}, which inhibits Cyclin E/CDK2, leading to de-phosphorylation of pRb. Senescent signals induce the expression of p16\textsuperscript{INK4A}, which, in turn, inhibits Cyclin D-CDK4/6 and prevents pRb phosphorylation and inactivation. pRb halts cell proliferation by suppressing the activity of the E2F transcription factor that stimulates the expression of genes that are required for cell cycle progression. E2F can also curtail proliferation by inducing p19\textsuperscript{ARF} expression, which engages the p53 pathway. Overall, there is a cross-talk between the p53/p21\textsuperscript{CIP1} and p16\textsuperscript{INK4A}/pRb pathways.

![Diagram of Senescence pathways](image-url)
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The p53/p21\textsuperscript{CIP1} pathway plays an essential role in human cells during replicative senescence as deletion of p21\textsuperscript{CIP1} (Medcalf et al., 1996; Brown et al., 1997; Pantoja and Serrano, 1999; Wei et al., 2001b) or inactivation of p53 by various strategies (Hara et al., 1991; Beausejour et al., 2003; Gire and Wynford-Thomas, 1998; Bond et al., 1994; Wei et al., 2003) is able to delay or abrogate replicative senescence. The p53/p21\textsuperscript{CIP1} pathway also plays an important role in telomere-independent premature senescence in human cells, including response to DNA damage (Di Leonardo et al., 1994), oxidative stress (Chen et al., 1998), or overexpression of oncogenic RAS (Serrano et al., 1997; Ferbeyre et al., 2000; Pearson et al., 2000). Oncogenic RAS-induced senescence is probably mediated by the production of reactive oxygen species (ROS) in a p53-dependent DNA damage response (Irani et al., 1997; Lee et al., 1999). Due to very long telomeres (Kipling and Cooke, 1990; Prowse and Greider, 1995; Greenberg et al., 1998), senescence induced in mouse embryonic fibroblasts (MEFs) is independent of telomere attrition and is probably mediated by oxidative stress induced by inadequate culture conditions (Sherr and DePinho, 2000; Parrinello et al., 2003). Inactivation of p53 is sufficient to prevent MEFs from entering senescence (Dirac and Bernards, 2003). However, MEFs from p21\textsuperscript{Cip1} null mice undergo senescence normally, suggesting p21\textsuperscript{Cip1} may not be a critical link downstream of p53 and other proteins may be involved during senescence in MEFs (Pantoja and Serrano, 1999).

Therefore, the induction of senescence by telomere attrition, DNA damage, oxidative stress, and oncogene activation converges on the p53/p21\textsuperscript{CIP1} pathway at least in some human and mouse cells, which is both necessary and sufficient to establish and maintain the senescence phenotype.

1.5.6.2 The p16\textsuperscript{INK4A}/pRb pathway

It has been shown that inactivation of both p53 and pRb is required to reverse senescence in human cells, indicating p53/p21\textsuperscript{CIP1} and p16\textsuperscript{INK4A}/pRb pathways function in parallel and perform partially redundant roles in senescence (Hara et al., 1991; Shay et al., 1991; Smogorzewska and de Lange, 2002; Voorhoeve and Agami, 2003).
p16\textsuperscript{INK4A} has been found to be increased dramatically in replicative senescent human cells (Wong and Riabowol 1996; Hara et al., 1996; Ohtani et al., 2001; Alcorta et al., 1996; Stein et al., 1999; Itahana et al., 2003). Overexpression of p16\textsuperscript{INK4A} triggers presenescent human cells to become senescent (McConnell et al, 1998; Dai and Enders 2000). However, in one study, functional inactivation of p16\textsuperscript{INK4A} did not prevent replicative senescence in human fibroblasts (Brookes et al., 2002, 2004; Wei et al., 2003), suggesting that p16\textsuperscript{INK4A} probably is not essential for replicative senescence of human fibroblasts (Beausejour et al., 2003; Itahana et al., 2003). Mounting evidence has shown that p16\textsuperscript{INK4A} is significantly induced during premature senescence in human cells in response to a variety of stimuli, including overexpression of oncogenes (Serrano et al., 1997; Brookes et al., 2002; Benanti and Galloway, 2004; Lowe and Sherr, 2003; Ohtani et al., 2001; Zhu et al., 1998; Lin et al., 1998), DNA damage (Robles and Adami, 1998; Smogorzewska and de Lange, 2002), and sub-optimal culture conditions (Sherr and DePinho, 2000; Ramirez et al., 2001; Romanov et al., 2001). Although the p53/p21\textsuperscript{CIP1} pathway is activated as an immediate response to DNA damage, cells that have been senescent for long periods no longer have high levels of p21\textsuperscript{CIP1} but often have high levels of p16\textsuperscript{INK4A} (Robles and Adami, 1998; Alcorta et al., 1996). The mechanism of increase of p16\textsuperscript{INK4A} as a response to DNA damage does not involve ATM or ATR (Alcorta et al., 1996; Krishnamurthy et al., 2004; Ohtani et al., 2001; Shapiro et al., 1998; Jacobs and de Lange, 2004, 2005), but potentially is related to p38\textsuperscript{MAPK} and the RAF/MEK/ERK pathway (Iwasa et al., 2003; Bulavin et al., 2004; Roux and Blenis, 2004; Zarubin and Han, 2005).

Despite the obvious significance of cell cycle mediators in the initiation of a block of cell proliferation, permanent growth arrest requires the formation of senescence-associated heterochromatin foci (SAHF) that were first described as a downstream consequence of high-intensity RAS signalling and telomere dysfunction (Narita et al., 2003). Simultaneously activation of p16\textsuperscript{INK4A} and p21\textsuperscript{CIP1} constitutively activate pRb during senescence (Stein et al., 1999; Sherr and McCormick, 2002). Active pRb is crucial for generating SAHF, which binds and silences E2F target genes coding for proliferation
(Narita et al., 2003). Once established, SAHF may become self-sustaining, no longer requiring p16\(^{INK4A}\) or pRb to maintain irreversible growth arrest state (Dai and Enders, 2000; Beausejour et al., 2003; Narita et al., 2003).

1.5.7 Secreted proteins in senescence

Senescent cells lose the capacity to proliferate, but remain metabolically alive and secrete various factors. Upregulation of the transcription of multiple secreted factors has been observed in senescent cells (Collado et al., 2005; Acosta et al., 2008; Kuilman et al., 2008) indicating those involved in immune response and inflammation (Yoon et al., 2004). The pattern of secreted protein expression in senescent cells has been termed the senescence-messaging secretome (SMS) (Ren et al., 2009; Kuilman and Peeper, 2009). Secreted factors from senescent cells include the key components of Wnt, Insulin-like growth factor 1 (IGF1), transforming growth factor-\(\beta\) (TGF\(\beta\)), plasmin, and interleukins (ILs), and they appear to act as signals to allow for communication both within and between cells. Upregulation of secreted proteins is observed in cells undergoing replicative senescence or OIS, regardless of their origin, either epithelial, fibroblasts, or endothelial (Collado et al., 2005; Kuilman et al., 2008; Shelton et al., 1999). Plasminogen activator inhibitor (PAI)-1, a secreted protein that has been regarded as a marker for senescence, is a transcriptional target of p53 that is necessary and sufficient for the establishment of replicative senescence (Kortlever et al., 2006). A tumour suppressive role for some of the factors secreted by senescent cells has been reported. Recently, in a genome-wide RNAi-based screen, IGFBP7 was identified to mediate senescence in melanocytes induced by oncogenic \(^{V600E}BRAF\) in an autocrine/paracrine manner (Wajapeyee et al., 2008). Importantly, the IGFBP7 promoter is methylated in a large number of \(^{V600E}BRAF\) positive melanomas, suggesting that loss of IGFBP7 may contribute to progression of nevi to melanomas. Injection of IGFBP7 protein into \(^{V600E}BRAF\)-expressing melanoma xenografts suppressed tumour growth, indicating the central role of the secreted protein in suppression of tumour development in response to \(^{V600E}BRAF\) expression (Wajapeyee et al., 2008).
Proinflammatory cytokines and chemokines secreted by senescent cells also have been reported to regulate senescent growth arrest (Acosta et al., 2008; Kuilman et al., 2008) and trigger an innate immune response that results in clearance of senescent lesions (Xue et al., 2007). It has been shown that IL-6 and IL-8 play causative roles in establishment and maintenance of senescent state (Acosta and Gil 2009; Kuilman and Peeper, 2009; Ren et al., 2009). IL-6 is upregulated during senescence induced by \textsuperscript{V600E}BRAF, and knockdown of IL-6 or its receptor IL-6R bypasses senescence induced by \textsuperscript{V600E}BRAF. The expression of IL-8 is also induced during OIS, and depletion of IL-8 expression resulted in similar effects as depletion of IL-6 (Kuilman et al., 2008). High levels of IL-8 expression are observed in the nonproliferative crypts in colon adenomas (Acosta et al., 2008; Kuilman et al., 2008), suggesting that these cytokines and chemokines function in vivo to promote or maintain the senescent phenotype of benign human tumours. Another study showed that the chemokine receptor CXCR2 (IL8RB) and many of its ligands are upregulated during OIS. They form part of a chemokine network reinforcing growth arrest in a p53-dependent manner. Enhanced expression of CXCR2 and its ligands occurs in different preneoplastic lesions with senescence and their downregulation or mutation may be necessary for progression of lung adenocarcinomas (Ascota et al., 2008).

1.6 Autophagy

1.6.1 Process of autophagy

Autophagy is a lysosome-dependent bulk degradation pathway for long-lived protein and damaged organelles within double-membrane autophagosomes, which eventually fuse with lysosomes for degradation (Klionsky and Emr, 2000; Klionsky, 2004). Autophagy occurs at a basal level in most cells, where it plays a housekeeping role in cytoplasmic quality control for elimination of protein aggregates and damaged organelles (Mizushima et al., 2008; Levine and Kroemer, 2008). Autophagy can also be induced above basal levels by several stimuli, including nutrient starvation, metabolic stress and low energy status, where autophagy is an important survival mechanism (Klionsky, 2004).
With respect to physiological functions and modes of delivery of the cytoplasmic cargo to the lysosomal lumen, there are at least three different types of autophagy in higher eukaryotes: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), all of which function in promoting proteolytic degradation of cytosolic components within lysosomes (Eskelinen and Saftig, 2009; Yu et al., 2008). Macroautophagy is the most studied mechanism of autophagy conserved among eukaryotic cells. During macroautophagy (hereafter called autophagy), cytoplasmic cargo is sequestered in a double membrane-bound vesicle, known as the autophagosome, and delivered to the lysosome and fused with the lysosome to form an autolysosome before degradation (Figure 1.12). The autophagosome is formed from nucleation and elongation of an isolation membrane or phagophore. The edges of the phagophore then fuse to form the autophagosome, a double-membraned vesicle that sequesters the cytoplasmic cargo. The outer membrane of the autophagosome fuses with the lysosome to form an autolysosome or autophagolysosome, where the autophagosomal contents together with the inner membrane, are degraded via acidic lysosomal hydrolases (Levine and Kroemer, 2008). Consequently, the resulting macromolecules are released back into the cytoplasm via permeases in the lysosome membrane, where they can be reused for building macromolecules and for cellular metabolism (Yang et al., 2006; Mizushima, 2007). In microautophagy, by contrast, cytosolic components are directly taken up by the lysosome itself through invagination of the lysosomal membrane. Both macroautophagy and microautophagy are able to engulf large structures through both selective and non-selective mechanisms. In chaperone-mediated autophagy, proteins containing a KFERQ-like peptide motif are directly translocated across the lysosomal membrane for degradation (Massey et al., 2006; Saftig et al., 2008).
Figure 1.12 Schematic representation of autophagy (Adapted from Pacheco and Lieberman, 2008)

Autophagy begins with nucleation of the phagophore which sequesters a portion of the cytoplasm including macromolecules or organelles. The phagophore expands to generate the double-membrane autophagosome. Upon completion, the outer membrane of the autophagosome subsequently fuses with the lysosome to form the autolysosome, where the internal materials are degraded. Following breakdown, the resulting macromolecules are released back into the cytosol for reuse.
1.6.2 Molecular machinery of autophagy

Autophagy was first described by Christian de Duve over 40 years ago based on the observation of the degradation of mitochondria and other intracellular structures within lysosomes (Deter and De Duve, 1967). Although the importance of autophagy is well recognised in mammalian systems, the molecular components of the autophagy machinery were first identified in yeast (Saccharomyces cerevisiae) and the molecular mechanisms underpinning the execution of autophagy have also been achieved in yeast (Levine and Klionsky, 2004; Nakatogawa et al., 2009; Klionsky, 2007). The molecular machinery underlying the autophagy process depends on the multiple AuTophaGy-related gene (ATG) products (Klionsky et al., 2003). By now, there are about 30 autophagy-related genes that have been identified and characterised by genetic screening in yeast (Kundu and Thompson, 2008; Suzuki and Ohsumi, 2007; Nair and Klionsky, 2005; Yorimitsu and Klionsky, 2005; Klionsky et al., 2003; Kawamata et al., 2005; Stasyk et al., 2006; Klionsky et al., 2010). Significantly, many of these genes are conserved throughout eukaryotes, with 16 orthologs identified in mammals (Cheong and Klionsky, 2008), implicating the importance of autophagy in response to starvation across to the eukaryotes (Nakatogawa et al., 2009).

It is generally accepted that the formation of the autophagosome occurs de novo through nucleation, assembly and elongation of a small membrane structure, termed phagophore or isolation membrane, rather than being generated by budding from the surface of a pre-existing organelle or sealing of a single piece of continuous membrane in mammalian cells (Noda et al., 2002 Seglen et al., 1987; Fensrud et al., 1995; Fensrud et al., 2004; Mizushima et al., 2001). Among all the ATG genes identified in yeast, there are more than 20 ATG genes encoding proteins that are essential for the execution of autophagy at various steps of autophagosome formation (Mizushima and Klionsky, 2007; Klionsky et al., 2003; Kawamata et al., 2005; Kabeya et al., 2007). These ATG proteins can be categorised into several groups according to their functions in the various steps of the pathway. The ATG1 complex is composed of ATG1, ATG13, and ATG17 and localises most upstream of all ATG proteins and downstream of the...
mammalian target of rapamycin (mTOR). The ATG1 complex is involved in control of early steps in autophagosome induction and initiation, and is negatively regulated by mTOR under nutrient rich conditions (Mizushima, 2010). Two ubiquitin-like conjugation systems comprise ATG12 and ATG8. The ATG12 conjugation system is composed of ATG12, ATG5 and ATG16; and the ATG8 conjugation system is composed of ATG8 and phosphatidylethanolamine (PE). These two conjugation system are key to autophagosome elongation (Ohsumi 2001; Ohsumi and Mizushima, 2004). ATG6/class III PI3K complex containing ATG6, class III PI3K Vps34, Vps15, and ATG14 controls the production of phosphoinositide signals that facilitate the first step towards the formation of the autophagosomes-the nucleation and assembly of the initial phagophore membrane (Petiot et al., 2000; Simonsen and Tooze, 2009). Following ATG1 activation, the ATG6/class III PI3K complex localises to the phagophore (Kihara et al., 2001) and subsequently is activated to produce PtdIns(3)P-enriched membrane domains (omegasomes) which act as platforms to recruit factors required for autophagosome assembly, including two ubiquitin-like conjugation systems and Atg9 to the phagophore (Suzuki et al., 2001, 2007; Suzuki and Ohsumi, 2007; Hara et al., 2008; Matsushita et al., 2007). Finally, a recycling system comprises ATG2, ATG9, and ATG18 that participates in transfer and recycling of ATG proteins from mature autophagosome to the growing autophagosome (Noda et al., 2002; Levine and Klionsky, 2004; Legakis et al., 2007; Xie and Klionsky, 2007).

1.6.3 Regulation of autophagy
Autophagy is active at basal levels in most cell types where it plays a housekeeping role in maintaining the integrity of intracellular proteins and organelles. Autophagy is strongly induced by starvation and is a key component of the adaptive response of cells and organisms to nutrient starvation that promotes survival (Levine and Klionsky, 2004). The regulation of autophagy by signalling pathways overlaps with the control of cell growth, proliferation, and cell death (Figure 1.13).
1.6.3.1 mTOR and autophagy

mTOR is a conserved serine/threonine protein kinase that regulates cell growth, nutrient import, protein synthesis and autophagy (Sarbassov et al., 2005a; Wullschleger et al., 2006). mTOR occurs in two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is rapamycin-sensitive and primarily regulates protein synthesis, nutrient import and autophagy (Sarbassov et al., 2005a; Wullschleger et al., 2006). mTORC2 is rapamycin-insensitive and appears to be involved in actin cytoskeleton regulation and Akt regulation (Sarbassov et al., 2004, 2005). It has been shown that mTORC1 represents the major negative regulator of autophagy in mammalian cells (Sabatini, 2006; Wullschleger et al., 2006). Components of the ULK1 (ATG1 in yeasts) complex are targets of mTORC1. It has been shown that mTORC1 is incorporated into the ULK1 complex (ULK1/ATG13/FIP200) in a nutrient-dependent manner and phosphorylates and inactivates both ULK1 and ATG13 resulting in inhibition of autophagy. Under starvation conditions or low energy status or in response to rapamycin, mTORC1 dissociates from the ULK1 complex, allowing activation of ULK1 and induction of autophagy (Chang and Neufeld, 2004; Hosokawa et al., 2009; Jung et al., 2009).

The mTORC1 signalling is regulated by growth factors, nutrient status and energy levels that are involved in modulation of autophagy (Wullschleger et al., 2006; Avruch et al., 2009; Polak and Hall, 2009; Sabatini, 2006). Firstly, mTORC1 plays a crucial role in response to growth factors via the class I PI3K/AKT pathway in inhibition of autophagy (Lum et al., 2005). It has been shown that growth factor signalling induces AKT-dependent phosphorylation of TSC2 and subsequent inactivation of the TSC1/TSC2 complex, thus allowing mTORC1 activation by Rheb. Activated mTORC1 promotes transcription and protein synthesis by phosphorylation of p70 S6 kinase (S6K) and eukaryotic initiation factor (eIF) 4E-binding protein 1 (4E-BP1) resulting in inhibition of autophagy (Potter et al., 2002; Inoki et al., 2002; Inoki et al., 2003; Arsham and Neufeld, 2006; Liang et al., 2007; Lum et al., 2005). Conversely, growth factor starvation inhibits the mTORC1 activity resulting in autophagy induction (Lum et al., 2005).
Figure 1.13 Signalling pathways involved in the regulation of mammalian autophagy

Autophagy is regulated by a complex signalling pathway network. Activation of growth factor receptor stimulates RAS, which activates the class I PI3K/AKT and the RAF/MEK/ERK pathways. AKT and ERK phosphorylate and inhibit the TSC1/TSC2 complex, leading to stabilisation of Rheb, which, in turn, activates mTORC1, causing inhibition of autophagy. ERK can also stimulate autophagy through stimulation of G alpha interacting protein (GAIP) and this process can be inhibited by amino acids via phosphorylation and inhibition of Raf-1. Metabolic stress stimulates autophagy by activating AMPK, which phosphorylates and activates TSC1/TSC2, leading to inactivation of mTORC1 and autophagy induction. Genotoxic stress stabilises and activates nuclear p53 (n), which stimulates autophagy through activation of AMPK or upregulation of DRAM. In contrast, cytoplasmic p53 (c) inhibits autophagy. BCL-2 proteins bind and inhibit the Beclin-1/Class III PI3K complex, causing inhibition of autophagy.
Secondly, mTORC1 also functions as a nutrient sensor. It has been shown that amino acid depletion stimulates autophagy by inhibition of the mTORC1 activity (Blommaart et al., 1995; Codogno and Meijer, 2005; Shintani and Klionsky, 2004). On the contrary, it has been shown that amino acid-mediated activation of mTORC1 and inhibition of autophagy is mediated by inhibition of the TSC1/TSC2 complex to relieve its inhibitory effect on Rheb or promotion of the association of Rheb with mTORC1 resulting in stimulation of mTORC1 (Roccio et al., 2006; Long et al., 2005), which is independent of AKT and class I PI3K (van Sluijters et al., 2000). Finally, mTORC1 also acts as an energy sensor mediated by AMPK kinase (Meijer and Codogno, 2004). Decreased ratio of ATP/AMP activates AMP-activated protein kinase (AMPK), which in turn, inhibits mTORC1 and protein synthesis through activation of the TSC1/TSC2 complex. Autophagy stimulated by mTORC1 downregulation results in increased ATP production via recycling nutrients to survive stress (Meijer and Dubbelhuis, 2004).

Emerging evidence has shown that mTORC2 is also involved in inhibition of autophagy through activation of AKT (Mammucari et al., 2007; Sarbassov et al., 2005b). mTORC2 activates AKT by phosphorylating AKT at Ser473 that in turn phosphorylates and inhibits FOXO3A transcription factor. It has been shown that FOXO3A transcription factor transcriptionally activate autophagy related genes leading to autophagy induction (Sarbassov et al., 2005b; Mammucari et al., 2007; Young et al., 2009).

1.6.3.2 p53 and autophagy

The tumour suppressor p53 has been shown to have a dual function in induction or inhibition of autophagy, depending on its subcellular localisation and types of stress (Figure 1.14) (Levine and Abrams, 2008; Green and Kroemer, 2009; Maiuri et al., 2010). It has been found that p53 activates AMPK, which in turn promotes autophagy through inhibition of the mTORC1 complex in response to genotoxins (Feng et al., 2005). Further studies indicated that p53 transactivates negative regulators of mTOR; the subunits of AMPK, AMPK-β1 and AMPK-β2, TSC2 and PTEN to induce autophagy (Feng et al., 2007). More recently, Sestrin1 and Sestrin2, two p53 target genes, have
been found to activate AMPK leading to autophagy induction upon genotoxic stress (Budanov and Karin, 2008; Maiuri et al., 2009). In addition, p53 has also been shown to induce autophagy through transactivating damage-regulated autophagy modulator (DRAM), a lysosomal protein that can trigger autophagy under genotoxic stress (Crighton et al., 2006; Criollo et al., 2009). Moreover, it has also been observed that p53 transactivates pro-apoptotic members of the BLC-2 family such as BAX, PUMA (Yee et al., 2009), BAD (Maiuri et al., 2007), and BNIP3 (Zhang and Ney, 2009) in autophagy induction. BH-3 only proteins and their mimetics were shown to promote autophagy by destabilising the interactions between BCL-2/BCL-xL and Beclin-1 (Lomonosova and Chinnadurai, 2008; Maiuri et al., 2007). Altogether, p53-mediated autophagy induction is believed to be regulated at the transcriptional level in response to genotoxic stress.

In contrast to the nuclear p53 functioning as a transcription inducer for autophagy, the cytoplasmic pool of p53 has been shown to suppress basal autophagy in unstressed cells (Tasdemir et al., 2008a, 2008b, 2008c; Morselli et al., 2008). It has been found that knockout, siRNA-mediated knockdown or pharmacological inhibition of p53 leads to induction of autophagy (Tasdemir et al., 2008a). Upon starvation, ER stress or mTOR inhibition by rapamycin, MDM2-dependent proteasomal degradation of p53 promotes autophagy, and this was reversed by MDM2 inhibition (Tasdemir et al., 2008a). It has also been shown that autophagy is inhibited by p53 in enucleated cells (Tasdemir et al., 2008a) and mutation or deletion of the DNA-binding domain of p53 has no effect on the suppression of autophagy (Morselli et al., 2008). Moreover, accumulation of some cancer-associated p53 mutants in the cytoplasm actively suppresses autophagy in human colon cancer cells (Tasdemir et al., 2008a, 2008b, 2008c; Morselli et al., 2008). Finally, expression of mutant p53 without nuclear localisation sequence was found to significantly inhibit autophagy, whereas deletion of the nuclear export signal which results in the accumulation of p53 within the nucleus totally abolishes autophagy inhibition by p53 (Tasdemir et al., 2008a; Morselli et al., 2008). These observations suggest that cytoplasmic localisation of both of WT and mutant p53 is indispensable for autophagy inhibition, independently of its transcriptional activity.
Figure 1.14 p53 and autophagy

p53 is downstream of both genotoxic stress and metabolic stress. Upon genotoxic stimulation, p53 becomes stabilised and activated as a transcription factor, and transactivates a number of genes with direct roles in autophagy induction, such as DRAM, or that encode inhibitors of mTOR activity, including AMPK b1/b2, TSC2, PTEN, Sestrin1/2, or pro-apoptotic BCL-2 proteins such as BAX, PUMA, BAD, and BNIP3. In response to metabolic stress, such as glucose starvation, p53 is phosphorylated by AMPK and induces G1 cell cycle arrest, thereby promoting cell survival. In the unstressed cell, silencing or inhibition of p53 induces autophagy. Therefore, p53 ties to autophagy as both a positive and negative regulator, depending on the presence of stress.
A decrease in the glucose level in the cell represents a common form of metabolic stress that can potently induce autophagy. Cross-talk between metabolic stress and the p53 signalling has been implicated (Feng et al., 2005; Jones et al., 2005). It has been reported that glucose starvation transiently phosphorylated p53 at Ser15 and activated it, but did not induce p53 transcriptional program or the stabilisation of the p53 protein. This phosphorylation was mediated by AMPK, which served as a fuel sensor in the cell and was activated by a high ratio of AMP/ATP (Feng et al., 2005). Phosphorylation of p53 at Ser15 is transient, being removed by α-4-PP2A phosphatase when glucose starvation continues (Feng et al., 2005). The activation of p53 by metabolic stress and AMPK eventually led to cell cycle arrest, thereby allowing cells to survive periods of nutrient deprivation (Jones et al., 2005). Cells that have undergone a p53-dependent metabolic arrest can rapidly re-enter the cell cycle upon glucose restoration (Jones et al., 2005). Altogether, these findings suggest that p53 may be involved directly in sensing the metabolic stress induced by energy deprivation (Feng et al., 2005; Jones et al., 2005).

1.6.4 Autophagy and cancer

In response to cellular stress, autophagy is crucial in preventing the accumulation of protein aggregates and damaged organelles. Failure to remove these cellular toxins contributes to tissue damage and chronic inflammation that would result in generating genome mutations leading to cancer initiation and development. Thus, activation of autophagy functions as a tumour suppression mechanism to prevent persistent tissue damage and chronic inflammation. On the other hand, autophagy serves as a survival mechanism for tumour cells upon metabolic stress, which is beneficial for tumour development. So autophagy has a dual function in tumourigenesis (Levine et al., 2007; Mathew et al., 2007a).

Although autophagy acts as a survival pathway used by both normal and tumour cells to survive starvation and stress, autophagy defects are frequently found in many human tumours. A number of autophagy genes such as Beclin-1, MAP1-LC3 (ATG8 in yeast),
*HsGSA7* (orthologue of *ATG7* in yeast) (Jin, 2006), and *UVRAG* (Ionov et al., 2004) have been reported to be frequently deleted in certain types of tumours. For example, the *Beclin-1* gene maps to a tumour-susceptibility locus on human chromosome 17q21 that is mono-allelically deleted in 40-75% of human breast cancers, ovarian cancers, and prostate cancers (Aita et al., 1999; Liang et al., 1999). Accordingly, decreased expression of Beclin-1 has been observed in human breast (Aita et al., 1999; Liang et al., 1999), ovarian, brain (Miracco et al., 2007), gastric cancer (Furuya et al., 2005) and melanoma (Miracco et al., 2010). On the contrary, ectopic expression of Beclin-1 was able to restore autophagy induced by starvation in breast cancer cells that lacked Beclin-1 expression, and this inhibited growth of tumour cells *in vitro* and tumourigenicity of human breast carcinoma cells in mouse xenograft models (Liang et al., 1999). *Beclin-1* heterozygous knockout mice (Liang et al., 1999; Qu et al., 2003; Yue et al., 2003), as well as mice deficient for other autophagy genes involved in autophagy induction including *Atg5* (Mathew et al., 2009), *Atg4c* (Marino et al., 2007), *Uvrag* (Liang et al., 2006), and *Bif-1* (Takahashi et al., 2007, 2008) have been shown to develop spontaneous tumours, which correlate with reduced autophagy. Moreover, immortal kidney and mammary epithelial cells derived from *Beclin-1*+/− mice with defective autophagy have been shown to have enhanced tumourigenesis in recipient mice (Karantza-Wadsworth et al., 2007; Mathew et al., 2007b). These data establish the link between deficiency in autophagy machinery and tumour development suggesting that autophagy may function as a tumour suppression mechanism (Jin and White, 2007; Levine 2007; Kundu and Thompson, 2008; Bialik et al., 2008; Levine and Kroemer, 2008).

Evidence to explain autophagy functioning in tumour suppression is emerging. White and colleagues et al., (2009) reported that autophagy may inhibit tumourigenesis through suppression of necrotic cell death and inflammation (Degenhardt et al., 2006). In apoptosis-defect epithelial cells, impairment of autophagy by mono-allelic deletion of *Beclin-1*, RNAi-mediated knockdown of *Beclin-1* or *ATG5*, or constitutive activation of AKT was shown to induce necrotic cell death upon metabolic stress (Degenhardt et al.,
2006). In tumours in vivo, this necrosis was associated with inflammation, activation of the cytokine-responsive NF-κB pathway and tumour progression (Degenhardt et al., 2006). Inflammation may provide a cell with a chronic wound healing response, where dramatic macrophage infiltration and production of chemokine and cytokine function to stimulate proliferation, tissue remodelling, and angiogenesis (Degenhardt et al., 2006; Jin et al., 2007; Jin and White, 2007; Mathew et al., 2007a). Thus, although it is well known that autophagy can promote cell survival, autophagy may serve as an important non-cell-autonomous tumour suppressor mechanism by limiting inflammation upon metabolic stress (Degenhardt et al., 2006; Mathew et al., 2007a).

1.6.4.1 Autophagy limits genome damage

It has been proposed that the tumour suppressive function of autophagy may also be linked to protect cells from DNA damage and chromosomal instability (Edinger and Thompson, 2003). Recent studies have strongly demonstrated that both Beclin-1 and ATG5 functioned as ‘guardians’ of the genome. Mono-allelic loss of Beclin-1 or deficiency in ATG5 in immortal epithelial cell lines impaired survival following metabolic stress and was accompanied by increased DNA damage, gene amplification, aneuploidy, and eventually led to tumourigenicity by increasing the mutation rate (Karantza-Wadsworth et al., 2007; Mathew et al., 2007b). It has been found that defective autophagy failed to turn over centrosomes (which result in multipolar division and chromosomal instability), damaged mitochondria (which increases ROS production and ROS-mediated DNA damage), ubiquitinated protein aggregates, or unfolded proteins in response to metabolic stress (Karantza-Wadsworth et al., 2007; Mathew et al., 2007b). Further studies demonstrated that failure to eliminate accumulation of p62/SQSTM1 that recognises ubiquitinated protein aggregates in autophagy-defective cells upon metabolic stress led to its binding and suppressing NF-κB. This would potentially increase ROS production by inhibition of the anti-oxidative transcriptional activities of NF-κB, which will eventually contribute to increased DNA damage and tumourigenesis (Mathew et al., 2009).
1.6.4.2 Autophagy-enabled senescence

As explained above, senescence has been proposed as an important tumour suppressor mechanism where cells undergo a state of permanent cell cycle arrest but maintain metabolically active (Campisi, 2005). OIS is a rapid and acute process triggered by activation of oncogenes (Collado and Serrano, 2006). Recently, it has been shown that autophagy is activated upon the induction of senescence by oncogenic \(G^{12V}\)HRAS and is required for the establishment of senescence (Young et al., 2009). In this study, a subset of ATG genes such as \(LC3B\), \(ATG5\), \(ATG7\), \(ULK1/2/3\) and \(UVRAG\) were upregulated during the senescence process by microarray analysis. It has also been shown that production of senescence-associated factors including IGFBP7, IL-6, and IL-8 is upregulated during autophagy-enabled senescence. Overexpression of \(ULK3\) was found to sufficiently induce autophagy and premature senescence. Conversely, inhibition of autophagy by knockdown of \(ATG5\) and \(ATG7\) attenuated the activation of autophagy and bypassed OIS by delayed production of IL6 and IL-8 (Young et al., 2009).

1.6.4.3 Autophagy in tumour promotion

It has also been shown that autophagy plays a pro-survival role for tumour maintenance and development against stressful conditions through its cellular protective and nutrient recycling functions (Ogier-Denis and Codogno, 2003; Gozuacik and Kimchi, 2004; Cuervo, 2004). Cancer cells that are located in the central areas of the tumour always encounter metabolic stress due to poor blood supply (Witz, 2008). In such stressful conditions, autophagy was shown to localise to these hypoxic regions most distal to blood vessels to recycle unessential cytoplasmic components for biosynthesis, thus enabling cancer cells to survive metabolic stress (Lum et al., 2005; Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007; Mathew et al., 2007a). In nutrient deprivation conditions, inhibition of autophagy by knockdown of ATG genes was shown to induce apoptosis in HeLa cells, suggesting a cell survival role of autophagy (Shintani and Klionsky, 2004; Boya et al., 2005). Similarly, growth factor depletion induced autophagy in apoptosis-defective \((Bax^{-/-}/Bak^{-/-})\) cells resulting in prolonged cell survival in an
autophagy gene-dependent manner (Lum et al., 2005). In response to hypoxia, autophagy was shown to selectively sequester damaged mitochondria, preventing these organelles from producing reactive oxygen species (ROS) and maintaining cell survival (Mathew et al., 2009; Zhang et al., 2008a). Furthermore, some cancer cells survive hypoxia via autophagy that involves selective degradation of cytoplasmic p62/SQSTM1 (Wilkinson et al., 2009). Interestingly, autophagy has been shown to be upregulated in response to cellular detachment from extracellular matrix, supporting the view that autophagy could serve a survival mechanism for detached tumour cells to survive anoikis, which may facilitate tumour cell dormancy, dissemination and metastasis (Lock and Debnath, 2008).

Most chemotherapeutic agents and radiotherapies induce cellular stress. The upregulation of autophagy has been frequently observed in tumour cells in vitro during anti-cancer treatment, which is evidenced by accumulation of autophagosomes (Maiuri et al., 2007). It has also been shown that inhibition of autophagy by chloroquine that impairs autophagic degradation in mice with c-Myc-induced lymphomas sensitised tumour cell death and tumour regression by enhanced efficacy of either p53 activation or DNA alkylating agent (Amaravadi et al., 2007). These observations suggest that autophagy may function as a cytoprotective mechanism in response to anti-cancer treatment, allowing the recycling of proteins and cellular components as nutrients for rapidly growing cancer cells (Ogier-Denis and Codogno, 2003; Gozuacik and Kimchi, 2004). Autophagy may also function to remove proteins or organelles that are damaged by anti-cancer treatment. Thus, autophagy-enabled cancer cell survival could result in increased aggressiveness and therapy resistance (Kondo et al., 2005). On this basis, inhibition of autophagy has been proposed as a therapeutic application in cancer treatment. siRNAs targeted against ATG genes or pharmacological inhibitors against autophagy have been shown to inhibit autophagy, resulting in accelerated cancer cell death (Maiuri et al., 2007; Abedin et al., 2007; Carew et al., 2007; Ding et al., 2009). Thus, combined use of autophagy inhibitors and anti-cancer therapies could maximise the effects of killing cancer cells (Kanzawa et al., 2004; Bursch et al., 1996; Opipari et al.,
2004; Longo et al., 2008; Apel et al., 2008; Tiwari et al., 2008; Ito et al., 2005; Amaravadi et al., 2007).

In conclusion, at the early stage of tumour development, autophagy may function as a tumour suppressor. At advanced stage of tumour development, autophagy may promote tumour progression (Kondo et al., 2005). Autophagy may also function as a cytoprotective mechanism in response to anti-cancer treatment. These findings suggest that autophagy inhibition rather than stimulation might be beneficial in treatment of advanced cancer.

1.7 Background to the project

BRAF is a component of the RAS/RAF/MEK/ERK signalling pathway that regulates multiple biological processes (Rapp et al., 2006). BRAF mutations have been found in ~7% of human cancers overall, with high frequency in certain type of human cancer, such as melanoma (~50-70%), papillary thyroid cancer (~30%), and colorectal cancer (~15%) (Davies et al., 2002; Garnett and Marais, 2004). Activating \(^{V600E}\)BRAF mutation is the most frequent BRAF mutation comprising a single amino acid substitution (V600E) (Davies et al., 2002). \(^{V600E}\)BRAF is a conventional oncogene as \(^{V600E}\)BRAF displays elevated kinase activity \textit{in vitro} (~500 fold greater than that of \(^{WT}\)BRAF), constitutively activating the MEK/ERK pathway, as well as enhanced transformation activity in fibroblasts and melanocytes (Davies et al., 2002; Wan et al., 2004).

To study the role of \(^{V600E}\)BRAf in tumour development, our laboratory generated conditional knockin mice. They are detailed below. Conditional gene targeting by using the Cre/LoxP system is a widely used method for studying gene function (Akagi, et al., 1997). The 38-kDa Cre recombinase, a member of integrase family from the bacteriophage P1, is the best characterized site-specific recombinase, which recognises a 34-bp specific sequence called locus of crossing over of P1 phage (LoxP) site (Figure. 1.15) (Sternberg and Hamilton, 1981). Each LoxP sequence consists of two 13-bp inverted repeats flanking an 8-bp non-palindromic spacer sequence. The spacer
determines the LoxP sequence orientation (Kuhn and Torres, 2002). Cre recombinase mediates recombination between two LoxP sequences.

Transgenic mice containing a conditional knockin mutation of \textsuperscript{V600E}Braf at the endogenous Braf locus were generated in our lab using the Cre/LoxP system after homologous recombination in embryonic stem (ES) cells (Mercer et al., 2005). In this model, the endogenous Braf locus was modified such that an LSL cassette was placed in intron 14. The LSL cassette consists of a mini-cDNA encoding exons 15-18 of the Braf gene, a neo\textsuperscript{R} gene, and three LoxP sites. At the 3' -end of the LSL cassette, the endogenous exon 15 was modified to include the T1799A mutation that expresses \textsuperscript{V600E}BRaf. These mice were termed Braf\textsuperscript{+/LSL-V600E}. In this arrangement, the wild-type BRaf protein is expressed with exons 15-18 being encoded by the mini-cDNA in the absence of Cre recombinase. The mutant \textsuperscript{V600E}BRaf protein is prevented from expression due to the two STOP sequences, represented by polyadenylation (PA) sequence located at the 3' -end of the mini-cDNA and at the 3' -end of the neo\textsuperscript{R} cassette. In the presence of the Cre recombinase, deletion of the mini-cDNA and neo\textsuperscript{R} cassette occurs such that \textsuperscript{V600E}BRaf is expressed (Figure 1.16). The advantage of this system is that the \textsuperscript{V600E}BRaf is expressed at physiological levels from its own promoter. By controlling the activity of the Cre recombinase, it is possible to mimic the situation that occurs in human cancer cells where one copy of the normal BRAF gene is mutated somatically to \textsuperscript{V600E}BRAF.

To induce the expression of \textsuperscript{V600E}BRaf \textit{in vivo}, these Braf\textsuperscript{+/LSL-V600E} heterozygous mice were crossed to heterozygous CMV-Cre mice that express the Cre transgene. Expression of \textsuperscript{V600E}BRaf was found in all tissues but resulted in embryonic lethality (Mercer et al., 2005). When interferon-inducible Mx1-Cre mice were crossed to Braf\textsuperscript{+/LSL-V600E} mice, expression of \textsuperscript{V600E}BRaf was observed in many tissues, but only liver and spleen displayed moderately increased proliferation. The mice developed nonlymphoid neoplasia of the histiocytic type, but died within four weeks due to bone marrow failure (Mercer et al., 2005). In primary MEFs derived from Braf\textsuperscript{+/LSL-V600E}...
Figure 1.15 Structure of the LoxP site

(A) The diagram indicates a LoxP sequence consisting of two 13-bp inverted repeats and an 8-bp nonpalindromic sequence which interrupts the two inverted repeats and provides orientation to the overall sequence. (B) Cre recombinase recognises two LoxP sites in tandem to excise the floxed gene.
The endogenous Braf locus was modified such that an LSL cassette was placed in intron 14. The LSL cassette consists of a mini-cDNA encoding exons 15–18 of the Braf gene with a β-globin splice acceptor sequence at the 5′-end of the mini-cDNA and a polyadenylation (PA) sequence at the 3′-end. The cassette also has a neoR gene driven by the PGK (phosphoglycerate kinase) promoter and a PA sequence at the 3′-end. The endogenous exon 15 was modified to include the T1799A (V600E) mutation. Three loxP sites are present in the LSL cassette; the first is between exon 14 and the mini-cDNA, the second between the mini-cDNA and the neoR cassette and the third between the neoR cassette and the modified exon 15. Expression of the Cre recombinase converts the BrafLSL-V600E allele into the BrafLox-V600E allele that expresses V600E Braf. Primers Braf-A (A), Braf-B (B), and Braf-C (C) are used to detect WT Braf, BrafLSL-V600E and BrafLox-V600E alleles by PCR.
embryos, expression of endogenous $V_{600E}BRaf$ induced morphological transformation, increased proliferation, and loss of contact inhibition. Overall, expression of endogenous $V_{600E}BRaf$ prominently induces proliferation in mice and partial transformation of some primary cell types (Mercer et al., 2005).

In a similar conditional knockin mouse model, expression of $V_{600E}BRaf$ was confined to the lung by intranasal instillation of an adenovirus expressing Cre recombinase (Dankort et al., 2007). $V_{600E}BRaf$-expressing mice developed multiple pulmonary tumours with adenoma morphology dependent on the MEK/ERK cascade. Most importantly, after an initial phase of rapid proliferation, there was a dramatic reduction in proliferative activity in the adenomas. These benign adenomas accumulated some senescence markers including p19$^{Arf}$ and Dec1, but not SA-$\beta$-gal. $V_{600E}BRaf$-induced benign lung adenomas rarely progressed to adenocarcinomas, unless either $p53$ or $Ink4a/Arf$ was inactivated (Dankort et al., 2007). This study supported the hypothesis that $V_{600E}BRAF$ can induce senescence that functions as a tumour suppressor mechanism in vivo.

Recently, a mouse model of melanoma was generated (Dhomen et al., 2009). In this study, $Braf^{+/LSL-V600E}$ mice (Mercer et al., 2005) were crossed to $Tyr::CreERT2$ mice to allow precise control of $V_{600E}BRaf$ expression in melanocytes in postnatal mice. After tamoxifen treatment, expression of $V_{600E}BRaf$ in mouse melanocytes stimulated skin hyperpigmentation due to increased melanocyte proliferation, the appearance of nevi harbouring senescent melanocytes positively stained with SA-$\beta$-gal, and development of melanomas. It was shown that p16$^{Ink4a}$ was not required to induce melanocyte senescence and its loss was not required for tumour development. This study established that $V_{600E}BRAF$ can be a founder mutation in melanoma. Similarly, Dankort et al (2009) showed that, after the induction of $V_{600E}BRaf$ expression, mice developed benign melanocytic hyperplasias that did not progress to melanoma. By contrast, the expression of $V_{600E}BRaf$ in combination with the silencing of the $PTEN$ tumour suppressor, promoted the development of a metastatic melanoma with 100%
penetrance. After a short latency period, melanomas metastasised to the lymph nodes and lungs (Dankort et al., 2009).

Finally, a mouse model of serrated colorectal cancer was developed by intercrosses between \( Braf^{+/LSL-V600E} \) (Mercer et al., 2005) and \( AhCre^{ERT} \) heterozygous mice that allow the expression of Cre recombinase in the proliferative cells of the intestinal crypt (Carragher et al., 2010). After injection of \( \beta \)-naphthoflavone (\( \beta \)-NF) and tamoxifen, expression of \( V600EBRaf \) induced a MEK-dependent burst of proliferation, leading to the formation of hyperplastic crypts. However, crypt hyperplasia was not sustained due to the induction of crypt senescence accompanied by upregulation of SA-\( \beta \)-gal and p16\(^{INK4a} \). Tumour progression occurred when \( Cdkn2a \) was knocked out or p16\(^{INK4a} \) was downregulated through enhanced CpG methylation of exon 1 in \( Cdkn2a \). This model is the first time to report that OSI occurs in the intestine, indicating \( V600EBRaf \) acts as an early genetic driver mutation in colorectal cancer.

1.8 Aims of the project

The aim of this project was to investigate the role of oncogenic \( V600EBRaf \) in tumour development. \( Braf^{+/LSL-V600E} \) mice and primary MEFs derived from heterozygous mice with or without Cre recombinase were employed to accomplish the following:

1. Establishment of the optimal conditions to efficiently induce \( V600EBRaf \) expression in MEFs by using the Cre\( ^{ERT} \) system. The role of oncogenic \( V600EBRaf \) in inducing cell proliferation and suppressing apoptosis in MEFs was assessed. The relevant \( V600EBRaf \) downstream targets and signalling pathways involved were characterised.

2. Investigation of the role of oncogenic \( V600EBRaf \) in early stage lung tumour development in mice, and specifically the senescence phenotype. The relevant downstream targets and signalling pathways were characterised using proteomics.

3. Investigation of the role of the cholesterol binding protein NPC2 in lung tumour suppression \textit{in vivo} and its growth inhibitory effect towards lung cancer cell lines \textit{in vitro}.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials
All chemicals and reagents were supplied by Fisher Scientific or Sigma-Aldrich unless otherwise stated. The H₂O used in all methods was always MilliQ water in sterile containers.

2.2 Methods
2.2.1 Molecular biology
2.2.1.1 Lysis of mouse tissues to extract genomic DNA
Mouse ear, tail or lung tissues were lysed by addition of 70 µl of 0.05 M NaOH, heated to 95°C for 15 min and neutralised by addition of 7 µl of 1M Tris-HCl [pH 7.5]. These samples were used for PCR genotyping.

2.2.1.2 Lysis of cells to extract genomic DNA
Cells were washed twice with sterile ice-cold PBS and an appropriate volume of DNA lysis buffer (50 mM Tris-HCl [pH 7.6], 1 mM EDTA, 100 mM NaCl, 0.2% [w/v] sodium dodecyl sulphate (SDS), 100 µg/ml fresh proteinase K) was added, then the mixture was incubated at 65°C for at least 1h. The DNA was precipitated by addition of absolute ethanol and pelleted by centrifugation at 13,000 rpm for 10 min at 4°C. The supernatants were discarded and DNA pellets were washed by addition of 70% [v/v] ethanol. The DNA was pelleted by centrifugation at 13,000 rpm for 10 min at 4°C. The ethanol was removed and DNA pellets were left to air dry and then resuspended in an appropriate volume of TE (Tris-EDTA) buffer (10 mM Tris-HCl [pH7.5], 1 mM EDTA) buffer and stored at 4°C.

2.2.1.3 Polymerase chain reaction (PCR)
8 µl of PCR ReadyMix (ABgene) was mixed with 1 µl of primers (Table 2.1) at a final concentration of 1 µM. 9 µl of this PCR mixture was placed in a 0.2 ml PCR tube and 1
µl of sample DNA was added. Each PCR reaction was overlaid with 10 µl of mineral oil. PCR reactions were performed on a GeneAmp PCR System 2700 (Applied Biosystems) or G-Storm™ PCR machine (Gene Technologies Ltd). The conditions for PCR were the following:

Initial denaturation of DNA at 94°C for 5 min followed by 35 cycles consisting of three steps and a final extension at 72°C for 10 min

Step 1  94°C for 1 min
Step 2  X°C for 1 min (Table 2.1)
Step 3  72°C for 1 min

2.2.1.4 Agarose gel electrophoresis

Between 0.8% and 3% pure Agarose MP (Roche Applied Science) in 1x TAE (Tris-acetate-EDTA) buffer (40 mM Tris base, 20 mM Acetic acid, 1 mM EDTA [pH 8.5]) was melted and poured into a gel mould containing a comb and left to set. The gel was immersed in an electrophoresis tank containing 1x TAE buffer. 10x DNA/RNA loading buffer (50% [v/v] glycerol, 0.4% [w/v] bromophenol blue, 0.4% [w/v] xylene cyanol) was added to each DNA sample before loading into separate wells. Samples were compared to a 1 kb DNA ladder (Life Technologies) for determination of size. Gels were run at 100 V until the DNA had separated sufficiently. The DNA bands in agarose gel were stained with 0.5 µg/ml ethidium bromide in TAE buffer, followed by visualisation using a Bio-Rad gel documentation system.

2.2.1.5 RNA Purification and reverse transcription

RNA was extracted from cells or lung tissues using the Qiagen RNAeasy kit (Qiagen) according to manufacturer’s instruction. DNA contaminants were removed by treatment of the RNA using the DNA-free™ kit (Ambion) according to manufacturer’s instruction. RNA samples were quantified using a BioPhotometer (Eppendorf) and the OD_{260/280} value was ensured between 1.8-2.0. First-strand complementary DNA (cDNA) synthesis was prepared in a 13-µl reaction mixture containing 1 µg of RNA from cells or 0.5 µg of RNA from lung tissues, 1 µl of 50 µM oligo(dT)$_{20}$ primer, 1µl of 10 mM dNTP and dH$_{2}$O.
The mixture was heated to 65°C for 5 min then cooled on ice. The following components of 4 µl of 5x First-strand buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseIN (Promega) and 1 µl of SuperScript® III (Invitrogen) were added to the mixture and heated to 50°C for 60 min to synthesise cDNA. The reaction was terminated by heating to 85°C for 15 min. The cDNA was used as the template in reverse transcription PCR (RT-PCR).

Table 2.1 Details of primers used for PCR genotyping

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5' to 3'</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>CreER(^T)-Fwd</td>
<td>GCC TGG TCT GGA CAC AGT GC</td>
<td>62°C</td>
</tr>
<tr>
<td>CreER(^T)-Rev</td>
<td>GGT TCA GCA TCC AAC AAG GC</td>
<td>62°C</td>
</tr>
<tr>
<td>Braf-A Fwd</td>
<td>GCC CAG GCT CTT TAT GAG AA</td>
<td>60°C</td>
</tr>
<tr>
<td>Braf-B Rev</td>
<td>GCT TGG CTG GAC GTA AAC TC</td>
<td>60°C</td>
</tr>
<tr>
<td>Braf-C Rev</td>
<td>AGT CAA TCA TCC ACA GAG ACC T</td>
<td>60°C</td>
</tr>
<tr>
<td>Npc2 PCR2-Fwd</td>
<td>TTT CCT CCC TAG TCA AAC TCA ACT</td>
<td>53°C</td>
</tr>
<tr>
<td>Npc2 PCR3-Fwd</td>
<td>CAT TCT CAG TAT TGT TTT GCC AAG</td>
<td>53°C</td>
</tr>
<tr>
<td>Npc2 PCR2 and</td>
<td>AGT GAG AAT TAT GGA CCC AGA CTC</td>
<td>53°C</td>
</tr>
<tr>
<td>PCR3-Rev</td>
<td>AGT GAG AAT TAT GGA CCC AGA CTC</td>
<td>53°C</td>
</tr>
<tr>
<td>Npc2 PCR1-Fwd</td>
<td>GCA CAC GTA GAG CTC AGA GAA TAA</td>
<td>55°C</td>
</tr>
<tr>
<td>Npc2 PCR1-Rev</td>
<td>TTC TCA CCA CCA CTA CTG TGT TTT</td>
<td>55°C</td>
</tr>
</tbody>
</table>

2.2.1.6 Semiquantitative reverse transcription PCR

Semiquantitative reverse transcription PCR reaction was performed using 1 µl of cDNA, 1 µM of each primer (Table 2.2) and PCR beads (GE Healthcare) in a final volume of 25 µl. The conditions for RT-PCR were 5 min at 94°C for initial activation, 35 cycles of 30 sec at 94°C for denaturation, 30 sec at annealing temperature, 30 sec at 72°C for extension and 10 min at 72°C for a final elongation. Gapdh was used as an internal control. The products were separated on 3% agarose gel by electrophoresis and stained with ethidium bromide, and visualised using a Bio-Rad gel documentation system.
Table 2.2 Details of primers used for RT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5' to 3'</th>
<th>Product size (bps)</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh-Fwd</td>
<td>AGG TCG GTG TGA ACG GAT TTG</td>
<td></td>
<td>60°C</td>
</tr>
<tr>
<td>Gapdh-Rev</td>
<td>TGT AGA CCA TGT AGT TGA GGT CA</td>
<td>140</td>
<td>60°C</td>
</tr>
<tr>
<td>Npc2-Fwd</td>
<td>TAT CCA CGA TGC GTT TTC TG</td>
<td></td>
<td>53°C</td>
</tr>
<tr>
<td>Npc2-Rev</td>
<td>TCA GGC TCA GGA ATA GGG AA</td>
<td>280</td>
<td>53°C</td>
</tr>
<tr>
<td>p16^{Ink4a}-Fwd</td>
<td>TCTGGAGCAGCATGGAGTCC</td>
<td>200</td>
<td>53°C</td>
</tr>
<tr>
<td>p16^{Ink4a}-Rev</td>
<td>TCGCAGTTCGAATCTGACC</td>
<td></td>
<td>53°C</td>
</tr>
<tr>
<td>p21^{Cip1}-Fwd</td>
<td>GTA CTT CCT CTG CCC TGC TG</td>
<td>180</td>
<td>57°C</td>
</tr>
<tr>
<td>p21^{Cip1}-Rev</td>
<td>TCT GCG CTT GGA GTG ATA GA</td>
<td></td>
<td>57°C</td>
</tr>
</tbody>
</table>

2.2.1.7 Quantitative real time PCR

Quantitative real time PCR reaction was performed using SYBR Green (Bio-Rad) for amplification detection in a MiniOpticon Real Time PCR system (Bio-Rad). The PCR reaction mixture was prepared using 1 µl of cDNA, 300 nM of each primer (Table 2.3), 12.5 µl of SYBR Green and 9.5 µl of dH₂O in a final volume of 25 µl. Each sample was amplified in triplicate. The PCR conditions were 5 min at 95°C for initial activation, 30 cycles of 30 sec at 95°C for denaturation, 30 sec at annealing temperature and 30 sec at 72°C for extension. All primers were designed in regions flanking introns to exclude possible DNA contamination (Table 2.3). For each primer pair, a temperature gradient was performed to determine the optimal annealing temperature for the amplification of each gene of interest. For each gene, a standard curve based on successive cDNA dilutions was performed at the established optimal annealing temperature and was used to calculate starting quantities. To ensure a thorough calculation, starting quantities of genes of interest were normalised to those of a housekeeping gene in the same plate. In this study, Gapdh was used as the internal control. After each qPCR, specificity of the amplification was monitored by a melting curve ranging from 55 to 95°C whereby a
single peak corresponding to the amplicon was present. Relative mRNA levels of genes of interest were calculated based on the C\textsubscript{T} (threshold cycle) values and corrected by the \textit{Gapdh} mRNA expression, according to the the \textit{2}\textsuperscript{-ΔΔC\textsubscript{T}} (Livak) method (Livak et al., 2001). The C\textsubscript{T} value refers to the cycle number at which the fluorescence of the amplified product appears above the background threshold that is within the exponential phase of amplification.

**Table 2.3 Details of primers used for quantitative real time PCR**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5' to 3'</th>
<th>Product size (bps)</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Bim\textsubscript{EL}-Fwd}</td>
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<td>60°C</td>
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<tr>
<td>\textit{Bim\textsubscript{EL}-Rev}</td>
<td>TCC TCC TTG GAC TTC TAG AC</td>
<td></td>
<td>60°C</td>
</tr>
<tr>
<td>\textit{Cyclin D1-Fwd}</td>
<td>CGG ATG AGA ACA AGC AGA CC</td>
<td>192</td>
<td>60°C</td>
</tr>
<tr>
<td>\textit{Cyclin D1-Rev}</td>
<td>TGG AAA GAA AGT GCG TTG TG</td>
<td></td>
<td>60°C</td>
</tr>
<tr>
<td>\textit{Gapdh-Fwd}</td>
<td>AGG TCG GTG TGA ACG GAT TTG</td>
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<td>60°C</td>
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<tr>
<td>\textit{Gapdh-Rev}</td>
<td>TGT AGA CCA TGT AGT TGA GGT CA</td>
<td>140</td>
<td>60°C</td>
</tr>
<tr>
<td>\textit{Igfbp7-Fwd}</td>
<td>GGC ATG GAG TGC GTG AAG AG</td>
<td>210</td>
<td>60°C</td>
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<tr>
<td>\textit{Igfbp7-Rev}</td>
<td>CTT GCT GAC CTG GGT GAT GG</td>
<td></td>
<td>60°C</td>
</tr>
</tbody>
</table>

**2.2.2 Cell culture**

**2.2.2.1 Medium and maintenance of cell lines**

All reagents were supplied by Invitrogen and plasticware was supplied by Nunc and Helena Biosciences unless otherwise stated.

Unless otherwise stated, all cell lines were routinely maintained in Dulbecco’s modified Eagle's medium (DMEM) with 4500 mg/litre D-glucose, supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin at 37°C with 5% \(\text{CO}_2\) in a humid incubator. CMT64 and CMT167 cells were maintained in DMEM,
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supplemented with 10% FBS, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Chinese hamster ovary (CHO) cells were maintained in DMEM/F12 (1:1), supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.

2.2.2.2 Production of primary MEFs from E14.5 mouse embryos

MEFs were obtained from E14.5 mouse embryos. In brief, mice were killed with CO₂ treatment and cervical dislocation. Then mice were swabbed in ethanol and the abdominal cell wall was cut into and the revealing viscera were removed. The uterus was dissected from the pregnant mice and placed into sterile phosphate buffered saline (PBS) (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄) at 4°C. Then the uterus was torn using forceps and each embryo was released from the placenta and amniotic fluids. This work was undertaken by Susan Giblett. The embryos were washed in PBS. The head and liver were cut and discarded; the tail was removed and kept for genotyping. The remaining tissues were then chopped into small pieces using a sterilised scalpel and placed into 15 ml tubes containing 4 ml of 0.25% [v/v] Trypsin/EDTA in PBS. The tubes were placed at 4°C for 6-18h to allow dissociation of the tissues followed by incubation at 37°C for 30 min. The tissues were collected by centrifuging at 1250 rpm for 4 min and Trypsin/EDTA was removed carefully. Growth media was added and the dissociated embryo was pipetted up and down until a cell suspension was obtained. The suspension was then plated out onto a 10 cm plate containing 10 ml of growth media. Cells were left to grow until confluent. A small amount of cells were kept for genotyping. All the remaining cells were passaged on to a 15 cm plate.

2.2.2.3 Passaging of cells

Cells were maintained at 37°C with 5% CO₂ in a humid incubator. Cells were passaged once they became 90% confluent. The growth media was removed and cells were washed by sterile PBS. PBS was removed and an appropriate volume of 0.05% [v/v] Trypsin/EDTA in PBS was added depending on the size of the plate. The cells were placed at 37°C for 5 min to generate a single cell suspension, followed by addition of
growth media to neutralise Trypsin/EDTA. An appropriate volume of resuspended cells were replated on a new plate supplemented with growth media.

2.2.2.4 Freezing down stocks of cell lines
Growth media was removed and cells were washed with sterile PBS. Then, cells were trypsinised, pelleted by centrifugation at 1500 rpm for 5 min and resuspended in the freezing media (5% [v/v] dimethylsulphoxide (DMSO) in growth media). Cells were transferred to cryovials, placed at -80°C and finally stored in liquid nitrogen.

2.2.2.5 Retroviral transfection of pBabe-Puro GFP-LC3 virus into CMT64 cells
Bosc23 cells were plated at 5x10^6 cells per plate onto 10 cm plates 24h prior to transfection. Transfection was carried out using Lipofectamine™ 2000 (Invitrogen) according to manufacturer's instruction. In brief, for each transfection, 12.5 µg of pBabe-Puro GFP-LC3 DNA (gift from Prof. Kevin Ryan) was mixed with 1 ml of OPTI-MEM-I medium without antibiotics; 90 µl of Lipofectamine™ 2000 was mixed with 1 ml of OPTI-MEM-I medium without antibiotics. Then the DNA was mixed with Lipofectamine™ 2000 and incubated at room temperature for 15-45 min. Bosc23 cells were washed twice with 5 ml of OPTI-MEM-I medium without antibiotics. The mixture of DNA and Lipofectamine™ 2000 was gently added onto Bosc23 cells and incubated at 37°C for 5h. 10 ml of OPTI-MEM-I medium containing 20% [v/v] FBS without antibiotics was gently added to Bosc23 cells and the incubation was continued for 19h. Culture media was removed and replaced with 6 ml of DMEM containing 20% [v/v] FBS without antibiotics. 24h later the viral supernatants were collected. 8 µg/ml of polybrene was added into the viral supernatants and this was filtered through a 0.2 µm low protein binding syringe filter to remove any detached Bosc23 cells. Virus was then infected directly into CMT64 mouse lung adenocarcinoma cells. Briefly, 2x10^5 CMT64 cells were plated onto 6 cm plates. Culture media was removed and replaced with 2 ml of virus and incubated for 24h. Viral media was removed and replaced with growth media for 48h. Fresh growth media containing 2 µg/ml of puromycin was added and media was changed every other day with puromycin selection over the next 2 weeks until colonies
appeared. Positive colonies were pooled to establish immortalised CMT64 cells expressing GFP-LC3.

2.2.3 Protein Analysis

2.2.3.1 Making whole cell lysates
Media was removed and cells were washed twice with ice-cold PBS. An appropriate volume of 1 x SDS lysis buffer (50 mM Tris-HCl [pH6.8], 2% [w/v] SDS and 10% [v/v] glycerol) was added directly onto cells. Cells were scraped off with a cell scraper and lysates were collected and transferred into an eppendorf tube. Each lysate was boiled at 100°C for 10 min and cooled down to room temperature. Protein concentration was measured with the Bicinchoninic Acid (BCA) protein assay kit (Thermo Scientific). In brief, on a 96-well plate, different amounts of Bovine Serum Albumin (BSA) (Thermo Scientific) were aliquoted to provide a concentration gradient of 0, 0.25, 0.5, 0.75, 1, 1.5, and 2 µg/µl diluted with 1 x SDS lysis buffer. 5 µl of cell lysates were aliquoted into individual wells in triplicate. The BCA™ working reagent was prepared freshly by mixing 50 parts of BCA™ reagent A (Thermo Scientific) with 1 part of BCA™ reagent B (4% cupric sulphate). 200 µl of the BCA™ working reagent was added for each test and the plate was incubated at 37°C for 30 min. The OD_{570} absorbance was determined using a Tecan GENios microplate reader and Magellan software. A standard curve was plotted according to the BSA concentration gradients versus the OD_{570} absorbance and the protein concentrations of the cell lysates were calculated using linear regression. Each cell lysate was prepared for electrophoresis by the addition of the loading buffer (0.4% [w/v] bromophenol blue in 50% [v/v] β-mercaptoethanol in dH₂O). Each sample was boiled at 100°C for 10 min and stored at -20°C.

2.2.3.2 Making clear protein lysates
Culture media was removed and cells were washed twice with ice-cold PBS. An appropriate volume of Gold lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5% [v/v] NP-40, 1% [v/v] Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride
hydrochloride (AEBSF), 250 μg/ml aprotinin and 50 μg/ml leupeptin) was added directly onto cells. Cells were scraped off with a cell scraper and lysates were collected and transferred into a pre-chilled eppendorf tube on ice for 15 min with occasional vortexing. The nuclei and insoluble materials were pelleted by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatants were transferred into fresh pre-chilled eppendorf tubes on ice. Protein concentrations were measured with the Bradford protein assay kit (Thermo Scientific). In brief, different amounts of BSA were aliquoted in triplicate to provide a concentration gradient of 0, 2, 4, 6, 8, and 16 μg/μl in Bradford protein solution (Thermo Scientific). 5 μl of the cell lysates were aliquoted into individual wells containing Bradford protein solution. The OD₅₉₅ absorbance was determined using a BioPhotometer (Eppendorf). A standard curve was plotted according to the BSA concentration gradients versus the OD₅₉₅ absorbance and the protein concentrations of the cell lysates were calculated using linear regression. Each cell lysate was prepared for electrophoresis by the addition of appropriate volume of 4x laemmli loading buffer (250 mM Tris-HCl [pH 6.8], 8% [w/v] SDS, 20% [v/v] β-mercaptoethanol, 40% [v/v] glycerol and 0.04% [w/v] bromophenol blue). Each sample was boiled at 100°C for 10 min and stored at -20°C.

2.2.3.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
6% to 15% separating polyacrylamide gels (Table 2.4), depending upon the size of the protein of interest, were made with a 5% stacking gel. Gels were placed in the Mini-protean II container (Bio-Rad) and immersed in SDS-PAGE running buffer (192 mM glycine, 25 mM Tris-base and 0.1% [w/v] SDS). 20 μg to 40 μg of each protein lysate was loaded per well. PageRuler™ Plus Prestained Protein Ladder (Fermentas) was loaded along with the protein lysates. The protein samples were electrophoresed at 100 V through the 5% stacking gel and 150 V through the separation gel using a Bio-Rad Power PAC300.
### Table 2.4 Composition of SDS-PAGE separating gels (10 ml)

<table>
<thead>
<tr>
<th></th>
<th>6%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>5.3 ml</td>
<td>4.6 ml</td>
<td>4.0 ml</td>
<td>3.3 ml</td>
<td>2.3 ml</td>
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<td>1.5 M Tris-HCl [pH 8.8]</td>
<td>2.0 ml</td>
<td>2.7 ml</td>
<td>3.3 ml</td>
<td>4.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>30% Acrylamide (Flowgen Bioscience)</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
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<tr>
<td>10% [w/v] SDS</td>
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<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>10% [w/v] APS</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
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<tr>
<td>TEMED</td>
<td>8 µl</td>
<td>6 µl</td>
<td>4 µl</td>
<td>4 µl</td>
<td>4 µl</td>
</tr>
</tbody>
</table>

#### 2.2.3.4 Western blotting

Both of nitrocellulose filters (Schleicher and Schuell) and filter papers (WhatMan®) were pre-soaked in the transfer buffer (192 mM glycine, 25 mM Tris base and 20% [v/v] methanol). The proteins were electroblotted onto 0.2 µm nitrocellulose filters at 10 V for 60 min using a semi-dry blotting apparatus (Bio-Rad). After transfer, blots were stained with Ponceau S Stain (0.5% [w/v] Ponceau S in 5% [v/v] acetic acid), and protein loadings were compared. Blots were washed in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.6] and 150 mM NaCl) containing 0.01% [v/v] Tween-20 (TBST) and non-specific sites were blocked by incubation of blots at room temperature with gentle shaking in 5% [w/v] non-fat milk powder (Marvel) or BSA (First Link (UK) Limited) in TBST for 1 h. If blots were to be subsequently incubated with p21$^{Cip1}$ or p16$^{ink4a}$ antibody, they were incubated with 5% [w/v] non-fat milk overnight at 4°C with gentle shaking. Then, blots were incubated with an appropriate dilution of primary antibody (Table 2.5) in 5% [w/v] non-fat milk or BSA in TBST at 4°C with gentle shaking. Blots were washed 3x 15 min in TBST and incubated with a 1:3500 dilution in TBST of the appropriate anti-mouse IgG-HRP or anti-rabbit IgG-HRP (Sigma-Aldrich) for 1h with gentle shaking at room temperature. After washing 3x 15 min with TBST, the enhanced chemiluminescence detection system (Thermo Scientific) was used to visualise antigen-antibody complexes by placing the blots onto plastic wrap, mixing an equal ratio of the SuperSignal West Pico Luminol/Enhancer Solution and the SuperSignal West
## Table 2.5 Primary antibodies used in western blotting

<table>
<thead>
<tr>
<th>Name of antibody</th>
<th>Molecular weight</th>
<th>Dilutions</th>
<th>Diluent</th>
<th>Species</th>
<th>Company</th>
</tr>
</thead>
<tbody>
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<td>p-4EBP1 (Ser65)</td>
<td>15-20 kDa</td>
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<td>Cell signaling</td>
</tr>
<tr>
<td>β-actin</td>
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<td>Sigma-Aldrich</td>
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<td>Cell signaling</td>
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<td>Rabbit</td>
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<td>Abcam</td>
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### Materials and Methods

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<th>Species</th>
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<td>Rabbit</td>
<td>Cell signaling</td>
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<tr>
<td>p53</td>
<td>53</td>
<td>1:1000</td>
<td>5% BSA</td>
<td>Mouse</td>
<td>Cell signaling</td>
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<td>Cell signaling</td>
</tr>
<tr>
<td>STC1</td>
<td>30</td>
<td>1:1000</td>
<td>5% milk</td>
<td>Rabbit</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

Pico Stable Peroxide Solution, and adding 1 ml of the mixture to each blot ensuring an even distribution for 3 min. Excess liquid was removed and blots were wrapped in Saranwrap and placed into a cassette. Various times of exposure to photographic film (Fuji) were performed at room temperature.

#### 2.2.3.5 Stripping Western blots

Western blots were incubated in stripping buffer (62.5 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, and 0.1 M β-mercaptoethanol (freshly added)) for 30 min at 50-60°C. Blots were washed twice for 5 min in TBST and re-blocked in 5% [w/v] non-fat milk or BSA before incubation with different primary antibodies.

#### 2.2.4 Assessment of cell proliferation, apoptosis and senescence

##### 2.2.4.1 BrdU/EdU incorporation

Cells were plated at 4 x 10^4 cells per well on cover slips onto 6-well plates. Cells were allowed to become 60% confluent and 10 µM Bromodeoxyuridine (BrdU; Roche Applied Science) or 5-ethynyl-2’-deoxyuridine (EdU, Molecular probes, Invitrogen) was added and cells were left in 37°C incubator for appropriate time. In BrdU assay, media containing BrdU was removed and cells were washed three times with washing buffer (Roche Applied Science) and then fixed by the addition of ethanol fixative (50 mM glycine in ethanol [pH 2.0]) for at least 20 min at -20°C. After washing 3x 15 min with
washing buffer, cells were incubated with a 1:10 dilution of an anti-BrdU working solution (Roche Applied Science) in PBS for 30 min at 37°C in a humid chamber. After washing 3x 15 min with washing buffer, cells were incubated with 1:10 dilution of an anti-mouse Ig fluorescein solution (Roche Applied Science) in PBS for 30 min at 37°C in a humid chamber.

In EdU assay, media containing EdU was removed and cells were fixed by addition of 1 ml of 3.7% formaldehyde in PBS for 15 min at room temperature. The fixative was removed and cells were washed twice with 1 ml of 3% BAS in PBS followed by permeabilisation in 1 ml of 0.5% Triton X-100 in PBS for 20 min at room temperature. Permeabilisation buffer was removed and cells were washed twice with 1 ml of 3% BSA in PBS followed by addition of 0.5 ml of Click-iT reaction cocktail (Molecular probes, Invitrogen) and incubation for 30 min at room temperature in dark. Reaction cocktail was removed and cells were washed once with 1 ml of 3% BSA in PBS.

After washing 3x 5 min with PBS, cells from BrdU or EdU assay were counterstained with 1:2000 dilution of 4',6-diamidino-2-phenylindole (DAPI) (Molecular probes, Invitrogen) in PBS for 30 min at room temperature. After three times washing with PBS, cells were mounted on microscope slides using Gel Mount (Sigma-Aldrich). A minimum total of 100 cells per field were counted and the percentage of BrdU/EdU positive cells were visualised using a fluorescence microscope (Nikon Eclipse TE300). Data were analysed by using the two-tailed unpaired t-test.

2.2.4.2 MTT assay

Cells were plated at 1,000 cells per well in triplicate onto 96-well plates. The blank control was growth media alone. 24h after plating, cells were treated with appropriate treatments over a time course of 1-5 days. At the designated time point, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was directly added at a final concentration of 1 mg/ml into the media and gently mixed before putting 96-well plates back into the incubator at 37°C. After 2h incubation, media was gently
removed without disturbing the dark purple formazan crystals formed during the reaction and then 100 µl of DMSO was added into each well. The dark purple crystals were mixed with DMSO before putting 96-well plates back into the incubator at 37°C. 30 min after incubation, plates were stirred at room temperature for 5 min. The OD$_{570}$ absorbance was determined using a Tecan GENios microplate reader and Magellan software.

2.2.4.3 Hoechst 33258 staining of apoptotic cells
Cells were plated at 5 x 10$^4$ cells per well onto 12-well plates. After treatment, adhering cells and floating cells were stained with 1 µg/ml of Hoechst 33258 (gift from Dr. Xiaoming Sun) in the media for 1h at 37°C. Apoptotic cells were identified as those with brightly stained and condensed nuclei. Images were acquired using a fluorescence microscope (Nikon Eclipse TE300) and analysed by Openlab software (Improvision).

2.2.4.4 Annexin V-FITC staining of apoptotic cells
Cells were plated at 1.5 x 10$^5$ cells per plate onto 6 cm plates. After treatment, floating cells in media were collected and placed in 15 ml tubes. Attached cells were washed with PBS once and PBS was collected and placed into the same tubes. 1 ml of 0.05% [v/v] Trypsin/EDTA in PBS was added to cells and cells were placed at 37°C for 5 min. 1 ml of growth media was added to neutralise trypsin/EDTA, and the cell suspensions were placed into the tube containing floating cells. Cells were pelleted by centrifugation for 5 min at 1,500 rpm. The supernatant was removed and discarded. The cell pellets were resuspended in 1 ml of growth media and placed in a humid incubator at 37°C for 30 min to allow the phosphatidyserine groups to re-equilibrate. 500 µl of cell suspension was removed from the tubes and discarded. Remaining cells were pelleted by centrifugation at 1500 rpm for 5 min. The supernatant was removed and discarded. Phosphatidylserine externalisation was quantified by resuspending cell pellets in 500 µl of 1x Annexin binding buffer (Bender MedSystems; 10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM CaCl$_2$) plus 5 µl of Annexin V-FITC (Bender MedSystems) followed by incubation for 10 min at room temperature in dark. 20 µg/ml of propidium iodide in
H₂O was added to cells to allow the measurement of membrane integrity. Cells were analysed by fluorescence-activated cell sorting (FACS, Becton Dickinson) in conjunction with CELLQuestPro software, with 5,000 events recorded per assay. Data were analysed by using the two-tailed unpaired t-test.

2.2.4.5 Caspase3/7 assay

Cells were plated at 1x10⁴ cells per well onto 96-well plates and allowed to adhere overnight before serum withdrawal for 24h. The measurement of caspase3/7 activity was carried out using an Apo-ONE homogeneous Caspase 3/7 kit (Promega) according to manufacturer’s instruction. In brief, cells were maintained in the media with appropriate treatment and at the end of the incubation period, 100 μl of Apo-ONE Caspase 3/7 reagent (Promega) was added per well of 96-well plates. Contents of wells were gently mixed using a plate shaker at 300-500 rpm for 1h at room temperature. The fluorescence of each well was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using Tecan GENios microplate reader and Magellan software.

2.2.4.6 Assessment of mitochondrial membrane potential by TMRE staining

Cells were plated at 1.5 x 10⁵ cells per plate onto 6cm plates. After treatment, floating cells in the media were collected and adhering cells were harvested by trypsinisation. Cells were pelleted by centrifugation at 1500 rpm for 5 min and incubated with 20 nM tetramethylrhodamine ethyl ester (TMRE, gift from Dr. Xiaoming Sun) for 20 min at 37°C. Cells were analysed by flow cytometry with 20,000 events recorded per assay. The fluorescence intensity of TMRE was monitored at 582 nm (FL-2). Data were analysed by using the two-tailed unpaired t-test.

2.2.4.7 Senescence-associated β-galactosidase (SA-β-gal) staining

Cells were plated at 1 x 10⁴ cells per well onto 12-well plates. The detection of SA-β-gal was carried out using a Senescence-associated β-galactosidase staining kit (Cell signalling) according to manufacturer’s instruction. In brief, the media was removed and
cells were washed twice with PBS. Then cells were fixed with 1x Fixative Solution (Cell signaling) for 15 min at room temperature. After washing twice with PBS, cells were stained with freshly prepared Staining Solution (1 ml of Staining Solution containing 930 µl of 1x Staining Solution, 10 µl of Staining Supplement A, 10 µl of Staining Supplement B, and 50 µl of 20 mg/ml X-gal in N-N-dimethylformamide (DMF)) and incubated at 37°C overnight. A minimum total of 100 cells per field were counted and the percentage of SA-β-gal positive cells were visualised using a phase contrast microscope (Nikon Eclipse TE300) equipped with a digital camera (Nikon Eclipse E800). Data were analysed by using the two-tailed unpaired t-test.

2.2.5 Fluorescence microscopy

Cells were plated at 2 x 10^4 cells per well on cover slips onto 12-well plates. After treatment, cells were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature. After washing 3x 5 min with 0.2% [w/v] BSA in PBS, cells were permeabilised by the addition of 0.2% [v/v] Triton X-100 in PBS for 5 min. After washing 3x 5 min with 0.2% [w/v] BSA in PBS, non-specific sites were blocked by the addition of 5% [w/v] BSA in PBS for 60 min. Then cells were incubated with an appropriate dilution of primary antibody (Table 2.6) in 1% [w/v] BSA in PBS for appropriate time at room temperature. After washing 3x 15 min with 0.2% [w/v] BSA in PBS, cells were incubated with an appropriate dilution of Alexa Fluor 488 goat anti-mouse/rabbit IgG or Alexa Fluor 594 goat anti-mouse/rabbit IgG (Molecular probes, Invitrogen) for 20 min at room temperature. After washing 3x 15 min with 0.2% [w/v] BSA in PBS, cells were stained with 1:2000 dilution of DAPI in PBS. After washing 3x 5 min with 0.2% [w/v] BSA in PBS, cells were mounted on microscope slides using Gel Mount (Sigma-Aldrich). Images were acquired using a fluorescence microscope (Nikon Eclipse TE300) and analysed by Openlab software (Improvision).
### Table 2.6 Primary antibodies used in fluorescence microscopy

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<th>Name of antibody</th>
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<td>BD Transduction Laboratories</td>
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<td>Rabbit</td>
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<td>Abcam</td>
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<tr>
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<td>21 kDa</td>
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<td>Rabbit</td>
<td>Santa Cruz</td>
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</tbody>
</table>

#### 2.2.6 Preparation of conditioned media from mouse lung tissues

Mouse lung tissues were excised and transferred into sterile ice-cold PBS. Lung tissues were weighed, chopped into 3 mm³ cube and washed twice in ice-cold 0.9% [w/v] NaCl to remove blood cells. The chopped lung tissues were passed through a 70 μm mesh cell strainer (BD Biosciences). The filtrates were collected and pelleted by centrifugation at 1500 rpm for 5 min. Supernatants were discarded and the pellets were resuspended in an appropriate volume of serum free media (SFM). Cell suspension was plated onto 6 cm plates in a final volume of 4 ml of SFM and incubated for 24h. The media was collected and centrifuged twice at 2000 rpm for 5 min to remove tissue debris and cell aggregates. Supernatants were transferred and concentrated using the Amicon® Ultra-4 Centrifugal Filter Units (Millipore) by centrifugation at 4000 rpm for 10 min. The volume of the media was concentrated in proportion to the weight of the individual lung tissue. This media was termed conditioned media (CM) and was stored at -80°C freezer.

#### 2.2.7 Proteomic analysis of conditioned media

Conditioned media (CM) was resolved on SDS-PAGE gels. After electrophoresis, gels were stained with Coomassie Blue (50% [v/v] methanol, 10% [v/v] acetic acid, 0.05% [w/v] Brilliant Blue R-250) and destained with Coomassie Blue destaining solution (50% [v/v] methanol, 10% [v/v] acetic acid in dH₂O) to visualise proteins in the gels. The gels were then sent to The Protein Nucleic Acid Chemistry Laboratory (PNACL) at the
University of Leicester for mass spectrometry. In brief, Bands of interest were excised from the gel and an in-gel trypsin digest was carried out. The band was destained using 200 mM ammonium bicarbonate in 20% acetonitrile, followed by reduction in 10 mM dithiothreitol (Melford Laboratories), alkylation in 100 mM iodoacetamide and enzymatic digestion in sequencing grade modified porcine trypsin (Promega) using an automated digest robot (Multiprobe II Plus EX, Perkin Elmer, UK). Liquid chromatography tandem mass spectrometry (LC-MS/MS) was carried out upon each sample using a 4000 Q-Trap mass spectrometer (Applied Biosystems). Peptides resulting from in-gel digestion were loaded at high flow rate onto a reverse-phase trapping column (0.3 mm i.d. x 1 mm) containing 5 µm C18 300 Å Acclaim PepMap media (Dionex) and eluted through a reverse-phase capillary column (75 µm i.d. x 150 mm) containing Jupiter Proteo 4µm 90Å media (Phenomenex) that was self-packed using a high pressure packing device (Proxeon Biosystems). The output from the column was sprayed directly into the nanospray ion source of the 4000 Q-Trap mass spectrometer. Fragment ion spectra generated by LC-MS/MS were searched using the MASCOT search tool (Matrix Science Ltd.) against the nucleotide contig sequences using appropriate parameters. The criteria for protein identification were based on the manufacturer’s definitions (Matrix Science Ltd.). Candidate peptides with probability based Mowse scores exceeding threshold ($p < 0.05$), and thus indicating a significant or extensive homology were referred to as ‘hits’. Comparison of the WTBRaf and V600E BRaf samples was carried out using Scaffold software (Proteome Sciences).

### 2.2.8 Preparation of blood plasma from mice

Mice were anesthetised and peripheral blood was obtained by cardiac puncture and collected in Vet-Tube (Li-Heparin) containing heparin sodium and gently mixed to avoid any blood clotting. The whole blood was centrifuged at 3500 rpm for 90 sec. Yellow supernatants containing blood plasma were transferred into fresh pre-chilled eppendorf tubes without disturbing the blood cell layers. The eppendorf tubes were centrifuged at 3500 rpm for 90 sec again to remove trace amount of blood cells. Blood plasma was transferred into fresh pre-chilled eppendorf tubes and stored at -80°C.
2.2.9 Purification of bovine NPC2 (bNPC2) protein from cow milk

All steps were carried out at 4°C unless otherwise mentioned. Whole cow milk was centrifuged at 6500 x g for 10 min to remove the fat layer. Skimmed milk was acidified to pH 3.5 by adding 1 M H₂SO₄ and the acidified milk was recovered by centrifugation at 6500 x g for 10 min. The supernatants were precipitated by gradually adding 400g/L of ammonium sulphate. After centrifugation at 6500 x g for 20 min, precipitated proteins were resuspended in 10 mM ammonium acetate [pH 7.0] followed by dialysing against 0.5 mM ammonium acetate [pH 7.0] with 6-kDa cut-off for 4-5 days. After dialysing, the samples were centrifuged at 6500 x g for 10 min and the supernatants were applied to a column of DEAE-Sepharose pre-equilibrated with 10 mM ammonium acetate [pH 7.0]. Flow-through fractions were collected, adjusted to pH 5.0 using glacial acetic acid, then applied to a column of CM-Sepharose pre-equilibrated with 10 mM ammonium acetate [pH 5.0] and eluted with a gradient of 10 mM-0.5 M ammonium acetate [pH 5.0] at a flow rate of 5 ml/min in 10 column volumes. The separation of proteins was monitored by measuring the absorbance at 280 nm. Flow-through fractions containing bNPC2 were pooled together based on their mobility on the SDS-PAGE gel, stained for Coomassie Blue and further analysed with a NPC2 antibody after western blot transfer. The pooled fractions containing bNPC2 were dialysed against 0.5 mM ammonium acetate [pH 5.0] using 6-kDa cut-off and further dialysed against sterile PBS. Finally, the bNPC2 protein was concentrated using Amicon® Ultra-4 Centrifugal Filter Units (Millipore) and quantified using UV absorbance at 280 nm.

2.2.10 Electron microscopy

Cells grown on collagen-coated cover slips in 12-well plates were washed with 200 mM sodium cacodylate [pH7.4] and 2 mM calcium chloride. Cells were fixed in 2.5% [v/v] gluteraldehyde in 100 mM sodium cacodylate [pH 7.4] and 2 mM calcium chloride, and post-fixed in 1% [w/v] osmium tetroxide in dH₂O followed by 0.5% [v/v] uranyl acetate. After ethanol dehydration, cells were infiltrated with a mixture of propylene oxide/agar low viscosity resin before embedding and polymerisation. Thin sections (80 nm) were
cut using a Reichert Ultracut S ultramicrotome, collected on copper mesh grids, counter stained with 2% [v/v] uranyl acetate and Reynolds' lead citrate and observed using a JEOL 1220 transmission electron microscope using an accelerating voltage of 80 kV. Digital Images were recorded using a SIS Megaview III Digital Camera with Analysis Software. This work was undertaken by Stefan Hyman and Natalie Allcock from Electron Microscopy Laboratory at the University of Leicester.

2.2.11 Statistical analysis

Results were analysed for statistical significance using analysis of variance with the Student’s unpaired two-tailed t test and in all cases p<0.05 was considered statistically significant.
CHAPTER 3

V600E BRAF INDUCES PROLIFERATION AND SUPPRESSES APOPTOSIS IN MEFs

3.1 Introduction

BRAF is a serine/threonine protein kinase that functions as a component of the RAS/RAF/MEK/ERK signalling pathway. Gain-of-function BRAF mutations have been found in ~50%-70% of malignant melanomas and V600E BRAF mutation is the most frequent mutation, representing more than 90% of BRAF mutations in human cancers (Davies et al., 2002; Garnett and Marais, 2004). V600E BRAF constitutively activates the MEK/ERK signalling pathway, leading to growth factor-independent melanoma cell proliferation (Wellbrock et al., 2004b; Cohen et al., 2002; Dong et al., 2003; Karasarides et al., 2004; Satyamoorthy et al., 2003), transformation of fibroblasts and immortalised melanocytes (Davies et al., 2002; Mercer et al., 2005; Wan et al., 2004; Michaloglou et al., 2005) and tumourigenicity in melanoma xenografts (Hoeflich et al., 2006; Hingorani et al., 2003; Karasarides et al., 2004; Wellbrock et al., 2004b; Sumimoto et al., 2004). Inhibition of V600E BRAF signalling resulting in blockage of cell proliferation, induction of cell cycle arrest or apoptosis, inhibition of invasive potential in vitro, and repression of the growth of melanoma xenografts in vivo (Hingorani et al., 2003; King et al., 2006; Tsai et al., 2008 Calipel et al., 2003; Karasarides et al., 2004; Salvatore et al., 2006; Sumimoto et al., 2006). These data demonstrate that V600E BRAF-mediated MEK/ERK signalling is necessary for the maintenance and progression of melanomas, and that cancers with this mutation are addicted to the oncogene.

The RAF/MEK/ERK signalling pathway plays a crucial role in the regulation of cell proliferation. Growth factor-dependent activation of ERK drives cell cycle progression mainly through stimulation of Cyclin D1 transcription. Activated ERK translocates to the nucleus and phosphorylates a number of pre-existing transcription factors, such as Fos and Jun family members that form AP-1 heterodimers, bind to Cyclin D1 promoter and directly transactivate it (Kerkhoff and Rapp 1997; Weber et al. 1997; Balmanno and
Cook, 1999; Shaulian and Karin, 2001). However, transient ERK activation is not sufficient to drive cell cycle progression as c-Fos protein is unstable and degraded (Murphy et al., 2002). When ERK signalling is sustained, c-Fos is phosphorylated and stabilised by active ERK and p90<sup>RSK</sup>, which is required for Cyclin D1 transcription leading to cell cycle progression (Murphy et al., 2002, 2004; Pages et al., 1993; Lavoie et al., 1996; Weber et al., 1997; Balmanno and Cook, 1999; Balmanno and Cook, 1999).

The strength of ERK signalling also influences cell cycle progression. It has been shown that low levels of ERK activity elicit cell cycle progression by inducing expression of Cyclin D1 and Cyclin E and suppression of p27<sup>Kip1</sup> (Woods et al., 1997). However, high levels of persistent ERK signalling can lead to cell cycle arrest by inducing high levels of p21<sup>Cip1</sup> that inhibits both Cyclin D/CDK4 and Cyclin E/CDK2 complexes (Woods et al., 1997). Therefore, the RAF/MEK/ERK cascade must be finely tuned to properly regulate gene expression that is a rate-limiting event for cell proliferation.

Constitutive activation of the RAF/MEK/ERK pathway can promote survival at different levels. During development, knockout mice for Craf (Mikula et al., 2001; Huser et al., 2001) or Braf (Wojnowski et al., 1997) results in embryonic lethality characterised by enhanced apoptosis in discrete tissues. It has been shown that activation of the RAF/MEK/ERK pathway influences the apoptotic machinery at the pre-mitochondrial and post-mitochondrial level. At the pre-mitochondrial level it has been shown that ERK inhibits the pro-apoptotic activity of BAD through p90<sup>RSK</sup>-mediated phosphorylation on Ser112 resulting in disassociation of BAD from BCL-2 sequestration (Zha et al., 1996; Bonni et al., 1999; Scheid et al., 1999). The RAF/MEK/ERK cascade also promotes rapid phosphorylation of BIM and subsequent degradation via the proteasome in the presence of growth factors (Ley et al., 2003, 2004; Luciano et al., 2003; Marani et al., 2004). The RAF/MEK/ERK pathway can also upregulate anti-apoptotic protein BCL-2, BCL-x<sub>L</sub> and MCL-1 (Liu et al., 1999; Leu et al., 2000; Jost et al., 2001) or stabilise them through phosphorylation thus preventing proteasome-dependent degradation (Dimmeler et al., 1999; Breitschopf et al., 2000; Luciano et al., 2003). At the post-mitochondrial level, caspase-9 is directly phosphorylated by activated ERK at
Thr125 resulting in inhibition of caspase-9 activity and promotion of cell survival (Allan et al., 2003). The BRAF-activated MEK/ERK cascade has also been shown to upregulate inhibitors of apoptosis (IAPs) to promote cell survival (Erhardt et al., 1999; Wiese et al., 2001). V600E BRAF induces the DNA-binding activity of NF-κB, resulting in upregulation of anti-apoptotic c-IAP-1, c-IAP-2 and XIAP (Palona et al., 2006). Role of CRAF in inhibition of apoptosis appears to be independent of MEK/ERK pathway (Chen et al., 2005; O’Neill et al., 2004).

To study the role of V600E BRaf in tumour development, our laboratory generated a conditional knockin mutation of Braf^{SL- V600E} in mice by using the Cre/LoxP system (Mercer et al., 2005). In order to temporally and spatially regulate the V600E BRaf expression in vivo and in vitro, the inducible CreER\textsuperscript{T} system was used in our study (Hayashi and McMahon, 2002). In the CreER\textsuperscript{T} system, the Cre recombinase is fused to the hormone-binding domain of a transcriptional inactive mutant (G525R) mouse estrogen receptor (ER) that is unable to bind estrogen, CreER\textsuperscript{T}, which is insensitive to its natural ligand 17β-estradiol but sensitive to the synthetic ligand, 4-hydroxytamoxifen (4-OHT) (Danielian et al., 1993; Littlewood et al., 1995; Hayashi and McMahon, 2002). The CreER\textsuperscript{T} fusion protein is usually inactivated in the cytoplasm and prevented from entering the nucleus by heat shock proteins 90 (Hsp90) binding in the absence of 4-OHT (Mattioni et al., 1994). In the presence of 4-OHT, 4-OHT binds to the ER domain, and CreER\textsuperscript{T} is released from Hsp90 sequestration, allowing translocation of CreER\textsuperscript{T} into the nucleus, where CreER\textsuperscript{T} participates in Cre-mediated recombination between two LoxP sites (Figure 3.1). In our study, \textit{Braf}^{\text{LSL-V600E}} heterozygous mice were crossed to CreER\textsuperscript{T+/-} heterozygous mice to generate \textit{Braf}^{\text{LSL-V600E}},\textit{CreER}^{T+/-} double heterozygous mice. In these mice, before Cre-mediated recombination, \textit{Braf}^{\text{LSL-V600E}} expresses wild-type BRaf protein due to the mini-cDNA encoding exons 15 to 18 of wild-type BRaf. After Cre-mediated deletion of the LSL cassette including the mini-cDNA, the \textit{Braf}^{\text{LSL-V600E}} allele is generated expressing V600E BRaf. The advantage of this system is that V600E BRaf is expressed from the endogenous \textit{Braf} promoter that mimics the somatic expression of V600E BRaf in human cancers.
3.2 Aims

Embryos from intercrosses between $Brat^{+/LSL-V600E}$ mice and $CreER^{T+/-}$ mice were used to generate primary mouse embryonic fibroblasts (MEFs). Primary MEFs were employed as a model to investigate the following:

1. Use of the CreER$^T$ system to activate $V600E$BRaf expression in MEFs
2. The role of oncogenic $V600E$BRaf in inducing cell proliferation in MEFs, and the downstream signalling pathways involved
3. The role of oncogenic $V600E$BRaf in suppressing apoptosis in MEFs, and the downstream signalling pathways involved
Figure 3.1 Activation of inducible CreER\(^T\) with 4-hydroxytamoxifen

(A) Cre recombinase is fused to hormone-binding domain of a mutant (G525R) mouse estrogen receptor (ER) that is 4-hydroxytamoxifen (4-OHT) inducible (ER\(^T\)). Expression of Cre\(^{ER,T}\) is driven by a chimeric promoter/enhancer of the cytomegalovirus immediate-early enhancer and the chicken β-globin promoter/enhancer. (B) In the absence of 4-hydroxytamoxifen (4-OHT), the Cre\(^{ER,T}\) fusion protein is bound to the heat shock protein Hsp90 and inhibited from entering the nucleus. Upon administering and binding of 4-OHT to the ER domain, Hsp90 dissociates from the complex and allows translocation of the Cre\(^{ER,T}\) into the nucleus where it mediates recombination using the two LoxP sites. PM, plasma membrane. NE, nuclear envelop.
3.3 Results

3.3.1 Induction of V600E BRaf expression in MEFs

3.3.1.1 Isolation of MEFs containing Brf^{LSL-V600E} and CreER\textsuperscript{T} alleles

Breeding colonies of Brf^{+/LSL-V600E} mice and CreER\textsuperscript{T+/0} mice were maintained. Primary MEFs were derived from embryos at E14.5 from intercrosses between Brf^{+/LSL-V600E} mice and CreER\textsuperscript{T+/0} mice according to standard methods (Huser et al. 2001). Each embryo was genotyped by using genomic DNA extracted from the embryo tail with appropriate primers (Figure 1.16). A typical PCR genotyping result is shown in Figure 3.2. Embryos 1, 3, 4, and 6 had the Brf^{LSL-V600E} allele which was amplified with primers Brf-A and Brf-B that generate a 140-bp product, while embryos 2 and 5 only had the WT Brf allele which was amplified with primers Brf-A and Brf-C that generate a 466-bp product. Embryos 1 and 3 contained the CreER\textsuperscript{T} allele. Taken together, embryos 1 and 3 were identified as being double heterozygous having the both Brf^{LSL-V600E} allele and CreER\textsuperscript{T} alleles. The other embryos represented important controls.

3.3.1.2 Induction of V600E BRaf expression using the CreER\textsuperscript{T} system

In order to temporally activate the expression of V600E BRaf, the CreER\textsuperscript{T} system was used in our study to mediate recombination. The CreER\textsuperscript{T} fusion gene consists of the entire coding region of the Cre recombinase gene fused to a hormone-binding domain of the mutant mouse estrogen receptor (ER) that is 4-OHT inducible (Hayashi and McMahon, 2002). To verify the Cre-dependent deletion of LSL cassette to generate Brf^{Lox-V600E} allele, PCR of genomic DNA derived from MEFs was used to assess the efficiency of recombination. The Brf^{+/LSL-V600E};CreER\textsuperscript{T+/0} MEFs were treated with 50 nM 4-OHT or ethanol control over a time course up to 96h. The levels of the recombined Brf^{Lox-V600E} allele (518 bp) increased and the levels of the Brf^{LSL-V600E} allele (140 bp) decreased in a time-dependent manner following administration of 4-OHT (Figure 3.3). The recombined Brf^{Lox-V600E} allele occurred as early as 5h after 4-OHT treatment and, at 96h, reached its full recombination. Thus, the expression of V600E BRaf is induced after 4-OHT treatment.
Figure 3.2. Detection of $\text{Braf}^{\text{LSL-V600E}}$, $\text{wtBraf}$ and $\text{CreER}^T$ alleles in mouse embryos

Genomic DNA was isolated from mouse embryo tails and standard PCR was performed to detect $\text{Braf}^{\text{LSL-V600E}}$, $\text{wtBraf}$ and $\text{CreER}^T$ alleles. A 140-bp product was amplified with primer $\text{Braf-A}$ and $\text{Braf-B}$ (primer names and positions refer to Figure 1.16) indicating the presence of the $\text{Braf}^{\text{LSL-V600E}}$ allele, and a 466-bp product was amplified with primer $\text{Braf-A}$ and $\text{Braf-C}$ indicating the presence of the $\text{wtBraf}$ allele. A 340-bp product with the Cre primers indicates the presence of $\text{CreER}^T$ allele. 1-6, six embryos; M, DNA marker.
Figure 3.3 *Braf*<sub>lox-V600E</sub> allele is generated upon 4-OHT treatment

*Braf<sup>+/LSL-V600E; Cre<sup>ERT<sup>+/0</sup></sup> primary MEFs were treated with 50 nM 4-OHT or ethanol control (EtOH) over a time course of 0, 5h, 16h, 24h, 48h, 72h and 96h and genomic DNA was isolated followed by standard PCR. A 518-bp product indicates the presence of the *Braf*<sub>lox-V600E</sub>, a 466-bp product indicates the presence of the *w7Braf* allele, and a 140-bp product indicates the presence of the *Braf<sup>LSL-V600E</sup>* allele. M, DNA marker.
3.3.1.3 Cre translocates to the nucleus to mediate recombination

Activation of the endogenous \(^{V600E}BRaf\) depends on the delivery of Cre recombinase from the cytoplasm to the nucleus and deletion of the LSL cassette. The CreER\(^T\) fusion protein is sequestered in the cytoplasm by Hsp90 binding. In the presence of 4-OHT, the interaction between the ER domain and Hsp90 is disrupted, allowing the CreER\(^T\) fusion protein to translocate to nucleus to mediate recombination. To monitor Cre translocation, \(Braf^{+/LSL-V600E};\text{CreER}^{+/0}\) primary MEFs were treated with 50 nM 4-OHT or ethanol control over a time course. Fluorescence microscopy was performed with an antibody for Cre. This showed that Cre accumulated in the nucleus as early as 5h after 4-OHT treatment and then continued to be present in the nucleus in a time-dependent manner, reaching a peak at 72h (Figure 3.4). Some of the Cre was exported back to the cytoplasm after 96h of 4-OHT treatment. In contrast, the Cre recombinase remained predominantly in the cytoplasm throughout the time course in the \(Braf^{+/LSL-V600E};\text{CreER}^{+/0}\) primary MEFs treated with ethanol control.

3.3.1.4 \(^{V600E}BRaf\) induces morphological transformation

\(^{V600E}BRAF\) is potent in transforming NIH 3T3 cells and immortalised melanocytes when ectopically expressed (Davies et al., 2002; Wan et al., 2004). To investigate the transformation effects of endogenous \(^{V600E}BRaf\), \(Braf^{+/LSL-V600E};\text{CreER}^{+/0}\) primary MEFs were treated with 50 nM 4-OHT to induce \(^{V600E}BRaf\) expression. This resulted in striking morphological transformation after 72h that was characterised by an elongated, highly refractile morphology and growth in a disorganised fashion with few cell-cell interactions (Figure 3.5). In contrast, \(Braf^{+/LSL-V600E};\text{CreER}^{+/0}\) primary MEFs with ethanol control treatment showed a typical non-transformed morphology over the time course. Moreover, loss of contact inhibition was observed in the transformed cells, as evidenced by the cells growing to a high density. Finally, transformed cells had disrupted actin cytoskeleton compared with ethanol-treated untransformed cells by Texas-Red Phalloidin staining (gift from Dr. Petra Kopp) (Figure 3.6). Therefore, the CreER\(^T\) system is able to efficiently induce endogenous \(^{V600E}BRaf\) expression upon 4-OHT treatment, leading to effective cell transformation.
**Figure 3.4 Cre recombinase translocates to the nucleus upon 4-OHT treatment**

*Braf^{LSL-V600E};CreER^{T2+}* primary MEFs were plated on coverslips in 12-well plates and were treated with 50 nM 4-OHT or ethanol control (EtOH) for a time course of 0, 5h, 16h, 24h, 48h, 72h and 96h. Cells were fixed in 4% PFA, washed and stained with Cre recombinase (green) antibody and counterstained with DAPI (blue) and examined by fluorescence microscope. Scale bar, 5 μm.
**BRaf induces proliferation and suppresses apoptosis in MEFs**

**Figure 3.5**

*BRaf inducible morphological transformation*

*BRaf* \(^{+/+}\);*CreER\(^{T2/0}\) and *Braf* \(^{+/LSL-V600E}\);*CreER\(^{T2/0}\) primary MEFs were treated with 50 nM 4-OHT or ethanol control over a time course of 0, 24h, 48h, 72h, and 96h. Cells were photographed by phase contrast microscopy (40 x magnification).
Figure 3.6 V600E-BRaf disrupts the actin cytoskeleton

*Braf*<sup>野生型/LSL-V600E;CreERT<sup>野生型/0</sup></sup> primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h. Cells were fixed in 4% PFA and stained with Texas Red-Phalloidin and examined by fluorescence microscopy. Scale bar, 15 μm.
3.3.2 \textsuperscript{V600E}BRaf induces cell cycle progression

3.3.2.1 \textsuperscript{V600E}BRaf promotes S phase progression

The RAF/MEK/ERK signalling cascade stimulates cell cycle progression. In order to examine DNA synthesis in response to \textsuperscript{V600E}BRaf expression, \textit{Braf}\textsuperscript{+/LSL-V600E};\textit{CreER}\textsuperscript{T+/0} primary MEFs were treated with 50 nM 4-OHT or ethanol control and S phase cell cycle progression was determined by assessing BrdU incorporation. At 16h and 24h after 4-OHT treatment, \textit{Braf}\textsuperscript{+/LSL-V600E};\textit{CreER}\textsuperscript{T+/0} primary MEFs showed significant increases in proliferation with ~50% and ~60% of cells entering S phase at 16h and 24h respectively, compared with ethanol control treatment in which ~30% and ~40% of cells entered S phase, respectively (Figure 3.7). Thus, MEFs expressing \textsuperscript{V600E}BRaf enter S phase at a faster rate from G\textsubscript{1} than MEFs expressing wild-type BRaf.

3.3.2.2 \textsuperscript{V600E}BRaf induces high levels of ERK phosphorylation and Cyclin D1 expression

To investigate why \textsuperscript{V600E}BRaf induces G\textsubscript{1}/S phase progression, we examined the expression of a number of known cell cycle regulators. Since constitutive activation of the RAF/MEK/ERK pathway is required to drive cell cycle progression through G\textsubscript{1} to S phase, first of all, we determined levels of phosphorylation of MEK and ERK expression after Cre activation. Immunoblot analysis showed that the \textit{Braf}\textsuperscript{+/LSL-V600E};\textit{CreER}\textsuperscript{T+/0} primary MEFs treated with 50 nM 4-OHT had high levels of MEK and ERK phosphorylation that were first detected after 48h of 4-OHT treatment (Figure 3.8). Interestingly, levels of phospho-ERK in \textsuperscript{V600E}BRaf-expressing MEFs slightly decreased at 72h and 96h after 4-OHT treatment compared with those of 48h; while levels of phospho-MEK stayed high, suggesting negative feedback acting at level of ERK may be involved in the \textsuperscript{V600E}BRaf/MEK/ERK signalling pathway.

Next we examined the expression of Cyclins and Cdks including Cyclin D1, Cyclin D2, Cyclin D3, Cyclin E, Cdk2, and Cdk4. As shown in Figure 3.8, high levels of Cyclin D1 were induced in a time-dependent manner in the \textit{Braf}\textsuperscript{+/LSL-V600E};\textit{CreER}\textsuperscript{T+/0} primary MEFs treated with 4-OHT, while CyclinD1 remained at a constantly low level in the ethanol
Figure 3.7 V600E BRaf induces DNA synthesis

Braf^{+/LSL-V600E; CreERT^{+/0}} primary MEFs were treated with 50 nM 4-OHT or ethanol control (EtOH) over a time course of 0, 2h, 4h, 8h, 16h and 24h. BrdU was added at the same time as the 4-OHT. Cells were counterstained with DAPI and BrdU positive cells were detected by immunofluorescence microscopy. Experiments were repeated three times. Error bars represent standard deviation.
Braf<sup>V600E</sup>-<sup>LSL-V600E</sup>:CreER<sup>T<sup>x/0</sup></sup> primary MEFs were treated with 50 nM 4-OHT or ethanol control over a time course of 0, 5h, 16h, 24h, 48h, 72h and 96h. Protein levels of PP-ERK, P-MEK, Cyclin D1, Cyclin D2, Cyclin D3, Cyclin E, Cdk2, Cdk4, p16<sup>Ink4a</sup>, p19<sup>Arf</sup>, p21<sup>Cip1</sup>, p27<sup>Kip1</sup> and p53 were assessed by immunoblotting. Actin was used as the loading control. Data are taken from a single experiment representative of three giving similar results.
Figure 3.9 Cre recombinase does not promote ERK phosphorylation and Cyclin D1 expression

*Braf<sup>+/+;CreER<sup>T+/0</sup></sup> primary MEFs were treated with 50 nM 4-OHT or ethanol control over a time course of 0, 5h, 16h, 24h, 48h, 72h and 96h. Protein levels of PP-ERK, P-MEK, Cyclin D1, Cyclin D2, Cyclin D3, Cyclin E, Cdk2, Cdk4, p16<sup>Ink4a</sup>, p19<sup>Arf</sup>, p21<sup>Cip1</sup>, p27<sup>Kip1</sup> and p53 were assessed by immunoblotting. Actin was used as the loading control. Data are taken from a single experiment representative of three giving similar results.
control treatment. Induction of CyclinD1 in \textit{V600E}Braf-expressing cells also showed a delayed profile of induction compared to the levels of phospho-ERK at late time points. There were no detectable alterations in Cyclin D2, Cyclin D3, Cyclin E, Cdk2 and Cdk4 levels. Similarly, \textit{Braf}^{+/+};\textit{CreER}^{T+/0} primary MEFs also displayed no obvious changes in the expression of proteins examined when treated with 4-OHT or ethanol control, this confirming that Cre recombinase itself plays no role in response by phospho-ERK or Cyclin D1 (Figure 3.9).

Cdk inhibitors (CKIs) play important roles in cell cycle progression. As shown in Figure 3.7, \textit{p16}\textsubscript{Ink4a}, \textit{p21}\textsubscript{Cip1}, and \textit{p27}\textsubscript{Kip1} were expressed at low levels and showed no significant differences between \textit{Braf}^{+/LSL-V600};\textit{CreER}^{T+/0} primary MEFs treated with 4-OHT or ethanol control. \textit{p53} was shown to be slightly induced after 4-OHT treatment in both \textit{Braf}^{+/LSL-V600};\textit{CreER}^{T+/0} and \textit{Braf}^{+/+};\textit{CreER}^{T+/0} primary MEFs at later time points. Thus, \textit{V600E}Braf promotes cell cycle progression mainly though inducing high levels of CyclinD1, but has no effect on the Cdk inhibitors. Altogether, the major effect of \textit{V600E}Braf in promoting G\textsubscript{i}/S phase cell cycle progression is induction of CyclinD1 expression, at least in primary MEFs.

3.3.2.3 \textit{V600E}Braf induces \textit{Cyclin D1} at the transcriptional level

It is known that ERK activation transcriptionally induces the \textit{Cyclin D1} gene, although other signalling pathways are recognised to affect Cyclin D1 protein stability (Diehl et al., 1998). In this study, the protein levels of Cyclin D1 were significantly induced by \textit{V600E}Braf. To determine whether the increase in the Cyclin D1 protein results from the induction of \textit{Cyclin D1} mRNA, qRT-PCR was performed. As shown in Figure 3.10, \textit{Braf}^{+/LSL-V600E};\textit{CreER}^{T+/0} primary MEFs treated with 50 nM 4-OHT expressed ~5-fold higher levels of \textit{Cyclin D1} mRNA at 48h than ethanol control treated cells. Thus, \textit{V600E}Braf significantly induces Cyclin D1 at the transcriptional level.
Figure 3.10 V600E B RAF induces Cyclin D1 transcription

Braf^{+/LSL-V600E;CreERT2+/0} and Braf^{+/+;CreERT2+/0} primary MEFs were treated with 50 nM 4-OHT or ethanol control (EtOH) over a time course of 24h and 48h, then Cyclin D1 mRNA expression were determined by qRT-PCR. Gapdh was used as the internal control for all samples. Relative abundance was determined by calculating the expression ratio (2^{ΔΔCT}) after normalising each C_T (threshold cycle) value for Cyclin D1 to the C_T value for Gapdh. Experiments were repeated three times in triplicate. Error bars represent standard deviation. Data were acquired in conjunction with Marcus Karlstetter.
3.3.2.4 Proteasomal inhibition stabilises Cyclin D1 protein

Cyclin D1 is a key regulator in the G\textsubscript{i}/S phase cell cycle progression. In the early G\textsubscript{i} phase, high levels of Cyclin D1 is required to activate Cyclin D-CDK4/CDK6 complex, which in turn phosphorylate Rb to release E2F transcription factors required for S phase progression. The protein levels of Cyclin D1 depend not only on increased transcription, but also on protein stability. Cyclin D1 is phosphorylated on Thr286 by GSK-3\textbeta, and then subjected to proteasome-dependent degradation (Diehl et al., 1998). To examine the effect of \textsuperscript{V600E}BRaf on Cyclin D1 protein stability, MG-132 and Epoxomicin (Calbiochem) were used to inhibit the proteasome. As shown in Figure 3.11, MG-132 significantly increased Cyclin D1 protein in \textit{Braf\textsuperscript{+/LSL-V600E};CreER\textsuperscript{T+/0}} primary MEFs treated with 4-OHT or ethanol control, while Epoxomicin only had little effect on Cyclin D1 levels. As MG-132 can increase the levels of Cyclin D1 in \textit{V600E}BRaf-expressing cells, this would suggest that \textit{V600E}BRaf itself has little direct effect on the stability of Cyclin D1 protein.

3.3.2.5 \textit{V600E}BRaf-induced morphological transformation is dependent on MEK

MEK inhibitor U0126 can specifically inhibit both active and inactive MEK1/2 kinases to downregulate the RAF/MEK/ERK pathway. The effects of U0126 on morphological transformation induced by \textit{V600E}BRaf in primary MEFs were examined. As shown in Figure 3.12, \textit{Braf\textsuperscript{+/LSL-V600E};CreER\textsuperscript{T+/0}} primary MEFs treated with 4-OHT for 72h underwent morphological transformation compared with ethanol treated cells, which maintained a non-transformed morphology. After addition of U0126, cellular transformation of \textit{Braf\textsuperscript{+/LSL-V600E};CreER\textsuperscript{T+/0}} primary MEFs was reversed to a normal appearance. Thus, morphological transformation induced by \textit{V600E}BRaf is dependent on MEK activity.

3.3.2.6 \textit{V600E}BRaf-induced Cyclin D1 expression is dependent on MEK

To determine the effects of MEK inhibition on cell cycle progression, the levels of phospho-ERK and Cyclin D1 were examined following U0126 treatment. As shown in Figure 3.13, MEK inhibition after U0126 treatment was confirmed by significant suppression of ERK phosphorylation in \textit{V600E}BRaf-expressing MEFs compared with
**Figure 3.11 inhibition of proteasome enhances Cyclin D1 stability**

*Braf<sup>+/−</sup>;Cre<sup>ERT<sub>0</sub></sup> and *Braf<sup>+/−</sup>;Raf<sup>LSL-V600E</sup>;Cre<sup>ERT<sub>0</sub></sup>* primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h. 5h prior to harvesting cells, 30 µM MG-132 (MG) or 0.5 µM Epoxomicin (Epo) were added into the media. Protein levels of PP-ERK and Cyclin D1 were assessed by immunoblotting. ERK2 was used as the loading control. Data are taken from a single experiment representative of three giving similar results.

![Image of protein levels](attachment:image.png)
Figure 3.12  MEK inhibition reverses morphological transformation

*Braf*+/LSL-V600E+/CreERT2+/0 primary MEFs were treated with 50 nM 4-OHT or ethanol control for 72h followed by addition of 10 μM U0126 or DMSO for 48h. Cells were photographed by phase contrast microscopy (40x and 100x magnification). Data are taken from a single experiment representative of three giving similar results.
Chapter 3
V600E Brf induces proliferation and suppresses apoptosis in MEFs

Figure 3.13 MEK inhibition suppresses Cyclin D1 induction by V600E Braf

Braf^{+/+};CreERT^{+/0} primary MEFs and Braf^{+/LSL-V600E;CreERT^{+/0}} primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h then synchronised to a quiescent state by serum starvation for 16h followed by serum stimulation for 8h with 10 µM U0126 or DMSO control. Protein levels of PP-ERK and Cyclin D1 were assessed by immunoblotting. ERK2 was used as the loading control. Data are taken from a single experiment representative of three giving similar results.

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Braf^{+/LSL-V600E;CreERT^{+/0}} MEFs

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DMSO control treatment. Induction of Cyclin D1 by \(^{V600E}BRaf\) was significantly inhibited by U0126 treatment compared with DMSO control treated cells. In control cells, Cyclin D1 expression was only partially suppressed by U0126 suggesting other pathways are involved in regulating Cyclin D1 in these cells. Thus, \(^{V600E}BRaf\) induces high level of Cyclin D1 expression dependent that is dependent on MEK activity.

3.3.3 \(^{V600E}BRaf\) suppresses apoptosis by inhibiting Bim\(_{EL}\)

3.3.3.1 \(^{V600E}BRaf\) suppresses apoptosis upon serum withdrawal

The RAF/MEK/ERK signalling pathway is known to induce a survival signal. It has been shown that activated MEK/ERK signalling by oncogenic RAF suppresses apoptosis at different levels. At the pre-mitochondrial level, activated ERKs directly phosphorylate the pro-apoptotic protein BIM at Ser65, resulting in degradation of BIM via proteasomal degradation pathway (Ewings et al., 2007; Ley et al., 2003; Ley et al., 2004; Weston et al., 2003). At the post-mitochondrial level, activated ERKs phosphorylate caspase-9 at Thr125, thus inhibiting caspase-9 activity and suppressing apoptosis (Allan et al., 2003).

To examine the anti-apoptotic effects of \(^{V600E}BRaf\) upon serum withdrawal, Hoechst33258 staining of nuclear DNA was employed. As shown in Figure 3.14, \(Braf^{+/LSL-V600E};CreER^{T+/0}\) MEFs treated with 4-OHT significantly reduced the proportion of cells displaying nuclear shrinkage and chromosome condensation, which are typical morphological features of apoptotic cells upon serum withdrawal compared with ethanol control treatment. To quantify the levels of apoptosis, annexin V-FITC staining was performed. \(Braf^{+/LSL-V600E};CreER^{T+/0}\) MEFs treated with 50 nM 4-OHT significantly suppressed apoptosis upon serum withdrawal by \(~40\%\) compared with ethanol treatment or \(Braf^{+/+};CreER^{+/0}\) MEFs treated with 4-OHT or ethanol (Figure 3.15 and 3.16). During apoptosis, caspases are activated and act as executors of apoptosis. To examine the effects of \(^{V600E}BRaf\) on caspase activation upon serum withdrawal, caspase-3/7 activity was determined. As shown in Figure 3.17, \(^{V600E}BRaf\) expression led to a significant reduction in caspase activity upon serum withdrawal. Thus, \(^{V600E}BRaf\) suppresses apoptosis induced by serum withdrawal in a caspase-dependent manner.
Figure 3.14  \textsuperscript{V600E}BRAf suppresses apoptosis upon serum withdrawal

\textit{Braf}^{+/+};\textit{CreER}^{T+/0} and \textit{Braf}^{LSL-V600E};\textit{CreER}^{T+/0} primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h. Cells were serum starved or remained in the growth media for 48h. Levels of apoptosis were assessed by Hoechst33258 staining and data were acquired by fluorescence microscopy. Scale bar, 65 \(\mu\)M.
Figure 3.15 \textsuperscript{V600E}BRaf suppresses apoptosis upon serum withdrawal

\textit{Braf}\textsuperscript{+/+};\textit{CreERT\textsuperscript{T+0}} and \textit{Braf}\textsuperscript{+/LSL-V600E};\textit{CreERT\textsuperscript{T+0}} primary MEFs were treated with 50 nM 4-OHT or ethanol control (EtOH) for 96h. Cells were serum starved or remained in the growth media for 48h. Levels of apoptosis were assessed by Annexin V-FITC staining and data were acquired by flow cytometry. Experiments were repeated three times. Error bars represent standard deviation.

* $p<0.01$
Figure 3.16 Histograms of Annexin V-FITC staining upon serum withdrawal

Braf\textsuperscript{+/+};Cre\textsuperscript{ERT1/0} and Braf\textsuperscript{+/LSL-V600E};Cre\textsuperscript{ERT1/0} primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h. Then cells were serum starved or remained in the growth media for 48h. Levels of apoptosis were assessed by Annexin V-FITC and propidium iodide (PI) staining and data were acquired by flow cytometry. Experiments were repeated three times. Histograms of Annexin V-FITC and PI staining are shown. X axis (FL2-H) represents PI staining and Y axis (FL1-H) represents Annexin V-FITC staining.
Figure 3.17 V600E BRaf suppresses caspase activation upon serum withdrawal

Braf^{+/LSL-V600E;CreERT2/+} primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h. Cells were serum starved or remained in the growth media for 24h. Caspase-3/7 activity was assessed by using the Apo-Caspase 3/7 Kit. Experiments were repeated three times. Error bars represent standard deviation.
3.3.3.2 \textsuperscript{V600E}BRaf maintains mitochondrial membrane potential upon serum withdrawal

During mitochondria-dependent apoptosis, the mitochondrial outer membrane permeabilisation (MOMP) is associated with the loss of mitochondrial inner membrane potential. To examine the anti-apoptotic effects of \textsuperscript{V600E}BRaf on the mitochondrial pathway upon serum withdrawal, the mitochondrial membrane potential ($\Delta \Psi_m$) was measured by using the TMRE dye which binds to mitochondria in a membrane potential-dependent manner. As shown in Figure 3.18, \textit{Braf$^{+/LSL-V600E};CreER^{T+/0}$} primary MEFs treated with 50 nM 4-OHT significantly protected the loss of mitochondrial membrane potential upon serum withdrawal by ~20% compared with ethanol control treatment or \textit{Braf$^{+/+};CreER^{T+/0}$} primary MEFs treated with 4-OHT or ethanol control. Thus, \textsuperscript{V600E}BRaf protects mitochondrial membrane potential and prevents subsequent caspase activation leading to inhibition of apoptosis. Taken these data together, \textsuperscript{V600E}BRaf suppresses apoptosis upon serum withdrawal at the pre-mitochondrial level.

3.3.3.3 \textsuperscript{V600E}BRaf suppresses apoptosis by degradation of Bim\textsubscript{EL}

The BCL-2 family of proteins control the mitochondrial membrane potential. BH3-only proteins directly activate BAX/BAK (Eskes et al., 2000; Wei et al., 2001) or bind to and inhibit BCL-2 proteins subsequently release BAX/BAK from BCL-2 sequestration (Huang and Strasser, 2000; Bouillet et al., 1999; Chen et al., 2005; Puthalakath and Strasser, 2002), resulting in mitochondrial outer membrane permeabilisation (MOMP). BIM, a pro-apoptotic BCL-2 family protein with BH3-only domain, is induced following withdrawal of survival factors and then promotes mitochondria-dependent apoptosis (Marani et al., 2002). A key survival pathway known to be induced by ERK is suppression of BIM expression. In the presence of serum, ERK targets Bim\textsubscript{EL} and promotes its phosphorylation and subsequent turnover through the proteasomal degradation pathway (Whitfield et al., 2003; Weston et al., 2003; Ewings et al., 2007). In order to examine the role of \textsuperscript{V600E}BRaf in suppressing apoptosis, a serum withdrawal time course was performed. \textit{Braf$^{+/LSL-V600E};CreER^{T+/0}$} primary MEFs treated with 4-OHT completely inhibited Bim\textsubscript{EL} expression, the extra-long isoform of Bim that is detected in
Figure 3.18 $^{\text{V600E}}$BRaf prevents loss of mitochondrial membrane potential upon serum withdrawal

$Braf^{+/+;\text{CreER}^{T+/-}}$ and $Braf^{+/+;\text{LSL-V600E};\text{CreER}^{T+/-}}$ primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h. Cells were serum starved or remained in the growth media for 48h and cells were stained with tetramethylrhodamine ethyl ester (TMRE). Data were acquired by flow cytometry. Experiments were repeated three times. Error bars represent standard deviation.
our system only, compared with ethanol control treatment where WT BRaf is expressed (Figure 3.19). By contrast, BimEL was expressed in a time-dependent manner upon serum withdrawal in the Brat+/+,CreERT+/0 primary MEFs treated with 4-OHT or ethanol control. To investigate whether the suppression of BimEL expression by V600E BRaf is due to transcriptional inhibition, qRT-PCR analysis of BimEL mRNA was performed. As shown in Figure 3.20, serum withdrawal caused only a modest increase in BimEL mRNA in Brat+/LSL-V600E;CreERT+/0 primary MEFs treated with ethanol control. Expression of V600E BRaf did not reverse the induction of BimEL mRNA upon serum withdrawal. Taking these data together, V600E BRaf significantly inhibits apoptosis by suppression of BimEL expression at the post-transcriptional level.

3.3.3.4 V600E BRaf induces proteasome-dependent degradation of BimEL

The degradation of BimEL is regulated via the proteasome (Ley et al., 2003, 2004). To determine whether V600E BRaf inhibits BimEL expression by inducing degradation of BimEL via proteasome-dependent pathway, the proteasome inhibitor MG-132 was added to serum free media. As shown in Figure 3.21, MG-132 completely blocked V600E BRaf-induced degradation of BimEL without affecting its phosphorylation judged by band shifting. This experiment confirmed that V600E BRaf-induced degradation of BimEL is via inhibition of the proteasomal degradation pathway.

3.3.3.5 MEK inhibition restores apoptosis suppressed by V600E BRaf

To determine whether V600E BRaf suppresses apoptosis in an ERK-dependent manner, MEK inhibition was applied. As shown in Figure 3.22, MEK inhibition alone had very little effect on inducing apoptosis in V600E BRaf-expressing MEFs and WT BRaf-expressing MEFs in the presence of serum. When combined with serum withdrawal, MEK inhibition significantly enhanced apoptosis in V600E BRaf-expressing MEFs by ~50% compared with serum withdrawal alone. Thus, V600E BRaf induced apoptosis suppression is MEK-dependent.
Figure 3.19 V600E BRaf promotes Bim<sub>EL</sub> degradation upon serum withdrawal

Braf<sup>+/+</sup>;CreER<sup>T+/0</sup> primary MEFs and Braf<sup>+/LSL-V600E;CreER<sup>T+/0</sup></sup> primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h followed by a serum withdrawal (SW) time course of 0, 4h, 8h, 16h, 24h, 48h and 72h. Protein levels of PP-ERK and Bim<sub>EL</sub> were assessed by immunoblotting. ERK2 was used as the loading control. Data are taken from a single experiment representative of three giving similar results.

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| Braf<sup>+/LSL-V600E;CreER<sup>T+/0</sup></sup> MEFs

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| Braf<sup>+/+</sup>;CreER<sup>T+/0</sup> MEFs
Figure 3.20 \textsuperscript{V600E}B\textsuperscript{RAf} does not suppress \textit{Bim}\textsubscript{EL} transcription upon serum withdrawal

\textit{Bra}f\textsuperscript{-/LSL-\textsuperscript{V600E},\textsuperscript{CreER}\textsuperscript{T+/-}} primary MEFs were treated with 50 nM 4-OHT for 96h followed by a serum withdrawal for 48h, then \textit{Bim}\textsubscript{EL} mRNA expression was determined by qRT-PCR. \textit{Gapdh} was used as the internal control for all samples. Relative abundance was determined by calculating the expression ratio (2\textsuperscript{-\Delta\Delta CT}) after normalising each C\textsubscript{T} value for \textit{Bim}\textsubscript{EL} to the C\textsubscript{T} value for \textit{Gapdh}. Experiments were repeated three times in triplicate. Error bars represent standard deviation.

\begin{figure}[h]
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\caption{\textsuperscript{V600E}B\textsuperscript{RAf} does not suppress \textit{Bim}\textsubscript{EL} transcription upon serum withdrawal}
\end{figure}

\begin{equation}
p > 0.05
\end{equation}
Figure 3.21 \textsuperscript{V600E} Braf induces proteasome-dependent degradation of Bim\textsubscript{EL}

\textit{Braf\textsuperscript{+/LSL-V600E;CreER\textsuperscript{T+/0}}} primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h followed by serum starvation for 24h. MG-132 (MG) at 30 \textmu M was added to the media 4h prior to harvesting cells. Protein levels of Bim\textsubscript{EL} were assessed by immunoblotting. ERK2 was used as the loading control. Data are taken from a single experiment representative of three giving similar results.
**Figure 3.22** MEK inhibition restores apoptosis suppressed by \(V_{600}E^{B}\)Raf

\(Bra{f}^{+/LSL-V_{600}E^{E}};{C}^{T+/0}\) primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h. Cells were serum starved or remained in the growth media for 48h. 5hs prior to harvesting cells, 10 \(\mu M\) U0126 was added to the media. Levels of apoptosis were assessed by Annexin V-FITC staining and data were acquired by flow cytometry. Experiments were repeated three times. Error bars represent standard deviation.
Figure. 3.23 MEK inhibition restores Bim_{EL} expression suppressed by V600E{BRaf}

Braf\textsuperscript{+/LSL-V600E;CreERT\textsuperscript{+/-}} primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h. Cells were serum starved or remained in the growth media for 48h. 4h prior to harvesting cells, 10 \mu M U0126 was added to the media. Protein levels of PP-ERK and Bim\textsubscript{EL} were assessed by immunoblotting. ERK2 was used as the loading control. Data are taken from a single experiment representative of three giving similar results.

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Braf\textsuperscript{+/LSL-V600E;CreERT\textsuperscript{+/-}} MEFs
3.3.3.6 MEK inhibition restores Bim\textsubscript{EL} expression suppressed by V\textsuperscript{600E}BRaf
To examine whether Bim\textsubscript{EL} expression is restored by MEK inhibition, levels of Bim\textsubscript{EL} were assessed. As shown in 3.23, combined U0126 with serum withdrawal treatment led to an increase in Bim\textsubscript{EL} expression in V\textsuperscript{600E}BRaf-expressing MEFs and this was faster migrating, suggesting less phosphorylation of Bim\textsubscript{EL}. Overall, our data show that V\textsuperscript{600E}BRaf suppresses apoptosis via induction of Bim\textsubscript{EL} degradation in a MEK-dependent manner.

3.4. Discussion
The CreER\textsuperscript{T} system was developed in order to temporally and spatially control the knockin or knockout of interested genes upon addition of 4-OHT (Hayashi and McMahon, 2002). In this study, the CreER\textsuperscript{T} system was used to regulate the conditional expression of V\textsuperscript{600E}BRaf in MEFs. Expression of the Braf\textsuperscript{Lox-V600E} allele was found to be totally dependent on induction of Cre recombinase activity after 4-OHT treatment. Recombination PCR showed that the recombined Braf\textsuperscript{Lox-V600E} allele occurred as early as 5h after 4-OHT treatment and reached its full recombination at 96h. This is consistent with the fluorescence microscopy data showing translocation of the Cre recombinase from the cytoplasm to the nucleus in a time-dependent manner. Thus, we have shown for the first time that the CreER\textsuperscript{T} system provides a convenient way to regulate expression of a conditional allele.

It has been shown that Cre-induced cytotoxicity resulting in growth inhibition is observed in cells (Loonstra et al., 2001; Silver and Livingston, 2001) and mouse tissues (Schmidt et al., 2000; Pfeifer et al., 2001). The primary cause for this seems to be the accumulation of DNA damage induced by the endonuclease activity of the Cre recombinase (Loonstra et al., 2001). In addition, leaky expression of Cre recombinase \textit{in vivo} has been reported in mouse models due to inappropriate nuclear translocation or proteolysis of the Cre-fusion protein (Nakamura et al., 2006; Nguyen et al., 2009; Vooijs et al., 2001). In the present study, we did not observe concomitant toxicity of the Cre recombinase, although p53 was found to be slightly increased at later time points.
Chapter 3

V600E BRAf induces proliferation and suppresses apoptosis in MEFs

(Figure 3.8 and 3.9) and this may be due to DNA damaging effects of the Cre recombinase. However, the effects of Cre on DNA damage were not directly measured in this study. Also, no spontaneous recombination was observed in Braf+/+/V600E;CreER+/0 primary MEFs without adding 4-OHT. These data suggest that the Cre recombinase itself plays no adverse role in our system.

4-OHT has been used as a potent estrogen receptor (ER) antagonist in the treatment of breast cancer, in which 4-OHT has cytostatic effects, inducing G$_0$/G$_1$ arrest, and cytotoxic effects by inducing apoptosis in vivo and in vitro (Chen et al., 1996; Osbourne et al., 1983; Perry et al., 1995; Bollig et al., 2007). In our study, 4-OHT did not induce adverse effects on primary MEFs as there were no obvious changes in apoptosis or the expression of phospho-ERK, Cyclin D1, and other cell cycle regulators in Braf+/+;CreER+/0 MEFs treated with 4-OHT compared with ethanol control treatment. These data confirm that there are no obvious side effects of 4-OHT observed in our study.

V600E BRAF is a traditional oncogene capable of transforming NIH 3T3 cells and immortal mouse melanocytes in vitro (Davies et al., 2002; Wan et al., 2004; Wellbrock et al., 2004b). However, overexpression of V600E BRAF was used in these previous studies. Here we showed that expression of endogenous V600E BRAf from a single knockin allele at physiological levels can induce typical morphological transformation in primary MEFs. We found that morphological transformation induced by V600E BRAf was MEK-dependent as it was reversed by MEK inhibition. These findings are consistent with previous studies in which MEK inhibition reverses transformation in melanoma cells expressing V600E BRAF (Klein et al., 2008) and in Madin-Darby canine kidney (MDCK) epithelial cells transformed by oncogenic ∆Raf-1:ER (Hansen et al., 2000). Our study with endogenous V600E BRAf shows that endogenous V600E BRAf is a potent transforming oncogene.

In our study, expression of endogenous V600E BRAf at physiological levels induced high levels of ERK phosphorylation and elicited a faster G$_1$/S phase cell cycle progression by
V600E BRAf induces proliferation and suppresses apoptosis in MEFs

inducing significant upregulation of Cyclin D1 expression at the transcriptional level and this was reversed by MEK inhibition. These data support the view that Cyclin D1 is a direct transcriptional target of the RAF/MEK/ERK signalling pathway via AP-1 transcriptional factors (Kerkhoff and Rapp 1997; Weber et al. 1997; Balmanno and Cook, 1999). Recently, microarray studies have shown that CCND1 and members of AP-1 transcription factors including FOS and FOSL1 are transcriptionally induced by V600E BRAF in melanoma cells and thus appear to be MEK-dependent targets of V600E BRAF (Packer et al., 2009; Pratilas et al., 2009). However, expression of endogenous V600E BRAf did not prevent Cyclin D1 degradation which was restored by proteasomal inhibition. These results are consistent with a previous study in which the stability of Cyclin D1 was found to be mediated by GSK-3β-dependent phosphorylation of Cyclin D1 at Thr286 and subsequent proteasome-dependent degradation (Diehl et al. 1998). In our study, Cyclin D1 is the only cell cycle regulator whose expression was dramatically induced following expression of V600E BRAf in MEFs. The role of other Cyclins and Cdks including Cyclin D2, Cyclin D3, Cyclin E, and Cdk2 were excluded. Together, our results suggest that expression of endogenous V600E BRAf promotes a faster G1/S phase cell cycle progression by significant induction of Cyclin D1 expression.

During V600E BRAf-induced G1/S cell cycle progression, no obvious changes were observed in expression of CKIs including p21Cip1 and p27Kip1. Woods et al., (1997) showed that induction of BRAf activity using ∆BRAf:ER expressing in NIH 3T3 cells induced cell cycle progression by induction of Cyclin D1 and Cyclin E and suppression of p27Kip1. However, expression of high levels of BRAf induced cell cycle arrest by induction of high levels of p21Cip1, independently of p53, that inhibited both Cyclin D/CDK4 and Cyclin E/CDK2 complexes (Woods et al., 1997). However, our data suggest that expression of endogenous V600E BRAf in MEFs induces G1/S phase cell cycle progression without affecting CKIs. The reason for the difference is not entirely clear but may be related to the level of expression of BRAf in each system.
In our study, expression of endogenous V600E BRaf induced high levels of ERK phosphorylation. However, this induction was not sustained as ERK phosphorylation slightly decreased at later time points while MEK phosphorylation stayed high. These results indicate negative feedback regulation of ERK. Several negative feedback mechanisms have been reported to inhibit the BRAF/MEK/ERK pathway including upregulation of Sprouty proteins (Tsavachidou et al., 2004; Yusoff et al., 2002), Dual specificity phosphatase (DUSPs) (Owens and Keyse, 2007), or direct phosphorylation of RAF by active ERK (Brummer et al. 2003; Dougherty et al., 2005). Microarray studies have shown that DUSP6 and Sprouty/Spred proteins are transcriptionally induced by V600E BRAF in melanoma cells and thus appear to be a consistent response to the oncogene across different cell types (Packer et al., 2009; Pratilas et al., 2009). The reason for induction of negative feedback regulators is not entirely clear but one possibility is that the suppression of high ERK signalling is necessary for the transforming effects of V600E BRAF as continued high levels of ERK activity might lead to induction of CKIs, as observed in the BRaf:ER system (Woods et al., 1997).

In our study, expression of endogenous V600E BRaf also induced a survival signal. V600E BRaf significantly suppressed apoptosis following serum withdrawal at the pre-mitochondrial level by preventing the mitochondrial membrane potential from dropping. Further analysis showed that expression of V600E BRaf dramatically downregulated the expression of BimEL following serum withdrawal at the post-transcriptional level which was rescued by MEK inhibition and proteasomal inhibition. These results are consistent with previous studies in which degradation of BimEL was observed to be MEK/ERK-dependent and required for evasion of apoptosis (Whitfield et al., 2003; Weston et al., 2003; Ewings et al., 2007; Ley et al., 2003, 2004). However, these studies typically involved the overexpression of RAF or MEK mutants or the use of conditional kinases that are not found in human tumours. Our study shows that endogenous V600E BRaf is also efficient in suppressing apoptosis by downregulation of BimEL expression through the MEK/ERK pathway.
At the post-transcriptional level, the RAF/MEK/ERK pathway has been found to be the major pathway involved in promoting rapid phosphorylation of Bim_{EL} at Ser65 (Ser69 in human) and subsequent degradation via the proteasome (Ley et al., 2003, 2004; Marani et al., 2004; Wickenden et al., 2008). We showed that when a proteasome inhibitor was applied, Bim_{EL} expression was restored in V600E BRAf-expressing cells and had reduced mobility, which probably corresponds to phosphorylated Bim_{EL} (Harada et al., 2004; Wang et al., 2004). When a MEK inhibitor was combined with a proteasome inhibitor, Bim_{EL} migrated more quickly, indicating less phosphorylation. These data suggest that expression of endogenous V600E BRAf is sufficient to turnover of Bim_{EL} upon serum withdrawal possibly through phosphorylation at Ser65. However, since a phospho-specific antibody against Bim_{EL} Ser65 was not available, we could not confirm whether the effects on Bim_{EL} were mediated through phosphorylation of this site or not.

Recently, BIM has been identified as a novel tumour suppressor (Bouillet., 2001; Egle et al., 2004; Tan et al., 2005) as homozygous deletions of the BIM locus has been found in ~40% of human mantle cell lymphomas (Tagawa et al., 2005; Mestre-Escorihuela et al., 2007). In our study, MEK inhibition rescued Bim_{EL} expression leading to a significant increase in apoptosis following serum withdrawal in V600E BRAf-expressing cells. We speculate that Bim_{EL} could be a therapeutic target in V600E BRAf-expressing tumour cells. It has been shown that MEK inhibitors or V600E BRAF inhibitors can be successfully used to induce apoptosis by inhibiting V600E BRAF signalling and stabilising Bim_{EL} in melanoma and colon cancer cells (Dankort et al., 2009; Cartlidge et al., 2008; Jiang et al., 2010; Wang et al., 2007; Sheridan et al., 2008; Boisvert-Adamo and Aplin, 2008; Wickenden et al., 2008). However, in our study, MEK inhibition alone did not induce obvious apoptosis, indicating that tumour cells with V600E BRAF mutation are sensitive to MEK inhibition (Solit et al., 2006). In addition, combined use of V600E BRAF inhibitors and proteasome inhibitors has been reported to restore Bim_{EL} expression and enhance cell death in vitro and in vivo (Jiang et al., 2010; Tan et al., 2005). Therefore, MEK inhibitors or V600E BRAF inhibitors in combination with proteasome inhibitors would give an additive or synergistic activity in chemotherapy by targeting Bim_{EL}. 

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Data presented in this chapter was published in *Oncogene* in 2008; see attached manuscript in Appendix B:

CHAPTER 4
IDENTIFICATION OF COMPONENTS OF THE SENESCENCE-MESSAGING SECRETOME IN THE LUNG EXPRESSING \(^{V600E}BRAF\)

4.1 Introduction

\(^{V600E}BRAF\) represents the most common BRAF mutation in melanomas and constitutively activates the MEK/ERK pathway, leading to growth factor-independent melanoma cell proliferation, transformation of immortalised melanocytes and tumourigenicity in melanoma xenografts (Hingorani et al., 2003; Karasarides et al., 2004; Wellbrock et al., 2004b). Inhibition of \(^{V600E}BRAF\) signalling blocked melanoma cell proliferation and induced apoptosis \textit{in vitro}, and suppressed growth of tumour xenografts \textit{in vivo} (Hingorani et al., 2003; Karasarides et al., 2004; Wellbrock et al., 2004b), demonstrating that \(^{V600E}BRAF\) is necessary for the maintenance and progression of melanoma.

It has also been shown that \(^{V600E}BRAF\) is associated with melanocyte senescence. \(^{V600E}BRAF\) mutation has been found in up to 80% of human nevi and these benign melenocytic lesions can remain unchanged for decades (Pollock et al., 2003). In addition, overexpression of \(^{V600E}BRAF\) induced classic OIS in human melanocytes and zebrafish melanocytes \textit{in vitro} (Gray-Schopfer et al., 2005; Michaloglou et al., 2005; Patton et al., 2005). Moreover, in a melanoma mouse model, it has been shown that \(^{V600E}Braf\) functions as a founder mutation that stimulates melanocyte proliferation and formation of nevi harbouring senescent melanocytes, and induce melanomas once senescence is overcome (Dhomen et al., 2009). Similarly, induction of senescence has also been observed in benign tumours induced by expression of endogenous \(^{V600E}BRAF\) in the lung and gastrointestinal duct in mouse models and tumours progress when senescence is surpassed (Carragher et al., 2010; Dankort et al., 2007). Together, these studies indicate that expression of \(^{V600E}BRAF\) induces senescence that functions as an important tumour suppressor mechanism in preventing tumour progressing.
Senescence-messaging secretome (SMS) has been recognised as a new effector mechanism of senescence, and it acts in an autocrine/paracrine manner to reinforce the senescent phenotype, allowing for communication between senescent cells and their microenvironment (Ren et al., 2009; Kuilman and Peeper, 2009). Induction of senescence requires secreted proteins including members of Wnt, IGF, transforming growth factor-β (TGFβ), plasmin, and interleukins (ILs) (Kuilman and Peeper, 2009). It has been shown that secreted protein IGFBP7, IL-6, and IL-8 mediates senescence induced by oncogenic V600E BRAF in an autocrine/paracrine manner, which functions as a tumour suppressor (Kuilman et al., 2008; Wajapeyee et al., 2008). Knockdown of IGFBP7, IL-6, or IL-8 bypasses OIS induced by V600E BRAF (Kuilman et al., 2008).

In our laboratory, in order to investigate the role of V600E BRAF in tumour development, Cre-LoxP system was used to generate a conditional knockin mutation of V600E Braf in mice, termed Braf\(^{+/LSL-V600E}\) (Mercer et al., 2005). The CreER\(^T\) system that is 4-OHT inducible was employed to regulate the expression of V600E Brf following activation of the Cre recombinase by addition of 4-OHT. Before Cre-mediated recombination, the Braf\(^{+/LSL-V600E}\) gene expresses wild-type BRAF due to the mini-cDNA encoding exons 15 to 18 of wild-type Braf. After Cre-mediated deletion of the LSL cassette including the mini-cDNA, the Braf\(^{Lox-V600E}\) allele is generated expressing V600E BRAF. Therefore, V600E Braf is expressed at physiological levels from its own promoter, closely mimicking the situation that occurs in human cancer cells where one copy of the normal BRAF gene is mutated somatically to V600E BRAF.

In work leading up to this project, Braf\(^{+/LSL-V600E}\) mice were crossed with CreER\(^{T\times\Omega}\) mice to generate Braf\(^{+/LSL-V600E}\);CreER\(^{T\times\Omega}\) double heterozygous (VE) mice (Figure 4.1A). These mice developed breathing difficulties and general wasting at about 2 weeks postpartum compared with Braf\(^{+/\Omega}\);CreER\(^{T\times\Omega}\) (WT) mice. The number of surviving mice decreased in a time-dependent manner and ~50% mice were dead by 3 months (Figure 4.1B). By crossing CreER\(^{T\times\Omega}\) mice with Rosa26R reporter mice, β-galactosidase positive staining was observed in the lung and liver indicating spontaneous
Cre-mediated recombination without 4-OHT treatment in these tissues (Figure 4.2A). Recombination PCR also confirmed that the recombined \textit{Braf}^{ex-V600E} allele was generated as early as 3 weeks and increased in a time-dependent manner in \textit{V600E}BRaf expressing lungs without administration of 4-OHT (Figure 4.2B). These results indicate that the expression of \textit{V600E}BRaf spontaneously occurred in the lung with the \textit{CreER}^T strain.

Haematoxylin and eosin staining showed that \textit{Braf}^{+/-LSL-V600E};\textit{CreER}^{+/-0} mice developed lung adenomas (Figure 4.3). All the histology and immunohistochemistry was undertaken by Susan Giblett. These tumours developed even in the absence of 4-OHT treatment due to leaky expression of Cre recombinase in lung tissues. Such lesions were not detected in WT mice with or without the \textit{CreER}^T allele. In \textit{V600E}BRaf expressing mice, tumours were not observed at birth. However, by 3 weeks papillary adenomas arose as a result of increased cell proliferation with a high percentage of Ki67 positive staining (Figure 4.4). The adenomas appeared to arise from alveolar ducts, expand outward and around bronchioles, and continue to proliferate such that there was extensive epithelial hyperplasia with time, forming larger adenomas at 10 weeks. These cells displayed morphological alterations including eosinophilic cytoplasm but did not undergo dysplastic changes.

Quantification of the cell number in lung adenomas indicated that adenomas continued to increase in size between 3 weeks to 8 weeks. At 10 weeks, there appeared to a growth restrain as cell number was halted (Figure 4.5A). Ki67 positive staining substantially increased from 3 weeks to 6 weeks indicating that massive proliferation occurred after \textit{V600E}BRaf expression (Figure 4.5B). However, the percentage of Ki67 positive staining was dramatically decreased at 8 weeks suggesting that proliferation had ceased (Figure 4.5B). So far we have not detected the progression of these adenomas to adenocarcinomas in mice.
Figure 4.1 Reduced survival of $Braf^{+/LSL-V600E}; CreER^{T+/0}$ mice

(A) $Braf^{+/LSL-V600E}$ mice were crossed with $CreER^{T+/0}$ mice to generate $Braf^{+/LSL-V600E}; CreER^{T+/0}$ double heterozygous mice. (B) The Kaplan-Meier plot shows survival of mice used in this study according to the percentage of surviving mice versus survival time (days). $Braf^{+/+}; CreER^{T+/0}$ mice and $Braf^{+/LSL-V600E}; CreER^{T+/0}$ mice were monitored by checking their health condition over a time course of three months.
Figure 4.2 Expression of \( \text{Braf}^{\text{Lox-V600E}} \) in \( \text{Braf}^{+/LSL-V600E};\text{CreER}^{T/+} \) mice

(A) Lung and liver tissues were taken from \( \text{Rosa26R};\text{CreER}^{T/+} \) mice aged 4 weeks and stained with X-gal for \( \beta \)-galactosidase to manifest the recombination event. Scale bar, 100 \( \mu \)m. (B) Genomic DNA was isolated from lung tissues of \( \text{Braf}^{+/+};\text{CreER}^{T/+} \) mice (WT) aged at 6 weeks and \( \text{Braf}^{+/LSL-V600E};\text{CreER}^{T/+} \) mice (VE) aged 3 weeks, 6 weeks, 8 weeks, 10 weeks, and 14 weeks. Recombination PCR was performed to detect \( \text{WTBraf} \) and \( \text{Braf}^{\text{Lox-V600E}} \) alleles without administration of 4-OHT. \( \text{Gapdh} \) was used as the internal control.
Figure 4.3 Expression of V600E BRaf induces lung tumours in mice

Histological sections were taken from lung tissues of Braf+/+;CreER<sup>T/v</sup> mice (WT) and Braf<sup>+/LSL-V600E;CreER<sup>T/v</sup></sup> mice (VE) and stained with haematoxylin and eosin. WT lung tissues were taken from mice aged 3 weeks and VE lung tissues were taken from mice aged 3 weeks, 6 weeks, 8 weeks, and 10 weeks. Scale bar in VE, 100 µm; WT, 200 µm. This work was undertaken by Susan Giblett.
Figure 4.4 Expression of $V_{600E}$BRaf induces cell proliferation in lung tumours

Histological sections were taken from lung tissues of $Braf^{+/+};CreERT^{+/0}$ mice (WT) and $Braf^{+/LSL-V600E};CreERT^{+/0}$ mice (VE) and stained with an antibody for Ki67. WT lung tissues were taken from mice aged 3 weeks and VE lung tissues were taken from mice aged 3 weeks, 6 weeks, 8 weeks, and 10 weeks. Scale bar in VE, 100 µm; WT, 200 µm. This work was undertaken by Susan Giblett.
Figure 4.5 Proliferation is not sustained in lung tumours expressing $^{V600E}_{BRaf}$

Lung tissues were taken from $Braf^{+/LSL-V600E;CreER^{+/0}}$ mice aged 3 weeks, 6 weeks, 8 weeks, 10 weeks, and 14 weeks. Cell number per adenoma in sections stained with haematoxylin and eosin (A) and percentage of Ki67 positive staining (B) were quantified. Error bars represent standard deviations. Above work was undertaken by Charles Foster.
Senescence-messaging secretome in the lung expressing $^{V600E}$BRaf

OIS is thought to play an important role in tumour suppression by preventing unscheduled proliferation of early neoplastic cells (Campisi, 2005; Collado and Serrano, 2006). In a similar conditional knockin mouse model of lung cancer, expression of $^{V600E}$BRaf induced cell proliferation leading to formation of lung adenomas that were histologically similar to our findings, followed by induction of senescence that prevent the adenomas from progressing (Dankort et al., 2007). The expression of senescence markers for p19$^{Arf}$ and Dec1 was detected in lung adenomas, but not for SA-β-gal (Dankort et al., 2007). These results suggest that induction of senescence might account for the cessation of proliferation in the lung induced by $^{V600E}$BRaf expression. Lung adenomas progressed to adenocarcinomas with increased proliferation and cancellation of senescence when $p53$ or Ink4a/Arf was inactivated (Dankort et al., 2007). Taken together, our data showed that massive cell proliferation accounts for the induction of adenomas in the lung following $^{V600E}$BRaf expression but proliferation is not sustained as senescence may occur that prevents the tumourigenesis, and additional molecular events are needed for the progression of adenomas to adenocarcinomas.

4.2 Aims
The work described above was undertaken prior to my PhD project. My goal was to use $Braf^{+/LSL-V600E; CreERT^{W/0}}$ double heterozygous mice as models to investigate the role of oncogenic $^{V600E}$BRaf in early stage lung tumour development, specifically the senescent phenotype, and to achieve the following:

1. Characterisation of the senescent phenotype in $^{V600E}$BRaf-induced lung tumours and the downstream signalling pathways involved
2. Preparation of conditioned media (CM) from WT lungs and lung tumours expressing $^{V600E}$BRaf to screen for secreted proteins that are expressed exclusively in the lung tumours by mass spectrometry
3. Validation of the secreted proteins identified from CM
4.3 Results

4.3.1 Senescence markers are induced in lung tumours expressing $^{V600E}BRaf$

4.3.1.1 Induction of p16$^{Ink4a}$, p21$^{Cip1}$ and Dec1 in lung tumours

As described in the introduction, expression of a conditional knockin mutation of $^{V600E}Braf$ developed lung adenomas in mice. After an initial burst of cell proliferation driven by expression of $^{V600E}BRaf$, proliferation ceased and this prevented the lung adenomas from progression. OIS has been proposed as a tumour suppressor mechanism at the pre-malignant stage (Campisi, 2005; Collado and Serrano, 2006). The p53/p21$^{CIP1}$ and p16$^{INK4A}$/pRb pathways are activated upon entry into the senescence and function as central integration points for the various signalling pathways leading to senescent growth arrest (Campisi, 2005; Campisi and d’Adda di Fagagna, 2007; Serrano et al., 1997). This led us to examine the expression of p16$^{Ink4a}$ and p21$^{Cip1}$ in the lung adenomas at first. Total RNA was isolated from the lung tissues of $^{Braf^+/+; CreER^{T/^0}}$ (WT) mice and $^{Braf^{+*/LSL-V600E; CreER^{T/^0}}}$ (VE) mice and RT-PCR analysis was performed. As shown in Figure 4.6, the expression of p16$^{Ink4a}$ and p21$^{Cip1}$ mRNAs was induced at 3 weeks and continued to increase in a time-dependent manner in the lung tumours expressing $^{V600E}BRaf$ compared with those of the wild-type (WT) lung, although their expression in adenomas was slightly decreased at 10 weeks. Dec1 is another senescence marker that has been detected in lung adenomas induced by conditional expression of $^{G12V}KRas$ and $^{V600E}BRaf$ in mice (Collado et al., 2005; Dankort et al., 2007). In our study, Dec1 was detected in the lung adenomas and its expression increased from 6 weeks to 8 weeks as assessed by immunohistochemistry (Figure 4.6). These data suggested that expression of $^{V600E}BRaf$ in the lung induces senescence and this could account for a decrease in cell proliferation and prevention of adenomas from progressing.
Figure 4.6 Senescence markers are induced in lung tumours expressing V600EBRaf

(A) Total RNA was isolated from lung tissues of Braf+/+;CreERT+/- mice (WT) aged at 6 weeks and Braf+/LSL-V600EB;CreERT+/- mice (VE) aged 3 weeks, 6 weeks, 8 weeks, and 10 weeks. RT-PCR of p16Ink4a and p21Cip1 was performed. Gapdh was used as the internal control. (B) Histological sections taken from lung tissues of Braf+/LSL-V600EB;CreERT+/- mice aged 6 weeks and 8 weeks were stained with an antibody for Dec1. Scale bar, 100 μm. This work was undertaken by Susan Giblett.
4.3.1.2 Igfbp7 does not play an important role in V600E BRaf-induced senescence in the lung

As recently reported, IGFBP7 is a secreted protein that is significantly induced during V600E BRAF-induced senescence and IGFBP7 protein can induce senescence when added exogenously through autocrine/paracrine pathways by inhibition of the BRAF/MEK/ERK signalling pathway (Wajapeyee et al., 2008). To examine whether Igfbp7 plays a role in inducing senescence in the lungs of our Braf+/LSL-V600E;CreERT2+/0 mice, immunoblot analysis was used to determine the expression of Igfbp7. The expression of Igfbp7 was not increased but slightly decreased at 3 weeks and 10 weeks in lung adenomas expressing V600E BRaf compared with the WT lung (Figure 4.7A). Consistently, the mRNA expression of Igfbp7 was decreased from 3 weeks to 10 weeks in a time-dependent manner by qRT-PCR analysis (Figure 4.7B), indicating Igfbp7 is not a transcription target for V600E BRaf in the lung. These data suggest Igfbp7 is not associated with the senescent phenotype in our lung system.

4.3.1.3 Interleukin-6 is not involved in V600E BRaf-induced senescence in the lung

As shown in the Haematoxylin and eosin staining (Figure 4.3), infiltrating macrophages emerged around the adenomas at 10 weeks suggesting that an inflammatory response was initiated. Proinflammatory cytokines and chemokines secreted by senescent cells have been reported to be required for senescence entry and maintenance (Acosta et al., 2008; Kuilman et al., 2008). It has been shown that Interleukin-6 (IL-6) is upregulated during V600E BRAF-induced senescence, and knockdown of IL-6 or its receptor IL-6R bypasses senescence induced by V600E BRAF. To determine whether IL-6 is involved in the senescence induced by V600E BRaf in adenomas, immunoblot analysis of IL-6 was performed. As shown in Figure 4.7A, the expression of IL-6 was induced at 8 weeks and 10 weeks in WT lungs, but not in VE lung adenomas. This experiment indicates that IL-6 might not play an important role in inducing senescence in the adenomas. The presence of IL-6 in some WT lung samples but not others may be related to levels of inflammation/respiratory infection in these animals that may occur in response to pathogens in environment.
Figure 4.7 Expression of Igfbp7 and Interleukin-6 in lung tumours expressing V600E Braf

Lung tissues were taken from Brarf+/+;CreERT2/0 (WT) mice and Brarf+/LSL-V600E;CreERT2/0 (VE) mice aged 3 weeks, 6 weeks, 8 weeks, and 10 weeks. (A) Protein levels of Igfbp7 and interleukin-6 (IL-6) were assessed by immunoblotting. Actin was used as the loading control. (B) Total RNA was isolated from above lung tissues. Igfbp7 mRNA expression was determined by qRT-PCR. Gapdh was used as the internal control. Relative abundance was determined by calculating the expression ratio \(2^{-\Delta\Delta CT}\) after normalising each CT value for Igfbp7 to the CT value for Gapdh. Error bars represent standard deviation.
Chapter 4 Senscence-messaging secretome in the lung expressing V600E BRaf

Taken together, these data showed that senescence is induced following the expression of V600E BRaf in the lung adenomas, but IGFBP7 and IL-6 are not involved in this.

4.3.2 The BRaf/MEK/ERK signalling pathway is downregulated in V600E BRaf-induced senescence in the lung

To investigate the signalling pathways involved in the transition from proliferation initiated at 3 weeks to induction of senescence at 6 weeks, immunoblot analysis of a time course of the lung tissues from WT mice VE mice was performed. As shown in Figure 4.8, levels of phospho-ERK were upregulated in VE lung adenomas compared with WT lungs at 3 weeks during the proliferative phase but levels of phospho-ERK decreased dramatically in VE lung adenomas during the senescent phase. BRaf itself displayed a similar profile as phospho-ERK, possibly indicating that decreased phospho-ERK expression resulted from downregulation of BRaf expression. However, phospho-MEK was not expressed in the same trend as phospho-ERK indicating a possible role for MAPK phosphatases. A panel of cell cycle associated proteins such as Cyclin D1, Cylin D2, Cyclin D3, Cyclin E, Cdk2, p27kip1, and c-Myc were immunoblotted. Their expression was consistently downregulated by V600E BRaf signalling, consistent with the reduction in ERK signalling. The only exception was Cyclin D1 whose expression was induced at 3 weeks and maintained at high levels throughout the time course. The increased Cyclin D1 may result from proliferation occurring during the expansion of the lung adenomas as well as in the infiltrating macrophages (Figure 4.3). Mac-2 is a marker for macrophages and its expression is increased in the VE lung. However, we failed to detect p16ink4a and p21cip1 expression by immunoblotting. Taken together, we conclude that the BRaf/MEK/ERK signalling pathway is activated in the initiation of lung adenomas then downregulated during senescence.

4.3.3 Autophagy is induced in senescent lung tumours

Autophagy is a lysosome-dependent bulk degradation pathway for long-lived proteins and damaged organelles within double-membrane autophagosomes, which eventually
Figure 4.8 The BRaf/MEK/ERK signalling pathway is downregulated in senescent lung tumours expressing V600EBRaf

Lung tissues were taken from \( \text{Braf}^{+/+};\text{CreER}^{T+/0} \) (WT) mice and \( \text{Braf}^{LSL-V600E};\text{CreER}^{T+/0} \) (VE) mice aged 3 weeks, 6 weeks, 8 weeks, and 10 weeks. Protein levels of BRaf, P-MEK, PP-ERK, Cyclin D1, Cyclin D2, Cyclin D3, Cyclin E, Cdk2, p27Kip1, c-Myc, and Mac-2 were assessed by immunoblotting. Actin was used as the loading control.
fuse with lysosomes for degradation (Klionsky and Emr, 2000; Klionsky, 2004). Although initially autophagy is identified as a survival mechanism upon metabolic stress, emerging evidence has shown that autophagy functions as an important tumour suppression mechanism in preventing persistent tissue damage and chronic inflammation (Mathew et al., 2007a; Levine and Kroemer, 2008). Recently, Young and colleagues reported that autophagy is activated upon the induction of senescence by oncogenic RAS and is required for the establishment of senescence (Young et al., 2009). It has also been shown that high levels of $^{V600E}$BRAF induce senescence and trigger autophagy in melanoma cells and melanoma xenografts (Maddodi et al., 2010).

To examine whether autophagy plays a role in $^{V600E}$Braf-induced senescence in lung adenomas in mice, immunoblot analysis of autophagy markers was performed. LC3 is a widely used marker for autophagy. The lipid-conjugated form of LC3 (LC3 II) localises to the membranes of autophagosomes indicating the number of autophagosomes. It can be separated from non-conjugated form (LC3 I) by immunoblotting since LC3 II migrates faster than LC3 I due to extreme hydrophobicity of LC3 II (Kabeya et al., 2000; Klionsky et al., 2008; Mizushima et al., 2010). As shown in Figure 4.9, levels of LC3 II significantly increased in VE lung adenomas in a time-dependent manner, and reached its peak with a ~1.4-fold increase at 10 weeks compared with WT lungs. These results indicate accumulation of autophagosomes in $^{V600E}$Braf-induced adenomas.

In addition, Beclin-1 (the mammalian orthologue of ATG6 in yeasts), which forms complex with class III PI3K Vps34, plays an important role in the nucleation of autophagosomes and assembly of the initial phagophore membrane (Petiot et al., 2000; Simonsen and Tooze, 2009). Levels of Beclin-1 were induced in VE lung adenomas at 10 weeks, but not present in earlier time points compared with the WT lungs.

It has been shown that mTORC2 activates AKT by phosphorylating AKT at Ser473 that in turn phosphorylates and inhibits FOXO3A transcription factor, which transcriptionally activate autophagy related genes (Sarbassov et al., 2005b; Mammucari et al., 2007; Young et al., 2009). The levels of phospho-Akt (Ser473) were determined by
Figure 4.9 Autophagy is induced in senescent lung tumours expressing V600E BRaf

Lung tissues were taken from Braf\textsuperscript{+/+}\textit;CreER}^{T+/-} (WT) mice and Braf\textsuperscript{+/LSL-}\textit;CreER}^{T+/-} (VE) mice aged 3 weeks, 6 weeks, 8 weeks, and 10 weeks. (A) Protein levels of LC3, Beclin-1 and p-Akt (Ser473) were assessed by immunoblotting. ERK2 was used as the loading control. (B) The ratio of LC3 II/LC3 I was quantified.
immunoblotting. As shown in Figure 4.9, phospho-Akt (Ser473) expression decreased at 3 weeks in VE lung adenomas compared with the WT lungs, and was not detectable from 6 weeks to 10 weeks, indicating inhibition of phospho-Akt (Ser473). This correlates with autophagy induction in the VE lung adenomas. Taken together, these results indicate that expression of \textsuperscript{V600E}BRaf induces autophagy in the lung adenomas.

4.3.4 Identification of secreted proteins in CM from senescent lung tumours

4.3.4.1 Preparation of CM from senescent lung tumours
Upregulation of secreted factors has been observed in OIS, such as IGFBP7, IL-6 and IL-8 (Wajapeyee et al., 2008; Kuilman et al., 2008; Young et al., 2009) and their expression in senescent cells has been termed SMS (Ren et al., 2009; Kuilman and Peeper, 2009). We hypothesise that senescent adenoma cells or infiltrating macrophages may produce secreted proteins that can induce or maintain the senescent phenotype. To test this hypothesis, CM was prepared from lung adenomas. As shown in Figure 4.10, lung tissues were taken from WT mice or VE mice at 6 weeks when senescence was induced. Lung tissues were chopped and passed through a cell strainer, and cells were collected and cultured in serum free media (SFM) overnight. To observe the differences within the CM from the WT and VE lungs, CM was subjected to the SDS-PAGE gel and stained with Coomassie Blue. As shown in Figure 4.11, there were more low molecular weight proteins in CM from VE lungs compared with WT lungs. These data indicated that the expression of a large number of proteins is induced by \textsuperscript{V600E}BRaf expression, and some of these may be secreted proteins.

4.3.4.2 Cell growth is inhibited by CM from senescent lung tumours
To test whether the CM from 6 weeks old lung adenomas has any effect on cell growth, MTT assay was performed. As shown in Figure 4.12, NIH 3T3 cell growth was inhibited by 1 \(\mu\)g/ml and 10 \(\mu\)g/ml of CM from VE lungs, although growth inhibition was also observed in 10 \(\mu\)g/ml of CM from WT lungs. These data suggest that factors that secreted in the CM by VE lung adenomas could inhibit cell growth and might mediate the molecular switch from proliferation to senescence following \textsuperscript{V600E}BRaf expression.
Figure 4.10 Preparation of conditioned media from lung tissues

Lung tissue was taken from $\text{Braf}^{+/+};\text{CreERT}^{+/-}$ (WT) mice and $\text{Braf}^{+/LSL-V600E};\text{CreERT}^{+/-}$ (VE) mice aged 6 weeks and was chopped and passed through a 70 µm cell strainer to make a cell suspension. Cells were cultured in serum free media (SFM) for 24h and media was collected and concentrated using Amicon® Ultra-4 Centrifugal Filter Units. This was termed conditioned media (CM).
Figure 4.11 Analysis of CM by SDS-PAGE

The volume of CM was normalised in proportion to the weight of the lung tissue. Equal volumes of CM from a WT lung (WT) and two individual lungs with adenomas (VE1 and VE2) were subjected to SDS-PAGE gels and gels were stained with Coomassie Blue. Bands from ~15-kDa (A), ~20-kDa (B), ~25-kDa (C), and ~37-kDa (D) regions (indicated in red rectangles) in the SDS-PAGE gel were chosen for liquid chromatography tandem mass spectrometry (LC-MS/MS).
**Figure 4.12 Cell growth is inhibited by CM from senescent lung tumours expressing $^{V600E}$$^{BRaf}$**

CM was prepared from lung tissues of $Braf^{+/+};CreER^{T+/-}$ (WT) mice and $Braf^{+/LSL-V600E};CreER^{T+/-}$ (VE) mice aged 6 weeks. Different concentrations of CM (1 µg/ml and 10 µg/ml) from WT and VE lung tissues were applied to NIH 3T3 cells and cell growth was assessed by MTT assay. Cells growing in growth media were used as a negative control. Error bars represent standard deviations.
4.3.4.3 Fractionation of proteins in CM by mass spectrometry

To screen for secreted proteins involved in inducing senescence, CM from WT and VE lungs was processed through SDS-PAGE. As shown in Figure 4.11, proteins at ~15-kDa, ~20-kDa, ~25-kDa and ~37-kDa showed the biggest difference in VE lungs versus WT lungs. Proteins within these fractions were eluted and subjected to LC-MS/MS. We reasoned that secreted proteins would be less than 40-kDa in size, so we concentrated on identifying low molecular weight proteins present in VE lung samples.

4.3.4.4 Identification of proteins in CM

After LC-MS/MS, 152 proteins were identified that were exclusively present in the CM from VE lungs according to the UniPort database. All of the proteins identified are listed in Table 4.1 (Appendix A). This showed a huge diversity in the proteins identified. According to their cellular functions, these proteins are involved in metabolism, such as Glutathione S-transferase kappa1 (Gstk1) and Cytochrome b5 (Cyb5); gene transcription, such as General transcription factor III C1 (Gtf3c1), T-box 6 (Tbx6); Stress response/hypoxia, such as Stanniocalcin 1 (Stc1) and Hypoxia up-regulated 1 (Hyou1); RNA processing related proteins, such as Heterogeneous ribonucleoprotein K (Hnrnpk) and polypyrimidine tract-binding protein 1 (Ptbp1); signal transduction, such as Mitogen-activated protein kinase 14 (Mapk14), Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein and epsilon (Ywhae) (alternatively 14-3-3 epsilon), Ras suppressor protein 1 (Rsu1); cell adhesion, such as Talin1 (Tln1), Actin-related protein 2 homolog (Actr2), Hyaluronan synthase1 (Has1); protein degradation, Cathepsin B (Ctsb), Cathepsin C (Ctsc); and immune response, Interleukin-25 (IL-25), Lectin galactose binding soluble 3 (Lgals3) (alternatively known as Mac-2).

4.3.4.5 Subcellular localisation of proteins identified in CM

Our study was aimed at identifying secreted proteins in the senescent lung CM. Secreted proteins are usually released into the extracellular space by cells. The subcellular localisation of the 152 proteins was determined by cross-reference to the Gene Ontology database (UniPort database and Ensembl database
This is shown in Figure 4.13. These categories are non-exclusive because a protein can be classified in more than one cellular compartment. A significant proportion of proteins (80.51%) were from intracellular locations including cytoplasm, cytoskeleton, Golgi, mitochondria, endoplasmic reticulum, nucleus, lysosome, proteasome. This could be due to disruption of the cells during preparation of the CM. As shown in Figure 4.13, of 152 proteins identified, 19 (8.05%) proteins were classified as extracellular proteins. These proteins included Galectin-1 (Lgals1), Niemann-Pick disease type C2 (Npc2), and phosphoglycerate kinase 1 (Pgk1) that are known to be secreted proteins. 8.05% of the proteins were classified as plasma membrane-associated proteins including Annexin A2 (Anxa2), Annexin A3 (Anxa3), and Fc receptor (Fcamr). 3.39% of the proteins were unclassified. Together, these data suggest that mass spectrometry has successfully been used to identify proteins in the CM following \( V600E \)BRaf expression, and this has identified a few secreted proteins.

Exosomes are small natural membrane vesicles released by a wide variety of cell types into the extracellular compartment by exocytosis and contain numerous extracellular proteins (Hegmans et al., 2008). Prediction of proteins presence in the exosomes was done based on Exocarta database (http://exocarta.ludwig.edu.au) (Mathivanan and Simpson, 2009). As shown in Table 4.1 (Appendix A), 67 proteins (44.1%) were present in exosomes such as Enolase 1, Cathepsin B, and Cathepin C.

### 4.3.4.6 Identification of secreted proteins in CM

Our aim was to find secreted proteins, induced by the expression of \( V600E \)BRaf, which may contribute to the senescent phenotype in lung adenomas in mice. However, most of the proteins (80.51%) we identified were not secreted. They included proteins in RNA processing, metabolic process and other cellular process, and most of them have not been related to cancer phenotype so far. To maximise the possibility of identifying secreted proteins, we setup the following two conditions. First, since signal peptides control the entry of virtually all proteins to the secretory pathway in both eukaryotes and prokaryotes (Gierasch, 1989; von Heijne, 1990; Rapoport, 1992), we assumed that a
Figure 4.13 Subcellular localisation of proteins identified in CM from senescent lung tumours expressing $V_{600}E_{B}R_a f$

The 152 proteins identified were cross-referenced with the Gene Ontology database (UniPort and Ensembl) to determine their subcellular localisation. The number of proteins (A) and the percentage of proteins (B) classified into the subcellular compartments are shown. The cytoplasmic proteins included those classified as cytoskeletal by Gene Ontology. The organellar designation includes proteins localised in the mitochondria, endoplasmic reticulum, the Golgi, the nucleus, peroxisomes, lysosomes, and ribosomes. Unclassified proteins are those that did not have a Gene Ontology classification or whose classification were ambiguous and thus could not be placed in the other four categories. There is redundancy in data as some proteins were placed in more than one compartment.
protein would possess a signal peptide cleavage site. Prediction of signal peptide cleavage sites was done using Ensembl. Second, we performed a literature search to identify proteins that were previously found to be in the extracellular fluids.

After intensive search, as shown in Table 4.2, of 152 proteins identified in total, we found 19 proteins (12.5%) containing a signal peptide cleavage site and 27 proteins (17.8%) were present in extracellular fluids as previously reported (Table 4.1, Appendix A). There were six protein including Cathepsin B (Ctsb), Chi3l1, Interleukin-25 (IL-25), Npc2, Rnase4, and Stc1 satisfied with containing a signal sequence and presence in extracellular fluids. Consistently, these six proteins were also classified as extracellular proteins by Gene Ontology. We speculate that senescent factors contributing to the induction of senescence in the lung following $^{V600E}$BRaf expression could exist in these identified secreted proteins.

4.3.4.7 Validation of secreted proteins
Of the 19 proteins with signal sequence, Stc1, IL-25, and Npc2 were selected for validation. Reason is that all are shown to be secreted into extracellular fluids.

Stanniocalcin 1 (Stc1) is a protein of interest identified through LC-MS/MS. STC1 is a glycoprotein that was first identified in fish as a hormone secreted by the corpuscles of Stannius, an organ unique to bony fish regulating calcium absorption and phosphate excretion (Chang et al., 2003). In mammals, STC1 is also a secreted glycoprotein (Jellinek et al., 2000) and is thought to have an autocrine or paracrine role (Chang et al., 2003). Enhanced STC1 gene expression has been found in hepatocellular, colorectal, and breast carcinomas and medullary thyroid cancers (Fujiwara et al., 2000; Gerritsen et al., 2002; Okabe et al., 2001; Watanabe et al., 2002; McCudden et al., 2004). In contrast, downregulation of STC1 expression was found in breast and ovarian cancer cell lines (Welch et al., 2002; Bouras et al., 2002; Kahn et al., 2000; Ismail et al., 2000). Recently, STC1 was shown to act in a negative feedback loop in the pro-survival ERK1/2 signalling pathway during oxidative stress (Nguyen et al., 2009).
Table 4.2 Proteins with signal sequence* identified in <40 kDa fractions from V600E BRAf senescent lung tumour CM

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Symbol</th>
<th>NCBI Accession no.</th>
<th>Mass (KDa)</th>
<th>Known function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abhydrolase 11</td>
<td>Abhd11</td>
<td>NP_660250</td>
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<td>Member of α/β hydrolase fold domain-containing family</td>
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<tr>
<td>Acid phosphatase 6</td>
<td>Acp6</td>
<td>NP_062774</td>
<td>48</td>
<td>Hydrolyzes lysophosphatidic acid to monoacylglycerol</td>
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<td>Ctsb</td>
<td>NP_031824</td>
<td>37</td>
<td>Lysosomal protease</td>
</tr>
<tr>
<td>Cathepsin C</td>
<td>Ctsc</td>
<td>NP_034112</td>
<td>52</td>
<td>Lysosomal protease</td>
</tr>
<tr>
<td>Chitinase 3-like 1</td>
<td>Chi3l1</td>
<td>NP_031721</td>
<td>43</td>
<td>Member of chitinase family of digestive enzyme</td>
</tr>
<tr>
<td>Cut divalent cation tolerance homologue</td>
<td>CutA</td>
<td>NP_080583</td>
<td>19</td>
<td>Copper tolerance in <em>E. coli</em></td>
</tr>
<tr>
<td>Cytochrome b5 reductase 3</td>
<td>Cyb5r3</td>
<td>NP_084063</td>
<td>34</td>
<td>Electron donor for cytochrome b5</td>
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<tr>
<td>FK506 binding protein 2</td>
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<td>NP_032046</td>
<td>13</td>
<td>Peptidyl-prolyl cis-trans isomerase activity</td>
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<tr>
<td>Hyaluronan synthase 1</td>
<td>Has1</td>
<td>NP_032241</td>
<td>65</td>
<td>Membrane-bound enzyme to produce hyaluronan</td>
</tr>
<tr>
<td>Hypoxia upregulated 1</td>
<td>Hyou1</td>
<td>NP_067370</td>
<td>111</td>
<td>Response to hypoxia/stress</td>
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<tr>
<td>Leucine rich repeat and fibronectin type III domain containing 4</td>
<td>Lrfn4</td>
<td>NP_700437</td>
<td>67</td>
<td>Integral membrane protein member of Lrfn family</td>
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<tr>
<td>N-acetylgalactosaminytransferase 12</td>
<td>Glt12</td>
<td>NP_766281</td>
<td>66</td>
<td>Catalyses the initial reaction in O-linked oligosaccharide biosynthesis</td>
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<td>Cholesterol binding protein</td>
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<td>NP_035162</td>
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<td>ER resident protein catalysing the formation and breakage of disulphide bonds</td>
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<tr>
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<tr>
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<td>Stc1</td>
<td>NP_033311</td>
<td>28</td>
<td>Calcium &amp; phosphate metabolism/oxidative stress</td>
</tr>
</tbody>
</table>

*Signal sequence prediction was based on Ensembl (www.ensembl.org)
As shown in Figure 4.14, Stc1 was exclusively expressed at high levels in CM from VE lungs but was not detectable in WT lungs. Consistently, the expression of Stc1 in the lung adenomas was significantly induced and maintained at high levels from 3 weeks to 10 weeks, whereas its expression was virtually absent in WT lungs (Figure 4.15). These data suggest expression of Stc1 is tumour-specific and may correlate with hypoxia or stress conditions in the adenomas.

Interleukin-25 (IL-25) is a proinflammatory cytokine belonging to IL-17 family, promoting the T helper 2 (T\(_h\)2)-type immune response by induction of other cytokines including IL-4, IL-5, and IL-13 in multiple tissues. IL-25 can induce NF-κB activation and stimulate the production of IL-8 in cell culture (Fort et al., 2001; Hurst et al., 2002; Pan et al., 2001; Kim et al., 2002; Lee et al., 2001). Emerging evidence has also shown that IL-25 is involved in allergic (Angkasekwinai et al., 2007; Tamachi et al., 2006), anti-tumour (Benatar et al., 2008) and anti-inflammatory activities (Owyang et al., 2006; Kleinschek et al., 2007).

The levels of IL-25 in the CM and lung adenomas were assessed. As shown in Figure 4.14 and 4.15, there were multiple bands detected in VE CM and VE lung tissues using a mouse specific antibody against IL-25 by immunoblotting, but different to its reported size (16-kDa). It has been known that IL-17 family members form glycosylated homodimers (Aggarwal and Gurney, 2002; Moseley et al., 2003; Hurst et al., 2002). In our study the bands migrating at higher molecular weight could be IL-25 homodimers or glycosylated forms. It is interesting that these slower migrating forms are very abundant in VE CM and VE lung tissues than controls. Further confirmation must be undertaken by knockdown experiments.

Taken together, these data show that Stc1 and IL-25 are secreted proteins and their expression is induced by \(^{V600E}BRaf\) in the lung adenomas.
Figure 4.14 Validation of secreted protein Stc1 and IL-25 in CM

CM was prepared from lung tissues of \( \text{Braf}^{+/+};\text{CreER}^{+/-} \) (WT) mice and \( \text{Braf}^{+/LSL-V600E};\text{CreER}^{+/-} \) (VE) mice aged 6 weeks. Protein levels of Stc1 and IL-25 in the CM were assessed by immunoblotting. Serum free media (SFM) was used as the negative control.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
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<td></td>
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<tr>
<td>SFM-2</td>
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<tr>
<td>WT/WT</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>WT/Cre</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>VE/Cre-2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Stc1

IL-25 16-kDa
Figure 4.15 Expression of secreted protein Stc1 and IL-25 in senescent lung tumours expressing $V_{600E}BRaf$

Lung tissues were taken from $Braf^{+/+};CREERT^{+/0}$ (WT) mice and $Braf^{+/LSL-V_{600E};CREERT^{+/0}}$ (VE) mice aged 3 weeks, 6 weeks, 8 weeks, and 10 weeks. Protein levels of Stc1, and IL-25 were assessed by immunoblotting. Actin was used as the loading control.
4.3.5 Characterisation of the cholesterol binding protein Npc2

4.3.5.1 Validation of Npc2 is induced in CM

Interestingly, the Niemann-Pick disease type C2 protein (Npc2), a 16-kDa secreted protein, was identified from the proteomic screen (Table 4.1). Npc2 protein is a small soluble protein that is related to Niemann-Pick disease type C (NPC) that is an autosomal recessive, progressive neurodegenerative disorder characterised by abnormal cholesterol trafficking and by intracellular accumulation of unesterified cholesteryl and glycosphingolipids in late endosomes and lysosomes (Vanier, 2010). NPC is caused by loss of function mutations in either the NPC1 or NPC2 gene, whose protein products mediate proper intracellular lipid transport (Vanier, 2010). NPC2 encodes a 151-amino acid proprotein with a cleaved 19-amino acid signal peptide that is localised to the lysosome (Naureckiene et al., 2000). Schematic representation of the human NPC2 gene and protein is shown in Figure 4.16. This protein, which is present at high levels in mammalian epididymal fluid (Kirchhoff et al., 1996; Fouchecourt et al., 2000) and bovine milk (Larsen et al., 1997), binds cholesterol (Okamura et al., 1999) and functions as a cholesterol transporter protein in vitro (Baker et al., 1993; Cheruku et al., 2006). Exogenous addition of purified NPC2 protein was able to correct the cholesterol-accumulation phenotype of human NPC2−/− fibroblasts via the mannose-6 phosphate receptor (MPR)-mediated endocytosis (Ko et al., 2003; Liou et al., 2006; Naureckiene et al., 2000).

To validate Npc2 as a secreted protein in our study, the expression levels of Npc2 in CM was determined by immunoblotting. As shown in Figure 4.17, Npc2 was significantly induced in the CM from VE lungs compared with WT lungs. There were two different isofromes detected by immunoblotting reflecting the known heterogeneity of the N-glycosylation of Npc2. This experiment confirmed that Npc2 is present in CM.
The human *NPC2* gene is composed of 5 exons. NPC2 protein is a small soluble 151-amino acid glycoprotein containing a 19-amino acid signal peptide. Four evolutionary constrained regions (A, B, C and D) are identified in the NPC2 protein. Constrained region D appears to be involved in cholesterol binding. There are three potential N-glycosylation sites identified, of which only Asn58 is important for proper targeting and function of the protein. Six cysteine residues are predicted to form three pairs of intramolecular disulfide bonds.
4.3.5.2 Npc2 is induced in senescent lung tumours

First, to determine the Npc2 expression, immunoblot analysis of the lung tissues from WT mice and VE mice over a time course was performed. As shown in Figure 4.18, levels of Npc2 were dramatically upregulated in VE lungs by ~10-fold at 3 weeks compared with WT lungs. Levels of Npc2 continued to be induced by ~20-fold at 6 weeks and reached its peak of ~35-fold at 8 weeks compared with WT lungs. At 10 weeks, levels of Npc2 decreased slightly, but still had a ~25-fold increase compared with WT lungs.

Second, the levels of Npc2 expression in WT lungs and VE lungs were examined by immunohistochemistry. As shown in Figure 4.19, the levels of Npc2 expression increased dramatically from 3 weeks to 6 weeks in VE lungs compared with WT lungs, consistent with immunoblot analysis. Npc2 expression was detected in alveolar macrophages and tumour cells as well as in the extracellular environment. These results suggest that Npc2 expression is significantly induced in the lung adenomas following V600EBRaf expression.

4.3.5.3 Npc2 is induced at the transcriptional and post-transcriptional level

To determine whether the increased Npc2 protein level results from an increase in transcription or not, RT-PCR analysis of Npc2 mRNA expression over a time course in the lung was performed. As shown in Figure 4.20, levels of Npc2 mRNA was increased from 3 weeks to 8 weeks by ~2-3 folds then decreased at 10 weeks in the VE lungs compared with WT lungs. Although this increase mirrors that of the Npc2 protein, the level of induction is much lower suggesting post-transcriptional effects are also contributing to this. These results indicate that Npc2 is induced at the transcriptional level following the expression of V600EBRaf in the lung adenomas, but post-transcriptional modification may stabilise the Npc2 protein.
Figure 4.17 Validation of Npc2 is induced in CM from senescent lung tumours expressing V600E BRaf

CM was prepared from lung tissues of Braf^{+/+};CreER^{T+/-} (WT) mice and Braf^{+/LSL-V600E};CreER^{T+/-} (VE) mice aged 6 weeks. Protein levels of Npc2 in the CM were assessed by immunoblotting. Serum free media (SFM) was used as the negative control.
Figure 4.18 Npc2 is induced in senescent lung tumours expressing $^{V600E}B\text{Raf}$

Lung tissues were taken from $Braf^{+/+};\text{CreER}^{T+/0}$ (WT) mice and $Braf^{+/LSL-V600E};\text{CreER}^{T+/0}$ (VE) mice aged 3 weeks, 6 weeks, 8 weeks, and 10 weeks. (A) Protein levels of Npc2 were assessed by immunoblotting. ERK2 was used as the loading control. (B) Quantitated expression of Npc2 was normalised to ERK2.
Figure 4.19 Npc2 is induced in senescent lung tumours

Histological sections taken from lung tissues of $Braf^{+/+};{\text{Cre}}ER^{T+/0}$ (WT) mice and $Braf^{+/LSL-V600E};{\text{Cre}}ER^{T+/0}$ (VE) mice aged 3 weeks and 6 weeks were stained with an antibody for Npc2. Scale bar, 100 $\mu$m. This work was undertaken by Susan Giblett.
Figure 4.20 Npc2 mRNA is induced in senescent lung tumours expressing \textsuperscript{V600E}BRaf

Total RNA was isolated from lung tissues of Braf\textsuperscript{+/+};CreER\textsuperscript{T+/0} (WT) mice aged at 6 weeks and Braf\textsuperscript{+/LSL-V600E};CreER\textsuperscript{T+/0} (VE) mice aged 3 weeks, 6 weeks, 8 weeks, and 10 weeks. (A) Npc2 mRNA expression was determined by RT-PCR. Gapdh was used as the internal control. (B) Npc2 mRNA levels were normalised to those of Gapdh by densitometric analysis.
4.3.5.4 Npc2 is found in blood plasma of mice with lung tumours

Npc2 was identified as a secreted protein in lung adenomas at 6 weeks by mass spectrometry. To examine whether Npc2 is secreted and present in the blood plasma, whole blood was collected from WT mice and VE mice and blood plasma was prepared over a time course of 3 weeks, 6 weeks, and 8 weeks. Immunoblot analysis was performed to detect Npc2 in the blood plasma. As shown in Figure 4.21A, Npc2 was present at high levels in the blood plasma of VE mice from 6 weeks to 8 weeks, but not 3 weeks. The protein was barely detected in blood plasma from WT mice. This result is consistent with previous study that NPC2 was detected in the plasma in human and mouse (Klein et al., 2005). Consistently, the expression level of Npc2 in blood plasma over the time course approximately correlated with the level of expression of the recombined $Braf^{\text{L-oX-V600E}}$ allele (Figure 4.21B). These data confirm that Npc2 is a secreted protein whose expression increases following the expression of $V600E$BRaf in lung adenomas.

4.3.6 Is Npc2 a downstream target for the BRaf/MEK/ERK signalling pathway?

As Npc2 is induced at the mRNA level and protein level following expression of $V600E$BRaf in the lung adenomas, we investigate that whether Npc2 is a downstream target for the BRaf/MER/ERK signalling pathway. To test this hypothesis, $Braf^{\text{f/LSL-V600E};\text{CreER}^{T+/-}}$ primary MEFs were treated with 50 nM 4-OHT to activate $V600E$BRaf expression and levels of intracellular Npc2 were assessed by immunoblotting. As shown in Figure 4.22, 4-OHT treatment significantly induced Npc2 expression compared with ethanol control treatment in the $Braf^{\text{f/LSL-V600E};\text{CreER}^{T+/-}}$ and $Braf^{+/+};\text{CreER}^{T+/-}$ primary MEFs. Npc2 present in the CM increased significantly from $Braf^{+/+};\text{CreER}^{T+/-}$ and $Braf^{\text{f/LSL-V600E};\text{CreER}^{T+/-}}$ primary MEFs upon 4-OHT treatment compared with ethanol control treatment. Taken together, these data suggest that Npc2 expression is induced by $V600E$BRaf, but partially dependent on Cre recombinase activity or 4-OHT treatment.
Figure 4.21 Npc2 is present in blood plasma of mice expressing V600E BRaf

Braf^{+/+}; CreER^{T2/0} (WT) mice and Braf^{+/LSL-V600E; CreER^{T2/0}} (VE) mice aged 3 weeks, 6 weeks, and 8 weeks were anaesthetised and whole blood was collected by cardiac puncture. Plasma was separated by centrifugation. (A) Protein levels of Npc2 in plasma were assessed by immunoblotting. (B) Genomic DNA was extracted from lung tissues followed by recombination PCR. The top band indicates the presence of the recombined \textit{Braf}^{\text{Lox-V600E}} allele, while the bottom band indicates the presence of the \textit{wtBraf} allele.
To avoid the interference with Cre recombinase, ∆BRaf:ER cells were used to test this hypothesis. In the ∆BRaf:ER cells, expression of the protein kinase domain of mouse BRaf is inducible by addition of 4-OHT resulting in constitutive activation of the BRaf/MEK/ERK signalling pathway (Pritchard et al., 1995). As shown in Figure 4.23, 4-OHT treatment induced constitutive activation of phospho-ERK in a time-dependent manner, indicating that the expression of the BRaf kinase domain is activated. However, Npc2 expression was not induced following 4-OHT treatment of ∆BRaf:ER cells. Together, these data would suggest that Cre recombinase induces Npc2 expression.

As AP-1 complex and Ets transcription factors are well known downstream targets for the RAF/MEK/ERK signalling pathway, PROSCAN Version 1.7 program (http://www-bimas.cit.nih.gov/molbio/proscan/) was used to search DNA binding sites for these transcription factors within Npc2 promoter region. However, no AP-1, Ets and other known transcription binding sequences were found in the promoter region of Npc2 gene (data not shown), indicating that Npc2 might not be a direct downstream target for the RAF/MEK/ERK signalling pathway.
**Figure 4.22 Npc2 is not specifically induced following activation of V600E BRAf in MEFs**

Braf<sup>+/+;CreER<sup>T<sup>+/0</sup></sup> (WT/Cre) and Braf<sup>+/+;LSL-V600E;CreER<sup>T<sup>+/0</sup></sup></sup> (VE/Cre) primary MEFs were treated with 50 nM 4-OHT or ethanol control for 96h. Cultured media was collected and concentrated as CM. (A) Levels of Npc2 in CM and levels of Npc2 and PP-ERK in the total lysates from MEFs were assessed by immunoblotting. ERK2 was used as the loading control.
Figure 4.23 Npc2 is not induced following activation of ΔBRaf:ER

ΔBRaf:ER cells were treated with 1 μM 4-OHT or ethanol control over a time course for 0, 10 min, 1h, 4h, 8h, 16h and 24h. Levels of Npc2 and PP-ERK were assessed by immunoblotting. ERK2 was used as the loading control.
4.4 Discussion

In our study, expression of an endogenous $^{V600E}$Braf allele at physiological levels induced lung adenomas in mice resulting from an initial burst of proliferation followed by cessation of proliferation and induction of senescence. Lung adenomas arose following spontaneous expression of $^{V600E}$Braf due to leaky expression of the CreER recombinase. Leak expression of Cre has been reported in various tissues using the CreER$^T$ system in mice (Vooijs et al., 2001; Sayama et al., 2010; Mao et al., 1999; Kemp et al., 2004; Haldar et al., 2009), resulting from a consequence of inappropriate nuclear transport or proteolysis of the Cre-fusion protein (Vooijs et al., 2001; Nakamura et al., 2006; Nguyen et al., 2009). This renders the conditional expression of target genes 4-OHT-independent.

During the proliferative phase of the lung adenomas, activation of the BRaf/MEK/ERK signalling pathway was essential for initiation and expansion of the adenomas, which was supported by elevated levels of phospho-ERK and an increase in Ki67 positive staining. Cyclin D1, a downstream target of the BRaf/MEK/ERK signalling pathway, was induced upon ERK activation, which may contribute to the cell proliferation observed in the lung adenomas. This result was consistent with a study of a conditional knockin $^{V600E}$Braf mouse model with adenoviral Cre carried out by Dankort and colleagues (Dankort et al., 2007). In this study, MEK inhibition completely prevented lung adenoma formation, indicating $^{V600E}$Braf-induced cell proliferation in the adenomas is dependent on MER/ERK signalling (Dankort et al., 2007). Similarly, when $^{V600E}$Braf was activated in a mouse model of colorectal cancer, $^{V600E}$Braf drove MEK-dependent crypt hyperplasia (Carragher et al., 2010). Together, these data suggest that activation of the BRaf/MEK/ERK signalling pathway is crucial for initiation and expansion of lung adenomas following $^{V600E}$Braf expression.

However, $^{V600E}$Braf-induced proliferation in the adenomas was not sustained. At 6 weeks, levels of phospho-ERK were significantly reduced and not detectable at 10 weeks. Consistently, Ki67 positive staining decreased at 8 weeks and only a few
proliferating cells were detected at 10 weeks. Growth arrest occurred in the lung adenomas and there was no progression to adenocarcinoma. OIS in vivo has been recognised as a potent barrier against oncogenic transformation by suppressing unscheduled proliferation of early neoplastic cells (Campisi, 2005; Collado and Serrano, 2006). It has been reported that expression of \( \text{V600E} \text{BRaf} \) in the lung (Dankort et al., 2007), gastrointestinal duct (Carragher et al., 2010), and in melanocytes (Dankort et al., 2009; Dhomen et al., 2009) induces benign tumours in mice accompanied by induction of senescence. In our study, induction of \( p16^{\text{Ink4a}} \) and \( p21^{\text{Cip1}} \) were detected by RT-PCR analysis, and upregulation of Dec1 was observed by immunohistochemistry. Our result supports the view that the p53/p21\(^{\text{Cip1}} \) and p16\(^{\text{INK4A}}\)/pRb pathways are activated upon entry into the senescence and function as two tumour suppressor pathways (Campisi, 2005; Campisi and d’Adda di Fagagna, 2007; Serrano et al., 1997). Consistent with our study, p16\(^{\text{INK4a}}\) was also detected in the lung adenomas induced by \( \text{G12V} \text{KRas} \) by immunohistochemistry (Collado et al., 2005). These results support the view that p16\(^{\text{INK4A}}\) is activated by Ets1 through the RAS/RAF/MEK/ERK pathway in senescence (Ohtani et al., 2001). As Dankort reported, \( p19^{\text{Arf}} \) was induced in \( \text{V600E} \text{BRaf} \)-induced senescence in mice and inactivation of \( p53 \) or \( \text{Ink4a/Arf} \) promoted adenomas progression (Dankort et al., 2007). We believe that the induction of \( p21^{\text{Cip1}} \) mRNA we observed may be due to upregulation of the \( p19^{\text{Arf}}/p53 \) pathway following oncogenic \( \text{V600E} \text{BRaf} \) expression in the lung. However, no \( p19^{\text{Arf}} \) expression has been shown to be induced in other studies with \( \text{V600E} \text{BRaf} \) expression in gastrointestinal crypts, mouse and human melanocytes (Carragher et al., 2010; Dhomen et al., 2009; Michaloglou et al., 2005). SA-\( \beta \)-gal is a common used senescence marker and it has been detected in \( \text{G12V} \text{KRas} \)-induced lung adenomas (Collado et al., 2005) and \( \text{V600E} \text{BRaf} \)-induced gastrointestinal crypt senescence (Carragher et al., 2010) and senescent nevi in mouse models (Dhomen et al., 2009). However, in our study we failed to detect SA-\( \beta \)-gal in the lung adenomas, which is consistent with the study carried out by Dankort et al., (2007). Thus, mechanism of OIS is tissue-specific.
Another line of evidence to explain the cessation of proliferation and induction of
senescence might be a link to autophagy. It has been shown that senescent human
fibroblasts contain increased number of autophagic vacuoles and SA-β-gal positive
staining (Gerland et al., 2003). In our study, we detected induction of autophagy
markers of LC3 and Beclin-1 in \textsuperscript{V600E}BRaf-expressing lung adenomas during the
senescent phase. In agreement with our study, it has been shown that overexpression
of \textsuperscript{V600E}BRAF inhibits growth of melanoma cells \textit{in vitro} and melanoma xenografts \textit{in vivo}
(Maddodi et al., 2010). Further analysis revealed that senescence is induced by
upregulation of SA-β-gal and p16\textsuperscript{INK4A} and downregulation of acetylated histone H3, and
most importantly, autophagy was triggered in melanoma cells and melanoma xenografts
by upregulation of LC3 (Maddodi et al., 2010). These data suggest that expression of
\textsuperscript{V600E}BRAF could induce senescence and trigger autophagy. Beclin-1 is an important
autophagy marker and is involved in initiation and nucleation of autophagosomes
(Petiot et al., 2000; Simonsen and Tooze, 2009), whose expression was induced
following \textsuperscript{V600E}BRaf expression, but only increased at the 10 weeks in the adenomas in
our study.

It has been shown that mTOR activation is required for growth of melanoma cells
containing \textsuperscript{V600E}BRAF mutation (Karbownikczek et al., 2008; Maddodi et al., 2009). We
found that phospho-Akt (Ser473) was downregulated following \textsuperscript{V600E}BRaf expression in
the adenomas. It has been found that AKT is phosphorylated and activated by mTORC2
at Ser473, and phospho-AKT (Ser473) is able to phosphorylate and inactivate FOXO3A
transcription factor that can transactivate key ATG genes involved in autophagy
induction (Sarbassov et al., 2005b; Mammucari et al., 2007). In agreement with our
result, Young et al., (2009) showed that phospho-AKT (Ser473) is downregulated during
\textsuperscript{G12V}HRAS-induced senescence accompanied by autophagy activation. Thus,
downregulation of phospho-Akt (Ser473) potentially correlates with induction of
autophagy in our study. Therefore, expression of \textsuperscript{V600E}BRaf in the lung not only induces
senescence but seems to trigger autophagy, possibly by inhibition of the mTOR
pathway.
Senescence-messaging secretome (SMS) has been recognised as a new effector mechanism of senescence that acts in an autocrine/paracrine manner to reinforce the senescent phenotype (Ren et al., 2009; Kuilman and Peeper, 2009). It has been shown in other systems that secreted protein IGFBP7, IL-6, and IL-8 mediate senescence induced by \( V^{600E} \)BRaf in an autocrine/paracrine manner and knockdown of these proteins bypasses OIS (Kuilman et al., 2008; Wajapeyee et al., 2008). The production of IGFBP7, IL-6, and IL-8 has been shown to be upregulated in autophagy-enabled senescence (Young et al., 2009). Defects in autophagy delayed IL-6 and IL-8 production leading to the bypassing of senescence (Young et al., 2009). These data led us to hypothesise that a senescent factor, which is secreted, is induced by \( V^{600E} \)BRaf activation to mediate induction of senescence and autophagy in the lung adenomas. However, we failed to detect IL-6, IL-8, or IGFBP7 in our study suggesting other factors could be involved.

Mass spectrometry has been used in this study to screen for secreted proteins contributing to induction of senescence by \( V^{600E} \)BRaf in the lung. CM from \( V^{600E} \)BRaf-expressing senescent lung adenomas was used as a source of protein. We identified 152 proteins in the CM from the lung adenomas rather than WT lungs. However, a significant proportion of proteins we identified were intracellular proteins involved in metabolism and catabolism and they most likely arose either from breakdown of cells or were secreted through exosomes. Compared with published proteomic data, the proteins identified in our proteomics in CM were also present in some lung cancer proteomic studies from mouse (Zhang et al., 2008b; Lee et al., 2009; Zhong et al., 2008) and human (Tian et al., 2007; Huang et al., 2006; Oh et al., 2001b; Seike et al., 2004; Kim et al., 2008; Xiao et al., 2005; Planque et al., 2009). Several proteins identified in our study have also been recognised as novel biomarkers in lung cancer. These data suggested that the secretome identified in CM from our mouse lung tumours expressing \( V^{600E} \)BRaf shares some features with those of other lung cancer proteomic studies in both human and mouse lung cancer.
Most of the proteins we identified are not secreted through known secretory pathways. After prediction of secreted proteins based on whether a protein containing a signal peptide cleavage site and presence in extracellular fluids or not, 19 secreted proteins were identified in the V600E BRaf CM. As expected, IGFBP7, IL-6, and IL-8 were not identified amongst them. This result supports the view that these proteins are not involved in induction of senescence in the lung model we have established here.

We confirmed that three proteins including Stc1, IL-25, and Npc2 are bona-fide secreted and upregulated in the V600E BRaf-expressing lung. We focused on Npc2 since it is a small soluble glycoprotein secreted at high levels in mammalian epididymal fluid (Kirchhoff et al., 1996; Fouchecourt et al., 2000), bovine milk (Larsen et al., 1997), and plasma and bile (Klein et al., 2006). It is involved in intracellular cholesterol binding and trafficking in late endosomes/lysosomes (Okamura et al., 1999; Ko et al., 2003; Liou et al., 2006; Cheruku et al., 2006). In our study, Npc2 was induced as early as 3 weeks of age and maintained at high expression levels at 6 weeks and 8 weeks in a time course analysis of the CM from lung adenomas. As a secreted protein, Npc2 was also detected in the blood plasma from mice expressing V600E BRaf, but not detectable from WT mice. Npc2 mRNA was induced by ~2-3 fold following V600E BRaf expression. However, the intracellular protein levels of Npc2 were induced by more than ~20 fold. These data suggest that induction of Npc2 in lung adenomas may be due to alterations in protein stability. In agreement with our speculation, recently, it has been shown that NPC2 has been identified as an interacting protein with the Nogo-B receptor (NgBR) through a yeast two-hybrid screen (Harrison et al., 2009). NPC2 protein levels increased in the presence of NgBR and NgBR enhanced NPC2 stability. Knockdown or deficiency in NgBR led to a decrease in NPC2 levels, increased intracellular cholesterol levels and a loss of sterol sensing (Harrison et al., 2009). Together, these data suggest that high levels of Npc2 are induced by V600E BRaf expression in the lung adenomas primarily at the post-transcriptional level. Finally, in a cell model, it was shown that Npc2 is induced by Cre recombinase activity suggesting that Npc2 expression may be a response to DNA damage.
The function of NPC2 in cholesterol binding and trafficking is well established (Ko et al., 2003; Sleat et al., 2004). Emerging evidence also suggests NPC2 may be a cancer biomarker. It has been shown that NPC2 is identified as a potential lung adenocarcinoma biomarker in human pleural effusion from lung adenocarcinoma patients using narrow-range peptide IEF/LC-MS/MS (Pernemalm et al., 2009). It has also been shown that NPC2 is identified in CM from human lung cancer cells (Planque et al., 2009) and in the CM from co-culture of mouse lung adenocarcinoma cells with mouse stromal cells (Zhong et al., 2008) by proteomic studies. It has been shown that a strong cytoplasmic expression of NPC2 is detected in lung adenocarcinomas, with a more prevalent granular pattern in cases with papillary differentiation than in cases with a prevalent glandular differentiation (Pernemalm et al., 2009). Apart from lung cancer, expression of the NPC2 gene has been detected in malignant melanomas by a microarray screen (McDonald et al., 2004). In another study, NPC2 gene was shown to be upregulated in thyroid papillary carcinoma compared to normal tissues by two-dimensional cDNA electrophoresis and immunohistochemistry (Asakawa et al., 2002). In addition, NPC2 was found in the ovarian cancer cell line proteome and ovarian cancer ascites proteome (Faca et al., 2008; Gunawardana et al., 2009; Kuk et al., 2009). However, a role of NPC2 in cancer development has not previously been analysed. Although a few NPC2 patients have been reported to develop cancer, they do not seem to have increased susceptibility to cancer but this may be because they die before adulthood which may preclude an analysis of whether they are more or less susceptible to cancer.

Regarding which types of cells produce NPC2, it has been shown that NPC2 is localised primarily in the alveolar macrophages in the normal human lung, but not in NPC2 patients, which was confirmed by co-localisation of the staining pattern of NPC2 with CD68 which stains macrophages, but not with the MNF116 which stains epithelial cells (Griese et al., 2009). These data provide immunohistochemical evidence to support the view that alveolar macrophages are likely the cells producing NPC2 protein. In our study, infiltrating macrophages occurred surrounding the lung adenomas as an inflammatory
response during the senescent phase. Moreover, Mac-2 was induced by \textsuperscript{V600E}BRaf in the lung adenomas by immunoblot analysis. Taken together, we speculate that alveolar macrophages are likely to be the cells producing Npc2 protein in our study, although immunohistochemistry shows that Npc2 does surround the alveolar type II pneumocytes. And recent data from our laboratory suggests that Npc2 may also be secreted by \textsuperscript{V600E}BRaf-expressing alveolar type II pneumocytes (Tamihiro Kamata, personal communication).

No previous reports have provided evidence for a role of NPC2 in induction of senescence. However, NPC2 is a mannose-6-phosphorylated protein and such protein can be transferred into cells via the cell surface mannose-6 phosphate receptor (MPR) (Ko et al., 2003). It is interesting to note that the IGF2R, also known as the cation-independent mannose-6 phosphate receptor (MacDonald et al., 1988), is involved in several senescence-associated signalling modules (Kuilman and Peeper, 2009). IGF2R is not only required for activation of latent TGF\(\beta\) by plasmin (Dennis and Rifkin, 1991) but also is implicated in IL-6 signalling during senescence. Thus possibly, Npc2 may interfere with functioning of this receptor and affect senescence in this way. Alternatively, effects of Npc2 may be exerted after it has entered the cell possibly by affecting cholesterol distribution.

In summary, expression of endogenous \textsuperscript{V600E}BRaf at physiological levels induces epithelial hyperplasia in the lung, and leads to the formation of lung adenomas in mice. Proliferation is not sustained and subsequently senescence and autophagy are induced to prevent adenomas from progressing to adenocarcinomas. It has been found that the BRaf/MEK/ERK signalling pathway is upregulated during initiation of the lung adenomas and dramatically downregulated when senescence occurs. Senescence is primarily mediated by the \(p53/p21^{\text{Cip1}}\) and \(p16^{\text{Ink4a}}\) pathways. This is the first time that induction of autophagy is reported in mice expressing a conditional knockin \(\text{Braf}^{\text{Lox-V600E}}\) allele at physiological levels and autophagy induction is likely to be regulated by inhibition of the mTOR pathway. A number of secreted proteins were identified by mass spectrometry in
conditioned media from lung adenomas expressing $^{\text{V600E}}\text{B}Raf$, among which is the cholesterol binding protein Npc2. It has been found that the Npc2 protein is induced more than 20 fold in $^{\text{V600E}}\text{B}Raf$-expressing lung adenoma. As a secreted protein, Npc2 was also detected in blood plasma from $^{\text{V600E}}\text{B}Raf$-expressing mice. Further studies of the role of Npc2 in senescence and autophagy are provided in Chapter 5.
5.1 Introduction

Senescence was first described in normal human diploid fibroblasts as a state of irreversible cell cycle arrest after a finite number of divisions, which was termed replicative senescence (Hayflick, 1965). Senescence can also be triggered prematurely by a variety of cellular stress including oncogene activation, inadequate culture conditions, and DNA damaging agents, and is referred to as premature senescence (Ben-Porath and Weinberg, 2004; Toussaint et al., 2002). Now, senescence has been recognised as a cellular stress response and functions as a powerful tumour suppressor mechanism both in vitro and in vivo in preventing neoplastic transformation (Campisi, 2005; Collado and Serrano, 2006). The p53/p21^{CP1} and p16^{INK4A}/pRb pathways are frequently activated upon entry into senescence and have been accepted as master regulators of senescence. Inactivation of these two pathways abrogates the senescent phenotype and promotes proliferation (Campisi, 2005; Collado and Serrano, 2006). Consistently, these two pathways are frequently disabled in cancer cells owing to mutations or deletions, contributing to neoplastic transformation (Sherr and McCormick, 2002; Ben-Porath and Weinberg, 2004).

Upregulation of secreted proteins is observed in cells undergoing replicative senescence or OIS (Collado et al., 2005; Kuilman et al., 2008; Shelton et al., 1999). It has been also shown that the induction of senescence requires several secreted proteins, including the key members of Wnt, IGF1, TGFβ, plasmin and interleukins (ILs); they appear to act as signals to allow for communication between senescent cells and their microenvironment (Kuilman and Peeper, 2009). Collectively, these senescent factors are termed senescence-messaging secretome (SMS) (Ren et al., 2009; Kuilman and Peeper, 2009). A tumour suppressive role for some of the factors secreted by senescent cells has been reported. Secreted protein IGFBP7 was identified to mediate senescence in human diploid fibroblasts and melanocytes induced by oncogenic
V600E BRAF in an autocrine/paracrine manner (Wajapeyee et al., 2008). It has also been shown that proinflammatory cytokines IL-6 and IL-8 are upregulated during V600E BRAF-induced senescence, and knockdown of IL-6, its receptor IL-6R, or IL-8 bypasses senescence induced by V600E BRAF (Kuilman et al., 2008). Altogether, these studies indicate that senescence can be induced by intracellular and extracellular factors that actively suppress tumourigenesis.

In addition to senescence, emerging evidence has shown that autophagy functions as a potent tumour suppressor mechanism (Jin and White, 2007; Levine, 2007; Kundu and Thompson, 2008; Bialik et al., 2008; Levine and Kroemer, 2008). It has been shown that Beclin-1−/− mice (Liang et al., 1999; Qu et al., 2003; Yue et al., 2003), Atg5−/− mice (Mathew et al., 2009) and Atg4c−/− mice (Marino et al., 2007) develop spontaneous tumours, which is correlated with reduced autophagy. These studies suggest that impairment of autophagy promotes tumour development by activation of local inflammation (Degenhardt et al., 2006) or induces chromosomal instability (Karantza-Wadsworth et al., 2007; Mathew et al., 2007b). Recently, it has been shown that autophagy-enabled senescence links these two distinct tumour suppressor mechanisms (Young et al., 2009). In this study, autophagy was activated upon G12V HRAS-induced senescence and was required for the establishment and maintenance of senescence by production of secreted protein IGFBP7, IL-6, and IL-8 (Young et al., 2009). Overexpression of ULK3 was found to sufficiently induce autophagy and premature senescence. Inhibition of autophagy by knockdown of ATG5 and ATG7 attenuated the activation of autophagy and bypassed senescence through delayed production of IL-6 and IL-8 (Young et al., 2009).

In the previous chapter, the secreted protein Npc2 was identified in conditioned medium (CM) from V600E BRAf-expressing senescent lung tumours by mass spectrometry. NPC2 is a small lysosomal glycoprotein secreted at high levels in mammalian epididymal fluids, cow milk, bile, and blood plasma (Kirchhoff et al., 1996; Larsen et al., 1997; Klein et al., 2005) and it functions as a cholesterol binding and trafficking protein in late
endosomes/lysosomes (Infante et al., 2008; Okamura et al., 1999; Ko et al., 2003; Liou et al., 2006). Loss of function mutations in \( \text{NPC2} \) gene are the cause of Niemann-Pick type C2 disease, a fatal autosomal recessive neurovisceral disorder characterised by accumulation of cholesterol in late endosomes/lysosomes (Naureckinene et al., 2000). Exogenously addition of purified NPC2 protein can be delivered to lysosomes via mannose-6 phosphate receptor (MPR)-mediated endocytosis and restore cholesterol levels in \( \text{NPC2}^{+/} \) fibroblasts from patients (Ko et al., 2003; Sleat et al., 2004).

It has been shown that the \( \text{NPC2} \) gene expression is upregulated in thyroid papillary carcinoma by two-dimensional cDNA electrophoresis (Asakawa et al., 2002). In melanomas, the expression of \( \text{NPC2} \) gene was detected in the advanced-stage melanomas by a microarray screen (McDonald et al., 2004). In addition, NPC2 was found in the ovarian cancer cell line proteome and ovarian cancer ascites proteome (Faca et al., 2008; Gunawardana et al., 2009; Kuk et al., 2009). Regarding to lung cancer, it has been shown that NPC2 was identified as a biomarker in the pleural effusion from lung adenocarcinoma patients (Pernemalm et al., 2009), in the CM from human and mouse lung cancer cells (Planque et al., 2009; Zhong et al., 2008) by proteomic studies. However, the role of NPC2 in human cancer is unknown.

In chapter 4, it was found that Npc2 protein is highly expressed in the lung adenomas and blood plasma from mice expressing \(^{\text{V600E}}\text{BRaf} \). Interestingly, the induction of Npc2 expression in the lung adenomas correlated with the cessation of proliferation and induction of senescence and autophagy. These results led us to investigate whether Npc2 functions as a senescent factor that prevents lung adenomas from progressing. Although there is no report that provides indication to its potential functions in cancer biology until now, we are trying to address this in a model of lung cancer.
5.2 Aims

To address the role of the cholesterol binding protein NPC2 as a potential tumour suppressor in lung cancer, bovine NPC2 (bNPC2) protein was purified from cow milk, recombinant human NPC2 (rhNPC2) protein was prepared and human NPC2 (hNPC2) protein was overexpressed in Chinese hamster ovary (CHO) cells.

1. Investigation of the role of the NPC2 protein in forms of bNPC2 and rhNPC2 in suppression of cell growth of non-small cell lung cancer (NSCLC) cells in vitro and overexpression of hNPC2 in suppression of cell growth of CHO cells in vitro, and the downstream signalling pathways involved

2. Assessment of the effect of knockout of Npc2 on progression of lung tumours induced by \textsuperscript{V600E}BRaf \textit{in vivo}
5.3 Results

5.3.1 Bovine NPC2 (bNPC2) suppresses growth of lung cancer cells

5.3.1.1 Purification of bNPC2 from cow milk

To examine the role of NPC2 in tumour suppression, purified NPC2 was needed. It has been found that cow milk is an abundant source of NPC2, into which NPC2 protein is secreted (Larsen et al., 1997). The methodology to purify bovine NPC2 (bNPC2) was available (Larsen et al., 1997; Friedland et al., 2003). The sequence identity between human and bovine proteins is 80%, with another 11% being conserved substitutions (Friedland et al., 2003). The bNPC2 protein was successfully purified by a two-step procedure from cow milk according to the standard protocol (Friedland et al., 2003). The protein in the pooled flow-through fractions from a column of DEAE-Sepharose was separated by ion-exchange on a column of CM-Sepharose. bNPC2 eluted as a peak at 0.15 mM ammonium acetate (Figure 5.1). After dialysing, bNPC2 was concentrated and stored in sterile PBS at -80°C. As shown in Figure 5.2, purified bNPC2 was a ~17 kDa protein due to glycosylation. Finally, bNPC2 protein was validated by LC-MS/MS (Figure 5.3). Appropriately 4 mg bNPC2 protein was obtained from 1 litre cow milk. These data confirmed that bNPC2 was successfully purified from milk.

5.3.1.2 bNPC2 suppresses growth of lung cancer cells in vitro

To test whether bNPC2 has any inhibitory effect on cell growth, MTT assay was performed. In this study, two mouse lung adenocarcinoma cell lines, CMT64 and CMT167, together with NIH 3T3 cells were used. CMT cells were first established by Franks et al (1976) from a spontaneous alveolar lung adenocarcinoma of a C57BL female mouse and several sub-lines with different metastatic potential were subsequently isolated and characterised (Layton and Franks 1984; Franks and Layton, 1984). Adenocarcinoma is the most prevalent type of NSCLC representing 80% of all human lung cancers (Husain et al., 2005) and the mouse and human lung adenocarcinomas share many histological and molecular characteristics (Dragani et al., 1995; Nikitin et al., 2004; Meuwissen and Berns, 2005). CMT64 cells, which are the parental cells with moderate metastatic potential, and sub-line of CMT167 cells with
Figure 5.1 Purification of bovine NPC2 (bNPC2) by ion-exchange

Purification of bNPC2 was performed by ion-exchange on a column of CM-Sepharose. Proteins were eluted with a linear gradient of 0.5 M ammonium acetate pH 5.0 at a flow rate of 3 ml/min. Absorbance at 280 nm was measured. Fractions containing bNPC2 protein were pooled as indicated by the bar.
Figure 5.2 Coomassie Blue staining and immunoblot analysis of purified bNPC2 protein

Fractions 8-13 containing bNPC2 protein eluted from the column of CM-Sepharose were resolved on a SDS-PAGE gel and stained with Coomassie Blue (A) and immunoblotted (B) against a NPC2 polyclonal antibody provided by Prof. Peter Lobel.
Figure 5.3 LC-MS/MS identifies bNPC2 protein purified from cow milk

SDS-PAGE gel containing bNPC2 protein was excised and analysed by LC-MS/MS. bNPC2 protein was identified as the major protein with molecular mass 16,972 Da.
higher metastatic potential were used in the assay. As shown in Figure 5.4A, the growth of CMT64 cells was significantly inhibited with 100 µg/ml bNPC2 treatment giving a reduction of ~60% at 3 days and ~45% at 5 days compared with PBS control treatment. Similar results were obtained with CMT167 (Figure 5.4B) cells and NIH 3T3 cells (Figure 5.4C) with the same treatment. These results suggest that bNPC2 has a growth inhibitory effect on lung cancer cells and immortalised cells when added exogenously, but the mechanism needs to be further investigated.

5.3.1.3 bNPC2 does not induce apoptosis of lung cancer cells in vitro
To examine the mechanism of bNPC2 in growth inhibition, annexin V-FITC staining was performed. As shown in Figure 5.5, there was no obvious apoptosis occurring after bNPC2 treatment compared with PBS control treatment in CMT64 cells. However, these cells were sensitive to adriamycin-induced apoptosis. These data suggest that inhibition of growth by bNPC2 is not mediated by changes in apoptosis.

5.3.1.4 bNPC2 induces senescence of lung cancer cells in vitro
Cellular senescence has been proposed as a proliferating barrier in preventing tumourigenesis (Campisi, 2005; Collado and Serrano, 2006). Firstly, to examine whether bNPC2 is involved in inducing senescence, senescence-associated β-galactosidase (SA-β-gal) was applied. As shown in Figure 5.6, CMT64 cells stained positively for SA-β-gal and had a flattened and enlarged morphology after the bNPC2 treatment indicating that bNPC2 can be senescence-inducing when applied exogenously.

Secondly, to examine the signalling pathways involved in senescence induced by bNPC2, immunoblot analysis was performed to examine the p53/p21Cip1 pathway. Since there was no information regarding p53 status in the CMT64 cells, adriamycin treatment was performed to determine whether p53 is functional or not. As shown in Figure 5.7, levels of p53 were induced by adriamycin treatment in a dose-dependent manner. Accordingly, as it is a transcriptional target of p53, levels of p21Cip1 were induced in a
Figure 5.4 bNPC2 suppresses growth of lung cancer cells in vitro

CMT64 cells (A), CMT167 cells (B) and NIH 3T3 cells (C) grown in growth media were treated with bNPC2 at concentrations of 1 µg/ml, 10 µg/ml, and 100 µg/ml or PBS control for a time course of 5 days. The effect of bNPC2 on cell growth was assessed by MTT assay. Absorbance at 570 nm was measured. Error bars represent standard deviation.
Figure 5.5 bNPC2 does not induce apoptosis of lung cancer cells in vitro

CMT64 cells were treated with 100 µg/ml bNPC2 or PBS control, or left untreated in growth media for 72h. Cells treated with 0.25 µg/ml adriamycin for 24h were used as the positive control. Levels of apoptosis were assessed by Annexin V-FITC staining and data were acquired by flow cytometry.
p53-dependent manner. In addition, p19\textsuperscript{Arf} showed high basal levels of expression in CMT64 cells, and its expression further increased upon high dose of adriamycin treatment. These data confirm that the p53/p21\textsuperscript{Cip1} pathway is functional in CMT64 cells. As reported, p16\textsuperscript{Ink4a} is silenced due to promoter methylation in CMT64 cells (Patel et al., 2000; Herzog et al., 1996), there was no p16\textsuperscript{Ink4a} detected in CMT64 cells (Figure 5.8).

Finally, levels of p53 and p21\textsuperscript{Cip1} after bNPC2 treatment were determined by immunoblotting. As shown in Figure 5.8, both p53 and p21\textsuperscript{Cip1} were induced by 100 $\mu$g/ml bNPC2 treatment with a ~1.5-fold increase compared with PBS control treatment; while Cyclin D1 was significantly reduced after bNPC2 treatment. Induction of nuclear p21\textsuperscript{Cip1} was also confirmed by fluorescence microscopy (Figure 5.9). In addition, the senescence markers p19\textsuperscript{Arf} and Dec1 were also induced by bNPC2 treatment determined by fluorescence microscopy (Figure 5.9). Interestingly, changes in cell shape and a reduction of cell-cell interactions after bNPC2 treatment compared with PBS control treatment were visualised by E-Cadherin fluorescence staining (Figure 5.10). Finally, intracellular NPC2 level increased after bNPC2 treatment in a dose-dependent manner indicating bNPC2 protein is transferred into the cells (Figure 5.8). Taken together, these data suggest that the growth inhibitory effect of bNPC2 in cancer cells might result from senescence induced by bNPC2 treatment and the senescence might be regulated by the p53/p21\textsuperscript{Cip1} pathway.

5.3.1.5 bNPC2 induces autophagy of lung cancer cells \textit{in vitro}

Autophagy is a genetically regulated and evolutionarily conserved cellular mechanism in response to starvation or stress characterised by formation of double-membrane autophagosomes, in which cytoplasmic proteins and organelles are delivered to the lysosome for degradation (Levine and Kroemer, 2008). Recently, autophagy has been proposed to be required for the maintenance of senescence induced by G12V HRAS (Young et al., 2009) and, as a lysosomal protein, NPC2 was shown to induce senescence in our study, we wondered whether NPC2 is also involved in induction of autophagy.
Figure 5.6 bNCP2 induces senescence-associated $\beta$-galactosidase in lung cancer cells \textit{in vitro}

CMT64 cells treated with 100 $\mu$g/ml bNPC2 or PBS control for 72h. Senescence-associated $\beta$-galactosidase staining was performed. Cells were photographed by phase contrast microscopy (400x magnification).
Figure 5.7 The p53/p21<sup>Cip1</sup> signalling pathway is functional in lung cancer cells

CMT64 cells were treated with adriamycin at concentrations of 0.25 μg/ml, 0.5 μg/ml, and 1.0 μg/ml or DMSO control for 24h. Protein levels of p19<sup>Arf</sup>, p21<sup>Cip1</sup>, and p53 were assessed by immunoblotting. ERK2 was used as the loading control.
Figure 5.8 bNPC2 induces the p53/p21Cip1 signalling pathway in lung cancer cells

(A) CMT64 cells were treated with bNPC2 at concentrations of 1 µg/ml, 10 µg/ml and 100 µg/ml or PBS control for 72h. Protein levels of NPC2, p16\textsuperscript{Ink4a}, p21\textsuperscript{Cip1}, p53, and Cyclin D1 were assessed by immunoblotting. ERK2 was used as the loading control. M, medium control; Intra., intracellular. (B) Quantitated expression of p21\textsuperscript{Cip1} and p53 was normalised to ERK2.
Figure 5.9 bNPC2 induces senescence markers of lung cancer cells *in vitro*

CMT64 cells treated with 100 µg/ml bNPC2 or PBS control for 72h were stained with antibodies for p19\(^{Arf}\), p21\(^{Cip1}\), and Dec1, counterstained with DAPI, and examined by fluorescence microscopy. Scale bar, 10 µm.
Figure 5.10 bNPC2 reduces cell-cell interactions in lung cancer cells

CMT64 cells treated with 100 µg/ml bNPC2 or PBS control for 72h were stained with an antibody for E-Cadherin, counterstained with DAPI, and examined by fluorescence microscopy. Scale bar, 10 µm.
Firstly, following bNPC2 treatment of CMT64 cells, the presence of vesicle-like structures in cells was observed. As shown in Figure 5.11, huge numbers of vesicle-like structures were induced in the CMT64 cells treated with bNPC2, while there were nearly no vesicle-like structures visible in the medium or PBS control treatment. Cells containing vesicle-like structures also showed fewer cell-cell interactions. This experiment suggests that bNPC2 might induce autophagy in lung cancer cells.

Secondly, as a widely used marker of autophagy, LC3 was employed to detect autophagy by immunoblotting. The lipid-conjugated form of LC3 (LC3II) localises to the membrane of autophagosomes, which can be separated from the non-conjugated form (LC3I) by immunoblotting. As shown in Figure 5.12, levels of LC3 II accumulated following bNPC2 treatment with a ~1.7-fold increase at 100 µg/ml of bNPC2. In addition, Beclin-1 (the mammalian orthologue of ATG6 in yeasts), an important player in autophagosomes initiation when complexed with class III PI3K Vps34, was induced by bNPC2 treatment (Figure 5.12A).

Thirdly, the subcellular localisation of LC3 after bNPC2 treatment was examined by fluorescence microscopy. The CMT64 cells were stably infected with the pBabe-Puro-GFP-LC3 retrovirus and puromycin resistant colonies were selected. Prominent GFP-LC3 punctate staining was observed after bNPC2 treatment whereas a diffuse distribution of GFP-LC3 occurred in PBS control treatment (Figure 5.13).

Finally, mTOR is a major negative regulator of autophagy. Decreased mTOR activity correlates with induction of autophagy (Sabatini, 2006). Expression of p-4EBP1 and p-p70S6K (Thr389) was decreased after bNPC2 treatment compared with PBS control, indicating that mTORC1 is partially inhibited (Figure 5.12A). Moreover, mTORC2 has been reported to activate p-AKT by phosphorylation of Ser473, resulting in suppression of FOXO3A transcription factor-mediated induction of autophagy genes (Sarbassov et al., 2005b; Mammucari et al., 2007; Young et al., 2009). As shown in Figure 5.12, p-AKT
Figure 5.11 bNCP2 induces vesicle-like structures in lung cancer cells *in vitro*

CMT64 cells treated with 100 µg/ml bNCP2 or PBS control for 72h were photographed by phase contrast microscopy. Cells growing in growth media served as a control. Scale bar, 50 µm
Figure 5.12 bNPC2 induces autophagy of lung cancer cells *in vitro*

(A) CMT64 cells were treated with bNPC2 at concentrations of 1 µg/ml, 10 µg/ml and 100 µg/ml or PBS control for 72h. Protein levels of NPC2, LC3, p-4EBP1, and p-p70S6K (Thr389) were assessed by immunoblotting. ERK2 was used as the loading control. M, medium control. Short exp., short exposure; Long exp., long exposure. (B) The ratio of LC3II/LC3I was quantified.
Figure 5.13 bNPC2 induces redistribution of LC3 in lung cancer cells *in vitro*

CMT64 cells were infected with retroviral pBabe-Puro-GFP-LC3 vector and puromycin resistant colonies were selected and treated with 100 µg/ml bNPC2 or PBS control for 72h. Cells were fixed, counterstained with DAPI, and examined by fluorescence microscopy. Scale bar, 5 µm.
(Ser473) was found to be downregulated by bNPC2 treatment, indicating mTORC2 activity might be inhibited. Taken together, these data suggest that bNPC2 can induce autophagy in lung cancer cells, possibly through downregulation of mTOR activity.

5.3.2 Recombinant human NPC2 (rhNPC2) protein induces autophagy of lung cancer cells

5.3.2.1 Purification of rhNPC2 protein

After testing the growth inhibitory effects of bNPC2 on lung cancer cells, we also examined whether human NPC2 protein can achieve the same effect. To answer this question, rhNPC2 protein was purified from bacteria. Peter Lobel’s group had tried previously to express hNPC2 protein in *Escherichia coli*, but this resulted in misfolding due to formation of both intermolecular and incorrect intramolecular disulfide bonds (Friedland et al., 2003). However, we used *Escherichia coli* Oragami (DE3) strain (Novagen) which helps the protein to form proper disulfide bonds when expressed. The full-length human *NPC2* cDNA was cloned into pET151/D-TOPO vector (Invitrogen) and rhNPC2 protein with 6xHis-tag was purified by using a column of Ni-NTA in our laboratory. This work was undertaken by Bipin Patel. As shown in Figure 5.14, soluble rhNPC2 purified from supernatants of the culture showed a single band at around 20 kDa. This result suggests rhNPC2 protein was successfully purified.

5.3.2.2 rhNPC2 induces autophagy of lung cancer cells *in vitro*

To examine the effect of rhNPC2 protein on lung cancer cells, rhNPC2 protein was applied to CMT64 cells and cell morphology was monitored. As shown in Figure 5.15, growth inhibition was observed in CMT64 cells after 100 μg/ml rhNPC2 protein treatment. Cells were found to be separated from the colonies and vesicle-like structures formed in the cytoplasm, in a similar way to those observed by bNPC2 treatment. No obvious changes were observed at low concentration of rhNPC2 protein-treated cells compared with PBS control. These data suggest that rhNPC2 protein could inhibit lung cancer cell growth.
Figure 5.14 Purification of recombinant human NCP2 (rhNPC2) protein

Full-length human NPC2 (hNPC2) cDNA was cloned into pET151/D-TOPO vector. Recombinant hNPC2 (rhNPC2) protein was expressed in *Escherichia coli* Oragami (DE3) strain and soluble fractions containing hNPC2 were purified by a column of Ni-NTA. rhNPC2 protein was resolved on SDS-PAGE gel and stained with Coomassie Blue. Data were acquired by Bipin Patel.
Figure 5.15 rhNCP2 induces vesicle-like structures in lung cancer cells *in vitro*

CMT64 cells treated with rhNPC2 at concentrations of 1 µg/ml, 10 µg/ml, 100 µg/ml or PBS control for 72h. Cells were photographed by phase contrast microscopy. Cells growing in growth media served as a control. Scale bar, 100 µm
Figure 5.16 rhNCP2 induces autophagy of lung cancer cells *in vitro*

**(A)** CMT64 cells treated with rhNPC2 at concentrations of 1 µg/ml, 10 µg/ml, 100 µg/ml or PBS control for 72h. Protein levels of NPC2, LC3, p21<sup>Cip1</sup>, and p53 were assessed by immunoblotting. ERK2 was used as the loading control. M, medium control; Intra., intracellular. Short exp., short exposure; Long exp., long exposure. **(B)** The ratio of LC3 II/LC3 I was quantified.
Due to limited yields of rhNPC2 protein, growth assays were not carried out. Instead, immunoblot analysis of autophagy markers was performed (Figure 5.16). First of all, intracellular NPC2 level increased after rhNPC2 treatment, suggesting that rhNPC2 protein is transferred into the cell. Second, levels of LC3 II increased by a ~2-fold after rhNPC2 protein treatment compared with PBS control, indicating rhNPC2 could induce formation of autophagosomes. A modest increase in Beclin-1 was only observed at 10 \( \mu g/ml \) of rhNPC2 treatment. These data suggest that rhNPC2 could also induce autophagy in lung cancer cells.

5.3.2.3 rhNPC2 induces the p53/p21\textsuperscript{Cip1} signalling pathway in lung cancer cells

To examine whether rhNPC2 also influences the p53/p21\textsuperscript{Cip1} pathway, immunoblot analysis of p53 and p21\textsuperscript{Cip1} was performed (Figure 5.16). p21\textsuperscript{Cip1} expression was found to be increased at 1.0 \( \mu g/ml \) and 10 \( \mu g/ml \) of rhNPC2 treatment, yet slightly decreased at 100 \( \mu g/ml \) of rhNPC2 treatment. Accordingly, the levels of p53 increased in a similar pattern to p21\textsuperscript{Cip1} upon rhNPC2 treatment. Taken together, these data suggest that rhNPC2 protein can induce the p53/p21\textsuperscript{Cip1} pathway.

5.3.3 Overexpression of hNPC2 induces senescence of Chinese hamster ovary (CHO) cells

5.3.3.1 Overexpression of hNPC2 suppresses growth of CHO cells

In previous experiments, bNPC2 protein was found to induce senescence and autophagy in lung cancer cells; rhNPC2 protein is also able to induce autophagy. To further characterise the role of hNPC2 in growth inhibition, a hNPC2 overexpression system in Chinese ovary (CHO) cells was used. CHO cells stably overexpressing hNPC2 were generated by Liou et al., (2006) by transfection of CHO cells with a full-length human \textit{NPC2} cDNA. These cells were referred to as CHO-hNPC2 cells and used in the following study. Untransfected CHO cells were referred to as CHO-WT cells. Firstly, levels of NPC2 in CHO cells were examined by immunoblot analysis. As shown in Figure 5.17A, both intracellular and extracellular levels of NPC2 were significantly increased, confirming that hNPC2 is overexpressed in CHO-hNPC2 cells and it is a
Figure 5.17 Overexpression of hNPC2 suppresses growth of CHO cells

(A) Intracellular and extracellular levels of hNPC2 were assessed by immunoblotting of CHO-WT and CHO-hNPC2 cells. ERK2 was used as the loading control. Extra., extracellular; Intra., intracellular. (B) Cell growth was assessed by a cell counting assay over a time course in CHO-WT and CHO-hNPC2 cells. (C) DNA synthesis was assessed by EdU incorporation and data were acquired by fluorescence microscopy. Error bars represent standard deviation.
secreted protein. Multiple bands of hNPC2 were detected possibly due to glycosylation of the protein.

Secondly, cell growth was determined. As shown in Figure 5.17B, cell growth in CHO-hNPC2 cells was inhibited compared with that of CHO-WT cells using a cell counting assay. Reduced proliferation of CHO-hNPC2 cells by ~20% compared with CHO-WT cells was also confirmed by EdU staining as detected by fluorescence microscopy (Figure 5.17C). These data suggest that overexpression of hNPC2 suppresses CHO cell growth.

5.3.3.2 Overexpression of hNPC2 induces senescence of CHO cells

To examine whether reduced cell growth correlates with induction of senescence as previously shown with bNPC2 protein treatment, SA-β-gal staining was performed. As shown in Figure 5.18, SA-β-gal activity was significantly increased by ~15% in CHO-hNPC2 cells compared with CHO-WT cells. CHO-hNPC2 cells displayed typical morphological features of senescence. This experiment suggests that reduced cell growth in CHO-hNPC2 cells may result from induction of senescence.

5.3.3.3 Overexpression of hNPC2 induces autophagy of CHO cells

Induction of autophagy by bNPC2 treatment led us to examine whether autophagy could account for the growth inhibition caused by overexpression of hNPC2 in CHO cells. Firstly, the morphology of CHO-hNPC2 and CHO-WT cells was monitored. As shown in Figure 5.19, CHO-hNPC2 cells contained more intracellular vesicle-like structures compared with CHO-WT cells, indicating overexpression of hNPC2 might induce formation of autophagosomes.

Secondly, immunoblot analysis was performed to detect the autophagy marker, LC3. As shown in Figure 5.20, the ratio of LC3 II/LC3 I was significantly increased in CHO-hNPC2 cells with an increase of ~7-fold compared with that of CHO-WT cells. In addition, fluorescence microscopy analysis of endogenous LC3 showed that there was
high expression of LC3 with a punctuate pattern in CHO-hNPC2 cells compared with that of CHO-WT cells (Figure 5.21).

Finally, transmission electron microscopy was applied to directly detect autophagosomes in CHO-hNPC2 cells. The morphological hallmark of autophagy is the formation of the sequestering vesicle, termed an autophagosome (Yorimitsu and Klionsky, 2005). As shown in Figure 5.22, there was a significant difference in overall cell morphology between CHO-WT cells and CHO-hNPC2 cells. There were few vesicles in the CHO-WT cells (Figure 5.22a). However, CHO-hNPC2 cells appeared bigger in size and contained more vesicle-like structures within the cytoplasm (Figure 5.22b). Different stages of autophagic vacuole formation were observed in CHO-hNPC2 cells. As shown in Figure 5.22c and 5.22d, a double-membrane autophagosomes was present. An expanding autophagosomal membrane was shown in Figure 5.22f. An autophagolysosome with partially degrading materials was shown in Figure 5.22e. These data support the view that overexpression of hNPC2 induces autophagy in CHO cells. Taken together, we conclude that overexpression of hNPC2 can induce autophagy and this is accompanied by elevated senescence.

5.3.3.4 Overexpression of hNPC2 induces the p53/p21Cip1 signalling pathway in CHO cells

To examine the signalling pathway involved in the induction of senescence and autophagy, immunoblot analysis was performed. As shown in Figure 5.23, both p53 and p21Cip1 expression were significantly induced in CHO-hNPC2 cells by ~5-fold compared with those in CHO-WT cells. Fluorescence microscopy analysis also confirmed that levels of p21Cip1 were increased in CHO-hNPC2 cells compared with those in CHO-WT cells (Figure 5.23C). These data suggest that the p53/p21Cip1 pathway may account for the growth inhibition of CHO cells, which correlates with the induction of senescence and autophagy.
Figure 5.18 Overexpression of hNCP2 induces senescence of CHO cells

(A) CHO-WT and CHO-hNPC2 cells were fixed and stained for senescence-associated β-galactosidase. Cells were photographed by phase contrast microscopy. Scale bar, 50 µm. (B) The number of SA-β-gal positive cells was quantified. Error bars represent standard deviation.
Figure 5.19 Overexpression of hNCP2 induces formation of vesicle-like structures in CHO cells

CHO-WT and CHO-hNPC2 cells were photographed by phase contrast microscopy. Scale bar, 50 µm.
Figure 5.20 Overexpression of hNCP2 induces autophagy of CHO cells

(A) Protein levels of LC3 were assessed by immunoblotting in CHO-WT and CHO-hNPC2 cells. ERK2 was used as the loading control. (B) The ratio of LC3II/LC3I was quantified. Error bars represent standard deviation.
Figure 5.21 Overexpression of hNPC2 induces redistribution of LC3 in CHO cells

CHO-WT and CHO-hNPC2 cells were stained with an antibody for LC3, counterstained with DAPI, and examined by fluorescence microscopy. Scale bar, 10 µm.
Figure 5.22 Overexpression of hNCP2 induces formation of autophagosomes in CHO cells

Transmission electron microscopy was used to detect autophagosomes in CHO-WT and CHO-hNPC2 cells. Scale bars are indicated in individual figures. a, a typical CHO-WT cell; b-f, typical CHO-hNPC2 cells; c, an autophagosome in CHO-hNPC2 cells; d, a double-membrane autophagosome from c was shown in high-power magnification; e, an autophagolysosome containing partially degrading materials; f, an expanding autophagosomal membrane.
Figure 5.23 Overexpression of hNPC2 induces the p53/p21<sup>Cip1</sup> signalling pathway in CHO cells

(A) Protein levels of p21<sup>Cip1</sup> and p53 were assessed by immunoblotting in CHO-WT and CHO-hNPC2 cells. ERK2 was used as the loading control. (B) Quantitated expression of p21<sup>Cip1</sup> and p53 was normalised to ERK2. (C) CHO-WT and CHO-hNPC2 cells were stained with an antibody for p21<sup>Cip1</sup>, counterstained with DAPI and examined by fluorescence microscopy. Scale bar, 10 µm.
5.3.4 Knockout of Npc2 promotes growth of lung tumours expressing \textsuperscript{V600E}BRaf \textit{in vivo}

5.3.4.1 Generation of \textit{Braf}\textsuperscript{+/LSL-V600E};\textit{CreER}\textsuperscript{T\textsuperscript{+/0}};Npc2\textsuperscript{+/−} mice

In Chapter 4, high levels of NPC2 protein were induced by \textsuperscript{V600E}BRaf expression in lung adenomas and this was accompanied by sustained senescence and induction of autophagy in mice. In addition, purified NPC2 protein has been shown to induce senescence and autophagy in lung cancer cells \textit{in vitro}. It is worth knowing how the adenomas will behave if we knock out the Npc2 gene in mice. To answer this question, \textit{Braf}\textsuperscript{+/LSL-V600E};\textit{CreER}\textsuperscript{T\textsuperscript{+/0}};Npc2\textsuperscript{+/−} mice were generated. All of the mice breeding were set up by Susan Giblett. Npc2\textsuperscript{+/−} mice with mixed genetic background of 129/C57BL/BALB were obtained from Professor Peter Lobel (Sleat et al., 2004) and these mice were crossed with \textit{Braf}\textsuperscript{+/LSL-V600E} mice and \textit{CreER}\textsuperscript{T\textsuperscript{+/0}} mice to generate \textit{Braf}\textsuperscript{+/LSL-V600E};Npc2\textsuperscript{+/−} mice and \textit{CreER}\textsuperscript{T\textsuperscript{+/0}};Npc2\textsuperscript{+/−} mice, respectively. Finally \textit{Braf}\textsuperscript{+/LSL-V600E};\textit{CreER}\textsuperscript{T\textsuperscript{+/0}};Npc2\textsuperscript{−/−} mice were generated from intercrosses between \textit{Braf}\textsuperscript{+/LSL-V600E};Npc2\textsuperscript{+/−} mice and \textit{CreER}\textsuperscript{T\textsuperscript{+/0}};Npc2\textsuperscript{+/−} mice (Figure 5.24).

\textit{Npc2}\textsuperscript{+/−} mice have been reported as hypomorphs expressing 0-4% of residual protein in different tissues (Sleat et al., 2004). These mice were produced due to a consequence of aberrant homologous recombination during the targeting of the Npc2 gene resulting in the insertion of the neo selection marker and part of the left arm containing intron 1 into intron 2 (Figure 5.25A). To PCR genotype the Npc2-targeted mice, two separate PCR reactions were performed to detect wild-type and targeted Npc2 alleles. A typical PCR genotyping result is shown in Figure 5.25B. Mice 1, 3, and 6 had the product PCR3 (419 bp) which determines the presence of an Npc2 targeted allele but does not differentiate between heterozygotes and homozygous targeted mice. Mice 2, 4, 5, 6, and 7 had the product PCR1 (3257 bp) which determines the presence of a wild-type Npc2 allele but does not differentiate between homozygous wild-type and heterozygous mice. The product PCR2 (345 bp) represents the internal control amplified from both targeted and wild-type alleles. Taken together, mice 1 and 3 were identified as Npc2 homozygous knockout. Mouse 6 was Npc2 heterozygous knockout. Mice 2, 4, 5, and 7
Figure 5.24 Generation of $Braf^{+/LSL-V600E}:CreER^{T+/0}:Npc2^{-/-}$ mice

$Npc2^{-/-}$ heterozygous mice were crossed with $Braf^{+/LSL-V600E}$ mice and $CreER^{T+/0}$ mice, respectively, to generate $Braf^{+/LSL-V600E}:Npc2^{-/-}$ mice and $CreER^{T+/0}:Npc2^{-/-}$ mice. Then $Braf^{+/LSL-V600E}:Npc2^{-/-}$ mice were crossed with $CreER^{T+/0}:Npc2^{-/-}$ mice to generate $Braf^{+/LSL-V600E}:CreER^{T+/0}:Npc2^{-/-}$ mice. This work was undertaken by Susan Giblett.
Figure 5.25 Detection of wild-type Npc2 and Npc2 knockout alleles

(A) Targeted disruption of the mouse Npc2 gene (Sleat et al., 2004). Structure of Npc2, the targeting construct, and the targeted allele were shown. Gray bars depicted informative PCR products PCR1, PCR2, and PCR3 for Npc2 genotyping. (B) Genomic DNA was isolated from ears of 7 mice from intercrosses between Braf^{+/LSL-V600E};Npc2^{+/+} mice and CreERT^{+/0};Npc2^{+/+} mice. Standard PCR was performed to detect targeted disruption of the mouse Npc2 gene. The product PCR3 (419 bp) determines the presence or absence of a targeted allele but does not differentiate between heterozygotes and homozygous targeted mice. The product PCR2 (345 bp) represents the internal control amplified from both targeted and wild-type alleles. The product PCR1 (3257 bp) determines the presence or absence of a wild-type allele but does not differentiate between homozygous wild-type and heterozygous mice. PCR to detect the WT\textit{Braf}, Braf^{LSL-V600E} and CreERT alleles was shown.
were identified as Npc2 wild-type. When combined with the genotyping results from $Braf^{SL-V600E}$ and $CreER^T$ alleles, mouse 3 having the both $Braf^{SL-V600E}$ and $CreER^T$ alleles was identified as $Braf^{SL-V600E};CreER^{T+/-};Npc2^{-/-}$. Other mice represented important controls. The PCR genotyping results confirmed that $Braf^{SL-V600E};CreER^{T+/-};Npc2^{-/-}$ mice were successfully generated.

5.3.4.2 Knockout of Npc2 compromises survival in mice

Survival of Npc2 hypomorph mice was monitored. As reported, Npc2 hypomorph mice were born normally and weights dropping and a continuous tremor were first observed at ~55 days, and death of these mice occurred at ~130 days with 129/C57BL/BALB background resulting from ataxia and overall locomotor dysfunction (Sleat et al., 2004).

In our study, Npc2$^{+/+}$ mice and Npc2$^{-/-}$ mice were first observed to die at ~54 days postpartum and 50% of the Npc2$^{-/-}$ mice survived at ~90 days. However, Npc2$^{+/+}$ mice showed a better survival evidenced by 90% of mice survived beyond ~120 when compared with Npc2$^{-/-}$ mice (Figure 5.26). These data suggested that knockout of Npc2 gene reduces survival in mice. Next, lung tissues from Npc2$^{-/-}$ and Npc2$^{+/+}$ mice were examined. Accumulation of eosinophilic protein-rich material was observed in the alveoli from Npc2$^{-/-}$ lung by Haematoxylin and eosin staining (Figure 5.27A), consistent with previous study in Npc2 hypomorph mice with lipoproteinosis (Griese et al., 2010; Sleat et al., 2004). In addition, obvious proliferation in the lung was observed in Npc2$^{-/-}$ mice by an increase in Ki67 positive staining compared with Npc2$^{+/+}$ mice (Figure 5.31B). This might indicate the hyperplasia of alveolar cells that are associated with pulmonary alveolar lipoproteinosis (Bjurulf et al., 2008; Griese et al., 2010). Taken together, these data support that knockout of Npc2 increases cell proliferation companied by increase lipid storage in lung tissues in mice.

5.3.4.3 Knockout of Npc2 reduces survival in mice expressing $^{V600E}BRaf$

To investigate the effect of knockout of Npc2 on survival in mice expressing $^{V600E}BRaf$, mice from intercrosses between $Braf^{SL-V600E};Npc2^{+/+}$ mice and $CreER^{T+/-};Npc2^{-/-}$ mice were monitored by checking health conditions and a survival chart was plotted (Figure
Figure 5.26 Knockout of Npc2 reduces survival in mice expressing V600EBRaf

The Kaplan-Meier plot shows survival of mice used in this study according to the percentage of surviving mice versus survival time (days). Mice were monitored by checking health conditions over a time course. The $\text{Braf}^{+/\text{LSL-V600E}}$;$\text{CreER}^{+/0}$;$\text{Npc2}^{+/+}$ (VE;Npc2$^{+/+}$) group contained 11 mice; the $\text{Braf}^{+/\text{LSL-V600E}}$;$\text{CreER}^{+/0}$;$\text{Npc2}^{-/-}$ (VE;Npc2$^{-/-}$) group contained 7 mice; the $\text{Braf}^{+/\text{LSL-V600E}}$;$\text{CreER}^{+/+}$;$\text{Npc2}^{+/+}$ (VE;Npc2$^{+/+}$) group contained 12 mice; the Npc2$^{+/+}$ group contained 20 mice; the Npc2$^{-/-}$ group contained 3 mice; control mice included all the mice excluded from above (>100 mice). This work was undertaken by Susan Giblett.
**Figure 5.27 Knockout of Npc2 accumulates lipid storage and promotes proliferation in the lung**

Histological sections taken from lung tissues of $\text{Braf}^{+/+};\text{CreER}^{T00};\text{Npc2}^{+/+}$ mice ($\text{Npc2}^{+/+}$) and $\text{Braf}^{+/+};\text{CreER}^{T00};\text{Npc2}^{-/-}$ mice ($\text{Npc2}^{-/-}$) aged 27 days and stained with haematoxylin and eosin (A) and an antibody for Ki67 (B). Arrow indicates the eosinophilic protein-rich material. Individual scale bars are shown. Data were acquired by Susan Giblett.
When combined with the expression of \textsuperscript{V600E}BRaf, the onset of diseases characterised by breathing difficulties and general wasting was observed to arise as early as \( \sim 22 \) days postpartum in \textit{Braf}^{\text{+/-LSL-V600E,CreER}\text{T}^{+/-},Npc2^{-/-}} \) mice and \textit{Braf}^{\text{+/-LSL-V600E,CreER}\text{T}^{+/-},Npc2^{+/+}} \) mice. However, \textit{Npc2} heterozygous or homozygous knockout mice expressing \textit{V600E}BRaf reduced survival dramatically in a time-dependent manner and \( \sim 50\% \) of these mice survived at \( \sim 40 \) days. In contrast, \textit{Npc2}^{+/+} mice expressing \textit{V600E}BRaf showed a better survival and \( \sim 50\% \) of these mice survived by \( \sim 90 \) days. These data indicate that knockout of \textit{Npc2} significantly reduces the survival when combined with expression of \textit{V600E}BRaf in mice.

### 5.3.4.4 Knockout of \textit{Npc2} promotes growth of lung tumours expressing \textit{V600E}BRaf

To investigate whether reduced survival in \textit{V600E}BRaf-expressing mice combined with \textit{Npc2} heterozygous or homozygous knockout correlates with the progression of lung adenomas, histology was performed. Haematoxylin and eosin staining showed that large numbers of papillary adenomas arose in the lung from \textit{Npc2}^{-/-} mice at 18 days postpartum compared with those of \textit{Npc2}^{+/+} mice (Figure 5.28). The adenomas appeared to continue proliferation such that there was extensive hyperplasia with time, forming larger adenomas and merging of the adenomas occurred at 27 days in \textit{Npc2}^{-/-} mice compared with \textit{Npc2}^{+/+} mice. From 47 days to 54 days, more advanced adenomas formed and more infiltrating macrophages and blood vessels occurred in \textit{Npc2}^{-/-} mice compared with \textit{Npc2}^{+/+} mice (Figure 5.28). However, cell counting assays showed that the number of tumours per mouse lung was not altered between \textit{Npc2}^{+/+} and \textit{Npc2}^{-/-} mice (Figure 5.29A). However, the number of cells per tumour was significantly increased in \textit{Npc2}^{-/-} mice compared with those of \textit{Npc2}^{+/+} mice (Figure 5.29B). These data suggest that growth of the adenomas following knockout of \textit{Npc2} is primarily due to cell proliferation in the adenomas expressing \textit{V600E}BRaf. However, the cells within the adenomas from \textit{Npc2}^{+/+} and \textit{Npc2}^{-/-} mice displayed similar morphology characterised with eosinophilic cytoplasm but did not undergo dysplastic changes. So far we have not detected the progression of these adenomas to adenocarcinomas. In addition, there was no tumour metastasis observed in other organs (data not shown). Taken together,
Figure 5.28 Knockout of Npc2 promotes growth of lung adenomas expressing V600E BRaf

Histological sections taken from the lungs of $Braf^{+/LSL-V600E;CreER^{T+/0};Npc2^{+/+}}$ (Npc2$^{+/+}$) mice, $Braf^{+/LSL-V600E;CreER^{T+/0};Npc2^{+/-}}$ (Npc2$^{+/-}$) mice, and $Braf^{+/LSL-V600E;CreER^{T+/0};Npc2^{-/-}}$ (Npc2$^{-/-}$) mice aged 18 days, 27 days, 47 days, and 54 days were stained with haematoxylin and eosin. Scale bar, 200 µm. Data were acquired by Susan Giblett.
Figure 5.29 Knockout of Npc2 increases cell number in lung tumours expressing V600EBRaf

Histological sections taken from the lungs of Braf^+/LSL-V600E;CreERT^+/0;Npc2^+/+ (Npc2^+/+) mice aged 20 days and 27 days, Braf^+/LSL-V600E;CreERT^+/0;Npc2^+-/+ (Npc2^+-/+) mice aged 30 days, and Braf^+/LSL-V600E;CreERT^+/0;Npc2^+-/-(Npc2^-/-) mice aged 27 days and 30 days were stained with haematoxylin and eosin. The number of tumours per mouse lung (A) and the number of cells per tumour (B) were counted. Data were acquired by Susan Giblett.
these data support the view that knockout of Npc2 increases the cell proliferation in adenomas induced by V600EBRaf and reduces survival in mice, but is not sufficient to promote progression to adenocarcinomas.

5.3.4.5 Knockout of Npc2 does not alter the BRaf/MEK/ERK signalling pathway in lung tumours expressing V600EBRaf

The observation that knockout of Npc2 accelerated lung adenomas growth led us to examine the BRaf/MEK/ERK signalling pathway in Npc2 knockout lung adenomas. First of all, the expression of NPC2 protein in homozygous and heterozygous knockout was confirmed by immunoblotting. It has been reported that Npc2 homozygous knockout mice are hypomorphs that express 0-4% of Npc2 protein in different tissues (Sleat et al., 2004). In the lung, Npc2 protein was undetectable (Sleat et al., 2004). Consistent with this, no Npc2 protein was detected in VE/Npc2−/− homozygous knockout adenomas, while ~50% Npc2 protein was detected in VE/Npc2+/− heterozygous knockout adenomas compared with wild-type and VE/Npc2+/+ lung tissues (Figure 5.30). We observed that BRaf expression was not recovered in VE/Npc2−/− homozygous or VE/Npc2+/− heterozygous knockout adenomas at 47 days and 54 days compared with VE/Npc2+/+ adenomas. The expression of CRaf and effectors of the BRaf/MEK/ERK signalling pathway including phospho-ERK, phospho-MEK, Cyclin D1, Cyclin D2, Cdk2, Cdk4, Cdk6 remained similar or still inhibited in VE/Npc2−/− homozygous or VE/Npc2+/− heterozygous knockout adenomas compared with those in VE/Npc2+/+ lung adenomas. Taken together, these data suggest that BRaf/MEK/ERK signalling is not altered in VE/Npc2−/− heterozygous knockout and VE/Npc2+/− homozygous knockout adenomas.

5.3.4.6 Knockout of Npc2 downregulates the p53/p21Cip1 signalling pathway in the lung tumours expressing V600EBRaf

As upregulation of proliferation was observed in Npc2 knockout adenomas, we next examined the senescence pathway. As p16ink4a is not detectable by immunoblot analysis of lung tissues, expression of p53 and p21Cip1 was determined (Figure 5.31). During the proliferative phase, levels of p53 were not altered and p21Cip1 was upregulated in Npc2
Figure 5.30 Knockout of Npc2 does not alter the BRaf/MEK/ERK signalling pathway in lung tumours expressing V600EBRaf

Lung tissues were taken from Braf\(^{+/+}\);CreER\(^{T0;0}\);Npc2\(^{+/+}\) (WT) mice, Braf\(^{+/LSL-V600E}\);CreER\(^{T0;0}\);Npc2\(^{+/+}\) (VE/Npc2\(^{+/+}\)) mice, Braf\(^{+/LSL-V600E}\);CreER\(^{T0;0}\);Npc2\(^{+/+}\) (VE/Npc2\(^{+/+}\)) mice, and Braf\(^{+/LSL-V600E}\);CreER\(^{T0;0}\);Npc2\(^{+/-}\) (VE/Npc2\(^{+/-}\)) mice aged 27 days, 47 days, and 54 days. Protein levels of NPC2, BRaf, CRaf, PP-ERK, P-MEK, Cyclin D1, Cyclin D2, Cdk2, Cdk4, Cdk6, and p-AKT (Ser473) were assessed by immunoblotting. ERK2 was used as the loading control.
Figure 5.31 Knockout of Npc2 downregulates the p53/p21^{Cip1} signalling pathway in the lung expressing V600E BRaf

Lung tissues were taken from Braf^{+/+};CreER^{T0;G};Npc2^{+/+} (WT) mice, Braf^{+/+};LSL-V600E;CreER^{T0;G};Npc2^{+/+} (VE/Npc2^{+/+}) mice, Braf^{+/+};LSL-V600E;CreER^{T0;G};NPC2^{+/+} (VE/Npc2^{+/+}) mice, and Braf^{+/+};LSL-V600E;CreER^{T0;G};Npc2^{-/-} (VE/Npc2^{-/-}) mice aged 27 days, 47 days, and 54 days. Protein levels of p19^{Arf}, p21^{Cip1}, p27^{Kip1}, and p53 were assessed by immunoblotting. ERK2 was used as the loading control.
knockout adenomas. However, at later time points there was a consistent downregulation of p53 and p21<sup>Cip1</sup> in Npc2 heterozygous and homozygous knockout adenomas compared with Npc2 WT adenomas. Similarly, CKI p27<sup>Kip1</sup> was also found to be downregulated at 47 days and 54 days in the Npc2 heterozygous and homozygous knockout adenomas. However, p19<sup>Arf</sup> levels did not consistently change. These data suggest that increased proliferation in Npc2 knockout adenomas might result from downregulation of the p53/p21<sup>Cip1</sup> pathway at later stages (47 days and 54 days) when senescence is normally induced. Suppression of p27<sup>Kip1</sup> may also contribute to proliferation at these stages. Thus, knockout of Npc2 might enable lung adenomas escape from senescence which would result in enhanced proliferation.

5.3.4.7 Knockout of Npc2 does not prevent autophagy in lung tumours expressing V600EBRaf

As shown previously, increased proliferation in lung adenomas might result from an escape from senescence when Npc2 is knocked out. It is also interesting to know whether autophagy is also prevented by Npc2 knockout. Firstly, the effect of Npc2 knockout in mice without the V600E<sup>Braf</sup> oncogene on autophagy was examined by immunoblot analysis of LC3 expression in lung tissues. As shown in Figure 5.32, the ratio of LC3 II/LC3 I was not significantly altered in Npc2<sup>-/-</sup> samples compared with Npc2<sup>+/+</sup> and Npc2<sup>+/−</sup> samples, although there is a trend towards an increase.

Secondly, the effect of Npc2 knockout in the presence of V600E<sup>BRaf</sup> on autophagy was examined. As shown in Figure 5.33, the levels of LC3 conversion were similar in Npc2<sup>+/−</sup> adenomas compared with Npc2<sup>+/+</sup> or Npc2<sup>−/−</sup> adenomas. Taken together, these data suggest that knockout of Npc2 is not sufficient to prevent autophagy in lung adenomas expressing V600E<sup>BRaf</sup>, indicating other factors are likely involved in this phenotype.
Figure 5.32 Expression of LC3 in Npc2 deficient mice

(A) Lung tissues were taken from Npc2⁺⁺⁺, Npc2⁺⁺⁻, and Npc2⁻⁻⁻ mice aged 20 days, 22 days, 23 days, 27 days, 54 days, and 100 days. Genotypes of mice are indicated. Protein levels of NPC2 and LC3 were assessed by immunoblotting. ERK2 was used as the loading control. 
(B) The ratio of LC3 II/LC3 I was quantified. Error bars represent standard deviation.
Figure 5.33 Knockout of Npc2 does not prevent autophagy in lung tumours expressing V600E BRaf

Lung tissues were taken from \( \text{Braf}^{+/+};\text{CreERT}^{0/0};\text{Npc2}^{+/+} \) (WT) mice, \( \text{Braf}^{+/LSL-V600E};\text{CreERT}^{+/-};\text{Npc2}^{+/+} \) (VE/Npc2\(^{+/+}\)) mice, \( \text{Braf}^{+/LSL-V600E};\text{CreERT}^{+/-};\text{Npc2}^{+/+} \) (VE/Npc2\(^{+/+}\)) mice, and \( \text{Braf}^{+/LSL-V600E};\text{CreERT}^{+/-};\text{Npc2}^{-/-} \) (VE/Npc2\(^{-/-}\)) mice aged 27 days, 47 days, and 54 days. Protein levels of LC3 were assessed by immunoblotting. ERK2 was used as the loading control. Short exp., Short exposure; Long exp., Long exposure.
5.4 Discussion

In Chapter 4, it has been shown that expression of V600E BRaf in mice induces lung adenomas that are prevented from progression as senescence occurs and this is accompanied by induction of autophagy. The cholesterol binding protein Npc2 was identified in the CM from adenomas through mass spectrometry, and its expression was significantly induced following expression of V600E BRaf. It has been shown that Npc2 is a secreted protein. Here the role of Npc2 in tumour suppression was investigated.

It has been shown that NPC2 protein is secreted in mammalian epididymal fluids, cow milk, bile, and blood plasma (Kirchhoff et al., 1996; Larsen et al., 1997; Klein et al., 2005) and its function in cholesterol binding and trafficking is well established (Okamura et al., 1999; Ko et al., 2003; Liou et al., 2006; Cheruku et al., 2006). Interestingly, elevated expression of NPC2 gene and protein in certain types of human cancers including melanomas, thyroid papillary carcinoma, ovarian cancer, and NSCLC has been reported through microarray screen or proteomic studies (Asakawa et al., 2002; McDonald et al., 2004; Pernemalm et al., 2009; Faca et al., 2008; Gunawardana et al., 2009; Kuk et al., 2009; Planque et al., 2009; Zhong et al., 2008). However, there has been no direct evidence demonstrating the role of NPC2 in cancer biology. NPC2 patients die in childhood which prevents assessment of its role in cancer although a few have been reported to develop hepatocellular carcinoma (Birch et al., 2003; Pennington et al., 1996). The present study shows for the first time that a cholesterol binding protein NPC2 has growth inhibitory effects on lung cancer cells in vitro and that knockout of Npc2 in mice reduces senescence and promotes lung tumour growth in vivo. These data provide new evidence that secreted NPC2 protein can function as a potential tumour suppressor.

The role of NPC2 in inhibition of lung cancer cell growth was examined. Bovine NPC2 (bNPC2) protein was successfully purified from cow milk. bNPC2 was shown to induce an inhibitory effect on lung cancer cell growth. And further studies confirmed that the p53/p21Cip1 signalling pathway is activated following bNPC2 treatment, together with
increased expression of senescence markers including SA-β-gal activity, p19\textsuperscript{Arf} and Dec1. Similarly, recombinant human NPC2 protein was also shown to induce a growth retardation and upregulation of the p53/p21\textsuperscript{Cip1} signalling pathway in lung cancer cells. We also overexpressed human NPC2 in CHO cells and showed inhibition of growth and induction of the p53/p21\textsuperscript{Cip1} pathway and the senescence marker SA-β-gal.

In our study, when Npc2 was knocked out in mice expressing \textsuperscript{V600E}BRaf, the progression of adenomas was promoted due to increased cell proliferation compared with adenomas expressing WT Npc2. As expected, suppression of the p53/p21\textsuperscript{Cip1} signalling pathway was observed in Npc2 knockout adenomas indicating downregulation of senescence. This is consistent with the \textit{in vitro} data. These data suggest that increased proliferation may correlate with decreased senescence and that loss of Npc2 potentially suppresses the senescence phenotype in lung adenomas. However, the cells within the adenomas from Npc2\textsuperscript{+/+} and Npc2\textsuperscript{-/-} mice displayed similar morphology characterised with eosinophilic cytoplasm but did not undergo dysplastic changes. So far we have not detected the progression of these adenomas to adenocarcinomas. In addition, there was no tumour metastasis observed in other organs. Finally, it should be pointed out that these data are only obtained from two mice, one of which is Npc2\textsuperscript{+/+} heterozygous and so we are currently awaiting more animals to confirm our findings.

Autophagy has been reported to be required for the establishment and maintenance of senescence induced by oncogenic RAS (Young et al., 2009). In this study, autophagy-enabled senescence was accompanied with induction of autophagy related genes including LC3\textsuperscript{B}, ATG5, and ATG7 (Young et al., 2009). Consistently, in our study, it has been shown that bNPC2 protein, rhNPC2 protein and overexpression of human NPC2 protein induce vesicle-like structures in lung cancer cells and CHO cells. Further studies confirmed that autophagy is induced by NPC2 protein treatment. It has been shown that an increased ratio of LC3 II/LC3 I is induced as detected by immunoblotting, a punctuate staining of GFP-LC3 is visualised by fluorescence microscopy, and autophagic vacuoles including autophagosomes and autophagolysosomes were
detected by transmission electron microscopy (TEM) following NPC2 treatment. These data confirm induction of autophagy by NPC2 treatment. Moreover, expression of downstream targets of mTORC1, p-4EBP-1 and p-p70S6K decreased; p-Akt (Ser473), a target for mTORC2, was also downregulated by bNPC2 treatment. These data suggest that mTOR activity is inhibited, consistent with the well known role of mTOR in suppression of autophagy (Lum et al., 2005). In our study, levels of Cyclin D1 were observed to decrease after NPC2 treatment. This is also likely due to inhibition of mTOR signalling because Cyclin D1 is well known to be regulated by mTOR signalling (Sabatini, 2006). Several studies have shown that chemotherapeutic agents induce autophagy in NSCLC cells resulting in inhibition of cell proliferation (Zhang et al., 2008c; Ding et al., 2009b; Lian et al., 2009; Fang et al. 2009) by inhibition of AKT activity and mTOR signalling (Fu et al., 2009). On basis of these observations, we propose that autophagy induced by NPC2 may be regulated by the mTOR pathway at least in lung cancer cells.

How does exogenous NPC2 protein induce autophagy? First, it has been shown that purified rhNPC2 protein can be delivered to the lysosome via mannose-6 phosphate receptor (MPR)-mediated endocytosis and restores cholesterol levels in human NPC2−/− fibroblasts (Ko et al., 2003; Liou et al., 2006). This study suggests that exogenous NPC2 protein could efficiently enter the cell. This was confirmed in our study as elevated levels of intracellular NPC2 were observed when exogenous NPC2 protein was added to the cell (Figure 5.8A and 5.16A). Second, overexpression of NPC1 (a genetic complementation group accounting for Niemann-Pick disease type C) in CHO cells increased total cellular cholesterol level (Millard et al., 2000). We speculate that NPC2 may have similar role in upregulation of cellular cholesterol level when overexpressed. There is evidence to show that overloaded cholesterol induces autophagy with an increase in LC3 II and accumulation of autophagic vacuoles in smooth muscle cells where autophagy acts as a cellular defence mechanism via degradation of dysfunctional mitochondria and ER against apoptosis and necrosis (Xu et al., 2010). Thus, it is possible that exogenously adding NPC2 protein or
overexpression of NPC2 in CHO cells significantly induces intracellular cholesterol level resulting in induction of autophagy.

To further dissect mechanisms of cholesterol overload in induction of autophagy, a role of p53 has been suggested from our study. p53 is a well known tumour suppressor that senses genotoxic stress to induce growth arrest, senescence, apoptosis, and autophagy leading to inhibition of tumour cell growth (Maiuri et al., 2010). p53 is also shown to mediate metabolic stress such as glucose starvation-induced cell cycle arrest in which p53 is phosphorylated and activated by AMPK that is a cellular energy sensor (Feng et al., 2005; Jones et al., 2005). Emerging evidence has shown that p53 is also involved in sensing lipotoxicity such as excessive levels of intracellular cholesterol (Bazuine et al. 2009). In our study, we consistently observed that p53 is induced by NPC2 treatment whether in the form of exogenous bNPC2, exogenous rhNPC2 or overexpression of endogenous hNPC2. NPC2 treatment may induce lipotoxicity and thus p53 activation through overload of cholesterol leading to the induction of senescence and autophagy. Under these circumstances, autophagy may act as a cell survival mechanism that protects cells from lipotoxicity since no apoptosis was detected upon bNPC2 treatment in lung cancer cells. As a well known target for p53, expression of p21$^{\text{Cip1}}$ was consistently increased upon NPC2 protein treatment. We conclude that the p53/p21$^{\text{Cip1}}$ pathway is activated to mediate autophagy induction, mitigating lipotoxicity triggered by NPC2-induced cholesterol overload.

However, our in vivo study has demonstrated that knockout of Npc2 in mice expressing $^\text{V600E-BRaf}$ does not prevent autophagy. When Npc2 was knocked out in mice without the $^\text{V600E-Braf}$ oncogene, there is a trend towards an increase in autophagy in the normal lung. It has been shown that knockout of Npc2 in mice is characterised by accumulation of cholesterol in late endosomes/lysosomes (Sleat et al., 2004). It has also been shown that blockage of cholesterol efflux from lysosomes/endosomes by pharmacological inhibitors that mimics NPC phenotype, is able to activate autophagy with an increase in LC3 II and a decrease in mTOR activity in human fibroblasts (Cheng et al., 2006). Thus,
accumulation of high levels of cholesterol in lysosomes may disturb the normal function of lysosomes, resulting in impaired fusion of autophagosomes with lysosomes, a reduction in turnover of autophagosomes and an increase in LC3 II (Cheng et al., 2006; Ishibashi et al., 2009; Liao et al., 2007).

In summary, we have provided evidence for the first time that the secreted NPC2 protein functions as a tumour suppressor by inhibition of lung cancer cell growth. It has been shown that the growth inhibitory effect of NPC2 on lung cancer cells results from induction of senescence and autophagy. Senescence is primarily mediated by the p53/p21\(^{Cip1}\) pathway and autophagy is likely to be regulated in a mTOR–dependent manner. It has also been shown that the p53/p21\(^{Cip1}\) pathway is involved in autophagy induction. It has been found that knockout of \(Npc2\) contributes to growth of the lung adenomas expressing \(^{V600E}BRaf\) with increased proliferation and reduced senescence by downregulation of the p53/p21\(^{Cip1}\) pathway. However, autophagy is not prevented by knockout of \(Npc2\) in lung adenomas. Knockout of \(Npc2\) in mice allows continued proliferation of autophagic cells suggesting that other factors are involved. It has been shown that perturbation of homeostasis in NPC2 levels by exogenous NPC2 treatment or knockout of \(Npc2\) might induce autophagy \textit{in vitro} and \textit{in vivo} in lung cancer. However, NPC2 is not found to be mutated or under-expressed in tumours. As p53 is commonly mutated in human lung cancer, it would predict that the effects of NPC2 on tumour suppression could be overcome by p53 mutations.
6.1 Induction of $^{V600E}\text{BRaf}$ expression using the CreER$^T$ system in MEFs

To study the role of $^{V600E}\text{BRaf}$ in tumour development, our laboratory generated a conditional knockin mutation of $\text{Braf}^{\text{LSL-V600E}}$ in mice by using the Cre/LoxP system (Mercer et al., 2005). In the present study, we showed that the CreER$^T$ system (Hayashi and McMahon, 2002) can be used to induce endogenous $^{V600E}\text{BRaf}$ expression at physiological levels after 4-hydroxytamoxifen treatment in MEFs. After Cre-mediated deletion of the LSL cassette, including the mini-cDNA, the $\text{Braf}^{\text{Lox-V600E}}$ allele was generated expressing $^{V600E}\text{BRaf}$. In primary MEFs, expression of endogenous $^{V600E}\text{BRaf}$ induced morphological transformation dependent on MEK activation (Figure 3.12).

$^{V600E}\text{BRaf}$ induced high levels of ERK phosphorylation and a faster G1/S phase progression associated with a significant upregulation of Cyclin D1 expression at the transcriptional level that was reversed by MEK inhibition. This result is consistent with the view that Cyclin D1 is a direct transcriptional target of the RAF/MEK/ERK signalling pathway via AP-1 transcription factors (Kerkhoff and Rapp 1997; Weber et al. 1997; Balmanno and Cook, 1999). We found that consistent with previous findings (Diehl et al., 1998), Cyclin D1 protein stability was mediated by the proteasomal degradation pathway, but this was not inhibited by $^{V600E}\text{BRaf}$. Roles of other Cyclins, Cdns and CKIs in G1/S phase progression induced by $^{V600E}\text{BRaf}$ were excluded in our study.

$^{V600E}\text{BRaf}$ also suppressed apoptosis at the pre-mitochondrial level following serum withdrawal by inducing a drastic downregulation in BimEL expression at the post-transcriptional level and this was rescued by MEK inhibition and proteasomal inhibition. Our results are consistent with previous studies (Whitfield et al., 2003; Weston et al., 2003; Ewings et al., 2007; Ley et al., 2003, 2004). A phospho-specific antibody against BimEL Ser65 the ERK target was not available to us, and so we could not confirm
whether or not the effects on Bim\textsubscript{EL} were mediated through phosphorylation of this site or not. The results of above work were published in Wickenden et al., 2008 and this manuscript is included at the end of this thesis.

**6.2 V600E\textsubscript{BRaf} induces senescence in the lung in mice**

We showed that expression of an endogenous V600E\textsubscript{Braf} allele at physiological levels induces lung adenomas in mice resulting from an initial burst of proliferation mediated by the BRaf/MEK/ERK pathway. This was followed by cessation of proliferation and induction of senescence that prevented lung tumours from progressing. This result is consistent with the view that oncogene-induced senescence functions as a tumour suppressor mechanism (Campisi, 2005; Collado and Serrano, 2006). This has also been found in mouse models of melanomas (Dankort et al., 2009; Dhomen et al., 2009), colon cancer (Carragher et al., 2010) and other studies of lung cancer (Dankort et al., 2007) when endogenous V600E\textsubscript{BRaf} is expressed at physiological levels. In the lung we found that senescence is primarily mediated by the p53/p21\textsuperscript{Cip1} and p16\textsuperscript{Ink4a} pathways, consistent with previous studies showing that inactivation of p53 or Cdkn2a results in abrogation of senescence and progression of benign tumours to malignancies (Dankort et al., 2007).

**6.3 V600E\textsubscript{BRaf} induces autophagy in the lung in mice**

Autophagy has been increasingly recognised as a tumour suppressor mechanism (Jin and White, 2007; Levine 2007; Levine and Kroemer, 2008). Induction of autophagy was observed in the lung adenomas expressing V600E\textsubscript{BRaf} by an increase in the ratio of LC3 II/LC3 I and upregulation of Beclin-1. This is the first time that autophagy induction has been reported in a mouse model of lung cancer expressing endogenous V600E\textsubscript{BRaf}. It has been shown that when V600E\textsubscript{BRAF} is overexpressed in melanoma cells, autophagy is induced in vitro and in melanoma xenografts by upregulation of LC3 resulting in inhibition of cell growth (Maddodi et al., 2010). Similarly, autophagy-enabled senescence was reported by Young and colleagues (2009) during oncogenic G12V\textsubscript{HRAS}-induced senescence in cell lines in vitro. SMS components including IL-6, IL-
8 and IGFBP7 were found to be induced during autophagy in this report.

6.4 Npc2 is induced by $V^{600E}_{BRaf}$ in senescent lung tumours

The recent discovery of several secreted proteins, including IGFBP7, IL6, and IL-8 as autocrine/paracrine mediators of oncogene-induced senescence provides strong support for the existence of the senescence-messaging secretome (SMS) (Kuilman et al., 2008; Wajapeyee et al., 2008). However, we failed to detect IGFBP7 and IL-6 expression in our lung study. We undertook a screen for senescent factors in $V^{600E}_{BRaf}$-induced senescent lung tumours and identified secreted protein Npc2, a cholesterol binding and trafficking protein (Ko et al., 2003; Naureckinene et al., 2000). The expression of Npc2 in $V^{600E}_{BRaf}$ lung adenomas was significantly induced at the post-transcriptional level; this is possibly mediated by the Nogo-B receptor (NgBR) by mechanisms that are currently not clear (Harrison et al., 2009). Npc2 expression correlated with the cessation of proliferation and induction of senescence and autophagy in the lung. We showed that Npc2 is detected in alveolar macrophages, tumour cells and the extracellular environment by immunohistochemistry. In previous studies, it has been shown that NPC2 is primarily localised in alveolar macrophages in human normal lung, but not epithelial cells (Griese et al., 2009). However, more recent studies in our laboratory have shown that Npc2 is secreted by infiltrating macrophages and alveolar type II pneumocytes (Tamihiro Kamata, personal communication).

6.5 Npc2 induces senescence and autophagy in lung cancer cells in vitro

The role of Npc2 in tumour suppression was investigated further. Purified bovine NPC2 (bNPC2) was found to induce the expression of senescence markers including SA-β-gal, p19$^{Arf}$, p21$^{Cip1}$, and Dec1 in lung cancer cells in vitro when added exogenously. In addition, bNPC2 also induced autophagy as indicated by an increase in LC3 II/LC3 I and downregulation of the mTOR pathway following its addition exogenously. Consistently, recombinant human NPC2 (rhNPC2) protein also induced autophagy when applied to lung cancer cells in vitro. In agreement with this, senescence and autophagy was also induced when human NPC2 was overexpressed within CHO cells.
It was consistently found that the p53/p21\textsuperscript{Cip1} pathway was induced by NPC2 treatment, whether applied exogenously to cells or everexpressed within.

6.6 Knockout of Npc2 abrogates senescence but not autophagy in lung tumours in vivo

The ability of NPC2 to induce senescence and autophagy in vitro, led us to investigate knockout of Npc2 in mice expressing V\textsuperscript{600E}Braf. Knockout of Npc2 in WT lungs without the V\textsuperscript{600E}Braf oncogene compromised the survival of mice. However, no tumours arose in these mice and they died of ataxia and overall locomoter dysfunction, with features of Niemann-Pick disease Type C2 (Sleat et al., 2004). When Npc2 was knocked out in lung tumours expressing V\textsuperscript{600E}Braf, there was a greater level of cell proliferation compared to lung tumours expressing Npc2 and this was associated with downregulation of the p53/p21\textsuperscript{Cip1} signalling pathway. However, the tumour cells did not display dysplastic changes, no adenocarcinomas were observed in the lung, and no metastasis was found in other organs. Moreover, autophagy was not prevented when Npc2 was knocked out. It is thus likely that Npc2 is for restricting growth of lung tumours but is not the only factor involved in maintenance of autophagy.

6.7 A model for the role of Npc2 in tumour suppression

On the back of the data presented here, we propose a model for the role of Npc2 in suppression of lung tumour development induced by V\textsuperscript{600E}Braf. As summarised in Figure 6.1, expression of V\textsuperscript{600E}Braf in the lung induces cell proliferation resulting in development of benign lung adenomas. However, proliferation is not sustained as senescence and autophagy is induced that prevents adenomas from progressing. During the senescent phase, a senescence-messaging secretome (SMS) is induced by V\textsuperscript{600E}Braf. Secreted protein Npc2 is significantly induced following V\textsuperscript{600E}Braf expression in senescent lung tumours and is released into the extracellular space. Extracellular Npc2 is transferred into the cell in an autocrine/paracrine manner and this may be via mannose-6 phosphate receptor (MPR)-mediated endocytosis or in conjunction with low-density lipoprotein (LDL). High levels of Npc2 within cells may dramatically
increase the intracellular cholesterol level resulting in cholesterol overload or lipotoxicity that can activate the p53/p21\(^{\text{Cip1}}\) signalling pathway resulting in induction of senescence. Autophagy is also induced to reduce excessive cholesterol in order to maintain cholesterol homeostasis. When \(Npc2\) is knocked out, senescence is abolished and cell proliferation is initiated by downregulation of the p53/p21\(^{\text{Cip1}}\) signalling pathway. However, knockout of \(Npc2\) does not prevent autophagy. Several questions remain unanswered. For example, it will be important to address:

- whether \(Npc2\) overload leads to a lipotoxic response
- how this activates p53
- how autophagy is induced

Also it is important to address if p53 mutant tumours are resistant to \(Npc2\) overload, we would predict that this is the case as \(NPC2\) is not found to be deleted or under-expressed in tumours, but clearly, effects of \(NPC2\) could be overcome by p53 mutation.
Figure 6.1 A proposed model for the role of Npc2 in tumour suppression

Expression of \textsuperscript{V600E}BRaf in the lung induces adenomas and oncogenic stress. High levels of Npc2 protein is produced by stressed alveolar type II pneumocytes and alveolar macrophages and secreted into the extracellular space. It is then transferred into cells in an autocrine/paracrine manner via mannose-6 phosphate receptor (MPR)-mediated or low-density lipoprotein (LDL)-mediated endocytosis. Npc2 senses cellular stress of overloaded cholesterol and induces senescence through activation of the p53/p21\textsuperscript{Cip1} pathway that prevents lung adenomas from progressing. This may be through sensing lipotoxicity or ER stress from excessive cholesterol resulting in autophagy induction.

\begin{itemize}
  \item \textsuperscript{V600E}BRaf lung tumours
  \item Oncogenic stress
  \item Lipotoxicity
  \item ER stress
  \item Autophagy
  \item Senescence
\end{itemize}
Table 4.1 Proteins identified in <40KDa fractions from \textsuperscript{V600E}BRaf senescent lung tumour conditioned media.

Signal sequence prediction was based on Ensembl (www.ensembl.org) and presence in exosomes was based on Exocarta database (http://exocarta.ludwig.edu.au). Ex fluids* indicates previous reports of presence in extracellular fluids (serum, urine, pleural effusion, seminal fluid, sputum, tear duct). Proteins of >40KDa predicted M.W. may represent degradation products in the CM or \textit{bona-fide} fragments of larger proteins.
Table 4.1 Proteins identified in <40KDa fractions from V600E BRaf senescent lung tumour conditioned media. Signal sequence prediction was based on Ensembl (www.ensembl.org) and presence in exosomes was based on Exocarta database (http://exocarta.ludwig.edu.au). Extracellular fluids indicate previous reports of presence in extracellular fluids including serum, urine, pleural effusion, seminal fluid, sputum, tear duct. Proteins of >40KDa predicted M.W. may represent degradation products in the CM or bona-fide fragments of larger proteins. ER, Endoplasmic reticulum; PM, Plasma membrane.

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APPENDIX B

Publications:

Colorectal cancer cells with the BRAFV600E mutation are addicted to the ERK1/2 pathway for growth factor-independent survival and repression of BIM

JA Wickenden¹, H Jin², M Johnson¹, AS Gillings¹, C Newson¹, M Austin¹, SD Chell¹, K Balmanno¹, CA Pritchard² and SJ Cook¹

¹Laboratory of Molecular Signalling, The Babraham Institute, Babraham Research Campus, Babraham, Cambridge, UK and
²Department of Biochemistry, University of Leicester, Leicester, UK

The RAF–mitogen-activated protein kinase kinase 1/2–extracellular signal-regulated kinase 1/2 (RAF–MEK1/2–ERK1/2) pathway is activated in many human tumours and can protect cells against growth factor deprivation; however, most such studies have relied upon overexpression of RAF or MEK constructs that are not found in tumours. Here we show that expression of the endogenous BRAFV600E allele in mouse embryonic fibroblasts from conditional knock-in transgenic mice activates ERK1/2, represses the BH3-only protein BIM and protects cells from growth factor withdrawal. Human colorectal cancer (CRC) cell lines harbouring BRAFV600E are growth factor independent for the activation of ERK1/2 and survival. However, treatment with the MEK1/2 inhibitors U0126, PD184352 or the novel clinical candidate AZD6244 (ARRY-142886) overcomes growth factor independence, causing CRC cell death. BIM is de-phosphorylated and upregulated following MEK1/2 inhibition in all CRC cell lines studied and knockdown of BIM reduces cell death, indicating that repression of BIM is a major part of the ability of BRAFV600E to confer growth factor-independent survival. We conclude that a single endogenous BRAFV600E allele is sufficient to repress BIM and prevent death arising from growth factor withdrawal, and CRC cells with BRAFV600E mutations are addicted to the ERK1/2 pathway for repression of BIM and growth factor-independent survival.


Keywords: apoptosis; BIM; BRAF; colorectal cancer; ERK1/2

Introduction

Most normal cells are dependent upon growth factors for their survival and proliferation, whereas cancer cells are growth factor independent and can evade apoptosis (Hanahan and Weinberg, 2000). Apoptosis following withdrawal of growth factors is regulated by the BCL-2 protein family in which the pro-apoptotic BAX and BAK proteins are restrained by their interaction with the antiapoptotic BCL-2 family members, such as BCL-2 and MCL-1; this balance is disrupted by the binding of BH3-only proteins to BCL-2 or MCL-1, thereby releasing BAX or BAK to promote cell death (Cory et al., 2003; Chen et al., 2005; Willis et al., 2007). BIM is a BH3-only protein that promotes cell death following withdrawal of survival factors (Bouillet et al., 1999; Dijkers et al., 2000; Ewings et al., 2007a). Survival factors repress BIM expression by reducing its transcription (Dijkers et al., 2000; Gilley et al., 2003). In addition, the most abundant splice variant, BIML, is phosphorylated by extracellular signal-regulated kinase 1/2 (ERK1/2), preventing it from binding to its target pro-survival BCL-2 proteins (Ewings et al., 2007a; Ewings et al., 2007b) and promoting its proteasomal turnover (Ley et al., 2003; Luciano et al., 2003; Weston et al., 2003; Ley et al., 2004; Marani et al., 2004; Ley et al., 2005).

The RAF–mitogen-activated protein kinase kinase 1/2–MEK–ERK1/2 signalling pathway is activated in human tumours because of activating mutations in RAS or BRAF. BRAFV600E is found in 10–25% of human colorectal cancers (CRC) (Davies et al., 2002; Mercer and Pritchard, 2003), early in the adenoma to carcinoma sequence (Rajagopalan et al., 2002; Yuen et al., 2002), and cancer cells harbouring BRAFV600E typically exhibit high ERK1/2 activity. Although tumours accumulate numerous mutations, they evolve to be dependent on a specific oncogene(s) to maintain the malignant phenotype; a phenomenon termed ‘oncogene addiction’ (Weinstein, 2002). Several studies have shown that the activation of ERK1/2 can inhibit BIM expression and prevent cell death arising from growth factor withdrawal (Ley et al., 2003; Luciano et al., 2003; Weston et al., 2003; Marani et al., 2004; Ley et al., 2005).

Apoptosis; BIM; BRAF; colorectal cancer; ERK1/2
et al., 2004). However, these studies have typically involved the overexpression of RAF or MEK mutants or the use of conditional kinases that are not found in human tumours and may not faithfully mimic the effects of BRAFV600E.

Here we show that conditional activation of a single BRAFV600E allele in primary mouse embryonic fibroblasts (MEFs) from transgenic conditional knock-in mice is sufficient to repress BIM expression and protect against growth factor withdrawal. CRC cell lines with BRAFV600E fail to upregulate BIM or undergo apoptosis following growth factor withdrawal unless the ERK1/2 pathway is also inhibited; furthermore, death arising from inhibition of the ERK1/2 pathway is substantially reduced by the use of short hairpin RNA or small interfering RNA (siRNA) to BIM. These data suggest that CRC cells with BRAFV600E are addicted to the ERK1/2 pathway for the repression of BIM and growth factor-independent survival.

Results

Expression of a single endogenous BRAFV600E allele is sufficient to repress BIM and protect cells from growth factor withdrawal

BrafLSL-V600E mice conditionally express a knock-in mutation of BRAFV600E (Mercer et al., 2005); Cre recombinase activity is required to induce recombination of the Lox-STOP-Lox cassette and allow expression of BrafV600E (Figure 1a). We used the CreER<sup>TM</sup> system in which Cre is expressed as a fusion protein with a tamoxifen-responsive version of the hormone-binding domain of the oestrogen receptor (Hayashi and McMahon, 2002). Primary MEFs derived from Braf<sup>+/LSL-V600E;CreER<sup>TM</sup></sup> double heterozygote embryos were treated over a time course with 4-HT (4-hydroxytamoxifen). The CreER<sup>TM</sup> protein was stabilized and localized to the nucleus within 5 h (Figure 1b), but full recombination of the Braf<sup>LSL-V600E</sup> allele was not achieved until 96 h (Figure 1c). These studies show for the first time the efficient regulation of a floxed allele by the CreER<sup>TM</sup> protein and establish the conditions for maximal induction of the Braf<sup>LSL-V600E</sup> allele by 4-HT.

Primary MEFs derived from either Braf<sup>+/LSL-V600E;CreER<sup>TM</sup></sup> or Braf<sup>+/LSL-V600E;CreER<sup>TM</sup></sup> embryos underwent apoptosis following serum starvation. Inclusion of 4-HT to induce BrafV600E expression strongly inhibited this apoptotic response in Braf<sup>+/LSL-V600E;CreER<sup>TM</sup></sup> MEFs but not in Braf<sup>+/LSL-V600E;CreER<sup>TM</sup></sup> MEFs as measured by three different apoptosis assays (Figure 2a and Supplementary Figure S1). This protective effect of 4-HT was due to BRAFV600E-dependent activation of the MEK–ERK1/2 pathway as it was reversed by the MEK inhibitor U0126 (Figure 2b). 4-HT treatment was able to partially reverse the loss of mitochondrial membrane potential (MMP) arising from serum withdrawal in Braf<sup>+/LSL-V600E;CreER<sup>TM</sup></sup> MEFs but not in Braf<sup>+/LSL-V600E;CreER<sup>TM</sup></sup> MEFs (Figure 2c).

BCL-2 proteins control the loss of mitochondrial membrane potential, and BIM has been implicated in the death of MEFs following the loss of growth factors (Ewings et al., 2007a). Indeed, when Braf<sup>+/LSL-V600E;CreER<sup>TM</sup></sup> MEFs were serum starved, there was a striking increase in BIM expression, predominantly the BIM<sub>EL</sub> isoform, which was completely prevented by the inclusion of 4-HT (Figure 2a). Serum withdrawal from Braf<sup>+/LSL-V600E;CreER<sup>TM</sup></sup> MEFs caused only a modest increase in BIM mRNA levels, as judged by (quantitative) reverse transcription PCR (QRT-PCR), and 4-HT treatment did not reverse this (Figure 3b). However, the expression of BrafV600E did cause the MEK-dependent hyperphosphorylation of BIM<sub>EL</sub>, and the BRAFV600E-dependent downregulation of BIM<sub>EL</sub> was reversed by the inclusion of the proteasome inhibitor MG132 (Figure 3c); furthermore, MG132 potentiated the induction of BIM<sub>EL</sub> upon serum withdrawal. These results indicate that the expression of a single BrafV600E allele is sufficient to repress BIM expression and it does so largely by promoting the phosphorylation and proteasome-dependent turnover of BIM<sub>EL</sub> rather than by repressing BIM transcription.

Growth factor-independent survival in colorectal cancer cells with the BRAFV600E mutation is reversed by the inhibition of MEK1/2

Initial experiments revealed that COLO205 cells fail to increase caspase/DEVDase activity (Figures 4a and b) or die (Figure 4c) following serum withdrawal. Similar results were observed in three other BRAFV600E-positive CRC cell lines (J Wickenden and S Cook, unpublished observations). In contrast, when COLO205 cells were serum starved in the presence of U0126, caspase activation was strikingly enhanced and accelerated (Figure 4a) and there was a large increase in the number of dead cells (Figure 4c); this was also seen in HT29, LS411 and CO115 cells (summarized in Figure 4d). U0126 also induced some death in cells maintained in fetal bovine serum (FBS) in some instances (for example, COLO205 cells, Figures 4a and c). The effect of U0126 was dose dependent; half-maximal cell death being induced by 300 nM–1 μM U0126 (Figure 4e). In addition, the effect of U0126 was replicated by PD184352, a more selective MEK1/2 inhibitor (Figure 4f).

Although death arising from MEK inhibition was inhibited by the caspase inhibitor zVAD.fmk in HT29 cells, death of COLO205 (Figure 4f) and LS411 cells (Supplementary Figure S2A) was largely caspase independent (Figure 4f). zVAD.fmk was fully competent to inhibit caspase activity in COLO205 cells (Supplementary Figure S2B) and had no off-target effects on de-phosphorylation of ERK1/2 or expression of BIM (Supplementary Figure S2C). This indicates that although caspases are activated during MEK inhibitor-induced cell death in COLO205 and LS411 cells, death can proceed through an alternative pathway if caspase activation is blocked.

Inhibition of MEK1/2 results in de-phosphorylation and accumulation of BIM protein

BIM is rate determining for cell death following withdrawal of survival factors (Bouillet et al., 1999;

Cell survival and repression of BIM by BRAF<sup>V600E</sup>

JA Wickenden et al.
BIMEL was the major BIM isoform present in COLO205 and HT29 cells maintained in 10% FBS and migrated as a diffuse hyperphosphorylated series of bands (Figures 5a and b). Although serum starvation inactivates ERK1/2 and promotes BIM expression in fibroblasts (Weston et al., 2003; Ewings et al., 2007a), it did not inactivate ERK1/2 and caused little increase in BIM expression in CRC cells (Figures 5a and b). However, the addition of U0126 to cells (whether in 10% FBS or serum starved) resulted in the rapid de-phosphorylation of ERK1/2 and BIMEL and an increase in BIMEL levels. U0126 did not affect the antiapoptotic BCL-2 proteins, except at 24 h when decreased levels of BCL-2 and MCL-1 were observed (Figure 5a). BIM expression is also repressed by the phosphatidylinositol 3-hydroxykinase (PI3K)-dependent protein kinase B (PKB) pathway (Dijkers et al., 2000; Gilley et al., 2003), but U0126 did not influence PKB phosphorylation (Figure 5a). Furthermore, serum starvation caused de-phosphorylation of PKB but had little effect on BIM levels (Figure 5a); similar results were observed in LS411 and CO115 cells (Supplementary Figure S3A and B) despite the very strong constitutive activation of PKB in CO115 cells (Supplementary Figure S3C). These data demonstrate that BRAFV600E-positive CRC cells are growth factor independent for ERK1/2 activation and repression of BIM, this can be reversed by the administration of an MEK1/2 inhibitor and the ERK1/2 pathway is the dominant pathway for the repression of BIM in these cells.

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**Figure 1**  Regulation of endogenous BRAFV600E expression using CreERTM. (a) Induction of V600EBraf by CreERTM. Conversion of the LSL-BrafV600E allele expressing 3Braf to the Lox-BrafV600E allele expressing 3V600E is shown. This allele is described in full in Mercer et al. (2005). Mice heterozygous for BrafLSL-V600E and CreERTM alleles were intercrossed and MEFs were obtained from resulting E14.5 embryos. To induce activation of Cre, and thus LSL recombination, MEFs were treated with 4-HT. (b) Immunofluorescence of Cre. BrafLSL-V600E; CreERTM MEFs were treated over a time course of up to 96 h with 4-HT and immunostained with a Cre antibody (green), counterstained with DAPI (blue) and the images merged. 4-HT induces a rapid translocation of the CreERTM protein within 5 h, and it remains here throughout the time course. (c) PCR detection of LSL-BrafV600E recombination. BrafLSL-V600E; CreERTM MEFs were treated with 4-HT or carrier control for the time course indicated, genomic DNA was isolated and subjected to PCR with the primers described in Mercer et al. (2005). Recombination is first detected within 5 h of 4-HT treatment and continues to increase in level throughout the time course. By 96 h, recombination is almost complete as indicated by the virtual absence of the LSL-BrafV600E allele and the high level of the Lox-BrafV600E allele. DAPI, 4',6-diamidino-2-phenylindole; MEFs, mouse embryonic fibroblasts.
AZD6244 (ARRY-142886) is a potent and selective MEK1/2 inhibitor that is undergoing pre-clinical and clinical evaluation (Wallace et al., 2006; Davies et al., 2007) and which does not inhibit the ERK5 pathway (Balmanno et al., submitted). AZD6244 was able to cause a substantial increase in the basal expression of BIM, predominantly BIMEL, even in COLO205 cells maintained in FBS (Figure 5c). Treatment of HT29 cells with AZD6244 overcame growth factor-independent survival, resulting in substantial cell death (Figure 5d), and also caused the de-phosphorylation of BIMEL and enhanced its expression upon serum withdrawal (Figure 5e). Thus, the MEK1/2-specific clinical candidate, AZD6244, replicated our results with U0126.

Reduced BIM expression protects HT29 cells against death induced by U0126 and AZD6244

Growth factor withdrawal-induced cell death requires de novo protein synthesis in some systems (Weston et al., 2003). Indeed, cycloheximide protected cells from caspase activation arising from MEK inhibition (Supplementary Figure S4A), exacerbated the reduction in MCL-1 and also reduced both basal and induced expression of BIMEL (Supplementary Figure S4B). As BIM expression decreases and cells are protected under these conditions, we considered that BIM might contribute to MEK inhibitor-induced cell death. To test this, we used RNA interference to reduce BIM expression. HT29 cells formed the focus of the RNAi experiments as their death was largely caspase dependent (Figure 4f), and BIM is primarily involved in the activation of caspase-dependent death pathways.

To assess the function of BIM, we used lentivirus (Wiznerowicz and Trono, 2003) to deliver short hairpin RNA against BIM to HT29 cells. BIM protein expression was reduced by 50–60% though it was not possible to fully prevent the increase in BIMEL following treatment with serum-free medium (SF) with U0126 (SF + U0126) (Supplementary Figure S5A); other members of the BCL-2 family were unaffected. Overexposure of the BIM blot revealed knockdown of smaller BIM splice variants. A control virus expressing the same sequence but with four mismatches (BIM-MM) did not affect BIM protein levels. Treatment with SF + U0126 in WT cells increased the fraction of cells with sub-G1 DNA and this was significantly reduced in the BIM RNAi cells, whereas the BIM mismatch sequence had
no effect (Supplementary Figure S5B). As a control, cisplatin-induced cell death was unaffected by the short hairpin RNAs used.

This partial reduction in cell death could reflect partial knockdown of BIM, a partial function for BIM or adaptation during the selection of virally infected cells. To address this, we used siRNA oligos to knock down human BIM transiently (Figure 6a). Knockdown of BIM was complete under these conditions (Figure 6b) and reduced death arising from the combination of serum withdrawal and U0126 or AZD6244 by 60% (Figure 6c). The corresponding siRNA oligos from mouse BIM, used as a control (Figure 6a), failed to reduce BIM expression and had no effect on cell death (Figures 6b and c). Thus, BIM contributes in large part to cell death arising when HT29 cells are serum starved in the presence of U0126 or AZD6244.

**BRAF**<sup>V600E</sup> provides a constitutive MEK-dependent signal for BIM<sub>EL</sub> degradation

Transcription of BIM is repressed by the PI3K-dependent regulation of FOXO-3A (Dijkers et al., 2000; Gilley et al., 2003). However, the ERK1/2 pathway can also repress BIM mRNA levels in fibroblasts (Weston et al., 2003) and epithelial cells (Reginato et al., 2005). When HT29 cells were deprived of growth factors, we found that BIM mRNA expression increased after withdrawal of growth factors but this was not enhanced further by MEK inhibition (Supplementary Figure S5C). Together, these data indicate a relatively minor function for the ERK1/2 pathway in repressing BIM mRNA levels in HT29 cells.

BIM<sub>EL</sub>, the most abundant form of BIM in all four cell lines, undergoes proteasomal degradation after phosphorylation by ERK1/2 (Ley et al., 2003; Luciano et al., 2003; Ley et al., 2004; Marani et al., 2004); consequently, we examined the turnover of BIM<sub>EL</sub> in COLO205 and HT29 cells. Cells were serum starved in the presence of U0126 for 18 h to increase the level of BIM protein. Cells were then washed to remove U0126 and subjected to an emetine chase in SF media, with or without fresh U0126 (Figure 7a). In both cell lines, ERK1/2 was rapidly re-activated in fresh SF medium, resulting in the rapid phosphorylation and degradation of BIM<sub>EL</sub>; U0126 reversed all of these effects (Figure 7b). These results indicate that there is a strong, constitutive, MEK-dependent signal for degradation of BIM<sub>EL</sub> in CRC cells harbouring BRAF<sup>V600E</sup>.
Figure 4  Inhibition of MEK overcomes growth factor-independent survival in CRC cells with BRAFV600E. (a and b) COLO205 cells were serum starved (SF) or placed in fresh FBS-containing medium (FBS) with or without 20 μM U0126 as indicated. Cells were then assayed for DEVDase activity and each data point represents (a) the mean ± range of duplicate dishes of cells or (b) the mean ± s.e.m. pooled from at least three experiments performed in duplicate. (c) COLO205 cells were treated 30 h in the presence (FBS) or absence (SF) of serum, with or without 20 μM U0126 and analysed for the percentage of cells with sub-G1 DNA. (d) The indicated cell lines were treated for 30 h in the presence (FBS) or serum starved with 20 μM U0126 and analysed for the percentage of cells with sub-G1 DNA. (e) HT29 cells were maintained in FBS or serum starved in the presence of increasing concentrations of U0126 for 30h and analysed for the percentage of cells with sub-G1 DNA. (f) HT29 and COLO205 cells were maintained in FBS or serum starved with 2 μM PD184352 in the presence of increasing doses of zVAD.fmk for 30 h before being analysed for the percentage of cells with sub-G1 DNA. In (a), (e), and (f), results are mean ± s.d. from a single experiment performed with biological triplicates; similar results were obtained in two further experiments. In (b-d), results are the mean ± s.e.m. pooled from at least three experiments performed in duplicate ± s.e.m. *P<0.05; **P<0.01; ***P<0.001. CRC, colorectal cancer; FBS, fetal bovine serum.
ERK1/2-dependent phosphorylation of BIMEL also inhibits its binding to pro-survival BCL-2 proteins such as MCL-1 (Ewings et al., 2007a; Ewings et al., 2007b).

To investigate if BRAFV600E signalling could regulate BIMEL–MCL-1 complexes, COLO205 and HT29 cells were treated with SF + U0126 for 18h to induce BIM expression and the formation of BIMEL–MCL-1 complexes; cells were then washed to remove U0126 and placed in fresh SF media (Figure 7c). Reactivation of ERK1/2 was complete within 30min in COLO205 cells or 10min in HT29 (Figure 7d). In both cases, as the ERK1/2 was reactivated, BIMEL became phosphorylated and the amount of BIMEL recovered in MCL-1 IPs was reduced. In both cell lines, this dissociation of BIMEL from MCL-1 preceded any decrease in total BIMEL. For example, loss of BIMEL from MCL-1 IPs occurred within 30min in COLO205 cells at which point total BIMEL levels were unchanged; in HT29 cells, loss of BIMEL from MCL-1 IPs occurred within 10min and again total BIMEL levels were unchanged (Figure 7d).

Figure 5  MEK inhibition promotes de-phosphorylation and increased expression of BIMEL in CRC cells with BRAFV600E. COLO205 cells (a) and HT29 cells (b) were maintained in FBS or serum starved (SF) with or without 20μM U0126 before lysis and western blotting analysis as described in Materials and methods. Results are representative of three independent experiments for each cell line. Similar results were obtained in LS411 and CO115 cells (Supplementary Figure S4). (c) COLO205 cells maintained in FBS were treated with 2μM AZD6244 or 10μM U0126 for the indicated times and probed for BIM expression by western blot; ERK1 was used as a loading control. (d and e) HT29 cells were serum starved with or without 2μM AZD6244 for indicated times. (d) The percentage of cells with sub-G1 DNA was determined by flow cytometry and data shown are mean ± s.d. of biological triplicates from a single experiment representative of three. **P < 0.01. (e) Cell lysates were probed for BIM, MCL-1 and P-ERK1/2 by western blot; ERK1 was used as a loading control. Results are representative of three independent experiments. CRC, colorectal cancer; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum.
Indeed, the dissociation of BIMₘ from the MCL-1–BIMₘ complex is not due to ERK-dependent ubiquitination or proteasomal degradation of BIM (Ewings et al., 2007a). Study of the BCL-Xₐ–BIM complex showed a similar pattern of dissociation as seen with MCL-1 (data not shown). Thus, the constitutive activation of the ERK₁/₂ pathway by BRAF V600E in CRC cells promotes dissociation of BIMₘ from its pro-survival target proteins and its proteasomal degradation.

Discussion

Growth factor-independent cell proliferation requires that cancer cells evade growth factor withdrawal-induced cell death; indeed, these are both hallmarks of cancer cells (Hanahan and Weinberg, 2000). Presumably, tumour cells must evolve mechanisms to repress or tolerate BIM. Several studies have shown that activation of ERK₁/₂ can block BIM expression and prevent cell death arising from growth factor withdrawal but these have usually involved ectopic overexpression of RAF or MEK mutants that are not found in human tumours, raising concerns about their physiological relevance.
Here we have studied MEFs from knock-in transgenic mice that exhibit conditional expression of a single Braf\textsuperscript{V600E} allele and CRC cells harbouring a single BRAF\textsuperscript{V600E} allele; in both cases, these mutant oncoproteins are expressed from their endogenous promoters rather than being overexpressed.

The importance of the Lox-STOP-Lox system is best exemplified by studies of genetically engineered mice with \textit{K-ras\textsuperscript{G12D}} alleles (Frese and Tuveson, 2007). Although conditional overexpression of ectopic \textit{K-ras\textsuperscript{G12D}} promotes proliferation and tumour initiation in several tissues, these models do not always faithfully reproduce the development of human cancers with \textit{KRAS\textsuperscript{G12D}} mutations due to supraphysiological RAS signalling. In contrast, the expression of endogenous \textit{K-ras\textsuperscript{G12D}} alleles by crossing \textit{K-ras\textsuperscript{G12D}}/+ \textit{LSL-Kras\textsuperscript{G12D}} mice with appropriate cre transgenic mice provides exquisite temporal and spatial control over oncogene expression. In the case of the lung and pancreas, this leads to the development of benign adenomas and eventually adenocarcinomas with many of the histopathological and molecular characteristics of human non-small-cell lung carcinoma and pancreatic ductal adenocarcinoma, respectively (Jackson et al., 2001; Hingorani et al., 2003; Tuveson et al., 2004). These studies highlight the advantage of expressing oncogenes from endogenous alleles and are helping to define the relative importance of various RAS effector pathways in tumour development. An important advance in our study was the use of primary MEFs derived from Braf\textsuperscript{+/LSL-V600E;CreERT}\textsuperscript{+/} mice (Mercer et al., 2005). Treatment of these MEFs with 4-HT allowed full recombination of the Braf\textsuperscript{+/LSL-V600E} allele, the first time this has been demonstrated with the CreERT system for any floxed allele. Significantly, expression of a single Braf\textsuperscript{V600E} allele and resultant activation of the endogenous ERK1/2 pathway protected against growth factor withdrawal and completely blocked the otherwise substantial increase in BIM expression. The fact that this was observed in primary MEFs indicates that this pathway alone is sufficient to repress BIM expression and cell death.

Prompted by these results, we also examined CRC cell lines harbouring a single BRAF\textsuperscript{V600E} allele and exhibiting a strong constitutive activation of ERK1/2. We found that (i) these cells were growth factor independent for survival; (ii) inhibition of the ERK1/2 pathway promoted cell death and this was substantially dependent upon BIM and (iii) constitutive ERK1/2 signalling is responsible for repressing BIM expression and function. The ‘oncogene addiction’ hypothesis posits that tumours evolve an unusual dependence upon certain oncogenes and the signalling pathways they control to maintain the malignant phenotype; for example, CRC cells with mutations in \textit{KRAS} are addicted to the mutant KRAS oncoprotein (Shirasawa et al., 1993). We found that CRC cells with BRAF\textsuperscript{V600E} were growth factor independent for cell survival and this could be overcome by any of the three distinct MEK inhibitors (U0126, PD184352 or AZD6244). Indeed, in some cases, MEK inhibition alone could induce cell death in the presence of FBS, indicating the degree to which these cells have evolved an extreme dependency upon the ERK1/2 pathway for survival. This is all the more remarkable as some of these CRC cell lines also harbour PIK3CA mutations (HT29) and/or exhibit strong basal PKB activation (CO115 cells) (Supplementary Figure S3B), a pathway that promotes cell survival. This underlines the extent to which these cells are addicted to the ERK1/2 pathway for growth factor-independent survival and suggests that inhibition of ERK1/2 signalling may be particularly effective in killing BRAF\textsuperscript{V600E}-positive CRC cells. Similar conclusions have been drawn in BRAF\textsuperscript{V600E}-positive melanoma (Karasarides et al., 2004).

There is a prominent function for increased BIM expression in death arising from growth factor withdrawal in MEFs (Ewings et al., 2007a). We found that the expression of endogenous Braf\textsuperscript{V600E} was sufficient to block BIM expression in Braf\textsuperscript{+/LSL-V600E;CreERT}\textsuperscript{+} MEFs (Figure 3a). Similarly, all the four BRAF\textsuperscript{V600E}-positive CRC cell lines failed to increase BIM expression unless serum starvation was combined with MEK inhibition; indeed, in some cases, the administration of U0126 or AZD6244 to cells in complete medium was sufficient to increase BIM expression, indicating that these cells are addicted to the ERK1/2 pathway for repression of BIM, even when they are exposed to growth factor-rich FBS, which activates the PI3K–PKB pathway, possess PIK3CA mutations or exhibit strong basal PKB activity, such as CO115 cells (Supplementary Figure S3B).

BIM was specifically implicated in death arising from MEK inhibition by the use of a BIM-specific short hairpin RNA and two different BIM-specific siRNAs, which reduced cell death by at least 60%. However, whereas BIM is involved in death arising from MEK inhibition in these CRC cells, it may not be the only regulator. For example, inhibition of MEK in the presence of FBS caused de-phosphorylation of BIM\textsubscript{EL} and some increase in BIM expression but only a modest increase in cell death. This may indicate that there is a crucial threshold level of BIM required for cell death that is only achieved upon serum withdrawal and MEK inhibition or that other important regulators are also induced by serum withdrawal and MEK inhibition. In addition, even when the siRNA-mediated knockdown of BIM was complete (Figure 6b), this did not completely prevent cell death (Figure 6c), again suggesting that other regulators are operating in parallel; likely candidates might include BAD, which is regulated by both the ERK1/2–RSK and PKB pathways (Datta et al., 1997; Eisenmann et al., 2003; Boisvert-Adamo and Aplin, 2008).

We observed little evidence of regulation of BIM mRNA levels by the ERK1/2 pathway in either MEFs or CRC cells. Furthermore, in both cell systems, BIM\textsubscript{EL} was by far the most abundant isoform and was certainly the major isoform that was dynamically regulated by MEK inhibition. Among the canonical splice forms, BIM\textsubscript{EL} is unique in being subject to extensive multisite phosphorylation by ERK1/2, which targets it for polyubiquitination and proteasomal degradation. Indeed, the downregulation of BIM\textsubscript{EL} in MEFs was
reversed by MG132 (Figure 3c), and both COLO205 and HT29 cells exhibited a strong constitutive MEK-dependent signal for BIMEL degradation (Figure 7b). Although growth factor independent for ERK1/2 activity, the CRC cells remained growth factor dependent for PKB activation (Figure 5a), so inactivation of the PI3K–PKB pathway upon serum withdrawal may contribute to increases in BIM mRNA levels, perhaps by the activation of FOXO3A (Dijkers et al., 2000; Gilley et al., 2003). However, the fact that serum withdrawal alone caused little or no increase in BIM protein expression in 4-HT-treated MEFs or CRC cells suggests that any mature BIMEL that is expressed following serum withdrawal is rapidly phosphorylated by ERK1/2 and thereby degraded. Thus, ERK1/2-dependent turnover of BIMEL appears to be the dominant signal responsible for restraining BIM expression in both MEFs and CRC cells with BRAFV600E.

BAX and BAK are thought to be kept in check by the pro-survival BCL-2 proteins until liberated by binding of the BH3-only proteins to the pro-survival proteins (Chen et al., 2005; Willis et al., 2007). In addition to protein turnover, ERK1/2-dependent phosphorylation of BIMEL can prevent its binding to pro-survival BCL-2 proteins (Ewings et al., 2007a; Ewings et al., 2007b), whereas withdrawal of growth factors and/or inhibition of ERK1/2 promotes the association of BIMEL with MCL-1. Here we have demonstrated that this is also the case in CRC cells where washout of U0126 and re-activation of ERK1/2 resulted in the phosphorylation of BIMEL and its dissociation from MCL-1. Thus, the constitutive activation of ERK1/2 observed in COLO205 and HT29 cells drives dissociation of BIMEL from MCL-1 and subsequent BIMEL turnover.

The ability of BIM to promote apoptosis and its expression following withdrawal of growth factors suggest that BIM has some of the credentials of a tumour suppressor protein. Indeed, the loss of one Bim allele accelerates Myc-induced leukaemia in the mouse (Egle et al., 2004), and disruption of the BIM locus has been observed in various haematological malignancies, most notably in 17% of mantle cell lymphomas (Tagawa et al., 2005). In contrast, the disruption of the BIM gene seems to be rare in solid tumours and yet many tumour cell lines exhibit very low levels of BIM, suggesting that alternative mechanisms are employed to repress BIM. These may include epigenetic mechanisms in renal cell carcinoma (Zantl et al., 2007), but activation of signalling pathways downstream of oncogenes is emerging as one such mechanism. Mutant forms of EGFR can repress BIM expression in lung cancer, and this is overcome by EGFR inhibitors that are in clinical use (Costa et al., 2007; Cragg et al., 2007; Gong et al., 2007). Our demonstration that BIM is repressed in an ERK1/2-dependent manner in CRCs harbouring BRAFV600E suggests that increased expression of BIM may contribute to cell death in response to inhibitors of the BRAF–MEK–ERK1/2 pathway such as AZD6244. Finally, it is important to note that the inhibition of the ERK1/2 pathway appears to initiate parallel caspase-dependent and caspase-independent cell death pathways in COLO205 and LS411 cells. Future studies should seek to investigate the pathway of caspase-independent cell death in these cells as it may be important in the response of CRC cells to ERK1/2 pathway inhibitors.

Materials and methods

Cells and cell lines

Primary MEFs were prepared from E14.5 embryos as described earlier (Hüser et al., 2001). All studies were performed on MEFs following immediate isolation from the embryo. MEFs and the CRC cell lines, COLO205, HT29 and CO115, were maintained in Dulbecco’s Modified Eagle’s high-glucose Medium (DMEM, Invitrogen, Carlsbad, CA, USA). The LS411 cell line was maintained in RPMI (Invitrogen). Both media were supplemented with 10% FBS (Invitrogen), 20 mm l-glutamine (Invitrogen) and 100 U streptomycin (Invitrogen). All four CRC lines harbour the BRAFV600E mutation and have wild-type KRAS and mutant p53.

Antibodies

The following antibodies were used for western blotting: BCL-2 (Santa Cruz; 7382); BCL-XL (Cell Signalling Technology, Danvers, MA, USA; 2762); BIM (Chemicon, Temecula, CA, USA; AB17003); BIM (rat, for analysis of immuno-precipitates) (Calbiochem/Novabiochem, San Diego, CA, USA; AM53); MCL-1 (Santa Cruz, CA, USA; sc-819); phospho-Akt/PKB (Cell Signalling Technologies; 9271); Akt/PKB (Cell Signalling Technologies; 9272); phospho-ERK1/2 (Cell Signalling Technologies; 9106) and ERK1 (BD Biosciences, San Jose, CA, USA; 61003). The ERK2 antibody was a gift from Professor Chris Marshall (ICR, London).

Immunoprecipitation and western blot analysis

At the required times, cells were lysed in Triton-Glycerol (TG) lysis buffer and analysed by western blot as described earlier (Weston et al., 2003; Todd et al., 2004). For immunoprecipitation, cell extracts were normalized for equal protein loading and incubated at 4°C with MCL-1 antibody (sc-819) bound to protein A sepharose beads (Sigma, St Louis, MO, USA).

Apoptosis assays

Cell extracts were assayed for DEVDase activity as described earlier (Weston et al., 2003). The proportion of cells with hypo-diploid (sub-G1) DNA was assessed by propidium iodide staining and flow cytometry (Weston et al., 2003; Todd et al., 2004). The annexin V assay was performed as described earlier (Häser et al., 2001). Changes in mitochondrial membrane potential were examined by flow cytometric analysis of cells stained with tetramethylrhodamine ethyl ester (Molecular Probes, Eugene, OR, USA) (Scaduto and Grottyhann, 1999), a cell-permeable dye accumulating in mitochondria with unaltered membrane potential. Cells were harvested by trypsinization and 5 × 10⁶ cells were incubated with 20 nM tetramethylrhodamine ethyl ester for 20 min at 37°C followed by the analysis with FACScan (20000 cells per sample). The fluorescence intensity of tetramethylrhodamine ethyl ester was monitored at 582 nm (FL-2).

Quantitative reverse transcription PCR

Total RNA was extracted from cells using TRI-Reagent (Sigma) and reverse transcription PCR was performed according to the protocol supplied with the TaqMan Reverse
Transcription reagents (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase was determined to be the most stable housekeeping gene using the GeNorm protocol (Vandesompele et al., 2002) and the following primers were used: 5'-TGACCACTCAGTCTTAGC; 3'-GGCATGGACTTG GTCATGAG. Primers for human BIM were 5'-ACCTTC TGATGATACTGTTGTA and 3'-GGATTACCTG TGGCTCTGTC. The primers for mouse BIM were 5'- GTTCCTCAGGGATTTCTC and 3'-CAGATCTCAGG TTCTCTCCT. Quantitative PCR was performed using SYBR Green (Applied Biosystems, Foster City, CA, USA) in a Chromo-4 Thermal Cycler analysed with Opticon Software (BioRad, Hercules, CA, USA).

siRNA sequences and RNAi
For transient RNAi, the following oligos were used: human BIM1 GACCGAAGGTCTGAGCAATT; mouse BIM2 GCAAACCTCAGTGTAAAGT; mouse Bim1 GGAGGAA CCTGAAGATCTG; the mouse Bim sequence has four mismatches when compared with the human BIM and served as a specificity control. HT29 cells were plated the day before transfection at 2×10^6 per well (six-well tray) in pen/strep-free medium. Briefly, 500 pmol of each human Bim siRNA was mixed with OptiMEM media (1000 pmol for control mouse siRNA), and an equivalent volume of OptiMEM was combined with a CRUK programme grant number C1362/A6969. Work in the SJC lab was supported by the Association for International Cancer Research (AICR), AstaZeneca, BBSRC (BB/E02162X/1) and the Babraham Institute.

References


Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)
References


Amaravadi, R.K., Yu, D., Lum, J.J., Bui, T., Christophorou, M.A., Evan, G.I., Thomas-


References


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Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A.J., Barradas, M., Benguria,


Dankort, D., Filenova, E., Collado, M., Serrano, M., Jones, K. and McMahon, M. (2007) A new mouse model to explore the initiation, progression, and therapy of
References


References


References

and p16INK4a-beta in mouse lung tumor cells. Oncogene, 13, 1885-1891.
References


Ito, H., Daido, S., Kanzawa, T., Kondo, S. and Kondo, Y. (2005) Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma...


References


References


Meloche, S., Seuwen, K., Pages, G. and Pouyssegur, J. (1992) Biphasic and synergistic activation of p44mapk (ERK1) by growth factors: correlation


References


Sensi, M., Nicolini, G., Petti, C., Bersani, I., Lozupone, F., Molla, A., Vegetti, C., Nonaka,


Silver, D.P. and Livingston, D.M. (2001) Self-excising retroviral vectors encoding the


1281-1293.


