PROTEOMIC AND MOLECULAR CHARACTERISATION
OF TRAIL-INDUCED SIGNALLING COMPLEXES

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by

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Proteomic and Molecular Characterisation of TRAIL-Induced Signalling Complexes

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TRAIL selectively induces apoptosis in cancerous cells through the formation of the death-inducing signalling complex (DISC). Apoptosis can progress through either a Type I (direct procaspase-3 cleavage) or Type II (Bid cleavage) route. However, factors acting at the DISC, which may determine the selectivity of TRAIL and/or which pathway (Type I/II) is activated, are currently ill-defined. Therefore, the aim of this thesis was to investigate the initial phase of TRAIL signalling through protein purification, mass spectrometry and functional assays.

A tagged variant of FADD (DISC component) was stably expressed, allowing interactions with calpain small subunit 1 (calpain S1) and heat shock cognate 71 kDa protein (HSC70) to be identified by mass spectrometry. It was speculated that FADD and calpains may interact via calpain S1, whilst HSC70 may contribute to TRAIL-induced subcellular redistribution of FADD. Mass spectrometry of the TRAIL DISC identified the known components and a potential interaction between transferrin receptor and TRAIL-R1/-R2. It was hypothesised that this interaction may help target TRAIL-R1/-R2 and DISC for endocytosis. Increased TRAIL DISC formation in Type I versus Type II cells was unlikely to be the result of other proteins, since no significant differences in DISC composition were detected. Further studies indicated that low TRAIL DISC formation in the Type II Jurkat cell line was likely related to the absence of TRAIL-R1 expression and inefficient recruitment of FADD to TRAIL-R2. Functional assays suggested that Type I and Type II TRAIL DISC have a similar substrate preference. Therefore, in contrast to previous reports, these data suggest that the lower level of DISC formed in a Type II cell line does not preferentially cleave Bid to activate the intrinsic pathway. Instead lower caspase-8 activation at the Type II DISC results in overall less substrate cleavage, delaying apoptosis and thus enabling the intrinsic pathway to contribute to caspase-3 activation.
Acknowledgements

Firstly, I would like to thank Dr Marion MacFarlane and Dr Kelvin Cain for their unwavering help and guidance.

I am also extremely grateful to Mrs Rebecca Jukes-Jones, Dr Rob Boyd and Dr Claudia Langlais of the Protein Profiling facility for all of their work, assistance and helpful discussions. In particular, my thanks go to Mrs Rebecca Jukes-Jones for the mass spectrometry work she performed, to Dr Rob Boyd for his help with the sucrose density gradients and to Dr Claudia Langlais for her help with the size-exclusion columns. I would also like to thank Dr Nick Harper for the biotinylated TRAIL he provided for the DISC purifications.

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<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Annexin V</td>
<td>Annexin V-FITC</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphocytic leukaemia proto-oncogene 2</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology domains</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculovirus IAP repeat</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bTRAIL</td>
<td>Soluble biotinylated TRAIL</td>
</tr>
<tr>
<td>c-FLIP</td>
<td>Cellular FLIP</td>
</tr>
<tr>
<td>c-FLIP&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Cellular FLIP, long isoform</td>
</tr>
<tr>
<td>c-FLIP&lt;sub&gt;R&lt;/sub&gt;</td>
<td>Cellular FLIP, raji isoform</td>
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<tr>
<td>c-FLIP&lt;sub&gt;S&lt;/sub&gt;</td>
<td>Cellular FLIP, short isoform</td>
</tr>
<tr>
<td>C-TAP</td>
<td>C-terminal TAP tag empty vector</td>
</tr>
<tr>
<td>Calpain S1</td>
<td>Calpain small subunit 1</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CapLC</td>
<td>Micro-capillary high performance liquid chromatography</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine-dependent aspartyl specific protease</td>
</tr>
<tr>
<td>CBP</td>
<td>Calmodulin binding peptide</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine rich domain</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DED</td>
<td>Death effector domain</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signalling complex</td>
</tr>
<tr>
<td>DLG1</td>
<td>Disks large homolog 1</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
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<td>FADD</td>
<td>Fas-associated death domain protein</td>
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<td>FADD-TAP mixed population cell line</td>
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<td>Description</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FIST</td>
<td>Fas-interacting serine/threonine kinase</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>FT</td>
<td>FADD-TAP</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl-phosphatidylinositol</td>
</tr>
<tr>
<td>Hr</td>
<td>Hour/s</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSC70</td>
<td>Heat shock cognate 71 kDa protein</td>
</tr>
<tr>
<td>HSP90α</td>
<td>Heat shock protein 90-alpha</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td>IETD.AFC</td>
<td>Acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin</td>
</tr>
<tr>
<td>IVT</td>
<td>In vitro transcription/translation</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
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<tr>
<td>Min</td>
<td>Minute/s</td>
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<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilisation</td>
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<td>MS/MS</td>
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<tr>
<td>NSAF</td>
<td>Normalised spectral abundance factor</td>
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<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
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<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
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<td>Protein kinase C</td>
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<tr>
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<td>Quadrupole time of flight</td>
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<tr>
<td>RNF113B</td>
<td>RING finger protein 113B</td>
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<tr>
<td>SAF</td>
<td>Spectral abundance factor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<td>Abbreviation</td>
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<tr>
<td>Sec</td>
<td>Second/s</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SPOTS</td>
<td>Signalling protein oligomerisation structures</td>
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<td>tBid</td>
<td>Truncated Bid</td>
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<td>TNF receptor-associated factor</td>
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<td>TNF-related apoptosis-inducing ligand</td>
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<tr>
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<tr>
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<td>Vacuolar protein sorting-associated protein 35</td>
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<tr>
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<tr>
<td>ZVAD.FMK</td>
<td>Carbobenzoxy-Val-Ala-Asp-(O-methyl)-fluoromethylketone</td>
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Chapter 1: Introduction
1 Introduction

“Life is pleasant. Death is peaceful. It’s the transition that’s troublesome.”
Isaac Asimov (Science-fiction writer and biochemist 1920-1992)

1.1 Cancer

Cancer arises when normal cells acquire abnormal properties that enable them to grow and survive inappropriately. The transformation of a normal cell into a cancerous one is a self-perpetuating multi-step process, often involving genetic alterations that bestow tumorigenic properties. These have been referred to as the six hallmarks of cancer and include an ability to grow independently of growth promoting or inhibitory signals, infinite replicative potential and resistance to cell death (Hanahan & Weinberg, 2000). Once cells acquire these properties they are able to grow in an unregulated manner resulting in excessive cell numbers, tumour formation and cancer.

Conventional cancer treatments are surgery, radiotherapy and chemotherapy, all of which aim to remove or reduce the tumour. Radiotherapy and chemotherapy damage proliferating cells, inducing apoptotic cell death as a secondary effect. However, the action of these treatments is non-selective and can result in the death of healthy, rapidly dividing cells. This gives rise to the well known unwanted side effects of cancer treatment, such as hair loss. Furthermore, most tumour cells are already or eventually become resistant to radio/chemotherapy through the disruption of pathways that couple damage detection to cell death. For example, p53 receives information from numerous stress pathways and acts to preserve the genomic stability of the normal cell population. One way it achieves this is to induce apoptotic cell death of the damaged cell thus preventing its replication (Whibley et al, 2009). The action of p53 makes it a potent barrier to the development of cancer, and consistent with this p53 is mutated in more than 50% of cancers. This impedes the conversion of cancer treatment-induced damage into cell death and results in a poor response to cancer therapy.

Cancer was estimated to be responsible for 13% of deaths worldwide and 20% of those that occurred in Europe in 2004 (World Health Organization, 2008). In the UK, cancer has been the second most common cause of death in males since 1971, whilst in females it became the primary cause of death in 2006 (Office for National Statistics, 2009). Furthermore, the incidence of cancer is on the increase with 2.9 million European cases reported in 2004 and 3.2 million reported in 2006 (Ferlay et al, 2007). This, in conjunction with the limitation of current therapies, means that there is a desperate need for new and more effective treatments. These new therapies must
address the molecular cause of cancer whilst inducing minimal side effects. One agent that holds particular potential as a targeted cancer therapy is the death ligand TNF-related apoptosis-inducing ligand (TRAIL), which appears to specifically induce apoptotic cell death in cancer cells.

1.2 Apoptosis

1.2.1 The function and morphology of apoptosis

The term apoptosis was first used in 1972 to describe a form of programmed cell death that removes cells that are not required or are irreparably damaged (Kerr et al, 1972). Apoptosis is just one of several mechanisms through which cell death occurs (Kroemer et al, 2005), but unlike other forms it is a co-ordinated and energy dependant suicide process (Elmore, 2007). However, it is now becoming clear that apoptosis forms one end of a cell death continuum, with the passive and disorganised process of necrosis at the other. The type of cell death that occurs seems to depend on the intensity of the death stimulus, availability of ATP and availability of caspases (Chautan et al, 1999, Formigli et al, 2000, Zeiss, 2003).

Several characteristics distinguish apoptosis from other forms of cell death, these include volume reduction (pyknosis), chromatin condensation and recognition by phagocytes (Wyllie, 1987). The volume reduction of apoptotic cells is accompanied by an increase in cell density, compaction of organelles as well as cell surface convolution caused by proliferation of cellular membranes. Chromatin condensation results in nuclear shrinkage, the most characteristic feature of apoptosis, and eventually the DNA and nucleus fragment (karyorrhexis). The apoptotic cell is dismantled into apoptotic bodies, which are membrane-bound packets of cytoplasm and densely packed organelles. Apoptotic bodies are rapidly phagocytosed through the recognition of so-called “eat me” signals, the best characterised of which is phosphatidylserine (PS). During apoptosis the plasma membrane loses its asymmetrical composition, resulting in the exposure of PS. PS is recognised by a cell surface receptor on macrophages, and other cell lines, resulting in engulfment of the dying cell (Fadok et al, 2000). The retention of the cytosol within apoptotic bodies and rapid phagocytosis prevents an inflammatory reaction, and allows recycling of cell components (Cristea & Degli Esposti, 2004, Elmore, 2007, Wyllie, 1987).

Acting as the negative counterpart to mitosis, apoptosis plays an important role in maintaining normal cell populations. Thus, it is involved in many physiological
processes including immune function, wound healing as well as tissue development, differentiation, remodelling and homeostasis. However, dysregulation of apoptosis can lead to pathology and has been implicated in cancer, neurodegenerative diseases, autoimmune diseases and ischemia-associated injury (Elmore, 2007).

**1.2.2 The discovery of the gene and protein families involved in apoptosis**

The genetic basis for apoptosis was initially discovered in *Caenorhabdititis elegans* (*C. elegans*). In this worm 1090 somatic cells are generated during development and exactly 131 die through apoptosis at particular times (Sulston et al, 1983). The predictability of *C. elegans* cell death allowed the genes involved to be identified through mutation studies (Kinchen & Hengartner, 2005). Four proteins (Ced-3, Ced-4, Egl-1 and Ced-9) produce the core machinery that is responsible for cell death in *C. elegans* (Figure 1.1). Ced-3, Ced-4 and Egl-1 promote, whilst Ced-9 inhibits, cell death since loss of function mutations lead to a respective decrease or increase in apoptosis (Conradt & Horvitz, 1998, Ellis & Horvitz, 1986, Hengartner et al, 1992). Egl-1 acts upstream to inhibit Ced-9, which in turn inhibits the ability of Ced-4 to activate Ced-3 (Figure 1.1) (Conradt & Horvitz, 1998).

The discovery that previously identified proteins were the mammalian orthologues of Ced-3, Ced-4, Ced-9 and Egl-1 enabled the protein families essential for mammalian apoptosis to be pinpointed (Figure 1.1). Egl-1 is orthologous to pro-apoptotic members of the B-cell lymphocytic leukaemia proto-oncogene 2 (Bcl-2) family (Conradt & Horvitz, 1998), whilst Ced-9 is orthologous to Bcl-2, an anti-apoptotic Bcl-2 family member (Hengartner & Horvitz, 1994). Therefore, the Bcl-2 family is vital in the regulation of apoptosis with the balance between the opposing factions determining the fate of a cell. The mammalian orthologue of Ced-3 is the cysteine-dependent aspartyl specific protease (caspase) family (Yuan et al, 1993). This family of proteases are activated in response to apoptotic stimuli and are essential for the execution of apoptosis. Finally, Ced-4 is orthologous to a scaffold protein called Apaf-1 that is involved in the activation of caspases in response to intracellular stimuli (Zou et al, 1997).

**1.2.3 Apoptotic pathways**

Apoptosis is principally initiated by three pathways, the extrinsic (death receptor), the intrinsic (mitochondrial) or the perforin/granzyme pathway (Figure 1.2). Furthermore, stress at the endoplasmic reticulum (ER) can initiate an alternative pathway that results in activation of the intrinsic pathway (Elmore, 2007, Orrenius et al, 2003). The extrinsic
C. elegans cell death machinery

![Diagram of C. elegans cell death machinery]

Mammalian cell death machinery

![Diagram of mammalian cell death machinery]

Figure 1.1: The gene families involved in the cell death machinery of C. elegans and mammals.

Studies of apoptosis in C. elegans identified four genes/proteins that are central to the cell death machinery. Egl-1 is at the apex of the signalling pathway in C. elegans and is homologous to the pro-apoptotic Bcl-2 proteins of mammals. Egl-1 inhibits Ced-9, which is homologous to the anti-apoptotic Bcl-2 proteins. Ced-9 in turn inhibits activation of Ced-4 (Apaf-1 in mammals), which activates Ced-3/caspases. In mammalian cells, the anti-apoptotic proteins prevent the release of cytochrome c from mitochondria, which is required for Apaf-1 to form the apoptosome in order to activate caspase-9 and downstream effector caspases.
Figure 1.2: The extrinsic and intrinsic pathways leading to apoptosis.

The extrinsic pathway is activated when death ligands bind to their receptors. A conformational change in the receptor allows recruitment of an adaptor molecule, which binds the initiator procaspase to form the death-inducing signalling complex (DISC, upper black box). Recruitment of the initiator procaspases to the adaptor protein can be competitively inhibited by c-FLIP, which acts to regulate DISC activity. Activation of the initiator procaspase causes cleavage of downstream effector caspases through the caspase cascade.

The extrinsic pathway is able to initiate the intrinsic pathway through the cleavage of Bid into tBid, which activates Bak/Bax to induce mitochondrial outer membrane permeabilisation (MOMP). The intrinsic pathway is instigated by cellular stress signals (such as DNA damage) that, through a signalling cascade regulated by the Bcl-2 family, lead to MOMP. Cytochrome c released from the mitochondria, with (d)ATP, induces formation of the apoptosome (lower black box) that recruits and activates procaspase-9. Procaspase-9 activates the effector caspases and it is at this point that the two apoptotic signalling pathways converge. Activated caspases cleave various substrates leading to the dismantling of the cell and eventually apoptosis. Adapted from Ashkenazi, 2008.
pathway is initiated through the binding of death ligands to their cognate receptors, whilst the intrinsic pathway is activated by cellular stress or damage that is detected by members of the Bcl-2 family. These pathways do not exist in isolation since caspase-8 activated through the extrinsic pathway cleaves Bid, which in turn initiates the intrinsic pathway through the induction of mitochondrial damage (Figure 1.2) (Li et al, 1998). A common feature of both pathways is the formation of scaffold platforms that induce activation of caspases (Figure 1.2 black boxes) (Bratton et al, 2000).

1.3 Caspases
Fourteen caspases have been identified, so far, and 11 of these are present in humans. The 10 most important caspases are divided into three sub-families based upon sequence similarity. These sub-families represent inflammatory (caspases-1, -4, -5), cell death initiation (initiator caspases-2, -8, -9, -10) and cell death execution (effector caspases-3, -6, -7) caspases (Figure 1.3) (Cohen, 1997, Elmore, 2007, Fuentes-Prior & Salvesen, 2004). Upon activation, caspases are able to cleave a broad range of substrates, including each other. This means that activation of initiator caspases can lead to mass caspase activation through the caspase cascade, the molecular order of which has been elucidated for the intrinsic pathway (Figure 1.4) (Inoue et al, 2009, Slee et al, 1999a, Slee et al, 1999b). Cleavage of vital proteins by activated cell death caspases is responsible for the morphology observed during apoptosis (Fischer et al, 2003). For example, cleavage of gelsolin initiates actin depolymerisation and membrane blebbing (Kothakota et al, 1997), whilst cleavage of ICAD allows the release of CAD, a nuclease that causes DNA fragmentation (Enari et al, 1998). Caspase inhibition reduces the appearance of apoptotic morphology but often cannot prevent cell death; instead there is a shift to a caspase-independent form of cell death (Kroemer & Martin, 2005).

1.3.1 Caspase activation
To prevent inappropriate cell death caspases are expressed as inactive precursors (procaspases) that have a very low intrinsic activity and are composed of an N-terminal prodomain, and a large and small catalytic subunit (Figure 1.3). Therefore, to become catalytically competent all caspases require an activation event, which differs between the effector and initiator apoptotic caspases.

Effector caspases exist as anti-parallel dimers in their latent form and are activated through cleavage by other caspases. Removal of an N-terminal prodomain is a classic
Introduction

**Initiator Caspases**

Figure 1.3: The cell death initiator and effector caspases.

Caspases are composed of an N-terminal prodomain (area indicated in initiator caspases, or labelled ‘Pro’ in effector caspases), large catalytic subunit (blue boxes) and small catalytic subunit (red boxes). Initiator caspases possess long prodomains with CARD or DED domains that allow their recruitment into the protein complexes in which they are activated. Procaspases are cleaved after aspartic acid residues (D) to remove the prodomain and to separate the catalytic subunits, generating the mature enzyme that forms a heterotetramer. Cleavage of procaspase-8 at D374 generates the p43 and p12 intermediates, whilst cleavage at D210/216 removes the prodomain (p26). Cleavage of procaspase-10 at D415 generates the p12 catalytic subunit and p47 intermediate, whilst cleavage at D219 removes the prodomain (p25). Procaspase-9 is cleaved by caspase-3 at D330 to produce p37, whilst Apaf-1-dependent autocatalytic cleavage occurs at D315 to produce p35. D130 is a potential cleavage site within caspase-9 but cleavage at this site is not normally observed. Procaspase-3 is cleaved at D175 to produce p20 and then autocatalytically processes at D9 and D28 to produce the p19 intermediate and p17 mature subunits. Sequences and domain positions were taken from the UniProt database (www.uniprot.org) and correspond to the canonical sequence. Accession numbers were as follows: caspase-2, P42575-1; caspase-9, P55211-1; caspase-8, Q14790-1; caspase-10, Q92851-1; caspase-3, P42574-1; caspase-6, P55212-1; caspase-7, P55210-1. Additional cleavage site information was taken from Cohen, 1997.
Upon its activation, via the intrinsic pathway, caspase-9 initially cleaves and activates procaspase-3 and -7. These in turn cleave procaspase-6 and -2. Active caspase-3 is also able to feed back onto procaspase-9, resulting in the processing of procaspase-9. Finally, active caspase-6 is able to cleave procaspase-8 and -10. Caspases depicted in green are those that, based on their structure, have been classified as initiator caspases, whilst those depicted in blue have been classified as effector caspases (Figure 1.3). Taken from Inoue et al, 2009, Slee et al, 1999a and Slee et al, 1999b.
way of activating proteases (Neurath, 1989). However, removal of this domain appears to be a secondary event in caspase activation. For example, removal of the prodomain of caspase-3 is not responsible for its activation and is likely achieved through self-cleavage (Stennicke et al, 1998).

Four surface loops (L1 – L4) of caspases are responsible for forming the active site and substrate binding groove. L1 and L4 form the sides of the substrate binding groove, L3 forms the base and L2, which contains the active site cysteine, lies across the front. In addition this conformation is supported by a section of loop L2’ (L2 in the adjacent caspase) (Shiozaki & Shi, 2004). Structural work on procaspase-7 has shown that it is near identical to its active form, except that surface loops L2, L3 and L4 adopt a dramatically different conformation in the proform. This difference is because L2, which links the large and small catalytic subunits, is not cleaved in the proform (Figure 1.5 A) (Chai et al, 2001b). Cleavage within L2 at Asp 198 initiates a conformational change in the L2, L3 and L4 loops producing an intermediate structure, which can bind to the substrate (Figure 1.5 A ii). Upon binding of the substrate, the free N-terminal section of L2’ (from the adjacent cleaved caspase molecule) forms a loop bundle with L2 and L4. This stabilises the active site and results in full caspase activation (Figure 1.5 A ii). Thus, activation of effector caspase-7 occurs through intersubunit linker cleavage and, due to the strong sequence identity, this mechanism is likely conserved in the other effector caspases-3 and -6 (Chai et al, 2001b).

Initiator caspases differ from effector caspases in terms of their latent form and mode of activation. Unlike effector caspases, initiator caspases exist as inactive monomers that are activated upon recruitment to scaffold complexes. Recruitment into complexes is achieved via the caspase recruitment (CARD, caspase-2 and -9) or death effector (DED, caspase-8 and -10) domains that are present in the N-terminal prodomains of the initiator caspases (Figure 1.3). In the extrinsic pathway formation of the scaffold platform, known as the death-inducing signalling complex (DISC), is an apical event and catalyses the activation of caspase-8 and -10. In the intrinsic pathway the apoptosome, the formation of which is a later event downstream of mitochondrial perturbation, is responsible for the activation of caspase-9.

Originally it was thought that like effector caspases, initiator caspases were activated through cleavage between the large and small catalytic subunits. This was proposed to occur through an autocatalytic mechanism whereby the recruited caspases cleave
Figure 1.5: Activation of effector and initiator caspases.

A. Activation of procaspase-7 as an example of effector caspase activation, images taken from Chai et al., 2001b. The surface loops that form the catalytic site of caspase-7 are disordered in the proform (A i). Cleavage of L2 (intersubunit linker) allows the surface loops to reorganise (indicated by the red arrows in A i) into a productive conformation for catalysis (A ii). These changes permit substrate/inhibitor binding, upon which L2' (free N-terminus from neighbouring molecule) interacts with the surface loops to stabilise the active site.

B. Activation of initiator caspases at the DISC through proximity-induced dimerisation and cleavage (Adapted from Hughes et al, 2009). Procaspases are recruited to the DISC where the increase in local concentration induces dimerisation. Dimerised procaspases have a low level of activity and are able to cleave adjacent substrates (ie other procaspases and c-FLIPL). In the absence of cleavage this ‘primed’ DISC may activate cell survival signalling. Cleavage of the intersubunit linker leads to fully active caspase-8 (p43/p10) that is able to cleave a broad range of substrates, including Bid and procaspase-3, inducing apoptosis. Further cleavage after the DEDs releases the heterotetramer (p18/p10) into the cytosol, where it is able to cleave additional cellular substrates also leading to apoptosis.
and activate themselves. Although this autoproteolytic action does occur within the scaffold platforms (Yang et al, 1998) it has been suggested that it is not essential for initiator caspase activity (Acehan et al, 2002, Boatright et al, 2003, Stennicke et al, 1999), although a 99 % loss in caspase-8 activity was observed in the absence of proteolysis (Boatright et al, 2003). However, the linker regions (between large and small catalytic subunits) of caspase-8 and -9 have been found to be sufficiently long and flexible to allow formation of their active sites without cleavage (Keller et al, 2009, Stennicke et al, 1999).

Therefore, one model proposed for the activation of initiator procaspases at scaffold platforms is the induced-conformation model. In this model a conformational change at the active site results in caspase activation. For example, it has been suggested that, in addition to recruiting procaspase-9 through a CARD-CARD interaction, Apaf-1 may also bind to the homodimerisation surface of procaspase-9. This additional interaction results in stabilisation of the active site resulting in the activation of caspase-9 (Shi, 2004, Shiozaki et al, 2003). Alternatively, it has been proposed that the active site of procaspase-9 may be stabilised, inducing activation, through oligomerisation of caspase-9 monomers upon their recruitment to the apoptosome (Shi, 2004).

The second model for initiator caspase activation is proximity-induced dimerisation, which proposes that recruitment of initiator procaspases into the scaffold complexes increases the local concentration inducing dimerisation and activation. Indeed, it has been shown that, under conditions of increased concentration, caspase-8 will dimerise (Donepudi et al, 2003). The essential role of dimerisation in the activation of initiator caspases has been demonstrated by many studies. For example, kosmotropic salts, which induce protein association, have been shown to activate DED/CARD-deficient caspase-8/-9 and full length caspase-8, although the effect was greatest in the truncated proteins (Boatright et al, 2003, Hughes et al, 2009, Roy et al, 2008). Furthermore, mutation of the dimerisation site in full length caspase-8 prevented its activation in an in vitro Fas DISC model (Hughes et al, 2009), whilst transfection of dimerisation mutants of full length caspase-8/-9 prevented cellular apoptosis (Boatright et al, 2003, Hughes et al, 2009).

However, it has recently been demonstrated that, whilst dimerisation produces some level of caspase-8 activity, cleavage is in fact required for full activation and to stabilise the active dimer (Hughes et al, 2009, Keller et al, 2009). This concept led to a new model for initiator caspase activation at the DISC (Hughes et al, 2009) (Figure 1.5 B).
In the first ‘DISC priming’ step procaspase-8 is recruited to the DISC and structural rearrangement upon dimerisation permits a low level of activity. This level of activity is sufficient to cleave the neighbouring substrates c-FLIP<sub>L</sub> and caspase-8, and may permit cell survival signalling as cleaved c-FLIP<sub>L</sub> has been shown to induce NFκB activation (Kataoka & Tschopp, 2004, Yu & Shi, 2008). Subsequent removal of the linker between the catalytic domains produces the p43<sub>2</sub>/p10<sub>2</sub> dimer, resulting in maximal caspase activation, procaspase-3 and Bid cleavage, and apoptotic signalling (Hughes et al, 2009). Further cleavage between the DEDs and the large catalytic subunit results in the release of the heterotetramer (p18<sub>2</sub>/p10<sub>2</sub>) into the cytosol (Figure 1.5 B). The cleavage of procaspase-3 and Bid by DISC-bound caspase-8 in the study by Hughes et al is supported by previous work that also suggested active caspase-8 was formed at, and remained associated, with the DISC (Lavrik et al, 2003). However, this concept is in contrast with previous work that observed an inhibition of apoptosis upon prevention of heterotetramer release into the cytosol (Martin et al, 1998).

### 1.3.2 Structure and function of caspases

All caspases form a heterotetramer composed of two catalytic domains, which are each composed of a large (p20) and small (p10) catalytic subunit. The caspase heterotetramer has a specific topology, known as the caspase fold, which takes the form of a 12 stranded β-sheet (5 parallel and 1 anti-parallel from each catalytic domain) sandwiched between two layers of α-helices (Walker et al, 1994, Wilson et al, 1994). The catalytic domains of the caspase interact in an anti-parallel manner, which results in an active site being present at each end of the molecule (Figure 1.5 A ii). The catalytic machinery of caspases is composed of a catalytic dyad and an oxyanion hole. The dyad, which is responsible for catalysis of the substrate, was originally identified in caspase-1 as Cys 285 and His 237, both of which are in the p20 subunit and are conserved across most caspases (Fuentes-Prior & Salvesen, 2004, Wilson et al, 1994). The oxyanion hole, which is composed of Gly 238 and Cys 285, is important as it hydrogen bonds the P<sub>1</sub> residue of the substrate during cleavage (Blanchard et al, 1999, Fuentes-Prior & Salvesen, 2004, Walker et al, 1994).

Caspases are extremely specific enzymes that recognise a four amino acid cleavage site (N'-P<sub>4</sub>-P<sub>3</sub>-P<sub>2</sub>-P<sub>1</sub>-C') in the substrate and cleave after the P<sub>1</sub> residue, which must be aspartic acid (D) (Stennicke et al, 2000). Caspases use surface pockets S<sub>1</sub>-S<sub>2</sub>-S<sub>3</sub>-S<sub>4</sub> to correctly align the scissile bond of the substrate with the catalytic machinery. The S<sub>1</sub> and S<sub>3</sub> pockets are similar across the caspase family accounting for the general mammalian substrate recognition motif of X-E-X-D. Thus, the specificity of individual
caspases is determined by the $S_2$ and $S_4$ pockets (Fuentes-Prior & Salvesen, 2004).
In addition to being grouped by sequence similarity (Figure 1.3), caspases can also be
grouped according to their preferential recognition motif. Caspases-2, -3 and -7 were
found to prefer the recognition sequence DEXD, whilst caspases-6, -8 and -9 preferred
L/VEXD (Thornberry et al, 1997). However, there may be more flexibility in the
recognition site motif than originally thought as caspase-8 can accommodate an
aspartic acid residue in the $P_4$ position (Blanchard et al, 2000), whilst caspase-3 was
shown to cleave after the non-canonical recognition site SALD (Kipp et al, 2000).

1.3.3 Regulation of caspase activity
The activity of caspases-2, -3, -7 and -9 is regulated by a subset of the inhibitor of
apoptosis (IAP) family (Figure 1.2). This family, characterised by the presence of at
least one copy of the highly conserved baculoviral IAP repeat (BIR) domain, has eight
mammalian members but only one (XIAP) displays significant caspase inhibitory
action.

XIAP, which is one of the most well characterised IAPs, contains three BIR domains
and inhibits the activity of caspases-3, -7 and -9 (Chai et al, 2001a, Scott et al, 2005,
Srinivasula et al, 2001). Caspase-9 is inhibited by XIAP through a two site interaction
with BIR3 that prevents homodimerisation, locking the caspase in its inactive
monomeric form (Shiozaki et al, 2003, Srinivasula et al, 2001). Structural studies with
caspase-7 indicated that the linker peptide between BIR1 and BIR2 of XIAP prevented
the interaction between the caspase active site and the substrate (Chai et al, 2001a).
However, another study showed that an additional interaction between the small
catalytic subunit of caspase-3/-7 and BIR2 of XIAP was also required for inhibition
(Scott et al, 2005).

The action of IAPs is also subject to regulation by other proteins, for example the
inhibitory effect of XIAP can be reversed by the mitochondrial protein Smac (Figure
1.2). Smac can directly compete with caspases for binding to XIAP or can alter the
conformation of XIAP preventing its interaction with caspases. Either way, the result is
the release of caspases from the inhibitory interaction with XIAP, enabling their
1.4 The intrinsic pathway

The intrinsic pathway is induced through intracellular proteins, such as p53, in response to stress stimuli such as DNA damage and growth factor withdrawal (Figure 1.2). These stimuli cause a series of intracellular signalling events that result in permeabilisation of the outer mitochondrial membrane (MOMP), loss of mitochondrial membrane potential, caspase activation and cell death. The manner in which MOMP is initiated has not been fully defined, although the currently favoured model is the formation of a lipid pore by oligomerised Bax/Bak (Chipuk et al, 2006, Kuwana et al, 2002). MOMP is considered to be the pivotal event in the intrinsic cell death pathway since it results in the release of proteins that aid either caspase activation (cytochrome c, Smac, Figure 1.2) or DNA fragmentation (CAD, Endonuclease G). In the event that caspase activation is blocked, the resulting disruption of mitochondrial function as well as DNA fragmentation ensures that the cell eventually dies.

Bax and Bak are members of the Bcl-2 family, and together this family of proteins are key controllers of mitochondrial integrity (Figure 1.2). The Bcl-2 family is divided into two opposing factions, with some members enhancing (pro-apoptotic) and other inhibiting (anti-apoptotic) apoptosis. All members of the Bcl-2 family possess at least one of the four α-helical Bcl-2 homology domains (BH1-4) that are important for interactions between the family members (Muchmore et al, 1996, Sattler et al, 1997). The anti-apoptotic members (such as Bcl-2, Bcl-XL, Mcl-1, A1, and Bcl-W) usually possess all four BH domains, whilst the pro-apoptotic members possess either BH1-3 or just BH3. This allows the pro-apoptotic members to be further classified into multi-domain (those that possess BH1-3, such as Bax and Bak) and BH3-only (such as Bid, Bad, Puma, and Noxa) proteins.

The pro-apoptotic BH3-only proteins are upstream sentinels that respond to specific death stimuli and can induce apoptosis through direct (activators eg Bid) or indirect (sensitizers eg Puma, Noxa) activation of Bax and Bak (Figure 1.2) (Kuwana et al, 2002, Kuwana et al, 2005, Wei et al, 2000). Activation of BH3-only proteins is achieved through a variety of processes, such as upregulation of transcription (Puma, Noxa are upregulated by p53 (Nakano & Vousden, 2001, Oda et al, 2000)), post-translational modification (Bad is dephosphorylated (Zha et al, 1996)) or alteration of subcellular location (translocation of tBid to mitochondria (Desagher et al, 1999, Wei et al, 2000)).
The activity of pro- and anti-apoptotic Bcl-2 members is regulated through heterodimerisation, as the structure of Bcl-XL demonstrated that BH1-3 form a long hydrophobic cleft into which the BH3 domain of pro-apoptotic proteins can bind (Liu et al, 2003, Muchmore et al, 1996, Sattler et al, 1997). Thus, upon receipt of a death signal the sensitising BH3-only proteins compete for the hydrophobic cleft in the anti-apoptotic proteins releasing the activating pro-apoptotic proteins, which results in the formation of the Bax/Bak lipid pore (Figure 1.2) (Letai et al, 2002). Bax and Bak can functionally substitute for each other but the presence of at least one is vital for the progression of the intrinsic pathway. Absence of both Bax and Bak causes apoptosis induced by various stimuli to become blocked at the level of the mitochondria (Wei et al, 2001).

Following MOMP, proteins that reside in the space between the outer and inner mitochondrial membranes (such as Smac and cytochrome c) are released. Smac, as discussed earlier is important for relieving XIAP inhibition of caspases, whilst cytochrome c is vital for apoptosome formation (Figure 1.2). The apoptosome is a large protein complex that takes the shape of a seven spoke wheel and is composed of Apaf-1, cytochrome c, (d)ATP and caspase-9 (Acehan et al, 2002). Binding of cytochrome c and hydrolysis of (d)ATP induces conformational changes in Apaf-1 that allow oligomerisation and apoptosome formation (Riedl et al, 2005, Riedl & Salvesen, 2007). There are two models for the exact formation of the apoptosome but in both the CARD domain of Apaf-1 forms a central ring to which procaspase-9 is recruited, resulting in its activation (Diemand & Lupas, 2006, Yu et al, 2005). Active caspase-9 remains bound to the apoptosome and cleaves effector caspases resulting in their activation, the caspase cascade (Figure 1.4) and apoptotic cell death.

1.5 The extrinsic pathway

The extrinsic pathway (Figure 1.2) is initiated when death ligands (such as tumour necrosis factor (TNF)α, FasL, TRAIL) interact with their cognate receptors (TNF-R1, Fas, TRAIL-R1, TRAIL-R2, see later) resulting in the formation of the multi-protein DISC (Figure 1.6). The DISC was first described in 1995 as a complex of proteins (designated CAP 1-4) that assembled at the activated death receptor, Fas (Kischkel et al, 1995). CAP 1 and 2 were identified as the non-phosphorylated and phosphorylated forms of FADD, which migrate at different molecular weights (Kischkel et al, 1995), whilst CAP 3 and 4 were later found to be the full length and cleaved forms of the initiator caspase, caspase-8 (Muzio et al, 1996). The current general model for DISC
Figure 1.6: The Fas or TRAIL death-inducing signalling complex (DISC).

The DISC is composed of FasL/TRAIL (death ligands, green), Fas/TRAIL receptors (death receptors blue), initiator procaspases (purple) and c-FLIP_L (c-FLIP_L is depicted in beige). DISC formation is mediated through homotypic interactions between death domains (DD, oblongs) or death effector domains (DED, hexagons). FADD is recruited to the death receptor through DD interactions, whilst initiator caspases and/or c-FLIP_L are recruited to FADD through DED interactions.
formation is that, following death ligand-receptor interaction, an adaptor protein (FADD for Fas and TRAIL DISC or TRADD for TNF DISC) is recruited to the cytoplasmic death domain (DD) of the receptor. This adaptor recruits procaspase-8, procaspase-10 and/or other regulatory proteins (such as c-FLIP and RIP) to produce the DISC (Figure 1.6). Once recruited to the DISC, the initiator procaspases are activated as discussed earlier. Although the absolute involvement of initiator caspase cleavage in their activation is debated, it does occur following recruitment into the DISC via a two step mechanism. In procaspase-8 the first cleavage after Asp 374 generates the p43/41 and p12, which is rapidly converted into p10 (Figure 1.3 and Figure 1.5 B). The second cleavage event at Asp 216 cleaves p43/41 into the p26/24 prodomain (remains bound to the DISC) and the p18 subunit that is released into the cytosol as a heterotetramer with the p10 subunit (Figure 1.5 B) (Medema et al, 1997). Active caspase-8/-10 is able to initiate the caspase cascade, through cleavage of procaspase-3, and the intrinsic pathway, via Bid cleavage, ultimately resulting in cell death (Figure 1.2). Activation of the intrinsic pathway through caspase-8-mediated Bid cleavage acts as an amplification loop to the extrinsic pathway, resulting in greater caspase activation that is either required (Type II cells) or not (Type I cells) for apoptosis to occur.

1.5.1 Type I and Type II death receptor signalling

Overexpression of anti-apoptotic Bcl-2 family members, and thus blockade of the intrinsic pathway, has been shown to prevent Fas-induced apoptosis in some cell lines. These cell lines were designated as Type II cells, whilst those that were unaffected by the overexpression of anti-apoptotic Bcl-2 family members were designated Type I cells (Jaattela et al, 1995, Scaffidi et al, 1998). Type I and II cells were proposed to be equally sensitive to Fas-induced apoptosis, based on the amount of DNA fragmentation observed following treatment with Fas antibody for more than 10 hr. However, Type I cells formed large amounts of DISC and rapidly activated caspase-8 and -3, whilst Type II cells formed comparatively little DISC and had delayed caspase activation (Scaffidi et al, 1998). It has been suggested that the small amount of active caspase-8 generated at a Type II DISC is not sufficient to directly activate caspase-3. Thus it has been suggested that full length Bid is the preferred substrate for a Type II DISC, causing the dependency on the mitochondrial amplification loop. In contrast, in a Type I cell it is proposed that the greater level of active caspase-8 generated at the DISC allows direct activation of caspase-3 (and the caspase cascade), resulting in independence from the intrinsic pathway (Figure 1.7) (Barnhart et al, 2003).
In Type I cells ligation of death receptors results in the formation of a large amount of DISC and thus activation of a large amount of caspase-8/-10. Active initiator caspases are able to directly cleave and activate caspase-3 leading to the caspase cascade and apoptosis. Active initiator caspases are also able to cleave Bid resulting in activation of the intrinsic pathway but this is not required for apoptosis to proceed.

In Type II cells, a lower level of DISC is formed resulting in reduced activation of caspase-8/-10. The level of active initiator caspases is not sufficient to directly activate caspase-3. However, Bid can be cleaved leading to activation of caspase-9 through the intrinsic pathway. The apoptosome is then able to amplify the activation of caspase-3 leading to the caspase cascade and apoptosis. Thick arrows indicate the proposed predominant events, whilst thin arrows indicate the minor events, which occur in each cell type.
The Type I/II model is supported by many studies where alteration of proteins involved in the intrinsic pathway affected Type II, but not Type I cells. For example, a reduction in caspase-9 expression protected Type II cells, but not Type I cells, from death receptor apoptosis (Samraj et al, 2006). Furthermore, in Bid or Bax/Bak deficient mice the Type II hepatocytes, but not the Type I thymocytes, were protected against Fas-induced apoptosis (Lindsten et al, 2000, Wei et al, 2001, Yin et al, 1999).

Although originally described in response to Fas activation, the Type I/II model has been shown to exist in the TRAIL-induced apoptosis, but it is unclear whether it is also present in TNF-mediated apoptosis (Barnhart et al, 2003). Bcl-2 and Bcl-XL attenuated TRAIL-induced apoptosis in Type II but not in Type I cells (Fulda et al, 2002). In addition, blockade of the intrinsic pathway through Bax knockout (LeBlanc et al, 2002), loss of caspase-9 expression (Samraj et al, 2006) or caspase-9 inhibition (Ozoren et al, 2000, Ozoren & El-Deiry, 2002) impaired TRAIL-mediated apoptosis in Type II cells.

Although many studies support the Type I/II model, others dispute its existence. Bcl-2 overexpression was shown to inhibit Fas or TRAIL-induced apoptosis in Type II cells but has also has been shown to either only delay apoptosis (Belka et al, 2000, Itoh et al, 1993, Suliman et al, 2001) or have no effect at all (Huang et al, 1999, Keogh et al, 2000, Rudner et al, 2001, Walczak et al, 2000). Explanations for the conflicting evidence for the Type I/II model have included the extent of Bcl-2 overexpression (Ruiz de Almodovar et al, 2001), the type of stimulus used (ligand versus antibody, crosslinked versus non-crosslinked) (Barnhart et al, 2003) and the strength of the stimulus (Rudner et al, 2005). The dependency of the Type I/II model on these various factors means that its importance in vivo is unclear.

Several reasons have been suggested for the difference between Type I and II cells, although many of these are controversial resulting in no definitive explanation. Suggestions for the apparent dependency of Type II cells on the intrinsic pathway amplification loop have included the blockade of DISC formation by an unidentified protein (Scaffidi et al, 1998), lower cell surface expression of receptors (Eramo et al, 2004, Sprick et al, 2000), lack of receptor localisation to lipid rafts (Legembre et al, 2005, Muppidi & Siegel, 2004, Siegel et al, 2004), formation of smaller receptor clusters upon activation (Algeciras-Schimnich et al, 2002, Soderstrom et al, 2005), delay or absence of DISC internalisation (Algeciras-Schimnich & Peter, 2003, Eramo et al, 2004), uncoupling from the actin cytoskeleton (Algeciras-Schimnich et al, 2002, Algeciras-Schimnich & Peter, 2003), altered expression levels of intracellular proteins
that regulate apoptosis (such as such as XIAP, caspase-3 and Smac) (Jost et al, 2009, Sun et al, 2002), a high expression level of caspase-9 and low activation rate of caspase-8 at the DISC (Okazaki et al, 2008), and a reduced availability of caspase-3 for cleavage by caspase-8 (Gilbert et al, 2008).

1.6 Death ligands and death receptors

The death ligands and receptors, which are responsible for mediating the extrinsic pathway, are members of the TNF or TNF receptor (TNFR) superfamilies. These families are large, encompassing 18 ligands and more than 20 receptors, and perform diverse biological functions including a central role in adaptive immunity.

The ligands of the TNF superfamily are expressed as type II transmembrane proteins that form a trimer and can be cleaved to produce a trimeric soluble form. This cleavage is mediated by distinct proteases and is essential for some ligands but can inhibit the function of others. The structure of the soluble form of TNFα demonstrated that the ligand monomer was an anti-parallel β-sheet sandwich, which formed a symmetrical bell-shaped trimer (Eck & Sprang, 1989). All TNF ligands share a C-terminal TNF homology domain, which is present in both transmembrane and soluble forms and is responsible for the propensity of the ligands to self-associate into trimers. Apart from this domain there is little similarity in the external surface of the trimeric ligands, explaining the specificity of each ligand for its receptor (Bodmer et al, 2002, Locksley et al, 2001). Furthermore, TRAIL is unique as it requires the presence of a zinc atom in the trimer interface for its stability and biological activity (Hymowitz et al, 2000).

The TNFR superfamily are type I membrane proteins that possess a variable number of characteristic extracellular cysteine rich subdomains (CRDs) (Figure 1.8). These cysteine rich sequences fold into modules that stack on top of each other producing an elongated structure, which binds the surface groove between adjacent subunits of the trimeric ligand (Banner et al, 1993, Hymowitz et al, 1999, Naismith & Sprang, 1998, Orlinick et al, 1997). In addition to providing a binding surface for TNF ligands the CRDs are involved in ligand independent self-association of the receptors into oligomers. CRD1 harbours the pre-ligand assembly domain (PLAD) that is responsible for self-association and is distinct from the CRD2/3 ligand binding region. PLADs have been identified in TNF, Fas and TRAIL receptors and are essential for receptor function
Figure 1.8: The Fas, TRAIL and TNF death receptors.

The Fas, TRAIL and TNF death receptors (blue) and corresponding ligands (green) are shown. The dark blue ovals represent the extracellular cysteine rich domains, whilst the light blue oblongs represent the death domains (DD). TRAIL-R3 and -R4 are classed as decoy receptors, which bind TRAIL and are proposed to inhibit apoptotic signalling through TRAIL-R1/-R2. Adapted from Locksley et al, 2001.

The protein interaction domain present in the cytoplasmic tail of the receptor is not universally conserved across the TNFR superfamily. This domain is essential for transmitting cell surface events into intracellular signalling cascades and determines which pathways are activated. Receptors with a TNF receptor-associated factor (TRAF) domain (such as TNF-R2) usually activate survival, proliferation and/or differentiation signalling pathways (Baud & Karin, 2001). However, the nine receptors that possess a DD (such as TNF-R1, Fas, TRAIL-R1 and TRAIL-R2, Figure 1.8) are classed as the death receptors, which induce apoptosis through the formation of DISC (Figure 1.6).

The 80 amino acid DD was originally identified through deletion mutagenesis of TNF-R1. Deletion of this region of the receptor prevented cell death signalling and has been identified in many other proteins, including Fas, TRAIL-R1/-R2, FADD and TRADD, enabling interactions to occur between these proteins (Boldin et al, 1995, Chinnaiyan et al, 1995, Feinstein et al, 1995, Hsu et al, 1995, MacFarlane et al, 1997, Pan et al, 1997a, Pan et al, 1997b, Tartaglia et al, 1993). The overall secondary structure of the DD is conserved between the DD proteins as well as in the DD superfamily, which includes the DED and CARD domains. All DDs are composed of six anti-parallel amphipathic α-helices, although there are differences in the size and orientation of the helices between individual proteins (Sukits et al, 2001). Despite the similar three-dimensional structure of the DD, it has been proposed that the death adaptors (FADD and TRADD) are able to discriminate between different death receptors through the use of specific binding surfaces (Sandu et al, 2005).

1.7 Death receptor signalling

Apoptosis induced through death receptors occurs as either the primary (Fas and TRAIL-R1/-R2) or secondary (TNF-R1) event. TNFα is a potent cytokine that plays an important role in inflammation, proliferation, differentiation and apoptosis. Ligation of TNF-R1 results in apoptosis through the recruitment of the adaptor molecule TRADD, which is able to recruit the additional mediators TRAF-2 and RIP or FADD and caspase-8 (Harper et al, 2003a, Hsu et al, 1995, Hsu et al, 1996a, Hsu et al, 1996b). TRAF-2 and RIP are recruited to TRADD to form the primary complex, which signals to cell survival through kinase pathways (Micheau & Tschopp, 2003). FADD and
caspase-8 are recruited to TRADD, to form a secondary apoptosis-inducing complex, which may either be a non-receptor-associated cytoplasmic complex (Micheau & Tschopp, 2003) or associated with TNF receptors in endocytic vesicles (Schneider-Brachert et al, 2004). The balance in the activity of the cell survival complex versus the death-inducing complex then determines whether the cell lives or dies upon exposure to TNF (Micheau & Tschopp, 2003).

In contrast to TNFα signalling, FasL and TRAIL primarily induce the formation of the DISC through the recruitment of FADD and caspase-8 to the receptors (Figure 1.6). However, Fas and TRAIL also induce formation of secondary cytoplasmic signalling complexes, which perform different functions (Lavrik et al, 2008, Varfolomeev et al, 2005). Fas stimulation leads to the formation of a secondary complex composed of FADD, c-FLIP_{L,S,R} and caspase-8, which appears to enhance apoptosis by amplifying caspase activation (Lavrik et al, 2008). However, similar to TNF complex I, the TRAIL-induced secondary complex, which is composed of FADD, caspase-8, RIP, TRAF-2, NEMO, and possibly TRADD, activates kinase pathways (Varfolomeev et al, 2005). RIP, which is essential for NFκB activation by TRAIL, has been detected in the TRAIL DISC suggesting that NFκB may also be activated through the primary DISC complex (Harper et al, 2001, Lin et al, 2000). Although it is unclear how universal this is since RIP was not identified in the DISC from the BJAB cell line (Kischkel et al, 2000). However, signalling to NFκB may occur through other routes upon TRAIL stimulation since recent proteomic screening identified the arginine methyltransferase PRMT5, which is capable of activating NFκB signalling, as a TRAIL-R1/-R2 interacting protein (Tanaka et al, 2009).

In TNF signalling the activity of the primary kinase-activating complex can hinder the effect of the secondary apoptosis-inducing complex, since NFκB activation can increase the levels of anti-apoptotic proteins, such as c-FLIP, Bcl-XL, and XIAP (Micheau & Tschopp, 2003). However, in TRAIL signalling the activity of the DISC is required for formation of the secondary complex and the kinase pathways activated by the secondary complex are not dominant inhibitors of TRAIL-induced apoptosis (Varfolomeev et al, 2005). In fact, NFκB activation upon TRAIL signalling is only predominant when the apoptosis signalling arm is blocked (Harper et al, 2001). TRAIL activation of kinase pathways has been shown to increase chemokine production, macrophage migration and secretion of inflammatory cytokines. This may promote phagocytosis of apoptotic bodies but could also lead to side effects following TRAIL treatment (Tang et al, 2009, Varfolomeev et al, 2005). Furthermore, activation of
TRAIL receptors in cells that are resistant to its apoptotic effects could enhance tumorigenic properties though activation of non-apoptotic signalling. Indeed, treatment of resistant cell lines with TRAIL can promote survival, proliferation, migration and invasion (Ehrhardt et al, 2003, Ishimura et al, 2006). Thus, the therapeutic use of TRAIL must be limited to those cancers that are sensitive, or can be sensitised, to its apoptotic effects.

1.8 TRAIL and TRAIL receptors

TRAIL was identified in 1995 (Wiley et al, 1995) and is a highly promiscuous death ligand that interacts with five receptors (Figure 1.8). TRAIL-R1, -R2, and -R4 are all classical type I transmembrane proteins, TRAIL-R3 is tethered to the membrane by a glycosyl-phosphatidylinositol (GPI) anchor, whilst the more evolutionary distant receptor osteoprotegerin (OPG) is secreted (Figure 1.8). Based on in vitro studies, TRAIL has a similar affinity for all of the receptors at 4 °C, however at 37 °C the rank-ordered affinity is TRAIL-R2 > TRAIL-R1 > TRAIL-R3 > TRAIL-R4 > OPG (Clancy et al, 2005, Truneh et al, 2000).

TRAIL-R1 and -R2 were identified in 1997, through sequence similarity searches, as members of the TNFR superfamily and are the only TRAIL receptors able to initiate apoptosis (MacFarlane et al, 1997, Pan et al, 1997a, Pan et al, 1997b). Post-translational modification of these receptors, through palmitoylation (TRAIL-R1) (Rossin et al, 2009) or glycosylation (TRAIL-R1 and -R2) (Wagner et al, 2007), is important for their function. Furthermore, TRAIL-R2 is expressed as two isoforms designated long (TRAIL-R2L) and short (TRAIL-R2S), which have a variable expression ratio but are functionally similar (Wang & Jeng, 2000).

TRAIL-R3 and -R4 have been classed as decoy receptors since they compete for TRAIL binding, albeit with a lower affinity (Clancy et al, 2005, Truneh et al, 2000), but cannot induce apoptosis due to their lack of complete intracellular domains. Indeed, overexpression of TRAIL-R3/-R4 in TRAIL sensitive tumour cells prevented TRAIL-induced apoptosis (Degli-Esposti et al, 1997a, Degli-Esposti et al, 1997b, Zhang et al, 2000). Although TRAIL-R3 and -R4 are both decoy receptors, the way in which they inhibit TRAIL-induced apoptosis through TRAIL-R1/-R2 is different. TRAIL-R3 is proposed to simply sequester TRAIL away from the other receptors (Merino et al, 2006), whilst TRAIL-R4 may reduce the activity of the TRAIL DISC. TRAIL-R4 has been shown to form a heteromeric complex with TRAIL-R2, which is mediated either
through the PLADs (Clancy et al, 2005) or via the ligand (Merino et al, 2006), and thus can become incorporated into the DISC. While the presence of TRAIL-R4 in the DISC would be predicted to reduce the number of complete receptor DDs, it did not appear to affect the recruitment of FADD or procaspase-8. Instead, the inclusion of TRAIL-R4 impaired the processing of procaspase-8 within the DISC and was therefore suggested to destabilise the structure of the DISC (Merino et al, 2006).

TRAIL-R1 and -R2 specific ligands have demonstrated that, even when both receptors are expressed, each can independently induce apoptosis and that the predominant signalling receptor can vary between cell lines (Kelley et al, 2005, MacFarlane et al, 2005). TRAIL-R2 was identified as the major contributor to apoptosis in cells from lung carcinoma, colon carcinoma and breast carcinoma as well as monkey hepatocytes (Kelley et al, 2005). However, in CLL and MCL cells TRAIL-R1 was shown to be the dominant receptor (MacFarlane et al, 2005). Although TRAIL-R1 and TRAIL-R2 can signal independently there is evidence that they may also co-operate to induce TRAIL signalling. Stimulation of both receptors has been shown to result in the formation of heteromeric complexes, although this occurs to a lower extent than the formation of homomeric complexes (Kischkel et al, 2000, Schneider et al, 1997). The homo- and hetero-receptor interactions could occur through the ligand, via PLADs (discussed earlier) and/or through the cytoplasmic domains of TRAIL-R1 and -R2 (Schneider et al, 1997).

1.8.1 Physiological role of TRAIL
TRAIL does not appear to have a role in embryonic development since TRAIL null mice develop normally with no gross phenotypic abnormalities (Cretney et al, 2002, Lamhamedi-Cherradi et al, 2003). However, TRAIL seems to be vital for immune function, with a role in tumour surveillance and negative selection. This was demonstrated through the TRAIL null mice, which were more susceptible to tumour initiation and metastasis, and possessed enlarged thymi with increased numbers of thymocytes (Cretney et al, 2002, Sedger et al, 2002). The role of TRAIL in negative selection may also explain the increased susceptibility of TRAIL null mice to autoimmune diseases (Lamhamedi-Cherradi et al, 2003) and is concurrent with the ability of TRAIL to inhibit autoimmune arthritis and autoimmune encephalomyelitis (Hilliard et al, 2001, Song et al, 2000). TRAIL has also been shown to be a negative regulator of erythropoesis (Zamai et al, 2000) and through OPG may help regulate bone resorption (Vitovski et al, 2007). However, it is unclear whether these are
physiologically relevant roles as no abnormalities were observed in the peripheral blood haematology or bones of TRAIL null mice (Sedger et al, 2002).

Mice only express one TRAIL receptor, which is most closely related to TRAIL-R2 (Wu et al, 1999), and removal of this receptor confers resistance to TRAIL-induced apoptosis in embryo fibroblasts (Finnberg et al, 2005). Similar to TRAIL null mice, TRAIL-R null mice also developed normally except for enlarged thymi, which implied the involvement of TRAIL-R in negative selection. However, in contrast to TRAIL null mice no increased susceptibility to autoimmune disease was observed, suggesting that TRAIL-R was not involved in TRAIL-mediated protection against these diseases (Finnberg et al, 2005). Furthermore, the involvement of the TRAIL-R in the tumour surveillance role of TRAIL has been controversial. In one study TRAIL-R deficient mice were protected from primary tumour formation (Finnberg et al, 2008), whilst this was not the case in another study (Grosse-Wilde et al, 2008). However, both studies suggested a role for TRAIL-R in suppressing cancer metastasis (Finnberg et al, 2008, 2003, Grosse-Wilde et al, 2008).

1.8.2 TRAIL as a cancer biotherapeutic

Therapies that activate the extrinsic pathway of apoptosis possess great therapeutic potential since they can activate caspases and induce apoptosis independently of the p53 status of the tumour. In addition, death receptors are widely expressed and components of the extrinsic pathway are rarely mutated in cancers (Ashkenazi, 2008). Agonists of the death receptors have a wide variety of applications. They can be used alone when the tumour is especially sensitive to their action but can also be used in combination with other new and traditional therapies to synergistically induce apoptosis, overcoming existing resistance and/or reducing the emergence of resistant cells.

Although in theory all death ligands should have therapeutic potential, their clinical development is limited by the selectivity for normal versus tumour cells. In vivo administration of Fas antibody or FasL resulted in massive hepatotoxicity and death of mice (Ogasawara et al, 1993, Tanaka et al, 1997), whilst TNF induced a lethal inflammatory response (Tracey et al, 1986, Van Molle et al, 2002). However, in vivo administration of TRAIL inhibited tumour progression without overt toxicity in either mice or non-human primates (Ashkenazi et al, 1999, Roth et al, 1999, Walczak et al, 1999).
The absence of TRAIL-induced side effects is attributable to its ability to selectively induce apoptosis in tumour cells but not in most normal cells (Bouralexis et al, 2005). However, TRAIL has been shown to induce apoptosis in normal hepatocytes (Jo et al, 2000, Lawrence et al, 2001), prostate epithelial cells (Nesterov et al, 2002), keratinocytes (Qin et al, 2001) and astrocytes (Walczak et al, 1999). It has been suggested that the apoptosis observed in these normal cell lines may have related to the form of ligand used as tagged TRAIL, which possesses a higher propensity to aggregate, was lethal to normal cells whilst untagged TRAIL was not toxic (Lawrence et al, 2001, Qin et al, 2001). Based on this, it was suggested that normal cells will undergo apoptosis upon high-order aggregation of the receptors, which can be achieved by aggregated tagged ligands (Ashkenazi, 2002, Lawrence et al, 2001). Therefore, normal cells could be envisaged to have a TRAIL signalling threshold, below which no apoptosis is observed and above which formation of extensively aggregated DISC results in cell death.

Clinical trials of recombinant TRAIL and receptor antibodies, such as Mapatumumab (TRAIL-R1) and Lexatumumab (TRAIL-R2), have shown treatment is well tolerated alone and in combination with other drugs (Ashkenazi, 2008, Hotte et al, 2008, Plummer et al, 2007). TRAIL gene transfer therapy has also been suggested as an alternative method to systemic administration of recombinant forms of TRAIL (Griffith et al, 2000). However, overexpression of TRAIL has been shown to induce resistance to TRAIL treatment, by reducing cell surface expression of TRAIL receptors; thus questioning the therapeutic use of TRAIL gene therapy (Wenger et al, 2006).

Most tumour cell lines are sensitive to TRAIL but it is now becoming apparent that many primary tumours are resistant to its apoptotic effects. However, if primary tumours can be selectively sensitised to its effects then administration of TRAIL can specifically target the tumour. Many drugs have been shown to sensitise tumour cells to, or synergise with, TRAIL through different mechanisms (Johnstone et al, 2008). For example, through upregulation of TRAIL-R2 expression (eg COX-2 inhibitors (He et al, 2008), doxorubicin (Wu et al, 2007), cisplatin (Duiker et al, 2009)), inhibition of overexpressed anti-apoptotic Bcl-2 proteins (BH3 mimetics (Hetschko et al, 2008)), or through improving DISC formation (eg ionising radiation (Verbrugge et al, 2008), depsipeptide (Inoue et al, 2004)). Histone deacetylase (depsipeptide) and proteosome (lactacystin) inhibitors were both able to selectively sensitise transformed lymphocytes, but not normal lymphocytes, to TRAIL (Inoue et al, 2004, Masdehors et al, 2000).
Therefore, careful evaluation of combination therapies as well as tumour sensitivity should permit targeted and safe treatment of cancers with TRAIL in the future.

1.8.3 TRAIL selectivity

The selectivity of TRAIL for inducing apoptosis in tumour cells is not fully understood, although several suggestions have been made. Initially it was thought that limited expression of the ligand or receptor may be responsible but this was found to not be the case since TRAIL and its receptors are widely expressed. The ability of the decoy receptors TRAIL-R3 and -R4 to inhibit apoptosis upon transfection lead to the suggestion that they regulate TRAIL sensitivity through competing for binding to TRAIL. In support of this, it has recently been shown that the ratio of TRAIL-R1 to TRAIL-R3/R4 in terms of surface expression or mRNA levels predicts TRAIL sensitivity in various cancer and normal cell lines (Buneker et al, 2009). However, in another report expression of TRAIL-R3 and -R4 did not correlate with the sensitivity of most melanoma cell lines; instead sensitivity correlated with expression of TRAIL-R2 (Zhang et al, 1999). However, expression of TRAIL-R2 did not predict sensitivity in all cell lines and those that were resistant despite receptor expression demonstrated reduced effector caspase activation. In these cell lines TRAIL resistance was attributed to a reduced release of mitochondrial Smac that was unable to remove the inhibition of XIAP (Zhang et al, 2001). In agreement with the melanoma cell line data, the sensitivity of different Jurkat cell line clones to TRAIL was attributed to TRAIL-R2 expression, caspase-8/-3 activation and the extent of RIP cleavage (Jang et al, 2003). The TRAIL resistance of several breast cancer cell lines has also been attributed to a loss of surface expression of TRAIL-R1 and -R2. This occurred through constitutive endocytosis of the receptors and therefore targeting the endocytosis machinery may be a potential method for restoring TRAIL sensitivity (Zhang & Zhang, 2008).

In another study surface expression levels of the TRAIL receptors across a panel of pancreatic or colorectal cancer cell lines did not correlate with sensitivity to TRAIL-induced apoptosis (Wagner et al, 2007). Sensitivity to TRAIL in these cell lines also did not correlate with the expression of anti-apoptotic proteins, such as c-FLIP, Bcl-2, Bcl-XL and XIAP, which have been implicated in regulating sensitivity (Newsom-Davis et al, 2009). However, a consistent correlation was found between TRAIL sensitivity and the expression of O-glycosylation enzymes (GALNT14, FUT6). These enzymes, which likely glycosylate TRAIL-R1 and -R2, showed a consistent correlation to TRAIL sensitivity and were expressed in sensitive but not in most resistant cell lines. Knock down of these enzymes decreased TRAIL sensitivity through reducing recruitment of
FADD and caspase-8 to the DISC. Thus the expression of these enzymes may be a potentially useful biomarker for rational TRAIL therapy (Wagner et al, 2007).

Many other proteins have been identified that can also regulate DISC formation and thus may affect TRAIL selectivity/sensitivity. An RNAi screen discovered that downregulation of SRP72 and GSK3α inhibited TRAIL-induced apoptosis, likely through affecting the DISC. Loss of SRP72 completely abrogated caspase-8 processing, leading to the suggestion that it is required for targeting of TRAIL receptors or other DISC components to the cell membrane (Aza-Blanc et al, 2003). IG20 has been shown to interact with TRAIL-R1 and -R2 increasing DISC formation and caspase-8 activation (Ramaswamy et al, 2004), whilst TRAIL receptor interaction with MADD (alternative splice form of the IG20) prevented caspase-8 activation (Mulherkar et al, 2007). The MUC1 oncprotein has also been shown to interact with caspase-8 and FADD, preventing caspase-8 recruitment to the DISC. It is believed that MUC1 may act in non-malignant cells to protect against caspase-8 activation, however its overexpression in many human tumours may induce resistance to death receptor-induced apoptosis (Agata et al, 2008). The DED protein PEA15 has been identified in the DISC of TRAIL resistant glioma cell lines, where it was proposed to prevent activation of the initiator caspases, resulting in the observed TRAIL resistance (Xiao et al, 2002). Furthermore activation of both protein kinase C (PKC) (Harper et al, 2003b) and protein kinase B (PKB) (Strauss et al, 2003) has been shown to reduce DISC formation resulting in resistance to death receptor-mediated apoptosis. The dependency of death receptor-mediated apoptosis on the DISC, and the ability of many proteins to modulate its formation, implicates a central role for the DISC in the regulation of TRAIL selectivity/sensitivity.

1.9 Components of the DISC

In its simplest form the Fas/TRAIL DISC can be considered to be composed of the death ligand, death receptor, FADD, initiator caspases (procaspase-8/-10) and the well documented regulator c-FLIP (Figure 1.6).

1.9.1 FADD

FADD (or Fas-associated death domain protein) was originally identified as important for Fas-induced apoptosis (Chinnaiyan et al, 1995). It is a bipartite molecule composed of an N-terminal DED and C-terminal DD. The DED of FADD is responsible for interacting with other DED proteins (such as procaspase-8/-10 or c-FLIP) and is also
involved in FADD self-association, whilst the DD of FADD interacts with the DD of Fas, TRAIL-R1, or TRAIL-R2 (Peter, 2000). The full length structure of FADD (191 out of 208 amino acids) showed that the six α-helical DD and DED (whose structures had been determined separately (Berglund et al, 2000, Eberstadt et al, 1998, Jeong et al, 1999)) are orientated in a tail-to-tail manner and are tethered together by a flexible loop (Figure 1.9 A i) (Carrington et al, 2006). Overall the structure of FADD is quite extended, which presumably leaves its interaction surfaces sterically unhindered allowing binding to other DD or DED proteins (Carrington et al, 2006, Jeong et al, 1999).

Deletion of FADD from cell lines demonstrated it is a vital component of the DISC and thus of Fas and TRAIL apoptotic signalling (Juo et al, 1999, Kuang et al, 2000, Sprick et al, 2000). However, FADD may also be involved in apoptotic signalling outside of the DISC. Following ER stress, ER-bound RTN3 has been shown to recruit FADD and caspase-8 to form an ER membrane complex that can initiate cell death (Xiang et al, 2006). Also adenylate kinase 2, released from the intermembrane space following MOMP, has been shown to form a complex with FADD and caspase-10 that could be involved in amplifying the apoptotic signal downstream of the apoptosome (Lee et al, 2007).

Due to its essential role in death receptor-mediated apoptosis, FADD was originally believed to be a cytoplasmic protein but in fact FADD has been found to be present in both the nucleus and cytoplasm. Treatment with TNFα increased cytoplasmic levels of FADD in a caspase and Bid dependent manner, which suggested a feed-forward loop to enhance apoptosis (Zhang et al, 2008). Furthermore, FADD has recently been shown to redistribute from the nucleus to the cytoplasm following treatment with Fas (Foger et al, 2009). The role of nuclear FADD is currently not clear, although it has been suggested that it may act as a safety mechanism preventing inappropriate DISC assembly (Foger et al, 2009). FADD has also been suggested to link DNA repair and apoptosis as it was found to be associated with methyl-CpG binding domain protein 4, which is involved in mismatch repair (Screaton et al, 2003), as well as with PIDD, which is also involved in the response to DNA damage (Telliez et al, 2000). Alternatively, nuclear FADD may participate in the suggested non-apoptotic functions of FADD (see later).

There are differing opinions on how the localisation of FADD is regulated. One suggestion is that nuclear localisation and export signals that reside in the DED may be
Figure 1.9: Structural elements of FADD.

Ai. Ribbon representation of the structure of FADD, taken from Carrington et al, 2006. Aii. Amino acid sequence with the location of the α-helices of the DED (light blue) and DD (dark blue). Red amino acids are those that have been suggested to be involved in death receptor binding, black bold confers specificity to Fas binding, pink confers specificity to TRAIL-R2, green are those specifically involved in the interaction with procaspase-8, brown residues form the ‘universal’ surface (FADD self-association and DED protein recruitment) and underlined residues form the RXDLL motif. B. Maps of the residues important for FADD-protein interactions. Red residues are some of those involved with Fas interaction, brown residues form the ‘universal’ surface, green are those specifically involved in the interaction with procaspase-8, and orange are some of the DED residues that have been proposed to be involved in receptor association. Map ii is a 180 ° vertical rotation of map i, map iii is 90 ° vertical rotation of map i and map iv is 150 ° vertical rotation of map iii. All maps taken (and adapted) from Carrington et al, 2006.
important (Gomez-Angelats & Cidlowski, 2003). However, the existence of these signal motifs has been disputed. Instead, it was proposed that phosphorylation of FADD is important in determining localisation, which may be mediated by the nuclear shuttling protein exportin-5 (Alappat et al, 2005, Screaton et al, 2003).

FADD was suggested to be phosphorylated by a cell cycle-regulated kinase (Scaffidi et al, 2000) and later casein kinase Iα was found to phosphorylate Ser 194 in vitro and in vivo (Alappat et al, 2005). In addition Fas-interacting serine/threonine kinase (FIST) has been shown to interact with the C-terminal domain of Fas and induce FADD phosphorylation (Rochat-Steiner et al, 2000). An increase in FADD phosphorylation has been shown to increase NFκB activity, but does not influence its recruitment to the DISC, and thus it has been suggested to be important for the non-apoptotic roles of FADD (Chen et al, 2005, Scaffidi et al, 2000). In support of this, chimeric FADD null or Ser 191 (Ser 194 in humans) mutant mice both possessed defects in the proliferation of T cells (Hua et al, 2003, Kabra et al, 2001, Zhang et al, 1998). This, in addition to other studies, has demonstrated that FADD is involved in cell cycle regulation and that its phosphorylation status is important for this role (Chen et al, 2005, Matsuyoshi et al, 2006, Osborn et al, 2007). Furthermore, FADD has been shown to have a role in embryogenesis. Disruption of the FADD gene resulted in prenatal death of the embryos, which possessed both defective cardiac development and abdominal haemorrhage (Yeh et al, 1998, Zhang et al, 1998). Therefore, FADD appears to be much more than an adaptor protein for the DISC.

1.9.2 Caspase-8

There are nine splice variants of caspase-8, of which only three are expressed (procaspase-8L (62 kDa), procaspase-8/a (55 kDa) and procaspase-8/b (53 kDa)). However, only procaspase-8/a and procaspase-8/b are recruited to the DISC where they are cleaved with similar kinetics (Scaffidi et al, 1997). Following release of the heterotetramer from the DISC, caspase-8 has been shown to cleave the prodomain between the tandem DEDs, releasing DEDa (DED1). DEDa/1 accumulates and translocates to the nucleus upregulating the expression of caspase-8, and possibly other apoptotic proteins, thus replenishing caspase-8 levels in a positive feedback loop (Yao et al, 2007).

Mutation of the caspase-8 gene in cell lines and mice indicated the vital role the enzyme plays in Fas, TNFα and TRAIL-mediated apoptosis (Juo et al, 1998, Sprick et al, 2000, Varfolomeev et al, 1998). Caspase-8 is also likely involved in non-apoptotic
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signalling and has been implicated in haematopoesis, cell motility and immune activation (Chun et al, 2002, Helfer et al, 2006, Senft et al, 2007, Varfolomeev et al, 1998). Furthermore, homozygous disruption of the caspase-8 gene in mice caused prenatal lethality. The caspase-8 null embryos possessed impaired heart muscle development and congested accumulation of erythrocytes. This phenotype was reminiscent of that in the FADD null embryos suggesting that these two proteins may co-operate to mediate signalling from other stimuli that is vital for embryogenesis (Varfolomeev et al, 1998, Yeh et al, 1998, Zhang et al, 1998). The non-apoptotic functions of FADD and caspase-8 do not relate to any known functions of the TNF receptor family and, as mentioned earlier, the removal of TRAIL, TRAIL-R, Fas or FasL from mice did not result in embryonic lethality (Adachi et al, 1996, Cretney et al, 2002, Karray et al, 2004, Lamhamedi-Cherradi et al, 2003). Therefore, it is possible that the non-apoptotic and embryogenesis roles of FADD and caspase-8 are mediated independently of the receptors that trigger their apoptotic role. However, it is possible that there is some redundancy between the death receptors and thus, without a double knock out of Fas and TRAIL-R, it is difficult to definitively state that the death receptors are not involved.

1.9.3 Caspase-10

Seven splice variants of caspase-10 (a-g) have been identified but only three isoforms of 59 kDa, 54 kDa and 31 kDa are expressed (Wang et al, 2007). All of the expressed proteins contain two DEDs but only the longer isoforms contain the large and small catalytic subunits (Sprick et al, 2002). Caspase-10 has been shown to be recruited to and activated at the Fas and TRAIL DISC (Kischkel et al, 2001, Sprick et al, 2002, Wang et al, 2001). Recruitment leads to cleavage producing p47/43 and p12 subunits and further processing releases the large (p23) and small (p12) subunits (Figure 1.3) whilst the prodomain (p25) remains associated with the DISC. Absence of functional caspase-10 has been implicated in the autoimmune disease Type II ALPS, demonstrating an important role in apoptotic signalling (Wang et al, 1999). However, its absolute contribution to apoptosis is unclear as an ability to functionally substitute for caspase-8 has been reported (Kischkel et al, 2001), but also disputed (Sprick et al, 2002). This disparity may be explained by the observation that caspase-10 was able to cleave caspase-8 substrates at overlapping but in several cases (eg Bid and procaspase-3) also at distinct cleavage sites. This indicates that, while there may be some overlapping function, the two caspases may also have non-redundant roles (Fischer et al, 2006).
1.9.4 c-FLIP

Cellular FLIP (c-FLIP) is a member of the DED family of proteins, which includes procaspase-8 and -10. It was discovered through sequence similarity to viral FLIP (v-FLIP), which inhibits both Fas and TRAIL-mediated cell death (Thome et al, 1997, Tschopp et al, 1998). Despite numerous splice variants of c-FLIP, only three isoforms are documented as being expressed. These are the 55 kDa long (c-FLIPL), 26 kDa short (c-FLIPS) and the 24 kDa raji (c-FLIPR) isoforms (Golks et al, 2005). These isoforms all contain two DEDs, but only c-FLIPL has a catalytic like domain and significant homology to procaspase-8/10. However, c-FLIPL is catalytically inactive since the catalytic dyad (Cys/His) is replaced by Tyr and Arg residues (Tschopp et al, 1998).

Similar to v-FLIP, c-FLIPS and c-FLIPR are potent inhibitors of death receptor-mediated apoptosis since their recruitment to the DISC prevents efficient recruitment and activation of initiator procaspases (Golks et al, 2005, Krueger et al, 2001b). However, the function of c-FLIPL is more complicated with reports of a pro-apoptotic (Goltsev et al, 1997, Guseva et al, 2008, Inohara et al, 1997, Shu et al, 1997) and anti-apoptotic (Irmler et al, 1997, Krueger et al, 2001b, Rasper et al, 1998, Scaffidi et al, 1999, Sharp et al, 2005) role. It is now believed that the expression level of c-FLIPL determines its effect. When highly expressed, c-FLIPL competes with procaspase-8 for recruitment to the DISC and prevents caspase activation. However, at lower expression levels c-FLIPL is recruited to the DISC with procaspase-8, where it promotes caspase activation through dimerisation and is subsequently cleaved into p43/p12 subunits (Boatright et al, 2004, Chang et al, 2002).

1.9.5 Other components

Aside from the well known DISC components, numerous DISC associated proteins have been suggested over the years. RIP, as mentioned earlier, has been shown to be present in the TRAIL DISC, where it acts to activate kinase pathways, (Harper et al, 2001) and also to be associated with Fas (Stanger et al, 1995). In addition to this, Daxx (Leal-Sanchez et al, 2007, Yang et al, 1997), caspase-2 (Lavrik et al, 2006), FLASH (Imai et al, 1999), DAP3 (Miyazaki & Reed, 2001) and FAF1 (Ryu et al, 2003) have all been suggested to be associated with either the Fas or TRAIL DISC. However, the involvement of many of these proteins in the induction of apoptosis through the DISC has been disputed (DAP3 (Berger & Kretzler, 2002); Daxx (Villunger et al, 2000); FLASH (Koonin et al, 1999)). Therefore, the only proteins that have been
consistently identified and are generally considered to be Fas/TRAIL DISC components are death receptors, FADD, initiator caspases-8/-10 and c-FLIP.

1.10 DISC formation at the cell membrane
A model of membrane-proximal death receptor signalling has been formulated based on Fas, which is the prototypic death receptor (Figure 1.10). Fas resides in the membrane as pre-associated receptor complexes to which FasL binds and within seconds induces the formation of SDS and β-mercaptoethanol stable microaggregates (Feig et al, 2007, Kischkel et al, 1995). The efficient formation of these microaggregates is facilitated by palmitoylation of Fas at Cys 199 (Feig et al, 2007). Palmitoylation of Cys 199 is also important for the association of Fas with Ezrin, which links Fas to the actin cytoskeleton and may allow recruitment of Fas into actin-cytoskeleton linked lipid rafts (Chakrabandhu et al, 2007). This linkage to the actin cytoskeleton is crucial since down-regulation of Ezrin has been shown to inhibit Fas-mediated apoptosis (Parlato et al, 2000).

The SDS stable microaggregates have been visualized as signalling protein oligomerisation structures (SPOTS) and require FADD recruitment and self-association for formation. SPOTS do not require caspase activation for formation and thus may serve to concentrate procaspase-8 at the plasma membrane, promoting its activation and leading to a low level of DISC formation (Siegel et al, 2004).

Following SPOTS formation, a particular 180 kDa species of the SDS stable aggregates clusters into large platforms (Figure 1.10). This process, known as capping, is dependent upon activation of caspases at the DISC, since it is prevented by inhibition of caspase-8 recruitment or activity. However, it has also been suggested that capping is not required for DISC assembly or apoptosis induction (Soderstrom et al, 2005). After capping, the complex is internalised through a clathrin-mediated pathway. Internalisation is vital for efficient Fas DISC formation and maximal caspase activation. Furthermore, it has been suggested that internalisation may also target DISC-bound active caspase-8 to intracellular substrates. In the absence of internalisation only a low level of Fas DISC is formed causing Fas-induced apoptosis to be inhibited and instead non-apoptotic signalling is initiated via the activation of NFkB and Erk1/2 pathways (Algeciras-Schimnich et al, 2002, Lee et al, 2006).

TRAIL DISC, like Fas DISC, has been shown to be internalised by clathrin-dependent endocytosis. However, blockade of internalisation did not inhibit, and actually
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**Figure 1.10: Membrane proximal events of Fas signalling**

Ligation of Fas leads to the rapid formation of SDS-stable microaggregates, to which FADD and procaspase-8 are recruited to form SPOTS and produce a low level of DISC formation. Fas may interact with the actin cytoskeleton through association with Ezrin, which is essential for DISC formation and internalisation. The microaggregate extensively clusters in an active caspase-8 dependent manner to produce a large platform. If this complex is not internalised it has the potential to activate non-apoptotic pathways but cannot fully induce apoptosis. If internalisation of the large platform occurs, then a large amount of DISC is formed during endosomal trafficking. This results in extensive caspase-8 activation leading to apoptosis. Adapted from Schutze et al, 2008.
enhanced, apoptotic signalling following stimulation with TRAIL. Therefore, internalisation is proposed to not be required for TRAIL DISC formation or apoptotic signalling (Austin et al, 2006, Kohlhaas et al, 2007). This implies that although similarities exist between the death receptors, there are likely to be subtle differences in the way that they induce apoptosis.

1.11 DISC assembly via protein-protein interactions

The assembly of the DISC is mediated through homotypic DD interactions between the receptor and FADD, followed by homotypic DED interactions between FADD and procaspase-8/-10/c-FLIP (Figure 1.6). However, the DED of FADD has also been shown to regulate the recruitment of FADD through direct interaction with Fas and TRAIL-R2 receptors. Mutation of specific DED residues (Figure 1.9 A ii red residues in DED and B iii orange residues), as well as removal of the DED altogether, decreased FADD binding to Fas and TRAIL-R2 (Thomas et al, 2002, Thomas et al, 2004a).

Mutation of the Fas DD has suggested the involvement of certain residues in its interaction with FADD. Mutation of the Fas DD at Val 238 (V238N, lpr mutation) prevents FADD recruitment, although this is likely to be caused by the structural disruption this mutation causes rather than a specific effect on the interaction with FADD (Eberstadt et al, 1997). However, the structure of the Fas DD was maintained upon mutation of Arg 234, Glu 240 and Asp 244 but there was a reduced FADD binding capacity (Huang et al, 1996). In addition mutation of Arg 234 and Asp 244 (as well as Ala 241) were found in Type Ia ALPS patients, where FADD recruitment to Fas is prevented resulting in lymphoproliferation (Martin et al, 1999).

Several studies have investigated the site in the FADD DD that is involved in DD interactions. Together they have demonstrated that it is quite extensive, encompassing all six DD α-helices (and even some DED residues, see above) (Figure 1.9 red residues). However, several DD residues were found to be vital for the interaction of FADD with Fas, TRAIL-R2 and TRADD. These were Arg 113, Arg 117, Asp 123 and Asp 175 (Figure 1.9 marked in red), suggesting that all FADD DD interactions include a common surface (Hill et al, 2004, Jeong et al, 1999, Sandu et al, 2005, Thomas et al, 2006). However, some residues appear to be important in specific interaction since mutation of Arg 142, Arg 166, Leu 172, Gln 169 disrupted FADD interaction with Fas (Hill et al, 2004) but they had no effect on the interaction of FADD with TRADD (Sandu et al, 2005). Furthermore, mutation of Val 108 (Figure 1.9 A ii black bold residue)
prevented FADD interaction with Fas but not with TRAIL-R2, whilst Arg 135 (Figure 1.9 A ii pink residue) has only been identified as important for interaction with TRAIL-R2 (Thomas et al, 2004a, Thomas et al, 2006).

The interaction between the DD of Fas and the DD of FADD had been proposed to occur through electrostatic attractions (Berglund et al, 2000). However, the recently published structure of the Fas-FADD DD complex demonstrated that a hydrophobic patch surrounded by polar residues forms the main interaction site on FADD (Scott et al, 2009). This site encompasses the DD α-helix 1 and 6 of the FADD DD and interacts with a corresponding hydrophobic patch in the Fas DD. Comparison of the uncomplexed and complexed Fas DD structures demonstrated that conformational changes occurred within this domain upon interaction with FADD. The structure of the complexed Fas DD was more open, likely allowing the exposure of residues important for the recruitment of FADD. Furthermore, these changes also permitted Fas-Fas interactions that lead to the formation of receptor dimers (Scott et al, 2009).

The Fas-FADD DD dimer was suggested to link adjacent Fas trimers inducing DISC clustering and stabilisation (Scott et al, 2009). The suggestion that adjacent complexes stitch together through Fas deviates from the idea of the DISC being an independent, trimeric complex (Figure 1.11 A). Furthermore, this stitching together of complexes may be aided by the recruitment of FADD, which has also been suggested to link adjacent complexes through self-association (Figure 1.11 B) (Sandu et al, 2006). This also explains the FADD-dependent nature of the SPOTS that form upon Fas stimulation (Section 1.10) (Siegel et al, 2004). The self-association of FADD is mediated by the DED but different residues have been suggested as important. One report demonstrated that an RXDLL motif (amino acids 72 to 76, Figure 1.9 A ii underlined residues) in the DED of FADD mediated the self-association (Muppidi et al, 2006). However, the RXDLL motif plays an important role in stabilising the DED fold and its mutation was shown to significantly alter the structure of FADD. Thus, it was argued that the observed loss of FADD self-association upon RXDLL mutation was caused by structural disorder (Carrington et al, 2006, Yang et al, 2005). In contrast, mutation of FADD in the DED at Phe 25 or Lys 33 did not compromise the stability of FADD, but did prevent self-association (Carrington et al, 2006). Thus, it was suggested that self-association of FADD was mediated by a ‘universal’ surface encompassing DED α-helix 2 and 3 (Figure 1.9 B iii, brown residues) (Carrington et al, 2006, Sandu et al, 2006).
Figure 1.11: Fas and/or FADD form bridges between receptor trimers and FADD recruits procaspase-8 in a perpendicular fashion.

A model for Fas and/or FADD mediated clustering of DISC and procaspase-8 recruitment. The Fas DD is shown in blue, FADD is orange and procaspase-8 is purple. DDs are oblong, DEDs are hexagons and the large and small catalytic subunits are teardrop shaped. A. The typical model of DISC formation where isolated trimeric ligand engages trimeric receptor leading to the recruitment of three molecules of FADD and procaspase-8. B. Model based on published data where Fas and/or FADD self-associate to form bridges between adjacent trimeric complexes, resulting in receptor clustering. C. Procaspase-8 would be recruited to the FADD bridge where two molecules of FADD can recruit two molecules of caspase-8 allowing caspase dimerisation and activation. Top views depict only the DDs of the receptors and do not show the ligand.
In addition to roles in receptor binding and self-association, the DED of FADD is essential for the recruitment of other DED proteins, such as procaspase-8, -10 and c-FLIP<sub>L/S</sub>, to the DISC. The structure of the Fas-FADD DD complex indicated that the conformational change in Fas causes a rearrangement in the relative positions of the DD and DED of FADD (Scott et al, 2009). This could lead to the exposure of residues allowing procaspase/c-FLIP recruitment that, due to the role of FADD in linking adjacent complexes, is likely to be perpendicular to FADD (Figure 1.11 C) (Carrington et al, 2006, Sandu et al, 2006, Scott et al, 2009).

The DED of FADD was shown to have two hydrophobic regions, one of which is conserved in procaspase-8, and in both viral and cellular FLIPs. The Phe 25 residue resides in the centre of the conserved hydrophobic patch in FADD and its mutation to Gly or Val prevented the interaction of FADD with procaspase-8 (Eberstadt et al, 1998). Furthermore, a DED conserved basic surface patch composed of residues Lys 33, Arg 34 and Lys 35 was also shown to be vital for the interaction of FADD with procaspase-8 and c-FLIP (Kaufmann et al, 2002). These residues, whose mutation did not affect protein folding, are part of the ‘universal’ surface (Figure 1.9 B iii, R34 labelled Q34) discussed above in reference to FADD self-association, implying that this area is important for general DED interactions. Although the ‘universal’ surface was involved with all FADD DED interactions, other residues (Ser 12, Arg 38, Asp 44 and Glu 51) have been found to be specific for the interaction of FADD with procaspase-8 (Figure 1.9 Aii and B iv green residues) (Carrington et al, 2006, Sandu et al, 2006).

An insight into DED interactions has been provided by the crystal structure of ν-FLIP, MC159, which contains two tandem DEDs that interact via hydrophobic patches (Yang et al, 2005). The sequence and structure of the DED of FADD is similar to that of DED2 of MC159, which uses α-helices 1 and 4 to interact with α-helices 2 and 5 of DED1 of MC159. Thus, FADD was suggested to interact with a DED of procaspase-8 in the same way that MC159 DED2 interacts with MC159 DED1. This concurs with the earlier observation that the residues specific for the FADD-procaspase-8 interaction (Figure 1.9 green residues) are mainly located in FADD DED α-helices 1 and 4 (Carrington et al, 2006, Sandu et al, 2006).

Since the DEDs of MC159 interact through hydrophobic patches and the hydrophobic residue Phe 25 was important in FADD DED interactions, it seemed likely that the FADD interaction site on procaspase-8 would be hydrophobic in nature. Furthermore, as the DED of FADD likely represented DED2 of MC159, the interaction site on the
DEDs of procaspase-8 must be in an equivalent position to the hydrophobic residues in MC159 DED1 (Phe 30 and Leu 31). Upon examination of the DEDs of procaspase-8, hydrophobic residues available for interaction with FADD were identified in DED2 (Phe 122/Lys 123). Mutation of these residues in procaspase-8 confirmed that they were vital for the interaction with FADD (Carrington et al, 2006, Yang et al, 2005). Thus, the DED of FADD is proposed to interact with DED2 of procaspase-8 through hydrophobic residues. Although only DED2 of procaspase-8 is proposed to mediate binding to FADD, both DEDs are required for successful recruitment of initiator caspases into the DISC (Tsukumo & Yonehara, 1999, Yao et al, 2007). Therefore, it has been suggested that DED1 may support the dimerisation of adjacent initiator caspases through the small catalytic subunit (Sandu et al, 2006).

1.12 DISC structure
The original model of the DISC was that one ligand trimer bound to one receptor trimer, causing recruitment of three molecules of FADD and three molecules of caspase-8 (Figure 1.11 A). A structure based model of a trimeric Fas-FADD interaction was published and predicted that either trimeric FADD or Fas could form the centre of the complex (Weber & Vincenz, 2001). However, this model did not fit with the apparent requirement of initiator caspase dimerisation for activation (see earlier). The requirement for two caspase molecules and the difficulty in demonstrating a 1:1 interaction between Fas DD and FADD DD in vitro lead to the suggestion of a different ratio of three receptors: two FADD: two caspase-8 molecules (Berglund et al, 2000). However, the discovery that hexameric, and not trimeric, FasL was required for DISC formation (Holler et al, 2003) initiated thinking that the DISC is not formed as individual trimers. Currently it seems likely that the DISC forms a ‘honeycomb’ structure with trimeric complexes stitched together by either Fas (Scott et al, 2009) and/or FADD (Sandu et al, 2006) (Figure 1.11 B). Since the bridge between trimers is formed by two Fas-FADD complexes there would be two initiator caspase binding sites, thus explaining how dimerisation of caspase-8 would be possible by trimeric receptors (Figure 1.11 C). Therefore in the honeycomb model the ratio of ligand:receptor:FADD:caspase-8 would be 1:1:1:1, as in the original isolated trimer model. However, the stoichiometry of the DISC has never been fully defined and interestingly, maximal caspase-8 activation was observed in an in vitro Fas DISC with a ratio of 1 Fas DD: 5 FADD: 3 caspase-8 molecules (Roy et al, 2008).
1.13 The aims of this thesis

TRAIL is of great scientific and clinical interest due to its apparent selectivity for inducing apoptosis in cancerous cells. However, there is still no definitive explanation for this selectivity and as yet there has been no systemic investigation of this pathway using proteomic analysis. Furthermore, there are several unanswered questions with regard to the stoichiometry of the DISC and why Type I/II cells respond differently to death receptor-mediated apoptosis. Therefore, the aim of this thesis was to address some of these important questions by characterising the initial phase of TRAIL signalling through protein purification, mass spectrometry and functional assays.

The first results chapter of the project investigated the interaction partners of FADD, a crucial component of the DISC, as well as other signalling complexes and pathways. FADD was tagged, transfected into a FADD null cell line and its ability to support TRAIL-induced apoptosis confirmed. Potential interacting proteins were identified by mass spectrometry of samples purified from the tagged FADD cell line under control and TRAIL treated conditions.

In the second results chapter the TRAIL DISC was isolated from Type I and Type II cell lines and its components analysed by mass spectrometry. This aimed to identify novel DISC-interacting proteins that might regulate DISC formation or activity. Identification of any such protein may help explain the selectivity of TRAIL, as compared to other death ligands, for inducing apoptosis in cancerous cells or why Type I and Type II cell lines form different amounts of TRAIL DISC.

In the third results chapter the differences between Type I and II cell lines was further investigated to elucidate an explanation for their different response upon apoptotic stimulation. The cell lines were characterised in terms of cell surface receptor and intracellular protein expression. Furthermore, the molecular size and functional characteristics of a Type I versus a Type II TRAIL DISC were investigated.
Chapter 2: Materials and Methods
2 Materials and Methods

2.1 Chemicals
All chemicals were of the highest quality and were purchased from Sigma (Gillingham, UK) unless otherwise stated.

2.2 Cell culture
All cell culture materials were from Invitrogen (Paisley, UK) except the plastic ware, which was from Grenier Bioone (Frickhausen, Germany). Four types of Jurkat T cells were cultured. Clone E6.1 was obtained from European collection of animal cell cultures (Wiltshire, UK), whereas the parental clone A3 (wildtype) and FADD null Jurkat cells (Juo et al, 1999) were a kind gift from Dr J Blenis (Harvard Medical School, Boston, USA). FADD-TAP Jurkat cells were produced by transfection of FADD null Jurkat cells with FADD-TAP vector. The BJAB cell line was kindly provided by Dr Andrew Thorburn (University of Colorado Health Sciences Center, Aurora, USA (Thomas et al, 2004b)). All cell lines were cultured in RPMI media supplemented with 10 % v/v FCS and 1 % v/v Glutamax™. FADD-TAP Jurkat cells were stably maintained in 400 μg/ml of the antibiotic G418 (Gibco™, Invitrogen). Cells were kept at 37 °C with 5 % CO2 in a humidified atmosphere and passaged every 3 to 4 days.

2.3 General methods

2.3.1 Assessment of protein expression in cell lines
Cell pellets were resuspended in 1 x sample buffer (62 mM Tris, 0.05 % w/v bromophenol blue, 15 % v/v glycerol, 2 % w/v SDS, 5 % v/v β-mercaptoethanol) to 0.01 x 10⁵ cells/μl and sonicated for 3 cycles (5 sec on, 5 sec off). Samples were boiled and analysed through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Several different types of gel were used for SDS-PAGE but the majority of experiments utilised the Mini-PROTEAN® II electrophoresis cell (BioRad, Hemel Hempstead, UK). Mini gels were prepared with a 4 % stacking gel (125 mM Tris (Roche, Newhaven, UK)-HCl pH 6.8, 0.1 % w/v SDS, 4 % acrylamide (ProtoGel®, National Diagnostics, Geneflow Ltd, Fradley, UK)) cast on top of a running gel (375 mM Tris-HCl pH 8.8, 0.1 % w/v SDS, variable acrylamide). The percentage of the running gel depended on the protein of interest, but was usually between 10 and 13 %. For experiments that...
Materials and Methods

generated a large number of samples, such as gel filtration and sucrose density gradients, the Criterion™ electrophoresis cell (BioRad) was used. Gels were either cast in empty 1.0 mm Criterion™ cassettes (BioRad) or 1.0 mm 4 - 20 % Criterion™ precast gels (BioRad) were used. Samples that were submitted for mass spectrometry were separated using the Novex mini XCell SureLock™ electrophoresis cell (Invitrogen) and 1.0 mm 4 - 20 % Tris-glycine precast gels (Invitrogen).

In all cases proteins were separated alongside either a SeeBlue® or SeeBlue® Plus2 pre-stained standard (Invitrogen). Gels were run in electrode buffer (25 mM Tris, 192 mM glycine (Fisher Scientific, Loughborough, UK), 0.1 % w/v SDS) at 50 to 100 V until the desired separation was achieved. After SDS-PAGE, gels were either blotted onto nitrocellulose membrane or stained with coomassie.

2.3.3 Western blot analysis
Following SDS-PAGE proteins were transferred to Hybond™-C Extra nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). The membrane was blocked for 1 hr at room temperature with 5 % w/v milk (Marvel, Nestlé, Surrey, UK) in Tris-buffered saline with Tween 20 (TBS-T, 2 mM Tris-HCl pH 7.6, 13.7 mM NaCl (Fisher Scientific), 0.1 % v/v Tween-20). Proteins of interest were visualised by incubation with appropriate primary and peroxidise-conjugated secondary antibodies (Table 2.1 and Table 2.2) followed by ECL™ Western blotting detection reagents (GE Healthcare). Membranes were incubated with primary antibodies for 1 to 4 hr at room temperature or overnight at 4 °C. Secondary antibodies were applied for 1 hr at room temperature.

2.3.4 Coomassie staining
Gels were washed with distilled water and then incubated overnight with colloidal coomassie stain. The stain was made by diluting 7 parts ProtoBlue™ Safe Colloidal Coomassie G-250 stain (National Diagnostics) to 3 parts absolute ethanol (Fisher Scientific). To reduce background staining, and improve protein band visualisation, the gel was destained in distilled water.

2.3.5 Bradford protein assay
The concentration of protein solutions was determined using the Bradford protein assay (Bradford, 1976). Bradford assay solution was produced though diluting 1 part protein assay concentrate (BioRad) with 4 parts distilled water. An appropriate amount of the protein solution was added to the Bradford assay solution and cuvettes (Sarstedt, Numbrecht, Germany) were inverted several times before the absorbance at
Table 2.1: Primary antibodies used for Western blot analysis during this project.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size (approx, kDa)</th>
<th>Type</th>
<th>Company</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>α-tubulin</td>
<td>60</td>
<td>Mouse monoclonal (DM1A)</td>
<td>Oncogene Research Products, Beeston, UK</td>
<td>1:1000</td>
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<tr>
<td>β-actin</td>
<td>42</td>
<td>Mouse monoclonal (AC-15)</td>
<td>Sigma</td>
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<tr>
<td>Active caspase-3</td>
<td>20, 19, 17</td>
<td>Rabbit monoclonal (5A1)</td>
<td>Cell Signaling Technology, New England Biolabs (UK) Ltd, Hitchin, UK</td>
<td>1:1000</td>
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<tr>
<td>Apaf-1</td>
<td>135</td>
<td>Mouse monoclonal (94408)</td>
<td>R &amp; D Systems, Abingdon, UK</td>
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<tr>
<td>Bak</td>
<td>30</td>
<td>Rabbit polyclonal (NT)</td>
<td>Upstate, Millipore, Watford, UK</td>
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<tr>
<td>Bax</td>
<td>20</td>
<td>Rabbit polyclonal (NT)</td>
<td>Upstate</td>
<td>1:500</td>
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<tr>
<td>Bcl-2</td>
<td>26</td>
<td>Mouse monoclonal (124)</td>
<td>Dako, Ely, UK</td>
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<tr>
<td>Bcl-XL</td>
<td>26</td>
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<td>BD Pharmingen, Oxford, UK</td>
<td>1:1000</td>
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<tr>
<td>Bid</td>
<td>22, 15, 13</td>
<td>Rabbit polyclonal</td>
<td>Kind gift from Dr X Wang, University of Texas, USA</td>
<td>1:2000</td>
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<tr>
<td>Calpain S1</td>
<td>28</td>
<td>Mouse monoclonal (ab54929)</td>
<td>Abcam, Cambridge, UK</td>
<td>3 µg/ml</td>
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<tr>
<td>Caspase-3</td>
<td>32, 20, 19, 17</td>
<td>Rabbit polyclonal</td>
<td>Kind gift from Dr D Nicholson, Merck Frost, Canada</td>
<td>1:10000</td>
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<tr>
<td>Caspase-8</td>
<td>55/53, 43/41, 18, 12, 10</td>
<td>Rabbit polyclonal</td>
<td>Kind gift from Dr X Sun, MRC Toxicology Unit, Leicester, UK</td>
<td>1:2000</td>
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<tr>
<td>Caspase-9</td>
<td>45, 35/37</td>
<td>Mouse monoclonal (5B4)</td>
<td>Medical &amp; Biological Laboratories, Woburn, USA</td>
<td>1:1000</td>
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<tr>
<td>Caspase-10</td>
<td>59/54, 47/43, 31, 25</td>
<td>Mouse monoclonal (4C1)</td>
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<td>1:1000</td>
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<td>c-FLIP</td>
<td>55, 43, 26</td>
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<td>Merck Frost Centre (Rasper et al, 1998)</td>
<td>1:3000</td>
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<td>DLG1 (SAP97)</td>
<td>130-135</td>
<td>Mouse monoclonal (2D11)</td>
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<td>1:200</td>
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<tr>
<td>FADD</td>
<td>24</td>
<td>Mouse monoclonal (1)</td>
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<td>Fas</td>
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<td>HSC70</td>
<td>71</td>
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<td>1:2000</td>
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<td>HSP90α</td>
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<td>Transferrin receptor</td>
<td>85</td>
<td>Mouse monoclonal (2)</td>
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<td>1:1000</td>
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<td>PKN1 (PRK1)</td>
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<td>Upstate</td>
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<tr>
<td>RNF113B</td>
<td>43</td>
<td>Mouse polyclonal</td>
<td>AbCam</td>
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### Materials and Methods

#### Table 2.1: Primary antibodies used for Western blot analysis during this project.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size (approx, kDa)</th>
<th>Type</th>
<th>Company</th>
<th>Dilution</th>
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<tr>
<td>Ser 194</td>
<td>28</td>
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<td>Cell Signaling Technology</td>
<td>1:1000</td>
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<tr>
<td>FADD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smac</td>
<td>22</td>
<td>Rabbit polyclonal</td>
<td>Kind gift from Dr X Sun</td>
<td>1:3000</td>
</tr>
<tr>
<td>TRAIL-R1</td>
<td>57</td>
<td>Rabbit polyclonal (CT)</td>
<td>Prosci Incorporated, Poway, USA</td>
<td>1:1000</td>
</tr>
<tr>
<td>TRAIL-R2</td>
<td>48, 40</td>
<td>Rabbit polyclonal</td>
<td>Cell Signaling Technology</td>
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<tr>
<td>TRAIL-R4</td>
<td>38</td>
<td>Mouse monoclonal (M445)</td>
<td>Kind gift from Immunex Corporation, Seattle, USA</td>
<td>1:1000</td>
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<tr>
<td>VPS35</td>
<td>91</td>
<td>Mouse monoclonal (2D3)</td>
<td>Abnova GmbH, Heidelberg, Germany</td>
<td>3 µg/ml</td>
</tr>
<tr>
<td>XIAP</td>
<td>57</td>
<td>Mouse monoclonal (28)</td>
<td>BD Transduction Laboratories</td>
<td>1:2000</td>
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</table>

#### Table 2.2: Secondary antibodies used for Western blot analysis during this project.

<table>
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<tr>
<th>Secondary Antibody</th>
<th>Company</th>
<th>Dilution</th>
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<tr>
<td>Goat anti-mouse IgG-peroxidase</td>
<td>Sigma</td>
<td>1:2000</td>
</tr>
<tr>
<td>Goat anti-rabbit immunoglobulins/HRP</td>
<td>Dako</td>
<td>1:2000</td>
</tr>
<tr>
<td>Rabbit anti-goat immunoglobulins/HRP</td>
<td>Dako</td>
<td>1:2000</td>
</tr>
</tbody>
</table>
595 nm was measured using a Lamdba 2 UV/VIS spectrophotometer (Perkin Elmer, Wellesley, USA). The protein concentration was calculated using a Bovine serum albumin (BSA) standard curve (1 - 8 μg/ml).

2.3.6 Size-exclusion chromatography
All size-exclusion experiments were conducted with the help of Dr Claudia Langlais, MRC Toxicology Unit. Gel filtration columns, Superose™ 6 PC 3.2/30 (GE Healthcare) and the Superdex™ 200 PC 3.2/30 (GE Healthcare) columns, were used in conjunction with the SMART® system (Pharmacia Biotech, now GE Healthcare). Both columns used the same basic running buffer that was composed of 20 mM HEPES-KOH pH 7.0, 5 % w/v sucrose, 0.1 % w/v CHAPS and 5 mM dithiothretol (DTT). To this 50 mM of NaCl was added for experiments using the Superose™ 6 column, whilst 150 mM was added when using the Superdex™ 200 column. Samples were applied to the SMART® system and eluted in 50 μl fractions at a rate of 40 μl/min. The elution fractions were analysed though SDS-PAGE separation and either Western blot or coomassie staining. All columns were calibrated using protein standards from both high and low molecular weight kits (GE Healthcare).

2.3.7 Assessment of apoptosis though annexin V and propidium iodide staining
Apoptosis was assessed using annexin V-FITC (Bender MedSystems, Buckingham, UK), which binds to externalised phosphatidylserine (King et al, 1998), and the vital dye propidium iodide (PI). Suspension cells (up to 200 μl) were added to annexin V buffer (10 mM HEPES-NaOH (Fisher Scientific) pH 7.4, 150 mM NaCl, 5 mM KCl (Fisher Scientific) 1 mM MgCl₂, 1.8 mM CaCl₂) to make a total volume of 1 ml. An appropriate dilution of annexin V was made, of which 1.5 μl was added to Jurkat cells whilst 15 μl was added to BJAB cells. The annexin V was incubated (at room temperature) with Jurkat cells for 8 min but left for 30 min with BJAB cells. Subsequently 1.5 μl of 50 μg/ml PI was added and the cells incubated on ice for 2 min. The level of annexin V and PI staining was assessed using the FacsCalibur™ machine (Becton Dickenson (BD), Oxford, UK) with an excitation/emission wavelengths of 488/525 nm (FITC) and 488/585 nm (PI). The data generated were analysed using the CellQuest Pro® software (Becton Dickenson). Figure 2.1 illustrates typical results following TRAIL treatment of Jurkat and BJAB cells.
Jurkat Cells

BJAB Cells

Figure 2.1: Forward/side scatter and annexin V/PI profiles of control and apoptotic cells.

Dot plots generated by the CellQuest Pro® software for the measurement of apoptosis in untreated or TRAIL treated Jurkat E6.1 and BJAB cells. The cells were either left untreated (control) or treated with 500 ng/ml of TRAIL (apoptotic) for 4 hr at 37 °C. Apoptosis was assessed though annexin V/PI staining as described in Section 2.3.7.
2.4 Generation and purification of FADD-TAP

2.4.1 Generation of tagged and untagged FADD plasmids

FADD was obtained via PCR amplification of pMal FADD-MBP (a kind gift from Dr Michelle Hughes, MRC Toxicology Unit) and was cloned in frame into the EcoR1 and Xho1 sites of three tandem affinity tag vectors (Figure 2.2). These vectors were pcDNA3-C-TAP tag easy (C-TAP), pcDNA3-V5/Prescission/His (V5/His) (both kind gifts from Dr Tencho Tenev and Dr Pascal Meier, Institute of Cancer Research, London, UK) and pcDNA4/TO-N-terminal TAP tag (N-TAP, a kind gift from Dr Justin Cross and Professor Julian Downward, Cancer Research UK, London, UK). The DNA sequence of the vectors was verified by sequencing analysis.

Mutagenesis was used to generate untagged FADD, by inserting a stop codon before the affinity tags, as well as the FADD phosphorylation mutants S10A, S167A and S194A. Mutagenic primers, shown below, were used in conjunction with the QuickChange® site-directed mutagenesis kit (Stratagene, La Jolla, USA) according to the manufacturers’ instructions.

**FADD-Stop-TAP primers**

5’ CGAACGCTCTGACTCGAGTCCATG 3’, 5’ CATGGAATCGAGTCAGGACGCTTCG 3’

**S10A primers**

5’ GGTGCTGCTGCACGCGGTGTCGTCCAGCC 3’, 5’ GGCTGGACGACACCGCGTGCAGCAGCACC 3’

**S167A primers**

5’ GGGGGGCCTCAGCCGGCGTGTCGTCAGCC 3’, 5’ GGCTGGACGACACCGCGTGCAGCAGCACC 3’

**S194A primers**

5’ GGAGTGGGGCCATGGCCCCGATGTCATGG 3’, 5’ CCATGACATCGGGGCCATGGCCCCACCTCC 3’

2.4.2 Sequencing of DNA vectors

DNA vectors were sequenced using the BigDye® v3.1 sequencing kit (Applied Biosystems, Warrington, UK) according to manufacturers’ instructions. Sequencing reactions were purified using Performa® DTR Gel Filtration Cartridges (Edge Biosystems, Gaithersburg, USA).
Figure 2.2: The three vectors, containing tandem affinity tagged FADD, which were generated. FADD was cloned into three different tandem affinity tag vectors, the C-terminal TAP tag vector (A), the C-terminal V5/His tag vector (B) and the N-terminal TAP tag vector (C). pCMV, Cytomegalovirus promoter; T7, T7 promoter; BGH pA, bovine growth hormone polyadenylation signal; MCS, multiple cloning site; TEV, tobacco etch virus protease cleavage site; CBP, calmodulin binding peptide; V5, V5 affinity tag; 3x PreScission, three PreScission protease cleavage sites; His, hexa-histidine affinity tag; 2x TetO2, tetracycline operator. Ampicillin was the bacterial selection antibiotic, whilst Neomycin (G418) and Zeocin were the mammalian selection antibiotics.
2.4.3 Cell-free protein synthesis
Proteins were synthesised in vitro (IVT) using a TNT® T7 coupled rabbit reticulocyte lysate system (Promega, Southampton, UK). Non-radioactive reactions were assembled and completed according to the manufacturers’ instructions.

2.4.4 In vitro reconstitution of the Fas DISC
Jurkat cell pellets of approximately 500 x 10^6 cells were collected and lysed for 45 min on ice in DISC lysis buffer (30 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 % v/v glycerol, 1 % v/v Triton X-100, 1 Complete™ Mini protease inhibitor cocktail tablet (Roche)/10 ml). Lysates were then cleared by centrifugation at 15,000 g for 30 min at 4 °C and 5 mg of the cleared lysate was used for the in vitro Fas DISC reconstitution. These were prepared with either wildtype cellular lysate or FADD null lysate supplemented with IVT-produced FADD or tagged FADD. The lysates were incubated in caspase assay buffer with 10 μg of glutathione sepharose beads (GE Healthcare) coated with GST alone or GST fused to the intracellular domain of the Fas receptor (kindly provided by Dr Michelle Hughes) as described previously (Harper et al, 2003a, Hughes et al, 2009). After overnight incubation at 22 °C on an end-to-end rotator, the beads were collected, washed with phosphate-buffered saline (PBS) and the proteins eluted by boiling in 2 x sample buffer (125 mM Tris-HCl pH 6.8, 0.1 % w/v bromophenol blue, 30 % v/v glycerol, 4 % w/v SDS, 10 % v/v β-mercaptoethanol). The supernatants and bead eluates were separated by SDS-PAGE and analysed by Western blot.

2.4.5 Electroporation
FADD null Jurkat cells were passaged 2 days prior to electroporation, such that the cell density would be approximately 0.3 x 10^6/ml. For each sample 3.5 x 10^6 cells were resuspended in 100 μl of Cell Line Nucleofector™ solution V (Amaxa Biosystems, Lonza, Cologne, Germany), mixed with 1 – 3 μg of DNA (pmaxGFP (Amaxa Biosystems) or FADD-containing vector) and electroporated using the C16 program of the Nucleofector™ device (Amaxa Biosystems). The electroporated cells were placed into 1.5 ml of conditioned media (containing 50 units of penicillin and 50 μg streptomycin (Invitrogen)) and incubated at 37 °C with 5 % CO₂ in a humidified atmosphere.

2.4.6 Selection of FADD-TAP and C-TAP transfected cells and single clone derived cell line production
FADD null cells transfected with FADD-TAP were selected by incubation with 1.4 mg/ml G418 for 1 month. The concentration of G418 was then reduced to a
Maintenance dose of 400 μg/ml. Stable single clones were produced by seeding the selected FADD-TAP cell population at 4 different cell densities (0.5, 1, 1.5 and 2 cells per well), in the presence of 700 μg/ml to 1.4 mg/ml G418, in 96 well plates. Once cells started to grow the clones most likely to have come from a single cell were cultured as described in Section 2.2.

2.4.7 Induction of apoptosis using soluble TRAIL and anti-Fas antibody

Transiently transfected cells with a density of approximately 1 x 10^6/ml were treated with 1 μg/ml of recombinant soluble TRAIL (TRAIL, generated in our laboratory as previously described (Harper & MacFarlane, 2008, MacFarlane et al, 1997)) or with 500 ng/ml mouse monoclonal anti-Fas antibody (Clone CH-11, Upstate). Cells were incubated with TRAIL or anti-Fas antibody for up to 6 hr and the extent of cell death was quantified by annexin V/PI staining.

2.4.8 Purification of FADD-TAP and associated proteins

The TAP purification method was modified from the original method (Puig et al, 2001, Rigaut et al, 1999). In the optimised protocol, empty PepClean™ C-18 spin columns (Pierce, Rockford, USA) were filled with 100 μl bed volume of washed IgG Sepharose™ 6 fast flow beads (GE Healthcare) and attached to a one way stopcock (World Precision Instruments Inc. Sarasota, USA). 100 x 10^6 FADD-TAP cells were lysed in 3 ml DISC lysis buffer on ice for 30 min and then cleared by centrifugation at 15,000 g for 30 min at 4 °C. The lysate was then loaded onto the column and allowed to pass through by gravity flow. Subsequently the beads were washed with 1x IPP150M buffer (30 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 % v/v Triton X-100, 1 Complete™ Mini protease inhibitor cocktail tablet /10 ml) and then with 1 x TEV cleavage buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 % v/v Triton X-100, 0.5 mM EDTA (Fisher Scientific), 1 mM DTT). AcTEV™ protease (500 U, Invitrogen) was added onto the beads in TEV cleavage buffer (1 x TEV cleavage buffer (Invitrogen), 1 % v/v Triton X-100, 1 mM DTT). After entering the beads the stopcock was closed to allow incubation of the TEV protease with the beads overnight at 4 °C. TEV cleavage released FADD-CBP that was eluted by washing the beads with 1 x TEV buffer. A total of 9 elution fractions were collected and those with the highest level of FADD-CBP, determined though SDS-PAGE and Western blot analysis, were pooled to use in the next purification step.

Pooled FADD-CBP eluate was diluted with 3 volumes of 1 x calmodulin binding buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 % v/v Triton X-100, 1 mM Mg(CH₃COO)₂ (Fisher Scientific), 1 mM imidazole, 2 mM CaCl₂, 10 mM β-mercaptoethanol) and an
extra 3 mM of CaCl₂ was added. The diluted eluate was incubated with 100 µl of washed calmodulin sepharose™ 4B beads (GE Healthcare) in a Poly-Prep Column (0.8 x 4 cm, BioRad) on an end-to-end rotator for 1 hr at 4 °C. The beads were washed with 1 x calmodulin binding buffer and FADD-CBP was eluted in 6 fractions using 1 x calmodulin elution buffer (as for binding buffer except 2 mM CaCl₂ replaced with 2 mM EGTA (Fisher Scientific)).

2.4.9 Immunoprecipitation of FADD
Immunoprecipitation of FADD from wildtype cells was used to validate interaction partners and FADD phosphorylation sites identified by TAP and mass spectrometry. Protein G Dynabeads® (50 µl, Dynal, Invitrogen) were incubated for 40 min at room temperature with 5 µg of FADD antibody (Clone A66-2, BD Pharmingen). The beads were washed with PBS and then with 0.2 M triethanolamine. The bound antibody was cross-linked to the beads though incubation with 20 mM dimethyl pimelimidate dihydrochloride for 30 min and the reaction stopped by adding 50 mM Tris-HCl pH 7.5 for 15 min at room temperature. Beads were washed and resuspended in DISC lysis buffer before addition to cleared lysate from 10 x 10⁶ cells. Lysate and beads were incubated overnight at 4 °C on an end-to-end rotator. After washing, bound proteins were eluted though incubation of the beads in elution buffer (200 mM glycine, 200 mM NaCl, pH 2.0) for 15 min at room temperature. Samples were neutralised using 2 M Tris-HCl pH 8.0 and eluted proteins were analysed by Western blotting or coomassie staining.

2.4.10 2-D gel electrophoresis
Protein samples were resuspended in sample preparation solution (6 M urea (Invitrogen), 2 M thiourea (Invitrogen), 4 % CHAPS, 64 mM DTT, 0.5 % IPG buffer pH 3 – 10 (GE Healthcare)) and applied to a 11 cm pH 3 – 10 non-linear ReadyStrip™ IPG strip (BioRad). The strip was covered with DryStrip cover fluid (GE Healthcare) and actively rehydrated with the sample overnight in the PROTEAN IEF cell (BioRad). Electrode wicks were inserted under the strip before running at 250 V for 2 hr, 8000 V for 1 hr and 8000 V for 27,000 Vhr. Before the second SDS-PAGE dimension the strip was incubated with DTT-containing (10 mg/ml) SDS equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3 % v/v glycerol, 2 % v/v SDS, 0.002 % w/v bromophenol blue) for 10 min and then for a further 10 min with iodoacetamide-containing (25 mg/ml, GE Healthcare) SDS equilibration buffer. After SDS-PAGE separation the proteins were transferred onto nitrocellulose membrane for Western blot.
2.5 Investigation of DISC

2.5.1 TRAIL DISC purification

Isolation of the TRAIL DISC was performed using a modified version of previously described methods (Harper et al, 2001, Sprick et al, 2000). In the optimised small scale purification 5 x 10^7 cells were treated with 500 ng/ml of soluble biotinylated TRAIL (bTRAIL, kindly provided by Dr Nick Harper, MRC Toxicology Unit (Harper & MacFarlane, 2008)). Soluble biotinylated TRAIL-R1 (R1-5) or TRAIL-R2 (R2-6) specific ligands (MacFarlane et al, 2005) were used to isolate the TRAIL-R1 or TRAIL-R2 DISC, respectively. Cells were incubated on ice for 1 hr followed by either 10 (BJAB) or 25 (Jurkat) min at 37 °C. Cell pellets were washed and lysed on ice for 30 min in 3.5 ml DISC lysis buffer.

Lysates were cleared by centrifugation at 15,000 g for 30 min at 4 °C and the resulting supernatant incubated for 17 hr at 4 °C on an end-to-end rotator with 50 μl magnetic M-280 streptavidin Dynabeads® (Dynal, Invitrogen). Beads were isolated from the supernatant and a sample of the supernatant was mixed with 10 x sample buffer (0.5 M Tris-HCl pH 6.8, 0.4 % w/v bromophenol blue, 15 % v/v glycerol, 16 % w/v SDS, 5 % v/v β-mercaptoethanol). The beads were washed prior to the elution of proteins, which was achieved by boiling in 1 x sample buffer. Proteins in the supernatants and bead eluates were separated by SDS-PAGE for analysis by Western blot. Any deviations from this protocol are indicated in the respective figure or figure legend.

In large scale experiments for DISC analysis by mass spectrometry this protocol was scaled up. DISC was identified by mass spectrometry when the experiment was increased 6 fold (BJAB cell line) or 60 fold (Jurkat E6.1 cell line).

2.5.2 Fas DISC purification

A total of 5 x 10^7 cells (BJAB or Jurkat) were treated with 1 μg/ml Flag-tagged Fas ligand (Alexis® Biochemicals, Axxora (UK) Ltd, Nottingham, UK), which had previously been cross-linked using 2 μg/ml ANTI-FLAG® M2 monoclonal antibody. Cells were incubated on ice for 1 hr followed by 25 min at 37 °C. Cell pellets were washed and lysed on ice for 30 min in 1 ml DISC lysis buffer. Lysates were cleared by centrifugation and the resulting supernatant incubated overnight at 4 °C on an end-to-end rotator with 50 μl magnetic Protein G Dynabeads®. Beads were isolated from the supernatant and washed prior to the elution of proteins though incubation at room temperature with non-reducing 2 x sample buffer (125 mM Tris-HCl pH 6.8, 0.1 % w/v
bromophenol blue, 30 % v/v glycerol, 4 % w/v SDS). The supernatants and bead eluates were separated by SDS-PAGE and analysed by Western blot.

2.5.3 Measurement of cell surface TRAIL receptor expression
BJAB or Jurkat cells (0.25 x 10^6) were resuspended in 10 % v/v goat serum in PBS and incubated with the appropriate phycoerythrin (PE) conjugated antibody (eBioscience, Insight Biotechnology Ltd, Wembley, UK) for 1 hr on ice in the dark. Cells were incubated with either anti-human TRAIL-R1 (DJR1, 5 ng/μl), anti-human TRAIL-R2 (DJR2-4 1.25 ng/μl) or mouse IgG1 kappa (isotype control, 5 ng/μl) antibody. Following incubation, cells were washed and the level of receptor expression analysed using the FACS Calibur™ machine with excitation/emission wavelengths of 488/575 nm and the CellQuest Pro® software.

2.5.4 Measurement of cell surface Fas expression
BJAB or Jurkat cells (1 x 10^6) were resuspended in 10 % v/v goat serum in PBS and incubated with 5 ng/μl of mouse monoclonal anti-Fas antibody (Clone CH-11) for 1 hr on ice. Cells were washed and resuspended in fresh 10 % goat serum. Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulins (F(ab')2 fraction, Dako) were added to a final concentration of 50 ng/μl. Following incubation, for 1 hr on ice, cells were washed and the level of receptor expression analysed using the FACS Calibur™ machine with excitation/emission wavelengths of 488/525 nm and the CellQuest Pro® software.

2.5.5 Separation of TRAIL DISC by sucrose density gradient centrifugation
Cells (100 x 10^6) were treated as described above to induce formation of DISC. The washed cell pellet was lysed in 2 ml of DISC lysis buffer and the lysate cleared by centrifugation. The cleared lysate was loaded onto a continuous 10 - 45 % sucrose gradient (10 - 45 % w/v sucrose, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1 % v/v Triton X-100) and centrifuged at 180,000 g for 17 hr at 4 °C. Following centrifugation the gradients were fractionated into 0.5 ml samples. To each of the fractions 16 μl of magnetic M-280 streptavidin Dynabeads® were added before overnight incubation at 4 °C. The beads were washed and the proteins eluted by boiling in 1 x sample buffer for SDS-PAGE separation. Protein standards from high and low molecular weight kits were used as molecular weight markers for the gradient fractionation.
2.5.6 Cleavage of procaspase-3, Bid and IETD-AFC by active caspase-8 within the TRAIL DISC

DISC was isolated from BJAB or Jurkat cell lines as described above, except the complex was not eluted from the beads. Instead, the beads were equally divided to assess the cleavage of recombinant procaspase-3 C163A, full length Bid and the fluorescent substrate Acetyl-Ile-Glu-Th-Asp-7-amino-4-trifluoromethylcoumarin (IETD.AFC). For all experiments the beads were resuspended in caspase assay buffer (100 mM HEPES-KOH pH 7.0, 10 % w/v sucrose, 0.1 % w/v CHAPS, 10 mM DTT).

Recombinant C163A mutant procaspase-3 (kind gift from Dr Michelle Hughes) or full length human Bid (R & D systems) was added to the beads to a final concentration of 49 nM and incubated at 37 °C for up to 3 hr. Procaspase-3 C163A and Bid were also added to 12.5 nM recombinant active caspase-8 (kind gift from Dr Michelle Hughes) in caspase assay buffer. The supernatant was removed from the beads and added to 10 x sample buffer so the extent of caspase-3 or Bid cleavage could be determined by Western blot. The beads were washed and bound proteins, also to be analysed by Western blot, were eluted though boiling in 1 x sample buffer.

The remaining beads, or recombinant active caspase-8, were mixed with 44 µM of IETD.AFC (Enzyme Systems Products, MP Biomedicals, Cambridge, UK) in a 96 well plate. The release of the fluorogenic group AFC was measured using a Wallac Victor 1420 Multilabel Counter (Wallac, Finland) and excitation/emission wavelengths of 400/505 nm. Samples were assayed for 99 cycles, with a 10 sec gap between each cycle. The cleavage rate of IETD.AFC, and therefore the activity of caspase-8, was determined by linear regression and expressed as pmol/min.

2.6 Proteomic analysis

2.6.1 Protein concentration

In large scale-up experiments for mass spectrometry it was necessary to concentrate the proteins so that the maximal amount of sample could be loaded onto SDS-PAGE gels. This was achieved using the UPPA-PROTEIN Concentrate™ kit (G Biosciences®, Web Scientific, Crewe, UK) according to manufacturer's instructions.
2.6.2 Shotgun proteomics

Shotgun proteomics was completed using liquid chromatography tandem mass spectrometry (LC-MS/MS), in collaboration with the Protein Profiling facility (MRC Toxicology Unit).

In-gel protein digestion

Gel slices (approximately 1-1.5 mm) were cut sequentially from the SDS-PAGE gel lanes and prepared for mass spectrometry by Ms Rebecca Jukes-Jones (MRC Toxicology Unit). In-gel protein digestion was completed by one of two methods. Gel slices were either processed using the Montage In-Gel DigestZP kit (Millipore) (Pluskal et al, 2002) or using PCR plates (Axygen Scientific Inc., Kinesis LM, St Neots, UK). Individual gel slices were destained in the destain buffers provided (Montage In-Gel DigestZP kit) or in 25 mM ammonium bicarbonate with 5 - 50 % v/v acetonitrile. Destained gel slices were then dehydrated using 100 % acetonitrile and subsequently rehydrated with porcine trypsin solution (Promega, sequencing grade). With the Montage In-Gel DigestZP kit, the dehydrated gel piece and trypsin were incubated for 3 hr at 37 °C, whilst incubation was completed overnight at 30 °C when the samples were processed in PCR plates. Tryptic peptides were extracted using either the extraction and elution buffers provided (Montage In-Gel DigestZP kit) or 0.2 % v/v trifluoroacetic acid. Extracted tryptic peptides were concentrated to dryness and then solubilised in 5 % v/v formic acid.

Mass spectrometry of tryptic peptides

Tryptic peptides were separated using a micro-capillary high performance liquid chromatography nanoLC system (CapLC, Waters, Elstree, UK). The separated peptides were loaded onto an Atlantis™ dC18, 5 μm OPTI-PAK™ trap column (Waters) in 0.1 % v/v formic acid for pre-concentration. The peptides were eluted using a linear gradient of acetonitrile (7 - 80 %) over 50 min and directly loaded onto an Atlantis™ dC18, 3 μm analytical column (Waters). Eluate from the analytical column was loaded into the Q-TOF (quadrupole time of flight) mass spectrometer (Waters) though a nanospray Z ion source. The mass-to-charge ratio (m/z) of the ionized peptides was analyzed, and based on the ion signal intensity, peptides were selected for fragmentation and sequencing by tandem mass (MS/MS) spectrometry. The peptide spectra generated were used for protein identification though comparison to the non-redundant SwissProt database (ftp://us.expasy.org/databases/) using the MASCOT program (Matrix Science, London, UK). The data generated was uploaded into
Scaffold software (Proteome Software Inc., Portland, USA) where the proteins identified could be analysed.

2.6.3 Analysis of protein hits
Mass spectrometry of all samples generated a vast amount of data. To ensure validation of potential interactors was manageable it was necessary to set up criteria to exclude unlikely hits. The criteria for the TAP and DISC data sets were different, but for all data sets the initial criteria was a 50 % probability of correct peptide and protein identifications.

Analysis of TAP mass spectrometry data
Keratin hits were removed since these were likely to be contamination during sample preparation. All proteins that were present in both samples were retained, however, any protein present in only one sample needed to be identified by more than 3 spectra for it to be kept in the protein list. This was because the identification of FADD varied between control and TRAIL treated sample by 3 assigned spectra. The highest spectral count for each protein was plotted against the protein identification probability to visualise any hits that would be worth further investigation.

Analysis of TRAIL DISC mass spectrometry data
Again keratin and actin hits were immediately excluded, as were carboxylases since they require biotin as a co-factor and therefore may have non-specifically bound to the streptavidin beads. The following criteria were adhered to unless a protein hit possessed a protein probability of greater than 95 %, in these cases violation of the criteria was allowed. The protein was required to have been identified by more than 1 spectra and must not have been identified in the control sample (unless greater than 95 % probability in the treated sample and much lower the untreated sample with a difference of more than 1 spectra). Proteins were excluded if they possessed a tissue or organelle specific expression, a specialised function that made them unlikely interactors or were secreted. Hits from the Jurkat DISC experiments were retained when present in the repeat where the greatest amount of eluate was separated by SDS-PAGE. Alternatively, the hit must have been identified in both of the other two repeats. Hits from the BJAB DISC experiments were excluded when they were only detected in the experiment where the lowest amount of eluate was separated by SDS-PAGE gel. The difference in spectral count between the control and the TRAIL treated sample for each protein was plotted against the protein identification probability (in the treated sample) to visualise any hits that would be worth further investigation.
2.6.4 Calculation of spectral abundance and normalised spectral abundance factors

The normalised spectral abundance factor (NSAF) (Blondeau et al, 2004, Paoletti et al, 2006) was calculated for each known component of the BJAB TRAIL DISC that was identified by mass spectrometry. The equations for the calculation of SAF and NSAF are as follows:

\[
SAF_k = \left( \frac{SpC}{MW} \right)_k
\]

\[
(\text{NSAF})_k = \frac{\left( \frac{SpC}{MW} \right)_k}{\sum_{i=1}^{N} \left( \frac{SpC}{MW} \right)_i}
\]

Where \( SpC \) is the number of MS spectra matching peptides from protein \( k \), \( MW \) is the molecular weight (kDa) of protein \( k \) and \( N \) is the number of proteins in the DISC.
Chapter 3: Purification and Mass Spectrometry of FADD-TAP
3 Purification and Mass Spectrometry of FADD-TAP

3.1 Introduction

FADD is a vital component of the Fas and TRAIL DISC (Juo et al, 1999, Sprick et al, 2000). However, previous work in our laboratory had shown that only a small amount of total cellular FADD is recruited to the TRAIL DISC (Harper et al, 2003a). This observation, and the involvement of FADD in other pathways, raised the question of what other proteins interact with FADD in control and TRAIL treated conditions. Thus, it was decided to attempt to identify FADD interacting proteins through mass spectrometry of FADD purified from control and TRAIL treated cellular lysates.

Tandem affinity purification (TAP), originally described in 1999, uses two purification steps and a specific cleavage elution step to produce a sample of high purity with reduced contamination from non-specific proteins. Furthermore, the purifications are performed under native conditions, allowing protein-protein interactions to be preserved (Puig et al, 2001, Rigaut et al, 1999). The principle of TAP is that two affinity tags are fused together by a protease cleavage site to produce a tandem affinity tag. This tag is attached to a bait protein that can then be sequentially purified, along with any interacting proteins, via the affinity tags present. The purity of the sample produced through TAP and its successful use in the identification of protein interactions (Bouwmeester et al, 2004, Martins et al, 2002) made it an attractive method for the purification of FADD for this study. Therefore, the aims of this chapter were to express tandem affinity tagged FADD in a cell line, purify it using the TAP method and to identify the interacting proteins present by mass spectrometry.

3.2 Generation of tandem affinity tagged FADD

Endogenous FADD interacts with proteins through both its N-terminal DED and C-terminal DD (Figure 1.6). Therefore, the size and position of the dual affinity tag was an important consideration, as it could potentially hinder any FADD-protein interactions. It has been reported that FADD can be tagged at the C-terminus without consequence (Muppidi et al, 2006). Thus, the original TAP tag (20 kDa) and a V5/His tag (6 kDa) were attached to the C-terminus to produce FADD-TAP and FADD-V5/His (Figure 3.1 A and B). The original TAP tag is composed of the IgG binding moieties of the *Staphylococcus aureus* Protein A (Protein A) separated from calmodulin-binding peptide (CBP) by a tobacco etch virus (TEV) cleavage site. The V5/His tag is composed of the V5 epitope (found in the P and V proteins of paramyxovirus SV5) separated from a hexa-histidine (His) tag by three Prescission™ cleavage sites. In
Figure 3.1: Three versions of tandem affinity tagged FADD were generated.

Representations of the three variants of tandem affinity tagged FADD. FADD was tagged at the C-terminus with either the original TAP tag (A) or a V5/His tag (B). In addition the original TAP tag was attached to the N-terminus of FADD (C). DED, death effector domain; DD, death domain; CBP, calmodulin binding peptide.

<table>
<thead>
<tr>
<th>Vector</th>
<th>FADD</th>
<th>FADD-TAP</th>
<th>FADD-V5/His</th>
</tr>
</thead>
<tbody>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 3.2: Recombinant untagged and tagged FADD produced by cell free in vitro expression.

A cell free in vitro transcription/translation (IVT) system was used to express either untagged or tagged FADD from 1 μg of vector DNA. Equal amounts were run on SDS-PAGE and the level of expression was determined by Western blot.
addition, FADD was also tagged at the N-terminus with the original TAP tag to produce TAP-FADD (Figure 3.1 C).

3.3 **In vitro produced tagged FADD variants can reconstitute the Fas DISC**

The ability of tagged FADD to bind to death receptors and recruit procaspase-8 was assessed using the cell free *in vitro* Fas DISC system generated within our laboratory (Hughes et al, 2009). An active Fas DISC can be formed in a cell free environment through incubating GST beads coated with the intracellular domain of Fas (GST-Fas) with a cellular lysate. Alternatively, the DISC can be completely reconstituted through incubating GST-Fas with recombinant FADD and procaspase-8. For the purpose of this study GST-Fas beads were incubated with either a wildtype cell lysate, or a FADD null cell lysate supplemented with *in vitro* expressed FADD, FADD-TAP or FADD-V5/His (Figure 3.2). TAP-FADD could not be included since it did not possess the T7 promoter required for *in vitro* transcription/translation (IVT).

*In vitro* expressed FADD, FADD-TAP and FADD-V5/His were all able to support formation of an active Fas DISC (Figure 3.3 lanes 4 to 6); although the amount formed was lower than with endogenous FADD (Figure 3.3 lane 2). All variants were recruited to the GST-Fas beads (Figure 3.3 A) and bound procaspase-8, which subsequently processed to p43/41 and p18 (Figure 3.3 B). However, FADD-TAP appeared to be the most promising variant as it recruited more procaspase-8 than FADD-V5/His, resulting in the generation of a higher amount of the p18 subunit.

3.4 **Transient expression of FADD-TAP restores Fas-induced apoptosis in FADD null cells**

All the FADD variants tested were capable of forming a reconstituted Fas DISC (Figure 3.3), but it was essential that they could also function in a cellular environment. To investigate this, all tagged FADD variants (and the empty vector controls, C-TAP, V5/His and N-TAP) were transfected into the FADD null Jurkat cell line. Preliminary experiments demonstrated that FADD null cells could be efficiently transfected with a range of DNA concentrations. Transfection efficiency, measured through GFP expression, was on average 67 % (± 1 %) with 1 µg DNA up to 79 % (± 1.7 %) with 3 µg DNA (data not shown).

\[ ^1 \text{Standard error of the mean (SEM)} (n=4) \]
Figure 3.3: *In vitro* expressed tagged FADD variants can reconstitute the Fas DISC.

Wildtype (WT) or FADD null cell lysates (in the presence or absence of *in vitro* expressed (IVT) protein) were incubated with GST or GST-Fas beads. The beads were washed and bound proteins eluted by boiling in 2 x sample buffer. A. Equal amounts of bead eluate (DISC) or supernatant were probed for FADD and GST (bead loading control). B. Unequal amounts of bead eluate (see table) or equal amounts of supernatant were probed for caspase-8. Bead loading controls are as shown in A. The asterix indicates a non-specific band in the FADD null cell lysates detected by the caspase-8 antibody. It is stronger in lane 5 as it coincides with FADD-TAP that, due to the presence of IgG binding moieties, is non-specifically detected by the secondary antibody. Blots shown are representative of two independent experiments.
FADD-TAP was well expressed following transfection of FADD null cells with 1 µg of DNA (Figure 3.4 A lane 4 and C lane 3). In contrast, low expression of FADD-V5/His and TAP-FADD was observed, even when 3 µg of DNA was used (Figure 3.4 A, lanes 6 and 8). Furthermore, FADD-V5/His was expressed from alternative start sites or was targeted for degradation on transfection, as three different molecular weight products were detected by Western blot (Figure 3.4 A lane 6). Upon treatment with death receptor agonists, the only transfected cell line that noticeably underwent apoptosis was the one transfected with FADD-TAP (Figure 3.4 B). Therefore, it was decided to continue solely with this construct.

The FADD-TAP transfected cell line underwent dramatically less apoptosis than the wildtype cell line (Figure 3.4 B). This could have been caused by either the transient nature of FADD-TAP expression or by the TAP tag interfering with the function of FADD. Therefore, untagged FADD was transfected into the FADD null cell line, and the sensitivity to death-mediated apoptosis was compared to that of cells transfected with FADD-TAP (Figure 3.4 C and D).

Preliminary experiments determined that 3 µg of DNA was required for optimal untagged FADD expression (data not shown). Transfection (with the appropriate amount of DNA) lead to approximately equal expression of endogenous FADD, transfected FADD and transfected FADD-TAP (Figure 3.4 C). The cells transfected with FADD or FADD-TAP were sensitive to the anti-Fas antibody, but to a lesser extent than the wildtype cell line (Figure 3.4 D). Wildtype cells were significantly more sensitive to Fas-induced apoptosis than those transfected with FADD (P<0.0001\(^2\)), which were in turn more sensitive than the FADD-TAP transfected cells (P=0.0032\(^2\), Figure 3.4 D). Overall the response to TRAIL was much lower across all cell lines tested, but the rank order of sensitivity was the same as that observed in response to anti-Fas antibody (Figure 3.4 D). The greatest decrease in sensitivity was observed between the wildtype cell line and the FADD transfected cell line. Therefore, it appeared that the major obstacle to fully restoring the function of FADD was the use of transient expression. Importantly this effect could be overcome through stable expression of FADD-TAP.

\(^2\) Statistics completed using unpaired Student’s t test, assuming normal distribution as sample size too small to test for normality or to use non-parametric tests.
Figure 3.4: FADD-TAP transiently transfected cells were sensitive to death receptor-mediated apoptosis.

FADD null cells were electroporated with the indicated quantity of vector DNA and incubated at 37 °C for 24 hr. A and C. Cell pellets were solubilised to determine FADD expression by Western blot; β-actin (B) and α-tubulin (C) were visualised as loading controls. B and D. Cells were treated with either 500 ng/ml anti-Fas antibody or 1 µg/ml TRAIL for 6 hr at 37 °C. The exposure of PS was used to quantify the percentage apoptosis and was measured by flow cytometry after annexin V/PI staining. A and B. Comparison of the sensitivity of FADD null cells transiently transfected with tagged variants of FADD. Results shown are from one experiment that is representative of three independent experiments where cells were transfected with different amounts of the FADD-V5/His and TAP-FADD vectors. C and D. Comparison of the sensitivity of FADD null cells transiently transfected with FADD-TAP or FADD. Western blot (C) is representative of two independent experiments, whilst for graph (D) n=3 and error bars are SEM. P values were calculated using Student’s t test. WT, wildtype.
3.5 Generation and characterisation of FADD-TAP cell lines

3.5.1 Generation of single clone cell lines that stably expressed FADD-TAP

Transient transfection experiments had identified FADD-TAP as the most promising bait protein but also suggested that it was essential to produce a cell line that stably expressed FADD-TAP (Figure 3.4). To achieve this, FADD null cells were transfected with FADD-TAP (or the empty vector control C-TAP) and selected through antibiotic treatment and serial dilution to produce single cell clones. The single clone FADD-TAP cell lines were screened for FADD-TAP expression and sensitivity to death receptor-mediated apoptosis. This extensive screening process produced two FADD-TAP single clone-derived cell lines (FADD-TAP A3 and D10) and several C-TAP (empty vector) cell lines. From the C-TAP cell lines, the D6 clone was chosen as it came from the highest dilution and was therefore the most likely to be a single clone-derived C-TAP cell line.

FADD-TAP expression in the single clone cell lines (A3 and D10) was much higher than in the FADD-TAP mixed population and wildtype cell lines (Figure 3.5 A, lanes 1, 4, 5 and 6). The FADD-TAP mixed population represents a cell line that was subjected to antibiotic selection but not serial dilution. In accordance with the lower expression of FADD-TAP, the mixed population cell line was not sensitive to death receptor-mediated apoptosis. These cells did not expose PS or initiate caspase cleavage following treatment with anti-Fas antibody or TRAIL (Figure 3.5 B and C lanes 11 and 12).

In contrast, there was significant induction of apoptosis in both of the FADD-TAP single clone cell lines by anti-Fas antibody and more importantly by TRAIL. Following treatment, the FADD-TAP A3 and D10 cell lines exhibited externalised PS (Figure 3.5 B) and procaspase-8 and -3 activation, as demonstrated by cleavage into smaller subunits (Figure 3.5 C lanes 14, 15, 17 and 18). Procaspase-8 was cleaved into the p43/41 and p18 subunits, whilst procaspase-3 was processed to its p19 and p17 subunits.

The FADD-TAP A3 and D10 cell lines were less sensitive to anti-Fas antibody than the wildtype cell line, which was reflected in a lower exposure of PS (Figure 3.5 B) and a reduced amount of caspase processing (Figure 3.5 C, lanes 2, 14 and 17). This was especially clear with the FADD-TAP A3 cell line, which was less sensitive than the FADD-TAP D10 cell line. Importantly, both FADD-TAP cell lines were as sensitive to TRAIL-induced apoptosis as the wildtype cell line. Following TRAIL treatment there
Figure 3.5: FADD-TAP single clone cell lines express FADD-TAP and are sensitive to death receptor-mediated apoptosis.

FADD null cells were electroporated with 3 μg of FADD-TAP or empty vector C-TAP and single clones cell lines were generated as described in Materials and Methods. A. Cell pellets were solubilised in 1 x sample buffer and an equal number of cells analysed by Western blot for FADD expression. B. Cells were treated with 500 ng/ml anti-Fas antibody or 1 μg/ml TRAIL for 6 hr at 37 °C. Percentage apoptosis represents the percentage of cells that were PS positive, error bars are SEM (n=6). C. Cell pellets were collected from post-treatment and an equal number of cells were analysed by Western blot for caspase-8 and -3 cleavage. Blots shown are representative of 2 independent experiments. FT, FADD-TAP.
was a dramatic increase in PS exposure (Figure 3.5 B) and caspase cleavage (Figure 3.5 C lanes 3, 15 and 18) in the single clone FADD-TAP cell lines.

3.5.2 Characterisation of the FADD-TAP A3 and D10 cell lines

FADD overexpressed in mammalian cells has been shown to form large filamentous structures (death effector filaments), which induce apoptosis through the recruitment of procaspase-8 (Siegel et al, 1998). The level of spontaneous apoptosis in the FADD-TAP single clone cells was very low (Figure 3.5 B), suggesting that expression of FADD-TAP itself was not lethal to the cells. However, to ensure that FADD-TAP was not aggregated within the cells I fractionated cellular lysates by molecular weight using size-exclusion chromatography.

FADD from wildtype cells peaked in fractions between the 25 and 43 kDa molecular weight markers indicating that it was likely to be present as a dimer, which would have a molecular weight of 46 kDa (Figure 3.6 fractions 23 to 26). FADD-TAP eluted across a range of molecular weights indicating the presence of monomeric, dimeric and possibly oligomeric species. However, the majority of FADD-TAP eluted in fractions 19 to 22 (between the 43 and 232 kDa molecular weight markers) suggesting that the predominant species of FADD-TAP was approximately dimeric (86 kDa) (Figure 3.6). The discovery of FADD and FADD-TAP at dimeric molecular weights agrees with previous studies that have demonstrated that FADD self-associates (Muppidi et al, 2006) and is present in mouse hepatocytes as a dimer (Zhang et al, 2008). Importantly, size-exclusion chromatography had demonstrated that the high expression of FADD-TAP did not cause a significant amount of aggregation.

The expression of a range of apoptotic regulators was not altered by the transfection of DNA, the selection process and/or the expression of FADD-TAP (Figure 3.7). The levels of the caspase inhibitor XIAP, the DISC regulator c-FLIP, initiator and executioner caspases (-9, -8 and -3), and members of the pro- and anti-apoptotic Bcl-2 families (Bcl-2, Bid and Bak) were not significantly different across all cell lines. Thus, expression of key regulators of apoptosis in the FADD-TAP cell lines was not different to that of the wildtype cell line.

Even though FADD-TAP A3 and D10 had been shown to be fully sensitive to TRAIL, it was important to confirm that FADD-TAP supported TRAIL DISC formation. The wildtype, FADD null and both stable FADD-TAP cell lines were treated with biotinylated soluble TRAIL (bTRAIL) to allow DISC purification. In this experiment the lysate from
Figure 3.6: FADD-TAP A3 and D10 single clone cell lines express predominantly non-aggregated, approximately dimeric FADD-TAP.

Lysates from each cell line were cleared by centrifugation and 670 μg of protein was loaded onto a Superose™ 6 column. Fractions of 50 μl were collected, separated by SDS-PAGE and FADD was visualised by Western blot. Standards shown were run separately when the column was calibrated.
<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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</tr>
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<tr>
<td>Cell Line</td>
<td>Wildtype</td>
<td>FADD Null</td>
<td>C-TAP D6</td>
<td>FADD-TAP A3</td>
<td>FADD-TAP D10</td>
</tr>
</tbody>
</table>

W. B. Ab

- FADD-TAP
- FADD

- c-FLIP<sub>L</sub>
- *
- c-FLIP<sub>S</sub>

Caspase-9
Caspase-8
Caspase-3
Bcl-2
Bid
Bak
α-tubulin

Figure 3.7: The FADD-TAP single clone cell lines do not have altered expression of proteins involved in apoptosis.

Cell pellets from each cell line were solubilised in 1 x sample buffer and an equal number of cells separated by SDS-PAGE. The expression of several key proteins involved in apoptosis was investigated by Western blot. The asterixed band is FADD-TAP that (due to the IgG binding moieties) is nonspecifically detected by the secondary antibody.
untreated controls was supplemented with bTRAIL, which allowed the purification of unstimulated TRAIL receptors, which were not associated with FADD or procaspase-8 (Figure 3.8 bead eluate lanes 1, 3, 5 and 7). An equal amount of TRAIL-R2 was precipitated from all the unstimulated lysates demonstrating that its expression had not been altered in the single clone cell lines. This result was further confirmed in the FADD-TAP D10 cell line by immunostaining and flow cytometry (Appendix 1).

Previously published data had demonstrated an obligatory role for FADD in TRAIL DISC formation (Sprick et al, 2000). In accordance with this only the cell lines that expressed FADD/FADD-TAP formed active TRAIL DISC (Figure 3.8 bead eluate lanes 2, 6 and 8) and underwent apoptosis (Figure 3.8 lower table). Both FADD-TAP A3 and D10 cell lines formed more TRAIL DISC than the wildtype cell line. Furthermore, this correlated with the increased level of apoptosis (as measured by PS exposure) observed in the FADD-TAP cell lines (Figure 3.8 lower table).

The DISC from both FADD-TAP cell lines contained a second, lower molecular weight band that was detected by the FADD antibody (Figure 3.8 marked by an asterix). This was possibly generated through cleavage of the full length FADD-TAP, either prior to DISC recruitment or within the DISC itself by active caspase-8. However, when FADD-TAP was incubated in vitro with active caspase-3, -6, -7 or -8 no degradation products similar to that seen in the TRAIL DISC were detected by Western blot (data not shown). Thus, it appears unlikely that the cleavage of FADD-TAP was caused by any of the caspases tested. Instead it is possible that another caspase or cellular protease was responsible for the cleavage of FADD-TAP.

Overall, the data obtained from the single clone FADD-TAP cell lines had proven that FADD-TAP could function in a similar manner to endogenous FADD in TRAIL-mediated apoptotic signalling.

3.6 Mass spectrometry of purified proteins from untreated and TRAIL treated FADD-TAP cells

3.6.1 Optimisation of the TAP methodology

A detailed TAP methodology (Figure 3.9) was published in 2001 (Puig et al, 2001). However, to ensure maximal recovery of FADD-TAP various parameters of this method were optimised as summarised in Table 3.1.
<table>
<thead>
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<th>Lane</th>
<th>1</th>
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<td>+</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**W. B. Ab**

**Bead eluate**

- **TRAIL-R2**
  - $R_{2_l}$
  - $R_{2_s}$

- **FADD**
  - $*$
  - FADD

- **Caspase-8**
  - Proform
  - p43/p41

**Supernatant**

- **FADD**
  - FADD

- **Caspase-8**
  - Proform

| % Apoptosis (6.5 hr) | 4 | 41 | 4 | 4 | 4 | 55 | 4 | 53 |

**Figure 3.8:** On exposure to TRAIL, FADD-TAP single clone cell lines form a functional TRAIL DISC.

Cells were treated with 500 ng/ml of bTRAIL for 1 hr at 4 °C followed by 25 min at 37 °C and the DISC formed was purified using streptavidin beads. This experiment differs from the method given in the Materials and Methods as 1.2 μg of bTRAIL was added to untreated cell lysate. Bead eluate (DISC) and supernatants (remaining after removal of beads) were loaded on to SDS-PAGE to assess the amount of TRAIL-R2, FADD and caspase-8 present by Western blot. The supernatant blots act as loading controls to ensure an equal number of cells were treated. Before lysis a small sample of cells were taken and kept at 37 °C for 6.5 hr for the assessment of apoptosis by annexin V/PI staining. These values are shown in the table at the bottom of the figure, where percentage apoptosis is the percentage of PS positive cells. The asterixed band is potentially a cleaved form of FADD-TAP present within the DISC.
Figure 3.9: The method for purification of FADD-TAP.

FADD-TAP was purified from FADD-TAP cellular lysates using the following method. Initially, IgG beads were used to capture FADD-TAP, through the Protein A tag, from cell lysates. Unbound proteins were removed through washing of the beads. FADD-TAP and interacting proteins were specifically eluted from the IgG beads using TEV, which cleaves at the canonical site between the Protein A and the CBP affinity tags. Cleavage converts FADD-TAP into FADD-CBP thus releasing it from the IgG beads. FADD-CBP eluted from IgG beads is further purified through capture onto calmodulin (CaM) beads. Further washing removes the remaining contaminants, including the TEV protease. Elution of FADD-CBP is achieved using EGTA, which chelates the calcium required for the CBP-CaM interaction.
Table 3.1: Aspects of the TAP method investigated and the optimal conditions determined for the purification of FADD-TAP.

<table>
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<th>Parameter Investigated</th>
<th>Published Condition</th>
<th>Optimal Condition</th>
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<td>500 U</td>
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<td>Cleavage incubation</td>
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<td>Elution of FADD-CBP from IgG beads</td>
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<tr>
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<td>200 µl</td>
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<td>Elution of FADD-CBP from CaM beads</td>
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<td>6 fractions of 300 µl</td>
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<tr>
<td>Concentration of final eluate</td>
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<td>UPPA-PROTEIN Concentrate™ kit</td>
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3.6.2 Purification of FADD-TAP from untreated and TRAIL treated FADD-TAP D10 cells

Characterisation of the two FADD-TAP cell lines had shown that they formed similar amounts of TRAIL DISC and had a similar sensitivity to TRAIL. Therefore, I decided to use the FADD-TAP D10 cell line for the mass spectrometry studies. This was based on the observation that FADD-TAP D10 was more sensitive to Fas-induced apoptosis and thus its sensitivity profile was closest to that of the wildtype cell line (Figure 3.5).

FADD-TAP was purified, using the optimised method (Table 3.1), from the lysate of both untreated and TRAIL treated FADD-TAP D10 cells. The samples generated were extremely clean with only 4 bands, representing monomeric and possibly dimeric FADD-CBP, identifiable by coomassie staining (Figure 3.10). The lower two bands corresponded to the monomeric molecular weight of FADD-CBP (28 kDa) and may have represented the phosphorylated and non-phosphorylated forms of the protein. However, obvious differences between the two samples were not evident by coomassie despite significant induction of apoptosis in the TRAIL treated cells (Figure 3.10 bottom table). The gel was analysed by mass spectrometry (LC-MS/MS) and the data generated uploaded from Mascot into the Scaffold software for further analysis. The Scaffold software provides a graphical interface that enables the user to interrogate the mass spectrometry data (Figure 3.11). Thresholds can be set that the identified proteins must meet to be displayed in the main list; thus permitting the exclusion of less confident hits. Furthermore, extensive information on each of the hits in the main list is available either in the main window or through the proteins tab (Figure 3.11). The information provided includes the protein probability (the likelihood that the peptide is correctly assigned to that protein), spectral counts and peptide coverage.

3.6.3 Analysis of FADD-TAP mass spectrometry data identified two novel FADD-TAP interacting proteins as well as a novel FADD-TAP phosphorylation site

Mass spectrometry of the TAP purified samples from the untreated and TRAIL treated FADD-TAP D10 cells identified 137 proteins in total. Some of the proteins identified were exclusive to one or other sample, whilst others were found in both samples. In agreement with the role of FADD in the DISC, caspase-8 was detected only in the TRAIL treated sample (Table 3.2 and Figure 3.12, protein/spot 18). However, it was only identified by 1 spectrum illustrating (as mentioned earlier) the very small amount of total FADD that is recruited to the Jurkat TRAIL DISC and thus directly interacts with caspase-8.
Figure 3.10: FADD-TAP can be purified from untreated and TRAIL treated FADD-TAP D10 cells.

FADD-TAP (FT) D10 cells were either left untreated or treated with 500 ng/ml of TRAIL for 1 hr at 4 ºC followed by 25 min at 37 ºC and then lysed in DISC lysis buffer. The lysate was cleared by centrifugation and then loaded onto an IgG column. Following column washing, 500 U of TEV protease was applied and left to cleave FADD-TAP overnight at 4 ºC. The following day FADD-CBP (cleaved FADD-TAP) was eluted and then incubated with calmodulin beads for 1 hr at 4 ºC. After washing of the beads, bound proteins were eluted using an EGTA-containing buffer and concentrated using the UPPA-PROTEIN Concentrate™ kit. Concentrated proteins were loaded onto 4 – 20 % gradient SDS-PAGE gel and after separation stained with coomassie. Before lysis a small sample of cells were taken from each sample and kept at 37 ºC for 6 hr for the assessment of apoptosis. These values are shown in the table at the bottom of the figure, where percentage apoptosis represents the percentage of PS positive cells.

<table>
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![SDS-PAGE gel with bands labeled as Dimeric FADD-CBP and FADD-CBP with markers and a table of percentage apoptosis values.]
Figure 3.11: Screen shot of the Scaffold software.

This is a screen shot of the data provided in the samples tab (on the left hand side) view of the scaffold software. All protein hits are listed in the main window with their full name, accession number, molecular weight and in which sample they were identified. Drop down menus at the top can be used to filter results based on the minimum protein/peptide probability or minimum number of peptides. The display options menu changes the number associated with the protein to the variable selected by the user. In this example it is the number of assigned spectra. More information about the protein hit, such as peptide coverage and spectra, can be obtained through the proteins tab (view not shown here).
Table 3.2: Mass spectrometry data for the 17 proteins (and caspase-8) identified, as potential FADD-TAP interactors, in TAP purified samples from untreated and/or TRAIL treated FADD-TAP D10 cells.

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<th>TRAIL</th>
<th>Assigned Spectra</th>
<th>Assigned Protein Probability (%)</th>
<th>Cleaved (Yes/No)</th>
<th>Unique Peptides</th>
<th>Sequence</th>
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Proteins were identified by mass spectrometry and analysed as described in the Materials and Methods. For those proteins identified by multiple peptides the number of spectra, the protein probability, the cleavage status and number of unique peptides are given. For those proteins identified by a single peptide additional information on the peptide sequence and Mascot ion score are provided. TH, protein identified in the SDS-PAGE gel higher than its expected molecular weight.
Figure 3.12: Analysis of the 17 proteins (and caspase-8) identified, as potential FADD-TAP interactors, by mass spectrometry of TAP purified cellular lysates from untreated and TRAIL treated FADD-TAP D10 cells.

Proteins identified by mass spectrometry were either retained or excluded based on their fulfilment of the criteria set out in Materials and Methods. For the remaining 17 proteins (and caspase-8) the highest spectrum count was plotted against the highest protein probability to discover hits that may be worthwhile following up. Green spots represent the proteins identified in the unstimulated sample only, blue spots represent those identified only in the TRAIL treated sample and red spots represent those identified in both samples. Those proteins deemed the best hits (as well as caspase-8) are labelled. The full protein list is shown in Table 3.2.
The list of 137 proteins was analysed according to the criteria set out in the Materials and Methods. Those that fulfilled all of the criteria were retained whilst those that did not (including caspase-8) were not considered further within this analysis, leaving a total of 17 proteins (Table 3.2). The highest spectral count for each of the remaining 17 proteins (and, for comparison, caspase-8) was then plotted against the highest assigned protein probability (Figure 3.12). This graph illustrated that there were 6 hits that were noticeably higher, in terms of spectral count and/or protein probability, than the others. These hits were heat shock cognate 71 kDa protein (HSC70, spot 7), FADD (spot 8), calpain small subunit 1 (calpain S1, spot 9), myosin light chain 2 (spot 10), myosin light chain kinase 2 (spot 11) and Ig kappa chain C region (spot 12).

Myosin light chain kinase 2 was likely identified as the CBP tag is derived from this protein, Ig kappa chain C region (contains an immunoglobulin domain) was likely a contaminant from the IgG beads and myosin light chain (an abundant protein often detected by mass spectrometry) was likely a non-specific contaminant. Thus, HSC70 and calpain S1 were the most promising candidates for true FADD-TAP interactors and thus warranted further analysis.

The identifications of FADD, calpain S1 and HSC70 by mass spectrometry were significant, with a 100 % protein probability assigned to each protein (Table 3.2 and Figure 3.12). The number of assigned peaks in the peptide spectrum, the number of spectra and the peptide coverage of the protein provide an indication to the accuracy of the protein identification. As these criteria increase so does the confidence in the protein identification. FADD, calpain S1 and HSC70 were identified by multiple spectra (with a range of assigned peaks) and multiple peptides, which produced good coverage of the protein sequence (Table 3.2 and Figure 3.13). Furthermore, the spectral counts of HSC70 and calpain S1 were higher than that of the known TRAIL-induced FADD binding partner, caspase-8. Therefore, by mass spectrometry, calpain S1 and HSC70 were putative FADD-TAP interactors.

Calpain S1 was identified in both the untreated and treated samples (Table 3.2). However, upon TRAIL treatment the association of FADD-TAP with calpain S1 appeared to decrease. This was based on the observation that the number of calpain S1 spectra, which provide an indication of protein abundance, was lower in the TRAIL treated than in the untreated sample (Table 3.2). In contrast, HSC70 was only identified in the treated sample and therefore appeared to only interact with FADD-TAP following exposure of cells to TRAIL (Table 3.2).
Figure 3.13: FADD, Calpain S1 and HSC70 were identified by mass spectrometry with good peptide coverage and spectra.

For each protein the top panel is the best peptide coverage obtained in its identification, whilst the lower panel is the best spectra in that peptide coverage. In the peptide coverage images, yellow highlighted peptides represent those that have been identified by mass spectrometry (green residues are modified). In the spectra images the peaks and sequences are colour coded, blue represent y ions, red represent b ions, black represent unidentified ions and green represent ions minus water.
In addition to the identification of two potential FADD interactors, the mass spectrometry data also unearthed a new phosphorylation site (Ser 167) within FADD (Figure 3.14). Phosphorylation at this site occurred in untreated and TRAIL treated cells suggesting that it was not the direct result of TRAIL treatment. FADD has previously been shown to be specifically phosphorylated at Ser 194 (Chen et al, 2005, Scaffidi et al, 2000). However, the absence of a trypsin cleavage site after Ser 194 means that a peptide containing this residue would not have been detected by mass spectrometry. Therefore, the phosphorylation of Ser 194 within FADD-TAP could not be detected or confirmed by mass spectrometry.

Scaffidi and Chen had mutated as well as removed the C-terminal cluster of serines (found between amino acids 189 and 208) and found that only Ser 194 was phosphorylated (Chen et al, 2005, Scaffidi et al, 2000). Therefore, it was possible that the TAP tag had interfered with Ser 194 phosphorylation resulting in alternative phosphorylation at Ser 167. However, an antibody specific to phosphorylated Ser 194 confirmed phosphorylation of both endogenous FADD and FADD-TAP at this site (Figure 3.15). Confirmation of the presence of the canonical 194 phosphoserine in FADD-TAP increased confidence that Ser 167 was a potentially novel phosphorylation site.

3.7 Validation of FADD-TAP phosphorylation sites and interactors

Mass spectrometry of the TAP purified samples had led to the discovery of the novel Ser 167 phosphorylation site and two potential FADD interactors (calpain S1 and HSC70). However, further investigation was required to ensure these discoveries were not the consequence of the attachment of the TAP tag to FADD.

3.7.1 Phosphorylation at novel sites, identified by mass spectrometry, may occur only in FADD-TAP

Mass spectrometry of immunoprecipitated FADD-TAP was employed to confirm phosphorylation at Ser 167 (Figure 3.16 A). Mass spectrometry of the band corresponding to the molecular weight of FADD-TAP identified peptides corresponding to FADD. However, a peptide containing Ser 167 was not detected. Instead there was complete coverage of the N-terminal serine cluster of FADD (amino acids 10 to 18, Figure 3.16 B), which had not been achieved in the mass spectrometry of TAP purified FADD-CBP (Figure 3.14). Interestingly, peptide coverage of the N-terminus of
Figure 3.14: Identification of the potential FADD-TAP phosphorylation site, Ser 167, by mass spectrometry of TAP purified FADD-CBP.

FADD-CBP purified from untreated and TRAIL treated FADD-TAP D10 cell lines was shown by mass spectrometry to be phosphorylated at Ser 167. The top panel for each sample shows the peptide coverage of FADD. Yellow sections represent the peptides identified by mass spectrometry, whilst green residues indicate those that are modified (methionine (M) is oxidised and serine (S) is phosphorylated). The positions of Ser 167 (green) and the known phosphorylation site Ser 194 (boxed) are also indicated.

The middle panels show the peptide sequences that identified Ser 167 as phosphorylated and the associated Mascot ion score. The lower panel for each sample is the only (untreated) or best (treated) spectra obtained for the peptide containing phosphorylated Ser 167. Spectral peaks and sequences are colour coded, blue represent y ions, red represent b ions and black represent unidentified ions.
Figure 3.15: FADD-TAP and endogenous FADD are phosphorylated at Ser 194.

Cell pellets from the FADD-TAP (FT) D10 and wildtype (WT) E6.1 Jurkat cell lines were solubilised. The levels of total (FADD antibody) or Ser 194 phosphorylated (Ser 194 FADD antibody) FADD, in an equal number of cells, were analysed by Western blot.
Figure 3.16: Mass spectrometry of immunoprecipitated FADD-TAP reveals another potential phosphorylation site at Ser 10.

FADD-TAP was immunoprecipitated from the FADD-TAP (FT) D10 cell line and mass spectrometry analysis revealed that Ser 10 was phosphorylated. A. Coomassie stained gel of immunoprecipitated FADD-TAP. B. The mass spectrometry data that identified Ser 10 as another phosphorylation site in FADD-TAP. The top panel shows the peptide coverage of FADD. The positions of Ser 10 (green), Ser 167 (boxed) and the known phosphorylation site Ser 194 (boxed) are indicated. The middle panel shows the peptide sequence that identified Ser 10 as phosphorylated and the associated Mascot ion score. The spectra for this peptide is shown in the lower panel where blue peaks and sequences represent y ions, black represent unidentified ions and green represent ions minus water. Ab, antibody.
FADD-TAP lead to the identification of another novel phosphorylation site at Ser 10 (Figure 3.16 B).

The experiments completed thus far had identified two potential phosphorylation sites within FADD-TAP (Ser 10 and Ser 167). To investigate potential phosphorylation at these sites in untagged FADD, Ser 10, Ser 167 and Ser 194 (the known phosphorylation site) were individually mutated to alanine residues to prevent phosphorylation.

Transient transfection of wildtype, S10A, S167A and S194A FADD into FADD null cells resulted in protein expression equivalent to that of endogenous FADD (Figure 3.17). Loss of Ser 194 phosphorylation in the S194A mutant, but not in any of the other FADD variants, was confirmed using the Ser 194 antibody (Figure 3.17 upper panel). In agreement with previously published data (Scaffidi et al, 2000) the upper molecular weight band of FADD was lost in the S194A FADD mutant (Figure 3.9 middle panel). However, this higher molecular weight band persisted in the S10A and S167A mutants. This indicated that phosphorylation of Ser 10 and 167 did not contribute to the increase in molecular weight that was related to phosphorylation of Ser 194. However, it was considered possible that phosphorylation at Ser 10 and Ser 167 could alter the isoelectric point of FADD rather than its molecular weight. Therefore, I decided to investigate the separation of the FADD variants by 2-D electrophoresis, which is able to identify post-translational modifications. Phosphorylation, the most common post-translation modification (Halligan et al, 2004), increases the acidity of a protein, decreasing its isoelectric point and causing a leftward shift of the protein upon separation by 2-D electrophoresis.

Cellular lysates from untransfected and FADD transfected cell lines were separated by 2-D electrophoresis and FADD was detected by Western blot (Figure 3.18). Previous 2-D electrophoresis of FADD from BJAB cells and lung adenocarcinoma samples identified 2 spots, one non-phosphorylated and the other phosphorylated (Chen et al, 2005, Scaffidi et al, 2000). In agreement with, this endogenous as well as transfected wildtype, S10A and S167A FADD were principally visualised as two spots at different molecular weights and isoelectric points (Figure 3.18 spots 1 and 2). A significant difference in spot pattern was observed with S194A FADD where, as with the 1-D Western blot, the higher molecular weight species (spot 1) was lost. Therefore, spot 1 represented Ser 194 phosphorylated FADD, whilst spot 2 most likely represented unmodified FADD.
Figure 3.17: Mutation of Ser 194, but not Ser 10 or Ser 167, alters the molecular weight of FADD.

FADD null cells were electroporated with 3 μg of empty or FADD-containing (wildtype (WT), S10A, S167A, S194A) vectors and incubated for 24 hr at 37 °C. After incubation non-transfected and transfected cells were pelleted and solubilised. An equal number of cells were separated by SDS-PAGE for the assessment of Ser 194 phosphorylated (Ser 194 FADD antibody) or total (FADD antibody) FADD expression.
Figure 3.18: Serine to alanine mutation of amino acid 194, but not of 10 or 167, alters the 2-D spot pattern of FADD.

Cellular lysates from an equal number of untransfected or FADD (wildtype, S10A, S167A, S194A) transfected cell lines were concentrated using the UPPA-PROTEIN Concentrate™ kit. The resulting protein pellets were resuspended in 2-D sample preparation solution and separated by isoelectric focusing on a 11 cm pH 3 – 10 non-linear ReadyStrip™ IPG strip. Following this, proteins were separated based on molecular weight and FADD was detected by Western blot.
The similarity in the 2-D spot pattern of wildtype, S10A and S167A FADD suggested that Ser 10 and 167 may not be phosphorylated in native FADD.

A smaller third spot was detected (Figure 3.18, spot 3), in all FADD expressing cells, with the same molecular weight as unmodified FADD but an isoelectric point similar to that of Ser 194 phosphorylated FADD. Since the absence of phosphorylation at Ser 10, 167 or 194 did not affect the detection of spot 3, it is possible that spot 3 represents a different and as yet unidentified post-translationally modified version of FADD.

3.7.2 The interaction between FADD-TAP and calpain S1 or HSC70, detected by mass spectrometry, was not evident by direct immunoprecipitation

Calpain S1 and HSC70 were earlier identified, by mass spectrometry, as potential FADD-TAP interactors. Western blot analysis confirmed that these proteins were expressed in the FADD-TAP D10 and wildtype E6.1 Jurkat cell lines and thus available for interaction with FADD-TAP/FADD (Figure 3.19 B). FADD-TAP (or FADD) was immunoprecipitated from untreated or TRAIL treated FADD-TAP D10 (or wildtype E6.1) cells to assess whether the interactions, detected by mass spectrometry, could also be identified through an alternative approach. FADD-TAP or FADD was identified by Western blot in the sample immunoprecipitated with anti-FADD antibody coated beads (Figure 3.19 A lanes 2, 3, 5 and 6). This corresponded to a decrease in the level of FADD-TAP/FADD in the post-bead incubation lysate (Figure 3.19 B). However, neither calpain S1 nor HSC70 were identified, by Western blot, in any of the immunoprecipitated samples from either cell line (Figure 3.19 A). This was surprising considering the compelling identification of these proteins by mass spectrometry (Figure 3.13). Therefore, it is possible that the interaction between FADD-TAP and calpain S1 or HSC70 was not preserved by the immunoprecipitation conditions. For example, the binding of FADD antibody to FADD-TAP may have in some way prevented the interaction with calpain S1 or HSC70.
Figure 3.19: The interaction between FADD-TAP and calpain S1 or HSC70 cannot be confirmed through immunoprecipitation.

Cells were either left untreated or treated with 500 ng/ml TRAIL for 1 hr on ice and 25 min at 37 °C. One set of untreated cellular lysates were incubated with Protein G beads coated with an isotype control antibody. The treated, and the second set of untreated, cellular lysates were incubated with Protein G beads coated with FADD antibody. Following overnight incubation at 4 °C the bound proteins were eluted using a low pH buffer. Samples of the bead eluate (A) and lysates, both pre- and post-bead incubation, (B) were separated by SDS-PAGE and then probed for the presence of FADD, calpain S1 and HSC70.
3.8 Discussion

This chapter has described the generation and characterisation of single clone cell lines that stably express FADD-TAP and the mass spectrometry analysis of FADD-TAP purified samples. These experiments, along with the validation studies completed to date, have revealed a number of important findings which are summarised as follows:

1. FADD null cells are sensitised to Fas-induced apoptosis through transient expression of FADD-TAP.
2. Stable expression of FADD-TAP in single clone cell lines lead to significant induction of apoptosis following treatment with anti-Fas antibody or TRAIL.
3. FADD-TAP single clone cell lines are fully sensitive to TRAIL and form an active TRAIL DISC.
4. FADD-TAP and interacting proteins could be purified from FADD-TAP D10 cell lysates with minimal contamination from non-specific proteins.
5. Mass spectrometry of FADD-TAP purified from the TRAIL treated cell line identified the bona fide FADD interactor caspase-8.
6. Mass spectrometry identified two previously unidentified FADD-TAP phosphorylation sites, Ser 167 and Ser 10.
7. Mass spectrometry of TAP affinity purified samples identified calpain S1 and HSC70 as potential FADD-TAP interactors.

3.8.1 Generation and characterisation of single clone FADD-TAP expressing cell lines

Generation of cell lines that stably expressed a tagged variant of FADD was essential for the identification, by mass spectrometry, of protein interactors under control and TRAIL treated conditions. FADD-TAP was selected to be the bait protein as in preliminary experiments its transient expression lead to the greatest sensitisation of FADD null Jurkat cells to Fas-induced apoptosis (Figure 3.4). Antibiotic and single clone selection of FADD null Jurkat cells transiently transfected with FADD-TAP resulted in the generation of the stably expressing FADD-TAP A3 and D10 cell lines. These cell lines formed DISC in response to stimulation with TRAIL and were as sensitive as wildtype Jurkat cells to TRAIL-induced apoptosis (Figure 3.5 and Figure 3.8). Optimisation of the TAP method allowed specific purification of FADD-TAP, with apparently very few non-specific proteins, from control and TRAIL treated FADD-TAP D10 cells. Mass spectrometry of these samples lead to the identification of two potential FADD-TAP phosphorylation sites (Ser 10 and 167) as well as two potential FADD-TAP interactors (calpain S1 and HSC70).
3.8.2 Identification of Ser 10 and 167 as potential phosphorylation sites in FADD-TAP

FADD is known to be phosphorylated at Ser 194 and, since this had no affect on the apoptotic function of FADD, it may be that FADD phosphorylation is instead important for its non-apoptotic roles (Chen et al, 2005, Hua et al, 2003, Scaffidi et al, 2000). Phosphorylation of FADD at Ser 194 could not be detected (for technical reasons) through mass spectrometry but two new phosphorylation sites in FADD-TAP were identified by mass spectrometry. Ser 167 was identified as a potential phosphorylation site through mass spectrometry of TAP purified FADD-CBP, whilst the Ser 10 site was identified through mass spectrometry of immunoprecipitated FADD-TAP. There was some variability in the FADD peptides identified by mass spectrometry, with peptide coverage of Ser 10 absent when Ser 167 was identified and vice versa (Figure 3.14 and Figure 3.16). This may be due to the observation that the peptides identified for a particular protein can vary between mass spectrometry runs and seems to depend upon protein abundance (with greater abundance producing higher peptide coverage).

Further investigation demonstrated that untagged FADD was apparently not phosphorylated at Ser 10 and 167, since mutation of these sites did not alter the 2-D spot pattern (Figure 3.18). This agreed with the NetPhos 2.0 (Blom et al, 1999) and PHOSIDA (Gnad et al, 2007) prediction programs that predicted FADD to be phosphorylated at Ser 194, but not at Ser 10 or Ser 167. Thus, it seems likely that phosphorylation at Ser 10 and Ser 167 was specific to FADD-TAP, which could be confirmed in the future by 2-D electrophoresis of wildtype, S10A and S167A FADD-TAP. If Ser 10 and Ser 167 were subsequently confirmed as specifically phosphorylated in FADD-TAP, then the TAP tag would almost certainly be the cause since it is the only difference between FADD and FADD-TAP. The two affinity tags within the TAP tag may have increased the association of FADD with a kinase leading to additional phosphorylation at non-canonical sites. However, any such interaction must have remained fairly transient since no kinases (that would likely be responsible for FADD-TAP phosphorylation) were significantly identified by mass spectrometry in both the TAP purified control and TRAIL treated samples. The kinase responsible for FADD-TAP phosphorylation could be casein kinase Iα, which has previously been shown to phosphorylate FADD at Ser 194 (Alappat et al, 2005). Alternatively glycogen synthase kinase 3 or serine/threonine-protein kinase Nek 6 could be responsible as according to PHOSIDA Ser 10, which is not predicted to be phosphorylated, is within a motif recognised by both of these kinases.
3.8.3 Identification of calpain S1 and HSC70 as potential FADD-TAP interactors

Mass spectrometry of TAP purified FADD-CBP identified calpain S1 and HSC70 as potential FADD-TAP interacting partners as well as identified the known FADD interactor, caspase-8 (Figure 3.12 and Table 3.2). In line with its role in DISC assembly, FADD-TAP was only associated with caspase-8 in TRAIL treated cells. However, the proportion of total FADD-TAP that was associated with caspase-8, and thus the DISC, in TRAIL treated cells was very low. This, and the absence of a mass spectrometry identification of TRAIL-R2 in the TRAIL treated sample, demonstrates the extremely low level of endogenous FADD that is recruited to the Jurkat TRAIL DISC. Calpain S1 was associated with FADD-TAP under control conditions and remained associated, although in a reduced amount, upon treatment of the cells with TRAIL. The reduced association of FADD-TAP with calpain S1, as judged by the spectral counts, may have been attributable to the interaction of FADD-TAP with HSC70, which only occurred in TRAIL treated cells.

The TRAIL-induced nature of the FADD-TAP-HSC70 interaction suggests that it may be related to apoptotic signalling and that an alteration must occur during TRAIL signalling to promote the association of these proteins. It has been suggested that the activity of HSC70, which is a target for tyrosine kinases, may be regulated by phosphorylation and that this could affect the protein-protein interactions of HSC70 (Egerton et al, 1996). Intriguingly, the level of HSC70 phosphorylation has been shown to increase during Fas-induced apoptosis (Gerner et al, 2000). Thus, one hypothesis could be that an increase in HSC70 phosphorylation also occurs during TRAIL-induced apoptosis and that this leads to an association with FADD-TAP through increasing the propensity of HSC70 to form protein-protein interactions.

HSC70 is a molecular chaperone and member of the heat shock protein family. It has many cellular roles including an involvement in protein folding (Fink, 1999), nuclear import/export of proteins (Kose et al, 2005, Mattaj & Englmeier, 1998, Okuno et al, 1993), targeting for protein degradation (Meacham et al, 2001), and disassembly of clathrin coats during receptor-mediated internalisation (DeLuca-Flaherty et al, 1990, Schmid et al, 1984). Due to its numerous roles, it is currently difficult to pinpoint the exact nature of the TRAIL-induced interaction of HSC70 with FADD-TAP. The TRAIL DISC is internalised through clathrin mediated endocytosis (Austin et al, 2006, Kohlhaas et al, 2007). Therefore, an interaction with FADD-TAP may target HSC70 to the internalised DISC-containing clathrin vesicles and allow it to perform its uncoating role. However, since other DISC components were either not identified (TRAIL-R2) or
were only present in low abundance (caspase-8) it remains to be determined whether HSC70 associates with the DISC through FADD-TAP. Alternatively, following TRAIL stimulation, HSC70 may target FADD for degradation as a way to regulate DISC signalling.

Interestingly, FADD has recently been shown to redistribute from the nucleus into the cytoplasm following the exposure of cells to Fas (Foger et al, 2009). FADD has been suggested to shuttle between the nucleus and cytoplasm through nuclear localisation and export signals (Gomez-Angelats & Cidlowski, 2003) or through a Ser 194 phosphorylation-dependent association with exportin-5 (Screaton et al, 2003). However, it is possible that upon TRAIL treatment HSC70, which is known to be involved in nuclear transport, associates with FADD-TAP to mediate its death receptor-induced redistribution.

The identification of the FADD-TAP-calpain S1 interaction in untreated and TRAIL treated cells suggests that it may be relevant for both non-apoptotic and apoptotic signalling. Calpain S1 is a small regulatory subunit that heterodimerises with the large catalytic subunits (m- and µ-calpain) to form calpains, which are calcium-dependent cysteine proteases. The function of the small subunit is unclear, although it has been suggested to act as a chaperone ensuring the correct folding and stabilisation of the large catalytic subunit (Meyer et al, 1996) and may either be involved (Zhang & Mellgren, 1996) or not (Suzuki & Sorimachi, 1998) in the proteolytic activity of calpain. Like FADD, calpains have been implicated in apoptosis and cell cycle regulation but are also involved in many other cellular processes (Goll et al, 2003).

Homoygous disruption of the FADD gene in mice resulted in defective cardiac development, abdominal haemorrhage and death of the embryos between E9 and E12.5 (Yeh et al, 1998, Zhang et al, 1998). Interestingly, homozygous disruption of the calpain S1 gene also resulted in cardiovascular defects, haemorrhaging and death by E11.5, as well as an accumulation of erythroid progenitors (Arthur et al, 2000, Zimmerman et al, 2000). It was suggested that the developmental defects of calpain S1 null embryos were possibly attributable to the loss of m- and µ-calpain activity, which resulted from the absence of calpain S1 expression. Thus, it appears possible that FADD and calpains may be involved in similar developmental pathways. It has already been suggested that, based on the similar phenotypes of FADD null and caspase-8 null embryos, FADD and caspase-8 mediate non-death receptor signalling that is vital for embryogenesis (Varfolomeev et al, 1998, Yeh et al, 1998, Zhang et al,
1998). Thus, it seems possible that calpain S1 with m- and/or µ-calpain could also be involved in the same non-death receptor signalling pathway. Interestingly, calpain S1 has also been identified as a significant inhibitor of TRAIL-induced apoptosis in a siRNA screen conducted in our laboratory by Dr Davina Twiddy (unpublished data). Based on the mass spectrometry data shown here, it is possible that the inhibitory effect of calpain S1 on TRAIL-induced apoptosis could be mediated through an interaction with FADD, possibly through reducing its recruitment to the DISC. Thus, it appears as though calpain S1 and FADD may interact in more than one signalling pathway although how this interaction affects the activity of calpains or the function of FADD is currently unclear.

Direct immunoprecipitation of FADD-TAP/FADD was unable to confirm the interaction of FADD-TAP/FADD with either calpain S1 or HSC70. This was possibly the result of the different experimental conditions used in the purification of FADD-TAP/FADD by immunoprecipitation versus TAP. Unfortunately within the time constraints of this thesis it was not possible to pursue the potentially interesting and novel interactions between FADD-TAP and calpain S1 or HSC70 further. However, future experiments could use Western blot to confirm the presence of calpain S1 and HSC70 in TAP purified samples. Also identification of calpain S1 and HSC70 (by mass spectrometry and/or Western blot) in TAP purified samples from the FADD-TAP A3 cell line would indicate that their interaction with FADD-TAP was not specific to the FADD-TAP D10 cell line. Reverse immunoprecipitation of calpain S1 and HSC70 could then be used to investigate whether these proteins interact with endogenous FADD. Once confirmed the functional relevance of the interactions could be investigated through time course, mutational, co-localisation and/or gene silencing studies.

The FADD-TAP cell lines generated in this study are useful tools that could be used in future projects. This project investigated the interactions of FADD untreated cells and those exposed to TRAIL for short period of time. However, in the future the cell lines could be used to investigate the interactions of FADD over longer periods of TRAIL treatment or even in different pathways. For example, the protein interactions of FADD during Fas-induced apoptosis, to which the FADD-TAP D10 cell line is more sensitive (Figure 3.5 B and C), could be investigated. Alternatively, another interesting avenue would be to use the FADD-TAP cell lines to investigate what proteins associate with FADD during different stages of the cell cycle, and how this might affect the role of FADD in cell cycle progression (Chen et al, 2005, Matsuyoshi et al, 2006, Osborn et al, 2007).
Chapter 4: Purification and Mass Spectrometry of TRAIL DISC
4 Purification and Mass Spectrometry of TRAIL DISC

4.1 Introduction

At present the selectivity of TRAIL for inducing apoptosis in cancerous cells is not fully understood. However, it is known that proteins acting at the level of the DISC can regulate death receptor-mediated apoptosis or even initiate survival signalling pathways. Furthermore, although it has been documented that the Type I BJAB cell line forms a greater amount of DISC, than the Type II Jurkat cell line, in response to FasL and TRAIL (Sprick et al, 2002), there is currently no conclusive explanation for this. Therefore, the aim of this chapter was to analyse the composition of the TRAIL DISC, from BJAB and Jurkat E6.1 cells, by mass spectrometry. Mass spectrometry has been previously employed to identify Fas DISC components (Kischkel et al, 1995, Muzio et al, 1996), but to date there has not been a systematic proteomic study of the TRAIL DISC. The previous use of mass spectrometry in the analysis of the Fas DISC meant that it should be possible to identify the components of the TRAIL DISC in this way. Identification of known components by mass spectrometry would confirm their involvement in the TRAIL DISC. However, the identification of novel interacting proteins would further current knowledge of the TRAIL signalling pathway and perhaps explain why more DISC is formed in a Type I than in a Type II cell line.

4.2 Biotinylated TRAIL is trimeric

The TRAIL DISC can be purified using biotinylated soluble TRAIL (bTRAIL) (Harper et al, 2001, Sprick et al, 2000), which has a strong interaction with streptavidin and thus can be efficiently recovered (Drakas et al, 2005, Green, 1975). TRAIL is routinely generated in E. coli, purified from cell lysates through the attached His tag and biotinylated to produce bTRAIL. However, a previous study showed that the attachment of a His tag to the TNF ligand TALL-1 resulted in the formation of higher order oligomers rather than the native trimeric form (Zhukovsky et al, 2004). The type of receptor stimulus used can affect the cellular response (Barnhart et al, 2003) and the oligomerisation status of TRAIL has also been shown to alter its potency (Berg et al, 2007, Kim et al, 2004). Therefore, it was important to initially characterise the oligomeric state of bTRAIL.

Three versions of soluble TRAIL (amino acids 95 to 281) have been generated in our laboratory; these are untagged TRAIL (TRAIL, kindly provided by Dr Michelle Hughes), bTRAIL and Strep-tagged TRAIL (Strep-TRAIL, both kindly provided by Dr Nick Harper). All of these were analysed by size-exclusion chromatography to investigate
whether the His tag or biotinylation affected the oligomerisation state of bTRAIL. TRAIL (27 kDa) contains T7 and His tags, bTRAIL (27 kDa) possesses the same tags but is biotinylated, and Strep-TRAIL (23 kDa) contains just a Strep II tag. Size-exclusion chromatography revealed that none of the TRAIL variants were highly aggregated. This was shown by the absence of major UV peaks (Figure 4.1 A) or coomassie staining (Figure 4.1 B) in the high molecular weight fractions. In fact all three ligands eluted in approximately the same position as the 67 kDa marker indicating that, under these conditions, they were trimeric. This corresponds to the known active conformation of members of the TNF-ligand superfamily (Locksley et al, 2001). Therefore, the DISC examined in this study was formed following stimulation with a soluble, trimeric and biotinylated TRAIL ligand.

### 4.3 Optimisation of DISC purification

Having established the trimeric conformation of bTRAIL it was important to optimise the DISC purification method. It was essential to maximise the amount of DISC isolated from cellular lysates to enhance the likelihood of identifying known and novel components by mass spectrometry. To achieve this, various parameters of the methodology were optimised and are summarised in Table 4.1. The most dramatic alterations to the methodology, discussed below, related to the way in which cells were exposed to bTRAIL.

Under normal conditions receptors at the cell surface are recycled through endocytosis. Furthermore, internalisation of TRAIL-R2 has also been shown to increase following stimulation with TRAIL (Austin et al, 2006), which may reduce the level of receptor available for ligation. Thus, it was hypothesised that blockade of endocytosis may increase the level of ligand-bound receptor and thus the absolute amount of DISC formed. Several reports investigating the internalisation of death receptor complexes have pre-chilled cells to 4 °C to inhibit endocytosis (Algeciras-Schimnich et al, 2002, Kohlhaas et al, 2007, Schneider-Brachert et al, 2004). Therefore, the effect of a ‘pre-chilling’ step on TRAIL DISC formation was investigated.

Untreated Jurkat E6.1 cells did not form TRAIL DISC since FADD and caspase-8 were absent from the bead eluate, despite detection in the supernatant (Figure 4.2 lane 1). Cells incubated with bTRAIL (without pre-chill) at 37 °C for 15 min formed a low level of TRAIL DISC and underwent apoptosis to a moderate extent (Figure 4.2 lane 2).
Three versions of recombinant TRAIL were subjected to size-exclusion chromatography to assess the oligomeric state of bTRAIL. The variants used were TRAIL (T7 and His tags), bTRAIL (T7 tag, His tag and biotinylated) and Strep-TRAIL (Strep II tag). The same amount of each TRAIL variant (100 µg) was loaded onto a Superdex™ 200 column and 50 µl fractions were collected for SDS-PAGE. A. Graphical representation of the chromatography runs with the absorbance at 280 nm plotted against time. B. Elution fractions 5 to 29 were separated by SDS-PAGE and coomassie stained to visualise the ligands. Standards shown were used to calibrate the column before the separation of TRAIL.

Figure 4.1: Biotinylated TRAIL (bTRAIL) is not aggregated and is approximately trimeric.
Table 4.1: Aspects of the DISC purification method investigated and the optimal condition determined.

<table>
<thead>
<tr>
<th>Parameter Investigated</th>
<th>Optimal Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of bTRAIL</td>
<td>500 ng/ml</td>
</tr>
<tr>
<td>Treatment conditions</td>
<td>Incubation with TRAIL for 1 hr at 4 °C followed by 25 min at 37 °C</td>
</tr>
<tr>
<td>Unstimulated control</td>
<td>Beads incubated with unstimulated lysate</td>
</tr>
<tr>
<td>Lysis time</td>
<td>30 min</td>
</tr>
<tr>
<td>Inclusion of ZVAD.FMK in lysis buffer</td>
<td>Not included</td>
</tr>
<tr>
<td>Type of streptavidin affinity beads</td>
<td>Magnetic</td>
</tr>
<tr>
<td>Ratio of cell to bead number</td>
<td>5 x 10^7 cells in 3.5 ml : 3.4 x 10^7 magnetic beads</td>
</tr>
<tr>
<td>Incubation on end-to-end rotator</td>
<td>Overnight at 4 °C</td>
</tr>
<tr>
<td>Elution technique</td>
<td>Boil in 1 x sample buffer for 5 min</td>
</tr>
</tbody>
</table>
Figure 4.2: Pre-chilling cells with bTRAIL increases the recruitment of FADD and caspase-8 to the TRAIL DISC.

Jurkat E6.1 cells were left untreated (lane 1) or treated, in various ways (see upper diagrams and table), with 500 ng/ml of bTRAIL (lanes 2 to 6) and the DISC isolated. Following treatment, the cells were lysed, 100 ng of bTRAIL was added to the untreated cell lysate, and the DISC or unstimulated receptor captured onto streptavidin beads. The amount of FADD and caspase-8 present in the bead eluate (DISC) and supernatants was assessed by Western blot. The supernatant blots act as loading controls to ensure equal numbers of cells were used. Before lysis a small sample of cells was taken and kept at 37 °C for 8 hr to assess the extent of apoptosis by annexin V/PI staining and flow cytometry (lower table), where percentage apoptosis represents the percentage of PS positive cells. The result shown is representative of two independent experiments.
A similar level of DISC formation and apoptosis was observed when cells were pre-chilled for 1 hr in the absence of bTRAIL before a 15 min 37 ºC incubation with bTRAIL (Figure 4.2 lane 3). However, pre-chilling the cells in the presence of TRAIL followed by a 37 ºC incubation (also in the presence of bTRAIL) lead to increased DISC formation and extensive apoptosis at 8 hr (Figure 4.2 lane 4).

TRAIL DISC has been shown to form at 4 ºC (Austin et al, 2006, Kohlhaas et al, 2007); thus it was possible that the 37 ºC incubation (after the pre-chill step) was not required for optimal DISC formation. However, removal of this step resulted in reduced DISC formation (Figure 4.2 lane 5). The level of DISC formation was also reduced when the pre-chill (1 hr 4 ºC) in the presence of bTRAIL (Figure 4.2 lane 4) was switched to a pre-warm (1 hr 37 ºC) in the presence of bTRAIL (Figure 4.2 lane 6). This indicated that the increased DISC formation observed in cells pre-chilled and incubated at 37 ºC with bTRAIL was not the result of increased exposure time to TRAIL. These data demonstrate that ligand-receptor interaction together with blockade of receptor internalisation leads to increased DISC formation upon subsequent activation of the receptor at 37 ºC.

Having demonstrated that the pre-chill protocol improved DISC formation in the Jurkat cell line, I then optimised the 37 ºC incubation time (Figure 4.3). Cells were pre-chilled with bTRAIL for 1 hr and then further incubated (in the presence of bTRAIL) at 37 ºC for between 5 and 35 min. Western blotting showed a time dependent increase in FADD recruitment and caspase-8 processing (Figure 4.3). However, maximal active DISC formation was achieved between 25 and 30 min (Figure 4.3 lanes 6 and 7). Therefore, for all subsequent Jurkat TRAIL DISC purifications the cells were treated with bTRAIL for 1 hr at 4 ºC followed by 25 min at 37 ºC.

**4.4 Mass spectrometry of Type II Jurkat TRAIL DISC**

To ensure purification of a sufficient amount of TRAIL DISC for analysis by mass spectrometry, the optimised isolation method was scaled up. Three large scale purifications were completed with between 0.6 and 3 x 10^9 Jurkat E6.1 cells. Western blots for TRAIL-R2, FADD and caspase-8 were completed, before analysis by mass spectrometry, to confirm successful purification of DISC from bTRAIL treated cells (Figure 4.4 A). The eluates were then separated by SDS-PAGE and coomassie stained (Figure 4.4 B). Since the Western blots identified DISC in the bTRAIL treated sample only (Figure 4.4 A lane 2), it was surprising to discover that coomassie staining
Figure 4.3: In Jurkat E6.1 cells maximal DISC is formed between 25 and 30 min at 37 °C.

Jurkat E6.1 cells were left untreated (lane 1) or treated with 500 ng/ml of bTRAIL (lanes 2 to 8) and the DISC isolated. Cells were pre-chilled for 1 hr with bTRAIL and then incubated at 37 °C for between 5 and 35 min in the presence of bTRAIL. Following treatment, the cells were lysed, 100 ng of bTRAIL was added to the untreated cell lysate and the DISC was captured onto streptavidin beads. The amount of FADD and caspase-8 present in the bead eluates (DISC) and supernatants was assessed by Western blot. Percentage apoptosis (% PS positive cells), assessed by annexin V/PI staining after 8 hr at 37 °C, was 50 % (on average) in the TRAIL treated samples.
Figure 4.4: Large scale TRAIL DISC purification from Jurkat E6.1 cells enables identification of known components by Western blot and other proteins by coomassie.

Jurkat E6.1 cells were left untreated or treated with 500 ng/ml of bTRAIL for 1 hr at 4 °C followed by 25 min at 37 °C and then lysed in DISC lysis buffer. DISC was captured from the lysate through overnight incubation at 4 °C with streptavidin beads. Proteins were eluted from beads through boiling with 1 x sample buffer (without bromophenol blue) and concentrated using the UPPA-PROTEIN-Concentrate™ kit. Concentrated eluates were separated by SDS-PAGE and either subjected to Western blot (A) or coomassie staining (B). Percentage apoptosis (lower table) was assessed by annexin V/PI staining and represents the percentage of PS positive cells. Results shown are representative of three independent experiments.
did not indicate any obvious differences between the control and treated samples (Figure 4.4 B). However, in agreement with the Western blot data mass spectrometry identified TRAIL, TRAIL-R2 and caspase-8 only in the bTRAIL treated sample (Table 4.2).

TRAIL, TRAIL-R2 and caspase-8, but not FADD or TRAIL-R1, were identified in all three of the experiments conducted (Table 4.2). TRAIL-R1 was not expected to be present as it is not detected at the surface of Jurkat cell lines (Appendix 1, Figure 5.3 and Sprick et al, 2000). Contrastingly, FADD should have been identified by mass spectrometry as Western blot had confirmed that it was present in the DISC sample submitted (Figure 4.4 A). A peptide \textit{NTEKENATVAHLVGALR}, amino acids 150 to 166) was assigned to FADD in the raw mascot data, but not in the Scaffold software. This peptide was assigned a score of 21, which fell below the significance threshold of 35 for that data set, and meant that FADD could not be confidently identified by mass spectrometry. This implied that FADD was less abundant than the other known DISC components (TRAIL, TRAIL-R2 and caspase-8), which were consistently identified by multiple peptides, multiple spectra and a protein probability of 100 % in all of the experiments conducted (Table 4.2). The clear identification of almost all known DISC components justified further evaluation of the Jurkat TRAIL DISC mass spectrometry data.

The criteria set out in the Materials and Methods were used to determine whether protein hits from the experiments should be investigated further and a list of 22 promising proteins was assembled (Appendix 2). For each of these proteins, the difference in the spectral count between the untreated and the TRAIL treated sample was plotted against the protein probability assigned in the treated sample (Figure 4.5). Proteins with either a high protein probability or a large increase in spectral count were identified as potentially worth further investigation. These proteins were 60S ribosomal protein L11, heat shock protein 90-alpha (HSP90α), 60S acidic ribosomal protein P0, serine/threonine-protein kinase N1 (PKN1), 40S ribosomal protein S18, and vacuolar protein sorting-associated protein 35 (VPS35). I decided not to pursue the ribosomal proteins further as I believed they were likely to be non-specific interactors. This was based on the observation that many other ribosomal proteins were identified in the untreated control. After thorough analysis of the mass spectrometry data (Appendix 2 and Figure 4.5) I was left with three potential interactors, which were HSP90α (a molecular chaperone (Pratt & Toft, 2003)), PKN1 (a serine/threonine kinase and
<table>
<thead>
<tr>
<th>Spot</th>
<th>Accession Number</th>
<th>Protein</th>
<th>PEA assignment (Yes/No)</th>
<th>Unique Peptides (%)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CASP8_HUMAN</td>
<td>Caspase-8 precursor</td>
<td>Yes</td>
<td>90</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>TNFR1_HUMAN</td>
<td>Tumor necrosis factor receptor ligand superfamily member 10 (TRAIL)</td>
<td>Yes</td>
<td>90</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>TRAILR-HUMAN</td>
<td>Tumor necrosis factor receptor ligand superfamily member 10 (TRAIL)</td>
<td>Yes</td>
<td>90</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>HSPA1A_HUMAN</td>
<td>Heat shock protein HSP 90-alpha</td>
<td>Yes</td>
<td>90</td>
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<tr>
<td>5</td>
<td>AKR1B1_HUMAN</td>
<td>Serine/threonine-protein kinase N1</td>
<td>Yes</td>
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<td>6</td>
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<td>Vacuolar protein sorting-associated protein 35</td>
<td>Yes</td>
<td>90</td>
<td>2</td>
</tr>
</tbody>
</table>

Proteins were identified by mass spectrometry and analyzed as described in the Materials and Methods. Data was provided for the number of spectra, the protein probability, the cleavage status, and the number of unique peptides. For those proteins identified by multiple peptides, the number of spectra, the protein probability, the cleavage status, and the number of unique peptides are provided. For those proteins identified by a single peptide, additional information on the peptide sequence and Mascot ion score are provided. UD, unable to determine position in SDS-PAGE gel.
Figure 4.5: Graphical analysis of the 22 proteins, identified through mass spectrometry in large scale Jurkat TRAIL DISC purifications, reveals potential interacting proteins.

Graphical analysis of the 22 proteins identified by mass spectrometry in the Jurkat TRAIL DISC that met the criteria described in the Materials and Methods. The increase in the treated spectrum count for each protein was plotted against the protein probability it was assigned in the treated sample. The spot numbers refer to the protein list in Appendix 2. Known DISC components are labelled in red and those that warranted further investigation are listed in the centre of the graph in black.

4 – 60S ribosomal protein L11
5 – Heat shock protein 90 α (HSP90α)
6 – 60S acidic ribosomal protein P0
7 – Serine/threonine-protein kinase N1 (PKN1)
8 – 40S ribosomal protein S18
15 – Vacuolar protein sorting-associated protein 35 (VPS35)
member of the PKC superfamily (Mukai, 2003)) and VPS35 (a component of the retromer complex that is involved in receptor recycling (Seaman, 2005)) (Table 4.2).

An indication of the accuracy of the protein identification can be provided by the number of assigned peaks in the peptide spectra and the peptide coverage of the protein. As these two criteria increase so does the confidence in the protein identification. The identifications of TRAIL, TRAIL-R2, and caspase-8 in the TRAIL DISC were based on excellent spectra and peptide coverage (Figure 4.6). There was less peptide coverage of HSP90α and PKN1 but the spectra contained many assigned peaks, so positive identification was a promising prospect (Figure 4.6). However, the best spectrum in the identification of VPS35 possessed only a few low intensity spectral peaks.

HSP90α, PKN1 and VPS35 were all detected in the Jurkat E6.1 cell line by Western blot (data not shown). However, out of these three proteins only HSP90α was detected in the Jurkat TRAIL DISC by Western blot (Figure 4.7 lane 2 and data not shown). HSP90α was identified by mass spectrometry in gel slices that corresponded to its molecular weight of 85 kDa. Concurrent with this HSP90α, was detected by Western blot in the TRAIL DISC at its expected molecular weight (Figure 4.7 lane 2). However, a band of similar molecular weight but slightly lower intensity was also identified in the untreated control (Figure 4.7 lane 1). This agreed with the mass spectrometry data that also identified HSP90α in the untreated samples, in lower abundance (reduced protein probability and number of spectra) than in the TRAIL treated sample (Table 4.2). Thus, it appeared that upon TRAIL treatment there was a low level of interaction between HSP90α and the Jurkat TRAIL DISC. However, the level of interaction was not sufficiently significant to warrant further investigation at that time.

In contrast to HSP90α, both PKN1 and VPS35 were identified by mass spectrometry in gel slices lower than their expected molecular weight, suggesting that these proteins were cleaved. VPS35 (92 kDa) was identified in the same gel slice as TRAIL-R2 (approximately 50 kDa), whilst PKN1 (104 kDa) was identified in gel slices lower than TRAIL (27 kDa). Therefore, it is possible that cleavage of these proteins rendered them unrecognisable by the antibodies used and prevented their detection in the TRAIL DISC sample by Western blot.
Figure 4.6: The best peptide coverage and spectra of the known, and potential, Jurkat E6.1 TRAIL DISC components identified by mass spectrometry.

For each protein the top panel is the best peptide coverage obtained in its identification, whilst the lower panel is the best spectra in that peptide coverage. In the peptide coverage images yellow highlighted peptides represent those that have been identified by mass spectrometry whilst green residues are those that have been identified with a modification (methionine (M) is oxidised). In the spectra images the peaks and sequences are colour coded, blue represent y ions, red represent b ions, black represents unidentified ions and green represent ions minus water or NH₃.
Figure 4.7: Western blot confirmed the presence of the known components, and a low level of HSP90\(\alpha\), in the Jurkat TRAIL DISC.

Jurkat E6.1 cells were left untreated or treated with 500 ng/ml of bTRAIL for 1 hr at 4 °C followed by 25 min at 37 °C and then lysed in DISC lysis buffer. DISC was captured from the lysate through overnight incubation at 4 °C with streptavidin beads and proteins eluted through boiling with 1 x sample buffer. DISC (bead eluate) from 25 x 10^6 cells was separated by SDS-PAGE for Western blot of TRAIL-R2, FADD, caspase-8, whilst DISC from 50 x 10^6 cells was used for HSP90\(\alpha\). A sample of the supernatant was included on each blot to ensure equal numbers of cells were used and as a positive control for the antibody. Percentage apoptosis (lower table) was assessed by annexin V/PI staining and represents the percentage of PS positive cells.
4.5 Mass spectrometry of Type I BJAB TRAIL DISC

4.5.1 Identification of potential DISC interaction partners

While mass spectrometry of Jurkat E6.1 TRAIL DISC identified TRAIL-R2 and caspase-8, it did not lead to a convincing identification of the obligatory TRAIL DISC component, FADD. Therefore, the optimised technique of purifying Jurkat TRAIL DISC through the bTRAIL produced only limited mass spectrometry detection of all known components. Therefore, I decided to move onto analysing the TRAIL DISC formed by the Type I BJAB cell line. As mentioned earlier, the BJAB cell line forms more TRAIL DISC than the Jurkat cell line (Sprick et al, 2002); thus the likelihood of identifying all of the known DISC components, as well as some additional novel interactors, by mass spectrometry was greatly increased.

The DISC purification method was extensively optimised earlier using the Jurkat E6.1 cell line. Therefore, the only aspect that was optimised for the BJAB cell line was the incubation time at 37 ºC (following the pre-chill with bTRAIL), which was found to be optimal at 10 min (data not shown and Figure 5.5). Using this approach three large scale DISC purifications were performed, with either 0.3 or 0.65 x 10^9 BJAB cells. Before analysis by mass spectrometry the presence of the DISC specifically in the TRAIL treated sample was confirmed through Western blot for TRAIL-R2, FADD and caspase-8 (Figure 4.8 A). As with the Jurkat E6.1 cell line, coomassie staining detected a similar protein band pattern in the samples from control and TRAIL treated BJAB cells. However, there was one very distinct band present only in the BJAB TRAIL DISC sample (Figure 4.8 B lane 2 marked by an arrow). This band represented bTRAIL since it was the correct molecular weight (27 kDa), was absent from the untreated sample (to which bTRAIL had not been added) and mass spectrometry identified TRAIL in this area of the SDS-PAGE gel.

Mass spectrometry of the BJAB TRAIL DISC was extremely successful since TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R4, FADD and caspase-8 were identified in all three experiments conducted (Table 4.3). In addition the known DISC regulator c-FLIP was identified in two out of three, whilst caspase-10 was discovered in one out of three treated samples (Table 4.3). TRAIL, TRAIL-R2, FADD and caspase-8 were all assigned a protein probability of 100 %, whilst TRAIL-R1 and c-FLIP were assigned a probability of 99 %. Caspase-10 was a little lower at 90 % and TRAIL-R4 was the lowest with a protein probability of just 67 % (Table 4.3).
Figure 4.8: Large scale TRAIL DISC purification from BJAB cells enables identification of known components by Western blot and other proteins by coomassie.

BJAB cells were left untreated or treated with 500 ng/ml of bTRAIL for 1 hr at 4 °C followed by 10 min at 37 °C and then lysed in DISC lysis buffer. DISC was captured from the lysate through overnight incubation at 4 °C with streptavidin beads. Proteins were eluted from beads through boiling with 1 x sample buffer (without bromophenol blue) and concentrated using the UPPA-PROTEIN-Concentrate™ kit. Concentrated eluates were separated by SDS-PAGE and either subjected to Western blot (A) or coomassie staining (B). Percentage apoptosis (lower table) was assessed by annexin V/PI staining and represents the percentage of PS positive cells. Results shown are representative of three independent experiments.
Table 4.3: Complete mass spectrometry data for the known and potential components of the BJAB TRAIL DISC.

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**Purification and Mass Spectrometry of TRAIL DISC**
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Proteins were identified by mass spectrometry and analysed as described in the Materials and Methods. Data represents 3 untreated controls and 3 bTRAIL treated samples. For those proteins identified by multiple peptides the number of spectra, the protein probability, the cleavage status and number of unique peptides are given. For those proteins identified by a single peptide additional information on the peptide sequence and Mascot ion score are provided.
The mass spectrometry data has provided conclusive evidence that TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R4, FADD, caspase-8, caspase-10, c-FLIP are bona-fide components of the BJAB TRAIL DISC. Therefore, I used the criteria set out in the Materials and Methods to further analyse the mass spectrometry data and this generated a final list of 52 proteins (Appendix 3). These proteins were then plotted, in the same way as those identified previously in the mass spectrometry of Jurkat TRAIL DISC, to pinpoint those that warranted further investigation (Figure 4.9). It was decided not to pursue proteins 9 (40S ribosomal protein S3), 11 (nuclear fragile X mental retardation-interacting protein 2) and 15 (GPI-anchored membrane protein 1). This was because although they were not identified in the control and treated samples of the same experiment, these proteins were identified in the control sample of at least 1 other experiment. However, transferrin receptor protein 1 (transferrin receptor), RING finger protein 113B (RNF113B), disks large homolog (DLG1), putative ATP-binding cassette transporter C13 and uncharacterised protein C15orf33 were all deemed worth further investigation (Figure 4.9).

As predicted, based on the high assigned protein probabilities, there was excellent peptide coverage and spectra for the known DISC components (Figure 4.10). There was also good peptide coverage of transferrin receptor (Figure 4.10). For DLG1, RNF113B, putative ATP-binding cassette transporter C13 and uncharacterised protein C15orf33 the peptide coverage of the protein and the number of peaks in the spectra were both quite low (Figure 4.10). However, I felt it was worthwhile investigating all of these proteins further. Antibodies were not available for uncharacterised protein C15orf33 and putative ATP-binding cassette transporter C13, so I decided to first investigate the potential involvement of transferrin receptor (involved in the cellular transport of iron (Testa et al, 1993)), DLG1 (a scaffold protein that may assemble T cell receptor signalling molecules and activate the MAPK p38 (Shaw & Filbert, 2009)) and RNF113B (a zinc finger containing protein) in the TRAIL DISC.

Western blot confirmed that transferrin receptor, RNF113B and DLG1 were expressed in BJAB cells (data not shown). However, out of these three proteins only transferrin receptor was detected by Western blot in the BJAB TRAIL DISC (Figure 4.11 lane 2). TRAIL-R1, TRAIL-R2, FADD, caspase-8, caspase-10 and c-FLIP_L/S were all also detected only in the TRAIL treated sample confirming active DISC formation and purification (Figure 4.11 lane 2). Interestingly, although TRAIL-R4 was not identified in the control sample by mass spectrometry (Table 4.3), some was detected in the control sample by Western blot (Figure 4.11 lane 1). Therefore, there appeared to be a small
Figure 4.9: Graphical analysis of 52 proteins, identified through mass spectrometry of large scale BJAB TRAIL DISC purifications, reveals potential interacting proteins.

Graphical analysis of the 52 proteins identified by mass spectrometry in the BJAB TRAIL DISC that met the criteria described in the Materials and Methods. The increase in the treated spectrum count for each protein was plotted against the protein probability it was assigned in the treated sample. The spot numbers refer to the protein list in Appendix 3. Known DISC components are labelled in red and those that warranted further investigation are listed in the centre of the graph in black.

- Caspase-8
- TRAIL-R1
- TRAIL-R2
- TRAIL Caspase-8
- Caspase-10
- TRAIL-R4

14 – Transferrin receptor protein 1 (Transferrin receptor)
17 – Uncharacterised protein C15orf33
18 – Putative ATP-binding cassette transporter C13
22 – RING finger protein 113B (RNF113B)
23 – Disks large homolog 1 (DLG1)
Figure 4.10 continued on next page
Figure 4.10: The best peptide coverage and spectra of the known, and potential, BJAB TRAIL DISC components identified by mass spectrometry.

For each protein the top panel is the best peptide coverage obtained in its identification, whilst the lower panel is the best spectra in that peptide coverage. In the peptide coverage images yellow highlighted peptides represent those that have been identified by mass spectrometry whilst green residues are those that have been identified with a modification (methionine (M) is oxidised). In the spectra images the peaks and sequences are colour coded, blue represent y ions, red represent b ions, black represent unidentified ions and green represent ions minus water or NH₃.
Figure 4.11: Western blot confirmed the presence of the known components, and transferrin receptor, in the BJAB TRAIL DISC.

BJAB cells were either left untreated or treated with 500 ng/ml of bTRAIL for 1 hr at 4 °C followed by 10 min at 37 °C and then lysed in DISC lysis buffer. DISC was captured from the lysate through overnight incubation at 4 °C with streptavidin beads and proteins eluted through boiling with 1 x sample buffer. DISC (bead eluate) from 25 x 10⁶ cells was separated by SDS-PAGE for Western blot of TRAIL-R1, TRAIL-R2, FADD, caspase-8 and c-FLIP, whilst DISC from 50 x 10⁶ cells was used for the other proteins. A sample of the supernatant was loaded onto each blot to ensure equal numbers of cells were used and as a positive control for the antibodies. The percentage apoptosis (lower table) represents the percentage of PS positive cells. The approximately 36 kDa band detected by the c-FLIP antibody in the supernatant sample may represent an alternative isoform of c-FLIP. The UniProt database (www.uniprot.org) states that there are 14 possible isoforms of c-FLIP, of which FLAME-1 beta (isoform 9, 30 kDa), FLAME-1 delta (isoform 10, 34 kDa) and Usurpin gamma (isoform 12, 33 kDa) are predicted to be similar sizes to the additional c-FLIP band detected.
amount of non-specific interaction between TRAIL-R4 and the streptavidin beads. However, there was a dramatic increase in the level of TRAIL-R4 in the treated sample indicating its specific inclusion in the DISC upon TRAIL treatment.

The absence of Western blot detection of RNF113B and DLG1 in the TRAIL DISC may be explained by the identification of these proteins by mass spectrometry in sections of the SDS-PAGE gel that did not correspond to their full length molecular weights (Table 4.3). RNF113B (36 kDa) was identified by mass spectrometry in a gel slice lower than FADD, whilst DLG1 (100 kDa) was mainly identified in the same gel slice as TRAIL, FADD and caspase-8 (approximately 26 kDa). Thus, as with PKN1 and VPS35 previously identified (by mass spectrometry but not Western blot) in the Jurkat TRAIL DISC, cleaved RNF113B and DLG1 may not have been recognised by antibodies used. However, unlike PKN1 and VPS35 that were identified by multiple peptides, RNF113B and DLG1 were only identified by one short peptide, which increased the likelihood of protein misidentification (Table 4.2 and Table 4.3).

4.5.2 Transferrin receptor associates with TRAIL receptors in the BJAB and Jurkat cell lines

Mass spectrometry and Western blotting of the BJAB TRAIL DISC had identified specific recruitment of TRAIL-R4, caspase-10, c-FLIPL/S and transferrin receptor. However, none of these proteins had been detected by mass spectrometry in the Jurkat TRAIL DISC (Table 4.2 and Appendix 2). Therefore, the recruitment of these proteins to the Jurkat TRAIL DISC was investigated by Western blot. Caspase-10, cleaved c-FLIP_L (p43) and transferrin receptor were all present in the Jurkat DISC, but TRAIL-R4 could not be detected (Figure 4.12 lane 2). Therefore, it is likely that caspase-10, c-FLIP and transferrin receptor were present in the Jurkat TRAIL DISC sample but, like FADD, were below the level required for identification by mass spectrometry.

The identification of transferrin receptor in the Jurkat TRAIL DISC was interesting as it suggested this interaction was not specific to the BJAB cell line. Further investigation revealed that transferrin receptor was also present when unstimulated TRAIL receptors were purified from BJAB and Jurkat E6.1 cell lines (Figure 4.13 lanes 2 and 5). The level of unstimulated TRAIL-R1 purified from BJAB cell lines was similar to that present in the DISC (Figure 4.13 lanes 2 and 3). However, the amount of TRAIL-R2 purified was dramatically higher in the BJAB and Jurkat TRAIL DISC than in the unstimulated
Figure 4.12: Caspase-10, c-FLIP and transferrin receptor, identified by mass spectrometry in the BJAB TRAIL DISC, were detected in the Jurkat E6.1 TRAIL DISC by Western blot.

Samples from the Jurkat E6.1 TRAIL DISC purification (shown in Figure 4.7) were re-run on SDS-PAGE gels to probe for proteins that were identified in the BJAB (but not the Jurkat) TRAIL DISC by mass spectrometry. The approximately 36 kDa band detected by the c-FLIP antibody in the supernatant sample may represent an alternative isoform of c-FLIP (see Figure 4.11).
Figure 4.13: Transferrin receptor was detected following the purification of unstimulated TRAIL receptors as well as in TRAIL DISC.

BJAB or Jurkat E6.1 cells were left untreated or treated with 500 ng/ml of bTRAIL for 1 hr at 4 °C followed by 10 (BJAB) or 25 (Jurkat) min at 37 °C. Following lysis, 0.34 µg/ml of bTRAIL was added to one untreated sample. Unstimulated receptors or DISC were subsequently captured through overnight incubation at 4 °C with streptavidin beads and proteins eluted through boiling with 1 x sample buffer. Unstimulated receptors or DISC (bead eluate) from 25 x 10^6 cells were separated by SDS-PAGE for Western blot of TRAIL-R1, TRAIL-R2, FADD, caspase-8, whilst that from 50 x 10^6 cells was used for Transferrin receptor blots. Blots shown are not the same exposure times for both cell lines. Percentage apoptosis (lower table) was assessed and represents the percentage of PS positive cells.
sample (Figure 4.13 lanes 2, 3, 5 and 6). Similarly, the level of transferrin receptor was also higher in the DISC compared to the unstimulated TRAIL receptor purification. This correlation, as well as the detection of transferrin receptor in unstimulated receptor and DISC purifications from Jurkat cells (that do not express TRAIL-R1), suggested that transferrin receptor may predominantly interact with TRAIL-R2.

4.5.3 Stoichiometry of the known BJAB TRAIL DISC components by mass spectrometry

Mass spectrometry is generally used to identify proteins within a particular sample. However, the data generated can also be used to quantitate the relative abundance of proteins and thus determine the stoichiometry of protein complexes (Paoletti et al, 2006, Zybailov et al, 2006). Therefore, I decided to use the mass spectrometry data obtained from the BJAB cell line to calculate the stoichiometry of the TRAIL DISC. Quantitation of the mass spectrometry data is achieved through the calculation of the normalised spectral abundance factor (NSAF) for each component within a complex. An NSAF for a particular protein is generated by dividing its spectral abundance factor (SAF, spectral count divided by molecular weight) by the sum of SAFs for all the proteins present in the complex (Section 2.6.4). For example, in the case of the DISC the NSAF for TRAIL-R1 would be calculated by dividing the SAF for TRAIL-R1 by the sum of the SAFs for TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R4, FADD, caspase-8, caspase-10 and c-FLIP. This normalisation accounts for variation between individual mass spectrometry runs. However, for the DISC this analysis is complicated by the fact that there are multiple isoforms of TRAIL-R2, caspase-8 and caspase-10 as well as the fact that both of these initiator caspases and also c-FLIP are cleaved into smaller subunits upon recruitment into the DISC. To take this into account while calculating SAF, I decided to use the molecular weight of the longest isoform of TRAIL-R2, caspase-8 and caspase-10 to ensure that the values represented the lowest possible abundance. In contrast, for c-FLIP I used the molecular weight of the cleaved form (43 kDa), which was the predominant species detected by Western blot in the BJAB TRAIL DISC (Figure 4.11). Average NSAFs were calculated for each known DISC component identified by mass spectrometry; except for caspase-10, where the NSAF was calculated using the data from the only experiment in which it was identified (Table 4.3 and Figure 4.14 A). The NSAFs demonstrated that the most abundant proteins in the DISC were TRAIL and caspase-8, while in comparison the amount of the TRAIL-R1, TRAIL-R2 and FADD within the DISC was much lower. TRAIL-R4, caspase-10 and c-FLIP were the least abundant proteins, which helps explain why
Figure 4.14: The normalised spectral abundance factor (NSAF) stoichiometry of the BJAB TRAIL DISC.

A. Spectral counts from all mass spectrometry runs of the BJAB TRAIL DISC were used to calculate an NSAF value for each known DISC component. For TRAIL, TRAIL-R1, -R2, -R4, FADD and caspase-8 n=3, for caspase-10 n=1 and for c-FLIP n=2. Error bars shown (not for c-FLIP and caspase-10) are SEM.

B. The NSAF for FADD was used to calculate the ratio of FADD to other DISC components, which were categorised, according to their mode of recruitment, into ligand, TRAIL receptors (TRAIL-R, -R1, -R2, -R4), DD recruited proteins (FADD) and DED recruited proteins (caspase-8, -10 and c-FLIP).
these proteins were identified in the BJAB TRAIL DISC with a lower protein probability (TRAIL-R4) or less consistently (caspase-10 and c-FLIP).

The DISC is formed through homotypic interactions between DDs and DEDs (Figure 1.6). Therefore, to determine the stoichiometry of the DISC the components were categorised according to their mode of recruitment. TRAIL-R1, -R2 and -R4 were classed together as TRAIL receptors, FADD was classed as the only protein recruited through DDs, whilst caspase-8, -10 and c-FLIP were categorised as proteins recruited through DEDs. Since the group that represented proteins recruited through DDs (ie FADD) was the least abundant, the NSAF of FADD was set to 1 and the NSAF for all other components was correspondingly adjusted. This produced a ratio of one FADD molecule to each of the other known DISC components, which illustrated that (based on the mass spectrometry data shown here) the BJAB TRAIL DISC is formed of approximately 3 ligands, 3 receptors (TRAIL-R1/-R2/-R4), 1 DD protein (FADD) and 4 DED proteins (caspase-8/-10 and c-FLIP) (Figure 4.14 B). However, by capturing the DISC through the ligand, there was a possibility that TRAIL receptor without associated FADD (and DED proteins) may have also been purified. This would have skewed the receptor to FADD ratio in favour of the TRAIL receptors. Therefore, the most intriguing result was the 1:4 ratio between FADD and the DED proteins, as this dramatically differs from the accepted 1:1 model of DISC formation (Sandu et al, 2006). The lower abundance of FADD in the Type I BJAB TRAIL DISC correlates with the absence of a mass spectrometry identification of FADD in the Type II Jurkat TRAIL DISC. Thus, FADD consistently appears to be sub-stoichiometric, in comparison to the DED proteins, in the TRAIL DISC purified from two different tumour cell lines.

4.6 Discussion

This chapter has described the analysis (by mass spectrometry and Western blot) of the composition of the TRAIL DISC purified from the Type II Jurkat E6.1 cell line and the Type I BJAB cell line. These experiments have:

1. Confirmed, by mass spectrometry, that TRAIL, TRAIL-R2 and caspase-8 are components of the Jurkat E6.1 TRAIL DISC.
2. Identified an extremely low level interaction between HSP90α and the Jurkat TRAIL DISC.
3. Identified a possible interaction between PKN1 and VPS35 and the Jurkat TRAIL DISC.
4. Confirmed, by mass spectrometry, that TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R4, caspase-8, caspase-10 and c-FLIP are components of the BJAB TRAIL DISC.

5. Identified an interaction between transferrin receptor and TRAIL receptors (TRAIL-R2 may predominate) in the BJAB and Jurkat cell lines, irrespective of TRAIL stimulation.

6. Detected a sub-stoichiometric recruitment of FADD to the TRAIL DISC in comparison to DED proteins.

4.6.1 DISC formation

It has been suggested that, unlike Fas DISC, TRAIL DISC forms independently of internalisation (Austin et al, 2006, Kohlhaas et al, 2007). In agreement with this, in the Jurkat cell line equal levels of DISC were formed under conditions were endocytosis was active (37 °C) or blocked (4 °C) (Figure 4.2 lane 2 and 5). Interestingly the combination of these two conditions, in the presence of bTRAIL, increased DISC formation and subsequent apoptosis (Figure 4.2 lane 4). Thus, it is possible that blockade of endocytosis by pre-incubation at 4 °C permits extensive ligand-receptor interaction and a low level of DISC formation. This low level of DISC is able to induce apoptosis in the absence of endocytosis; but is converted into greater DISC formation upon full activation of the receptors at 37 °C.

4.6.2 Identification of known TRAIL DISC components by mass spectrometry

TRAIL-R1/-R2/-R4, FADD, caspase-8/-10, and c-FLIP were identified by mass spectrometry (and confirmed by Western blot) as components of the TRAIL DISC in the Type I BJAB cell line (Table 4.3, Figure 4.9 and Figure 4.11). For the Jurkat TRAIL DISC, TRAIL-R2 and caspase-8 were unequivocally identified as components by mass spectrometry and Western blot, whilst FADD, caspase-10 and c-FLIP were detected in the complex by Western blot. This is the first demonstration by mass spectrometry that all (or some) these proteins (TRAIL-R1/-R2/-R4, FADD, caspase-8/-10, and c-FLIP) are components of the TRAIL DISC in the Type I BJAB (or Type II Jurkat) cell line. The detection of all of these proteins by mass spectrometry (and Western blot) in the TRAIL DISC from both cell lines validates previous studies that have also shown, by Western blot, their inclusion in the complex (Clancy et al, 2005, Jin et al, 2009, Kischkel et al, 2000, Merino et al, 2006, Sprick et al, 2002, Wachter et al, 2004).
The discovery of TRAIL-R4 in the BJAB (but not the Jurkat) TRAIL DISC was interesting as previous data had shown that TRAIL-R4 was not expressed on the surface of either BJAB or Jurkat cells (MacFarlane et al, 2002, Sprick et al, 2000). Thus, its inclusion in the BJAB TRAIL DISC suggests that TRAIL-R4 traffics to the BJAB cell surface upon TRAIL stimulation. In support of this, TRAIL-R3 and -R4 were found to relocate from the nucleus to the cytoplasm and cell surface of melanoma cells following TRAIL treatment (Zhang et al, 2000). This may also occur in the Jurkat cell line, however in this case TRAIL-R4 would not be readily detected due to the lower amount of TRAIL DISC formed in this cell line.

TRAIL-R4 identified in the BJAB TRAIL DISC sample may have been purified as TRAIL-bound receptor that, due to the absence of a complete DD, would not have been associated with either FADD or caspase-8. Alternatively, TRAIL-R4 could been purified as part of a heteromeric DISC with TRAIL-R1/-R2, since TRAIL-R4 has been previously shown to interact with both of these receptors and to be included in the DISC (Clancy et al, 2005, Merino et al, 2006). TRAIL-R4 is proposed to inhibit TRAIL-induced apoptosis through its interaction with TRAIL-R1/-R2, likely preventing full activation of initiator caspases at the DISC (Clancy et al, 2005, Merino et al, 2006). However, TRAIL-R4 was unlikely to be significantly affecting TRAIL-induced apoptosis in the BJAB cell line as the amount identified in the BJAB TRAIL DISC was minimal and after 4 hr BJAB cells were 70 to 80 % apoptotic (Figure 4.8, Figure 4.11 and Figure 4.13). However, it could be interesting in future to investigate whether knockout of TRAIL-R4 affects the amount of TRAIL DISC formed or its enzymatic activity.

Mass spectrometry of the BJAB TRAIL DISC lead to conclusive identification of FADD (Table 4.3 and Figure 4.9), but did not identify the other suggested DISC adaptor proteins TRADD or DAP3 (Miyazaki & Reed, 2001). This agreed with Western blot data that had previously shown that TRADD was not present in the BJAB DISC (Kischkel et al, 2000) and confirmed the direct recruitment of FADD to TRAIL-R1/-R2 in the BJAB cell line. Furthermore, neither TRADD nor DAP3 were identified by mass spectrometry in the Jurkat TRAIL DISC. Therefore, the absence of a conclusive mass spectrometry identification of FADD in the Jurkat TRAIL DISC was not due to its substitution in the DISC by an alternative and more abundant adaptor protein.

As FADD appeared to be the sole adaptor protein responsible for the recruitment of caspase-8 to the Jurkat TRAIL DISC, it was surprising that FADD had not been
unequivocally identified in the Jurkat TRAIL DISC by mass spectrometry when caspase-8 had been. Furthermore, although the Jurkat DISC was isolated from more cells (1.5 x 10^9 Jurkat versus 0.3 x 10^9 BJAB cells), TRAIL-R2 and caspase-8 were identified to a similar extent in the Jurkat and BJAB TRAIL DISC whilst FADD was only identified in the BJAB cell line (Table 4.2 and Table 4.3). This suggests that less FADD may be recruited to the Jurkat than the BJAB TRAIL DISC and it is possible that this is a contributing factor to the lower DISC formation reported in Jurkat (as compared to BJAB) cells (Sprick et al, 2002).

Future work could investigate an alternative purification method for the isolation of Jurkat TRAIL DISC, as simply increasing the number of cells from which the DISC was purified (using bTRAIL) did not result in a conclusive identification of FADD. To begin addressing this, a three step purification of TRAIL DISC was trialled using FADD-TAP (Chapter 3) and bTRAIL. The aim was to use TAP to improve the purity of the Jurkat TRAIL DISC sample and then to remove non-DISC associated FADD-TAP through purification against bTRAIL. It was hoped that this three step method would generate a sample that was sufficiently pure to allow all of the known Jurkat DISC components to be identified by mass spectrometry. However, the yield of DISC through this three step method was very low (data not shown) and thus it was deemed impractical for future mass spectrometry work. However, the purity of the sample generated by TAP (Chapter 3) demonstrates that a dual affinity tag can reduce non-specific contamination. Therefore, future work could instead analyse, by mass spectrometry, the Jurkat TRAIL DISC purified using dual affinity tagged versions of TRAIL.

4.6.3 Identification of novel TRAIL DISC components by mass spectrometry
Mass spectrometry of the Jurkat and BJAB TRAIL DISC identified several proteins whose interaction could not be confirmed through Western blot. These were PKN1 and VPS35 for the Jurkat, and DLG1, RNF113B, uncharacterised protein C15orf33 and putative ATP binding cassette transporter C13 for the BJAB TRAIL DISC. Uncharacterised protein C15orf33 and putative ATP binding cassette transporter C13 could not be investigated through Western blotting since no commercial antibodies were available for these proteins. For the remainder of the proteins, no Western blot signal was detected in the TRAIL DISC sample. This could mean that these proteins, especially those (DLG1, RNF113B) with weaker mass spectrometry identifications, may not be essential components of the TRAIL DISC. Alternatively, as PKN1, VPS35, DLG1 and RNF113B all appeared to be cleaved within the DISC, the antibody
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recognition site may have been destroyed preventing their detection within the complex by Western blot.

Previous studies on these proteins may hold the clues to their potential roles in TRAIL signalling. VPS35 is an essential component of the retromer complex, which is involved in the recycling of receptors from endosomes to the Golgi (Seaman, 2005). The vital role of VPS35 in receptor recycling was demonstrated by the mislocalisation of several receptors and endocytic proteins upon loss of the Drosophila VPS35 homologue (Korolchuk et al, 2007). Thus, it is possible that VPS35 is associated with the DISC and plays a role in the recycling of TRAIL receptors. Interestingly, VPS16 and VPS39 have been identified as required for TRAIL apoptosis in siRNA screens (Aza-Blanc et al, 2003, Ren et al, 2004). Therefore, it seems likely that the vacuolar sorting protein family play an essential role in TRAIL signalling, which is possibly through the regulation of TRAIL receptor trafficking.

PKN1 is a member of the PKC superfamily of serine/threonine protein kinases and has numerous roles in cell physiology (Mukai, 2003). The identification of PKN1 in the Jurkat TRAIL DISC is interesting since activation of PKC and PKN1 have previously been shown to regulate the recruitment of adaptor proteins to TRAIL-R1/-R2 and TNF-R2, respectively (Harper et al, 2003b, Kato et al, 2008). Furthermore, PKN1 has been shown to be catalytically activated through caspase cleavage (Takahashi et al, 1998) and contains a recognition motif (LGTD) similar to the preferred motif of caspase-8 (LETD, (Thornberry et al, 1997)). Therefore, it is possible that PKN1 associates with the TRAIL DISC in order to modulate DISC formation and is cleaved into lower molecular weight fragments by active caspase-8. This would explain why a cleavage fragment of PKN1 and not the full length protein was identified by mass spectrometry.

DLG1 is a scaffold protein with three PDZ domains (bind to the intracellular domain of transmembrane proteins) and an SH3 domain (binds the actin cytoskeleton). DLG1 has been suggested to link T cell receptor signalling machinery with cytoskeletal regulators and may promote receptor internalisation or recycling (Shaw & Filbert, 2009, Xavier et al, 2004). Therefore, it is possible that DLG1 was identified in the BJAB TRAIL DISC due to a role in the internalisation of the complex. However, as neither TRAIL-R1 nor TRAIL-R2 possess the consensus motif (S/T-X-V/L/I, (Yanagisawa et al, 1997)) required for interaction with PDZ domains, the association of DLG1 with the DISC is unlikely to be through direct recruitment to the receptors. Interestingly another scaffold protein (Homer1) has recently been shown to have a role in regulating TRAIL
susceptibility, possibly through an association with the DISC (Shin et al, 2009). Thus, it seems likely that scaffold proteins also play a vital role in TRAIL signalling.

Relatively little is known about the function of RNF113B, putative ATP binding cassette transporter C13 and uncharacterised protein C15orf33. Therefore, it is difficult to infer what roles these proteins may have in TRAIL signalling. RNF113B contains a RING finger domain that has been suggested to play a key role in NFκB and JNK activation (Kato et al, 2008) and ubiquitination (Lorick et al, 1999) pathways. Therefore, it is possible that it has a role in TRAIL-induced kinase activation. Putative ATP binding cassette transporter C13 contains sequences related to the ABC transporters (use ATP to transport substances across membranes) but lacks key motifs indicating that it may be a non-functional transporter and thus of unknown function.

Future work could further investigate the interaction of PKN1, VPS35, DLG1, RNF113B, putative ATP binding cassette transporter C13 and uncharacterised protein C15orf33 with the TRAIL DISC. This could be achieved through the use of alternative antibodies and/or gene silencing. Alternatively, TRAIL DISC could be isolated from cells in which caspase activity, and thus protein cleavage, is blocked either through the use of ZVAD.FMK or through the use of a Jurkat cell line that stably expresses the relatively inactive non-cleavable caspase-8 (Hughes et al, 2009). This may prevent cleavage of PKN1, VPS35, DLG1 and RNF113B and permit them to be identified in the TRAIL DISC by Western blot. Future investigation of PKN1 would be especially worthwhile as, out of the unconfirmed proteins, it was identified with the highest confidence (multiple spectra, multiple peptides and a 95 % protein probability) (Table 4.2, Table 4.3, Figure 4.6 and Figure 4.10).

HSP90α, identified in the Jurkat TRAIL DISC by mass spectrometry and Western blot (Table 4.2, Figure 4.5 and Figure 4.7), is the inducible isoform of the molecular chaperone HSP90. HSP90 is involved in maintaining protein stability and has been shown to have a role in apoptosis (Panner et al, 2007, Vanden Berghe et al, 2003). Furthermore HSP90α has previously been shown to be significantly recruited to the TRAIL DISC (Panner et al, 2007). This seemed to validate its identification (by mass spectrometry and Western blot) in the Jurkat TRAIL DISC. However, I did not find a dramatic increase in the amount of HSP90α present in the Jurkat TRAIL DISC compared to the untreated control (Figure 4.7). Therefore, HSP90α did not seem to represent a significant component of the Jurkat TRAIL DISC. Furthermore, HSP90α
was not identified by mass spectrometry in the BJAB TRAIL DISC, suggesting it is not significant component of the complex in this cell line either.

The difference in the amount of HSP90α identified within the TRAIL DISC between this study and the one by Panner et al may be related to the cell lines used or the DISC purification method. I have purified TRAIL DISC from the Jurkat E6.1 cell line using a biotinylated ligand; whilst Panner et al purified TRAIL DISC from transformed astrocytes through FADD immunoprecipitation (Panner et al, 2007). However, as no Western blots for other DISC components were shown by Panner et al it seems likely that their immunoprecipitated sample did not represent only the TRAIL DISC, but also proteins that interacted with FADD. Thus, the HSP90α interaction observed by Panner et al may have been with FADD rather than with the TRAIL DISC. However, no such interaction was identified by mass spectrometry of purified FADD-TAP (Chapter 3).

Transferrin receptor was originally identified by mass spectrometry in the BJAB TRAIL DISC. Importantly, further experiments demonstrated that this protein was also present in the Jurkat TRAIL DISC as well as associated with unstimulated TRAIL receptors (possibly specifically TRAIL-R2) isolated from the BJAB and Jurkat cell lines (Figure 4.11, Figure 4.12 and Figure 4.13). Transferrin receptor, usually involved in the uptake of iron, has been implicated in gambogic acid-induced apoptosis. This involved activation of caspase-8 and the cell death intrinsic pathway, but not through an association with known DISC components (Kasibhatla et al, 2005). Thus, the association of transferrin and TRAIL receptors appears to be a novel observation. Transferrin receptor may associate with TRAIL receptors either directly or may interact via through TRAIL. However, transferrin receptor lacks the CRDs that mediate death receptor interaction (through the PLAD) and death ligand binding (Clancy et al, 2005, Naismith & Sprang, 1998). Therefore, it is possible that the transferrin and TRAIL receptors instead interact via their cytoplasmic domains.

Transferrin receptor is the classic example of a cargo protein for clathrin-mediated endocytosis and thus is frequently used as a marker of this pathway (Le Roy & Wrana, 2005). TRAIL and its receptors have been shown to be primarily internalised through a clathrin-mediated endocytosis (Austin et al, 2006, Kohlhaas et al, 2007). Therefore, it is possible that transferrin receptor is involved in targeting TRAIL receptors/DISC for clathrin-mediated endocytosis. Alternatively, it is also possible that transferrin receptor was present in the same vesicle as internalised TRAIL receptors/DISC and thus was identified (by mass spectrometry and Western blot) due to co-purification rather than a
specific interaction. This point would need to be addressed in future work that could use various approaches (such as reverse immunoprecipitation, co-localisation studies and gene silencing) to confirm whether a direct interaction occurs between the receptors and investigate its potential relevance. Furthermore, the earlier suggestion that transferrin receptor predominantly interacts with TRAIL-R2 could be investigated through specific isolation of TRAIL-R1/-R2 using either TRAIL receptor specific ligands or the TRAIL-R2 null BJAB cell line (Natoni et al, 2007).

Using tandem mass spectrometry, Jin et al recently identified the E3 ligase CUL3, the deubiquitinase A20 and the ubiquitin binding polypeptide p62 as TRAIL DISC interacting partners (Jin et al, 2009). Furthermore, CUL3 was reported to polyubiquitinate caspase-8 that, instead of targeting it for degradation, promoted p62-mediated aggregation and full protease activation (Jin et al, 2009). The level of CUL3 detection by mass spectrometry was similar to that of the known DISC components c-FLIP and FADD, with all three proteins identified by a similar number of spectra. A20 was identified by fewer spectra, whilst p62 was identified by a greater number of spectra (Jin et al, 2009). Since FADD, c-FLIP, CUL3 and p62 appeared to be similarly abundant in the DISC isolated by Jin et al, it was surprising that mass spectrometry had not identified CUL3 and p62 in the BJAB TRAIL DISC described here. This discrepancy could mean that CUL3 and p62 are not associated with the TRAIL DISC in all cell lines (Jin et al used H460 cells whereas I used BJAB cells) or that these proteins did not associate with the BJAB TRAIL DISC under the treatment conditions that I used (Jin et al treated cells for 2 hr at 4 °C, I treated cells for 1 hr at 4 °C followed by 10 min at 37 °C). Furthermore I purified DISC (through bTRAIL) from total cellular lysate, whilst Jin et al immunoprecipitated DISC (through TRAIL-R1/-R2) from high molecular weight fractions of size-exclusion fractionated cellular lysates. Therefore, future work could use the methodology of Jin et al to investigate (through mass spectrometry and/or Western blot) whether CUL3 interacts with either the BJAB or Jurkat TRAIL DISC.

In summary, mass spectrometry and Western blot have detected a potential interaction between transferrin and TRAIL receptors in both the Type I BJAB and Type II Jurkat cell lines. Furthermore, there was no significant identification of a protein specific to either the Type I or Type II TRAIL DISC. Thus, on the basis of the data presented here it appears unlikely that the recruitment of an additional component accounts for the previously described difference in TRAIL DISC formation in these cell lines (Sprick et al, 2002).
4.6.4 DISC stoichiometry

For each component identified in the BJAB TRAIL DISC an NSAF value was calculated allowing the stoichiometry of the TRAIL DISC to be approximated (Figure 4.14). This demonstrated that in the BJAB TRAIL DISC the amount of FADD was sub-stoichiometric compared to the amount of DED proteins (procaspase-8/-10 and c-FLIP). This was in agreement with the recent TRAIL DISC mass spectrometry data from Jin et al, where FADD was also detected in a lower abundance than the other DISC components (Jin et al, 2009). In contrast, it has been suggested that a greater amount of FADD compared to caspase-8 is required for optimal in vitro Fas DISC formation (Roy et al, 2008). This may represent a difference in the formation of the Fas versus the TRAIL DISC or a difference in the calculation of stoichiometry. I have used the validated quantitative method of comparing NSAFs, whilst Roy et al used various concentrations of the components to determine the optimal stoichiometry for in vitro DISC formation. Also in support of the stoichiometry calculated here is the similarity between the observed ratio of 1 c-FLIP to 7 caspase-8 molecules (Figure 4.14 B) and the published suggestion of a ratio of 1 c-FLIP to 5 caspase-8 molecules (Chang et al, 2002).

FADD was the only adaptor protein that was identified, although in a sub-stoichiometric amount, through mass spectrometry of the BJAB TRAIL DISC. Thus, it appears unlikely that DED proteins are recruited by a different adaptor protein, suggesting that FADD is able to recruit more than one DED protein to the TRAIL DISC. Furthermore, the current model of DISC formation suggests that caspase-8 recruited to the DISC dimerises with an adjacent molecule to initiate activation (Boatright et al, 2003, Hughes et al, 2009). However, in this model the two caspase-8 molecules would be orientated in the same direction, which does not concur with the anti-parallel nature of dimeric caspsases, including caspase-8 (Blanchard et al, 1999). Therefore, based on this information, I propose a new model for procaspase-8/-10/c-FLIP recruitment to the TRAIL DISC (Figure 4.15).

In this model, FADD (recruited by its DD to TRAIL-R1/-R2) recruits one molecule of procaspase-8 (or other DED protein) through a hydrophobic interaction between its DED and DED2 of procaspase-8, as has been previously suggested (Carrington et al, 2006, Yang et al, 2005). This molecule of caspase-8 in turn recruits three other molecules through two dimerisation events, mediated by the small catalytic subunit and the DEDs, to produce the observed ratio of 1 FADD to 4 DED proteins (Figure 4.14 B).
Figure 4.15: A new model for the recruitment of procaspase-8/-10/c-FLIP to the TRAIL DISC.

In the new model of procaspase-8/-10/c-FLIP recruitment to the DISC, FADD recruits one molecule of procaspase-8 (or other DED protein) through the hydrophobic DED interaction. This molecule dimerises with a free molecule through the small catalytic subunits to produce an anti-parallel dimer. An additional interaction between the DISC-bound dimer and a free anti-parallel dimer, through charged or hydrophobic patches (Figure 4.16) results in the recruitment of four DED proteins to each molecule of FADD.
Previous studies have demonstrated that caspase-8 dimerises, upon an increase in protein concentration (Donepudi et al, 2003), through its small catalytic subunit (Boatright et al, 2003, Keller et al, 2009). Therefore, I propose that a local increase in the concentration of procaspase-8/-10/c-FLIP at the DISC leads to dimerisation of a DISC-bound and a free molecule, via the small catalytic subunit, resulting in the formation of an anti-parallel dimer (Figure 4.15). This would produce a ratio of 1 FADD to 2 DED proteins, so another anti-parallel dimer would have to be recruited (Figure 4.15) to produce the observed ratio of 1 FADD to 4 DED proteins (Figure 4.14 B).

The recruitment of another anti-parallel dimer could occur through an interaction between charged or hydrophobic patches of the DEDs. Structural work on the tandem DEDs of the v-FLIP MC159 identified charged surfaces on the DEDs. These were shown to mediate the interaction of MC159 with FADD and also proposed to allow interactions with other DED proteins (Li et al, 2006, Yang et al, 2005). These charged patches are conserved in FADD, procaspase-8 and c-FLIP (Li et al, 2006, Yang et al, 2005) and thus I propose that they may mediate the recruitment of an additional anti-parallel dimer to the DISC (Figure 4.16 A and B). Alternatively, an additional anti-parallel dimer could be recruited to the hydrophobic patch of DED1 of the DISC-bound DED protein (Figure 4.16 C). The hydrophobic patch of procaspase-8 DED1 has been suggested to be buried at the interface between DED1 and DED2, and thus not available for interaction with FADD (Carrington et al, 2006). However, no evidence for this was shown and no full length structure of caspase-8 has been determined. Therefore, it is possible that the hydrophobic patch of DED1 could mediate an interaction with either DED1 or DED2 of a free DED protein, resulting in its recruitment to the DISC (Figure 4.16 C). Whether through charged or hydrophobic patches, the recruitment of an additional anti-parallel dimer would produce the ratio of 1 FADD to 4 DED proteins calculated from the mass spectrometry data.

In support of my model, it has been suggested that both DEDs of initiator caspases (rather than just the FADD interacting DED2) are required for DISC recruitment (Tsukumo & Yonehara, 1999, Yao et al, 2007). Furthermore, numerous studies have identified interactions between c-FLIP and procaspase-8/-10 (Krueger et al, 2001a), with c-FLIP able to interact with procaspase-8 through both its DEDs and catalytic-like domain (Irmler et al, 1997). Also in support of procaspase-8/-10/c-FLIP-mediated self-recruitment, the DEDs of procaspase-8 and procaspase-10 have been shown to associate with themselves and with each other (Wang et al, 2001). The recruitment of procaspase-8 to the DISC through self-association would also explain how mass
In the new model of procaspase-8/-10/c-FLIP recruitment to the DISC (Figure 4.15) the DED protein dimer bound to FADD recruits an additional anti-parallel dimer through a DED mediated interaction via charged (A or B) or hydrophobic (C) patches. In this figure the DEDs of only one molecule of the anti-parallel dimer is shown. The free DED dimer (green hexagons) may interact through charged patches (red circles) with either the DED of FADD (orange hexagon) and one DISC-bound DED (purple hexagon) (A), or with both DEDs of the DISC-bound dimer (purple hexagons) (B). If the free dimer is recruited through a hydrophobic interaction (red bars), this could occur between DED1 of the DISC-bound molecule (purple hexagon) and DED1/2 of the free dimer (green hexagons) (C). Adapted from Li et al, 2006.

Figure 4.16: The potential interactions that may occur between DEDs in the suggested model of procaspase-8/-10/c-FLIP recruitment to the TRAIL DISC.
spectrometry identified caspase-8 to a similar extent in the Jurkat and BJAB TRAIL DISC (although from different number of cells, see earlier discussion), when FADD was only confidently identified by mass spectrometry in the BJAB TRAIL DISC.

This model of DISC assembly provides a novel insight into the recruitment of procaspase-8/-10/c-FLIP to the TRAIL DISC and could be further investigated in the future. The model proposed here has been based on the stoichiometry calculated from the BJAB TRAIL DISC mass spectrometry data. However, the stoichiometry of DISC components in other cell lines could be different, for example as mentioned earlier the mass spectrometry data obtained indicates that FADD may be less abundant in the Jurkat than in the BJAB TRAIL DISC. If this were true then the number of DED proteins recruited to each molecule of FADD would increase. Thus, it would be worthwhile assessing the TRAIL DISC stoichiometry in other cell lines so that differences in DISC formation could be identified and a more general model for DISC assembly formulated. Furthermore, the stoichiometry of the individual DISC components could be refined using a more exact method of mass spectrometry quantitation, such as through the use of the AQUA strategy (Kirkpatrick et al, 2005). Finally, mutational studies could be employed to determine whether the small catalytic subunit, hydrophobic patches or charged surface patches of procaspase-8/-10/c-FLIP mediate the recruitment of additional DED proteins to the DISC.
Chapter 5: Comparison of TRAIL DISC from Type I and Type II Cells
5 Comparison of TRAIL DISC from Type I and Type II Cells

5.1 Introduction

The Type I/II model was originally proposed based on the response of cells stimulated with anti-Fas antibody (Scaffidi et al, 1998) but has since also been described in TRAIL signalling (Fulda et al, 2002, Kim et al, 2003, LeBlanc et al, 2002, Samraj et al, 2006). Apoptosis in Type II cells can be blocked through overexpression of Bcl-2/Bcl-XL leading to the suggestion that in these cells the intrinsic pathway is required for apoptosis to progress. Type II cells form a relatively small amount of Fas DISC compared to Type I cells and have delayed caspase activation (Scaffidi et al, 1998, Schmitz et al, 1999). It has been suggested that the lower level of DISC formation in Type II cells prevents direct cleavage and activation of caspase-3. However, the proposal is that the Type II DISC is instead able to cleave Bid to activate the intrinsic pathway. Contrastingly, in a Type I cell line the greater Fas DISC formation permits direct cleavage of caspase-3 and activation of the caspase cascade, such that the intrinsic pathway is not required to amplify caspase activation (Barnhart et al, 2003, Scaffidi et al, 1998). Therefore, the level of Fas DISC formation is apparently vital in determining which pathways are activated upon stimulation of death receptors.

In Chapter 4 it was demonstrated that the molecular composition of the Type I and II TRAIL DISC was essentially identical. Therefore, based on the current data, the composition of the TRAIL DISC does not appear to contribute to the amount of complex formed upon TRAIL stimulation. This raised questions as to what other factors may determine whether a Type I or II response is observed following stimulation of TRAIL receptors. As discussed in the Introduction, various suggestions have been proposed to explain the Type I/II model (Figure 5.1). Thus, the aim of this chapter was to investigate some of these suggestions with respect to TRAIL signalling in the Type I BJAB and Type II Jurkat cell lines. Specifically cell surface receptor expression, the rate and extent of DISC formation, DISC substrate (procasparse-3/Bid) preference, as well as the expression and cleavage of proteins vital for activation/regulation of downstream apoptotic pathways would be investigated.
Comparison of TRAIL DISC from Type I and Type II Cells

Figure 5.1: Schematic representation of the possible mechanisms that may explain the requirement of the intrinsic pathway for death receptor-mediated apoptosis in Type II cells.

Following activation of death receptors, Type I cells form a large amount of DISC, display early activation of caspases and do not require the intrinsic pathway for apoptosis. In contrast, in Type II cells DISC formation is reduced, caspase activation is delayed and the activation of the intrinsic pathway is essential for death receptor-mediated apoptosis. The above scheme details various suggestions proposed to explain the Type II phenotype.
5.2 Type I BJAB cells form a greater amount of DISC and express more death receptors at the cell surface

In agreement with the Type I/II model (Scaffidi et al, 1998) the Type I BJAB cell line formed more Fas DISC than the Type II Jurkat E6.1 cell line upon exposure to antibody-crosslinked Fas ligand (Figure 5.2 A). In correlation with this, the BJAB cell line was found to express more Fas at its surface than the Type II Jurkat E6.1 cell line (Figure 5.2 B). This observation agrees with previously published data that also demonstrated lower cell surface expression of Fas in Type II cells (Eramo et al, 2004, McNees et al, 2002).

Having established that the amount of Fas DISC formed in the BJAB and Jurkat E6.1 cell lines corresponded with their proposed Type I/II status, I decided to investigate the level of TRAIL DISC formed in each cell line. TRAIL signalling in the BJAB cell line can occur through TRAIL-R1 and -R2, since both receptors are expressed on the cell surface (Figure 5.3). However, in the Jurkat E6.1 cell line TRAIL-R1 is not expressed at the cell surface and thus TRAIL signalling in this cell line is mediated via TRAIL-R2 (Figure 5.3). Furthermore, in agreement with a previous study (Sprick et al, 2000), the level of TRAIL-R2 cell surface expression in the Jurkat cell line was significantly (approximately 40 %) lower than in the BJAB cell line. Importantly, this is not due to a difference in cell size since the BJAB and Jurkat cells have a similar diameter of 13 and 12 µm, respectively.

The absence of TRAIL-R1 expression in the Jurkat cell line meant it was important to determine the relative contribution of TRAIL-R1/-R2 to DISC formation in the BJAB cell line. Thus, DISC was purified from cells treated with wildtype (stimulates TRAIL-R1 and -R2), TRAIL-R1 specific or TRAIL-R2 specific ligand (Figure 5.4). In agreement with the data shown for Fas (Figure 5.2 A), and in correlation with TRAIL-R1/-R2 cell surface expression levels (Figure 5.3), all three ligands induced greater TRAIL DISC formation in the BJAB cell line than in the Jurkat cell line (Figure 5.4).

As expected, based on the absence of detectable TRAIL-R1 cell surface expression, Jurkat E6.1 cells did not significantly form TRAIL DISC or undergo apoptosis upon treatment with the TRAIL-R1 specific ligand (Figure 5.4 lane 3). However, treatment of BJAB cells with the TRAIL-R1 specific ligand lead to significant DISC formation and apoptosis induction, which was not dramatically different to that observed in this cell line upon treatment with the wildtype ligand (Figure 5.4 lanes 6 and 7). However,
Figure 5.2: The Type I BJAB cell line forms more Fas DISC and has higher Fas cell surface expression than the Type II Jurkat cell line.

A. Jurkat E6.1 and BJAB cells were either left untreated or treated with 1 μg/ml of Flag-tagged Fas ligand crosslinked with 2 μg/ml of M2 antibody for 1 hr at 4 °C followed by 25 min at 37 °C, and then lysed in DISC lysis buffer. Following DISC capture onto Protein G beads, bound proteins were eluted with 2 x sample buffer (without β-mercaptoethanol) and separated by SDS-PAGE. The supernatant blots act as loading controls ensuring an equal number of cells were treated. B. Fas cell surface expression was assessed through incubation of the cell lines with either goat serum alone (no antibody and control antibody) or with goat serum containing anti-Fas antibody. Cells were then washed and incubated with either goat serum (no antibody) or with goat serum containing FITC-conjugated secondary antibody (control and anti-Fas antibody). After washing with PBS the fluorescence intensity was analysed by flow cytometry. The values represent the mean fluorescence intensity (geometric mean) with the SEM (n=3).
Comparison of TRAIL DISC from Type I and Type II Cells

Figure 5.3: Cell surface expression of TRAIL-R1 and -R2 is higher in the Type I BJAB cells than in the Type II Jurkat E6.1 cell line.

For TRAIL-R1/-R2 expression analysis, cells were incubated with either goat serum (no antibody) or with goat serum containing PE-conjugated isotype control or TRAIL receptor specific antibody. After washing with PBS the fluorescence intensity was analysed by flow cytometry. The numbers represent the mean fluorescence intensity (geometric mean) with the SEM (n=3).
Figure 5.4: The Type I BJAB cell line forms more DISC following activation of TRAIL-R1/-R2, either together or independently.

Jurkat E6.1 and BJAB cells were either left untreated or treated with 500 ng/ml of biotinylated wildtype (WT), TRAIL-R1 or TRAIL-R2 specific ligand. Cells were incubated for 1 hr at 4 °C followed by either 10 min (BJAB) or 25 min (Jurkat E6.1) at 37 °C and then lysed in DISC lysis buffer. DISC was captured through overnight incubation at 4 °C with streptavidin beads, proteins were eluted by boiling with 1 x sample buffer and separated by SDS-PAGE. Percentage apoptosis (lower table) was assessed by annexin V/PI staining and represents the percentage of PS positive cells. The blots shown are representative of 2 experiments, whilst the percentage apoptosis is the average of 2 experiments.
stimulation of BJAB cells with the TRAIL-R2 specific ligand resulted in a dramatic reduction in the recruitment of FADD and caspase-8 to the DISC (Figure 5.4 lanes 6 and 8). Furthermore, specific stimulation of TRAIL-R2 also reduced the level of apoptosis observed in the BJAB cell line to that observed in the TRAIL-R2 expressing Jurkat cell line. Therefore, despite expression of both TRAIL-R1 and TRAIL-R2, TRAIL-R1 appears to be predominantly responsible for FADD recruitment, DISC formation and subsequent apoptotic signalling upon exposure of BJAB cells to TRAIL. This observation may also explain why a greater amount of TRAIL-R1, compared to TRAIL-R2, was identified in the BJAB DISC by mass spectrometry (Figure 4.14). A small amount of TRAIL-R2 was recruited into the DISC formed upon stimulation of BJAB cells with the TRAIL-R1 specific ligand (Figure 5.4 lane 7), whilst a low level of TRAIL-R1 was recruited into the TRAIL-R2 specific DISC (Figure 5.4 lane 8). This finding suggests that stimulation of TRAIL receptors leads to heterocomplex formation, but that this only represents a small proportion of the total DISC formed.

Overall, the data had demonstrated that the level of TRAIL DISC formation was related to the level of TRAIL-R1/-R2 cell surface expression but also to whether TRAIL-R1, -R2 or both were stimulated. Therefore, in the cell lines tested, relative expression of TRAIL-R1 and TRAIL-R2 at the cell surface is likely to be an important factor in determining whether a Type I or Type II response is observed in response to TRAIL.

### 5.3 Type I BJAB cells form TRAIL DISC more rapidly than Type II Jurkat E6.1 cells

Previous studies have demonstrated rapid formation of the Type I Fas DISC (Medema et al, 1997, Scaffidi et al, 1997). Therefore, I decided to investigate the kinetics of TRAIL DISC formation in Type I and II cell lines. In these experiments, cells were pre-chilled (to block endocytosis) in the presence of bTRAIL for 1 hr and then incubated for an increasing amount of time at 37 °C. In both the Type I BJAB and Type II Jurkat cell lines there was significant DISC formation without the 37 °C incubation step (Figure 5.5 lanes 2 and 9). However, in both cell lines the amount of DISC formed at 4 °C was sub-maximal and increased upon incubation at 37 °C. Thus, maximal TRAIL DISC formation in the Type I and II cell lines tested required stimulation at 37 °C.

Formation of BJAB TRAIL DISC peaked after a 10 min incubation at 37 °C (Figure 5.5 lane 5). However, in agreement with data shown in Chapter 4 (Figure 4.3), the Jurkat E6.1 cell line required at least a 20 min incubation at 37 °C for the assembly of
Figure 5.5: The Type I BJAB cell line forms TRAIL DISC faster than the Type II Jurkat cell line.

Jurkat E6.1 and BJAB cells were either left untreated or treated with 500 ng/ml of bTRAIL. Cells were incubated for 1 hr at 4 °C followed by an increasing amount of time at 37 °C and then lysed in DISC lysis buffer. DISC was captured through overnight incubation at 4 °C with streptavidin beads, proteins were eluted by boiling with 1 x sample buffer and separated by SDS-PAGE. The exposures shown for DISC (bead eluate) from the BJAB and Jurkat E6.1 cell line are after different amounts of time. Percentage apoptosis (% PS positive cells), assessed by annexin V/PI staining after 4 hr at 37 °C, was on average 86 % and 31 % for TRAIL treated BJAB and Jurkat E6.1 cells, respectively.
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maximal TRAIL DISC (Figure 5.5 lane 13). Thus, total TRAIL DISC formation in the BJAB cell line is greater and occurs with faster kinetics than in the Jurkat E6.1 cell line. This implies that there is extremely efficient recruitment of FADD and caspase-8 to the Type I TRAIL DISC. TRAIL DISC purification using receptor specific ligands had already demonstrated that the majority of FADD (and caspase-8) in the BJAB DISC was recruited via TRAIL-R1 (Figure 5.4). Therefore, it was likely that the fast kinetics of FADD and caspase-8 recruitment to the BJAB TRAIL DISC was primarily achieved via the TRAIL-R1 receptor. Thus, the absence of detectable TRAIL-R1 surface expression may explain the delayed recruitment of FADD and caspase-8 to the TRAIL DISC in the Jurkat E6.1 cell line.

5.4 TRAIL DISC is a high molecular weight complex (> 700 kDa) in both Type I and Type II cell lines

The Fas and TRAIL DISC have previously been shown to be very high molecular weight complexes, with Fas DISC being in the region of 1 to 7 MDa (Feig et al, 2007, Wagner et al, 2007). Furthermore, clustering of activated Fas occurs in both Type I and II cells but has been suggested to be less extensive in Type II cells (Algeciras-Schimnich et al, 2002). Therefore, I decided to investigate if there was a difference in the molecular weight of a Type I and Type II TRAIL DISC. To do this TRAIL treated cellular lysates were fractionated using sucrose density gradients, and the TRAIL DISC present in each fraction was isolated using streptavidin beads. This demonstrated that TRAIL DISC formed in the BJAB and Jurkat E6.1 cell lines was of an extremely high molecular weight, greater than 700 kDa (Figure 5.6). BJAB TRAIL DISC was predominantly present in fractions 17 to 22 (Figure 5.6 A), whilst the Jurkat TRAIL DISC peaked between fractions 19 and 23 (Figure 5.6 B). Therefore, the Jurkat TRAIL DISC, which contains only TRAIL-R2, appeared to be more highly aggregated than the BJAB TRAIL DISC. This was an intriguing observation, which suggests that the overall amount of TRAIL DISC formed does not determine the extent of aggregation. The increased aggregation of TRAIL-R2, relative to TRAIL-R1, was also observed within the BJAB TRAIL DISC, with TRAIL-R2 peaking in the DISC at a higher molecular weight than TRAIL-R1 (Figure 5.6 A blue versus red box).

To investigate the aggregation of TRAIL-R2 further it was decided to directly compare the molecular weight of DISC formed in the BJAB cell line following treatment with either wildtype or the TRAIL-R2 specific ligand (Figure 5.7). This experiment confirmed that, upon treatment of BJAB cells with wildtype TRAIL, TRAIL-R2 peaked in higher
A - Type I BJAB

B – Type II Jurkat E6.1

Figure 5.6: TRAIL DISC formed in Type I and II cell lines is of a high molecular weight, but TRAIL-R2 DISC is even more highly aggregated.

BJAB (A) and Jurkat E6.1 (B) cells were treated with 500 ng/ml of bTRAIL. Cells were incubated for 1 hr at 4 °C followed by either 10 (BJAB) or 25 min (Jurkat E6.1) at 37 °C and then lysed in DISC lysis buffer. Cleared lysate was applied to a continuous 10 – 45 % sucrose density gradient and centrifuged for 17 hr at 38,000 g and 4 °C. The gradient was fractionated into 27 fractions and to each of these streptavidin beads were added for overnight capture of DISC. Proteins were eluted from the beads by boiling with 1 x sample buffer and separated by SDS-PAGE. The green boxed area indicates those fractions in which DISC components were found when unstimulated lysates were fractionated and incubated with streptavidin beads. The dashed boxed area indicates fractions that contain BJAB DISC. Red and blue boxes indicate the peaks of TRAIL-R1 and -R2, respectively. The asterixed band represents residual signal from TRAIL-R2 as this membrane was re-probed for caspase-8. The blots shown are representative of 2 (Jurkat cell line) or 3 (BJAB cell line) independent experiments. Molecular weight standards were run on a separate gradient and the positions shown are representative of three independent experiments.
Comparison of TRAIL DISC from Type I and Type II Cells

A- Wildtype ligand

Figure 5.7: Stimulation of TRAIL-R2, in the Type I BJAB cell line, causes the formation of a higher molecular weight homomeric complex.

BJAB cells were either left untreated or treated with 500 ng/ml of biotinylated wildtype (A) or TRAIL-R2 specific (B) TRAIL. Cells were incubated for 1 hr at 4 °C followed by 10 min at 37 °C and then lysed in DISC lysis buffer. DISC was purified from sucrose density gradient fractionated lysate as in Figure 5.6 and separated by SDS-PAGE for Western blot. The green boxed area indicates those fractions in which DISC components were found when unstimulated lysates were fractionated and incubated with streptavidin beads. The dashed boxed area indicates fractions that contain wildtype TRAIL DISC, whilst red and blue boxes indicate the peak of TRAIL-R1 and -R2, respectively. Molecular weight standards were run on a separate gradient and the positions shown are representative of three independent experiments.
Comparison of TRAIL DISC from Type I and Type II Cells

molecular weight fractions of the DISC (Figure 5.7 A blue box) compared to TRAIL-R1 (Figure 5.7 A red box). Furthermore, the TRAIL-R2 BJAB DISC (Figure 5.7 B blue box) lacked the lower molecular weight fractions of the wildtype TRAIL BJAB DISC (Figure 5.7 A fractions 15 to 17), demonstrating that indeed a TRAIL-R2 DISC is more highly aggregated. Interestingly, the peak of TRAIL-R2 in the wildtype TRAIL BJAB DISC (Figure 5.7 A fractions 18 to 21) and the TRAIL-R2 specific BJAB DISC (Figure 5.7 B fractions 19 to 21) was near identical. Therefore, the absence of TRAIL-R1 activation did not appear to significantly alter TRAIL-R2 aggregation. This observation, and that of separate TRAIL-R1 and TRAIL-R2 peaks consistently seen in the wildtype TRAIL BJAB DISC (Figure 5.6 A and Figure 5.7 A), supports the earlier suggestion that the TRAIL DISC is predominantly comprised of homomeric TRAIL-R1 or TRAIL-R2 complexes. These data are also consistent with the work by Kischkel et al who, based on the specific immunoprecipitation of TRAIL-R1 or TRAIL-R2, proposed that TRAIL stimulation predominantly results in the formation of homomeric receptor complexes (Kischkel et al, 2000).

In the TRAIL-R2 BJAB DISC (Figure 5.7 B), FADD was detected in fewer higher molecular weight fractions than in the wildtype TRAIL BJAB DISC (Figure 5.7 A). This suggested, in agreement with data shown earlier (Figure 5.4), that FADD recruitment to the BJAB TRAIL DISC was reduced upon specific stimulation of TRAIL-R2. Therefore, it appears as through TRAIL-R1 is responsible for the majority of FADD (and caspase-8) recruitment, and thus functional TRAIL DISC formation in the BJAB cell line.

In both cell lines some TRAIL-R1 and TRAIL-R2 were isolated from low molecular weight fractions (Figure 5.6 and Figure 5.7 A, fractions 7 to 10). This most likely represented ligand-bound receptor alone, and not DISC, as FADD and active caspase-8 were not significantly detected in these fractions. In the BJAB cell line the amount of these ligand-bound receptors was comparable to the amount of receptor seen in the high molecular weight DISC (Figure 5.6 A and Figure 5.7 A). This suggests that, once bound by ligand, a significant proportion of TRAIL receptor on the surface of BJAB cells is able to swiftly aggregate and/or recruit FADD (and caspase-8) to form the high molecular weight DISC. In contrast, in the Jurkat cell line a greater proportion of ligand-bound TRAIL-R2 was detected in the low molecular weight fractions (Figure 5.6 B fractions 8 to 10) than in the DISC (Figure 5.6 B fractions 19 to 23). Therefore, one possibility is that the low amount of TRAIL DISC formed in the Jurkat cell line may be related to inefficient receptor oligomerisation and/or recruitment of FADD and caspase-8 to TRAIL-R2. One contributing factor to this could be the low expression of
Comparison of TRAIL DISC from Type I and Type II Cells

TRAIL-R2 on the surface of Jurkat E6.1 cells (Figure 5.3), as in BJAB cells (where TRAIL-R2 surface expression is higher) specific stimulation of TRAIL-R2 resulted in its efficient movement into the high molecular weight DISC fractions (Figure 5.7 B).

Thus far the data had shown that Type I BJAB cells form a greater amount of DISC at a faster rate than the Type II Jurkat cell line. However, in both cell lines the TRAIL DISC formed was a high molecular weight complex, greater than 700 kDa. Furthermore, TRAIL-R2 appeared to form a more highly aggregated complex, whilst TRAIL-R1 was apparently required for efficient FADD recruitment to the BJAB TRAIL DISC.

5.5 Type I and Type II DISC have a similar substrate preference

Having characterised the formation of TRAIL DISC in the Type I BJAB and Type II Jurkat cell lines, I now decided to explore the catalytic activity of the DISC. Studies have shown that caspase-8 bound to either Fas or TRAIL DISC is catalytically active and able to cleave, among other substrates, procaspase-3 and Bid (Hughes et al, 2009, Lavrik et al, 2003, Wachter et al, 2004). It has been proposed that the amount of caspase-8 generated at a Type II DISC is not sufficient to activate caspase-3 directly; but is sufficient to cleave full length Bid and thus initiate the intrinsic apoptotic pathway (Barnhart et al, 2003, Scaffidi et al, 1998). Thus, it was decided to investigate whether the Type II TRAIL DISC was capable of cleaving procaspase-3, Bid or both.

The cleavage activity of the TRAIL DISC was assessed by Western blot (procaspase-3 C163A and Bid) or fluorogenic assay (IETD.AFC). Cysteine to alanine mutation of procaspase-3 inactivates the catalytic residue and thus any caspase-3 cleavage observed was the direct result of caspase-8 activity (generates p20 subunit) and not caspase-3 autocatalysis (generates p19 and p17 subunits) (Han et al, 1997). Bid cleavage in these assays was visualised through the generation of the C-terminal p15 (tBid) and, less abundant, p13 subunits (Fischer et al, 2006, Li et al, 1998). Finally, cleavage of IETD.AFC by caspase-8 leads to the release of the AFC fluorogenic group, the rate of which can be measured as IETDase activity.

TRAIL DISC was isolated from both the BJAB and Jurkat cell lines following a 1 hr pre-chill with bTRAIL and either a 10 min (optimal BJAB DISC formation, Figure 5.5) or 25 min (optimal Jurkat E6.1 DISC formation, Figure 4.3 and Figure 5.5) incubation at 37 ºC. The BJAB TRAIL DISC, isolated at either time point, was significantly more catalytically active than the Jurkat TRAIL DISC (Figure 5.8). Thus, generation of
A - Procaspe-3 cleavage

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Bioassay supernatant

% Apoptosis (4 hr) | 13 | 79 | 82 | 5 | 41 | 47 |

W. B. Ab

Active Caspase-3

B - Bid cleavage

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Bioassay supernatant

% Apoptosis (4 hr) | 12 | 78 | 82 | 6 | 37 | 41 |

W. B. Ab

Bid

C - IETD.AFC cleavage

Figure 5.8: Assay of bead-bound TRAIL DISC demonstrates that Type I BJAB DISC has more catalytic activity than a Type II Jurkat DISC.

BJAB and Jurkat E6.1 cells were either left untreated or treated with 500 ng/ml of bTRAIL. Cells were incubated for 1 hr at 4 °C followed by either 10 or 25 min at 37 °C and then lysed in DISC lysis buffer. Percentage apoptosis induced in these cells was measured by annexin V/PI staining (lower tables of A and B) and represents the percentage of PS positive cells. DISC was captured onto streptavidin beads and divided into two samples to assay the cleavage of caspase-8 substrates. Recombinant procaspase-3 C163A (A) or Bid (B) was added to one set of bead-bound DISC and incubated at 37 °C for 2 hr. Assay supernatants (non-complexed proteins) and bead elutes (DISC-bound proteins) were separated by SDS-PAGE and cleavage investigated by Western blot. Bead eluate blots shown are the same exposure time as supernatant blots. The second set of bead-bound DISC was assayed for cleavage of IETD.AFC. The increase in the fluorogenic cleavage product was measured and expressed as IETDase activity (C). The results shown in C are the average of 2 experiments and the error bars represent minimum and maximum values. U/S, unstimulated.
caspase-3 p20 (Figure 5.8 A) or tBid (Figure 5.8 B) in the assay supernatant was significantly higher following incubation with the BJAB TRAIL DISC as compared to incubation with the Jurkat TRAIL DISC. Importantly, neither procaspase-3 C163A nor Bid directly associated with the TRAIL DISC (Figure 5.8 A and B bead eluate). The greater catalytic activity of the BJAB TRAIL DISC likely correlated with the greater level of DISC formed in this cell line (Figure 5.4). To confirm that this was the case, assays were completed with a fixed amount of bead-bound Jurkat TRAIL DISC (from 50 x 10^6 cells) or an increasing amount of bead-bound BJAB TRAIL DISC (from 0.5 to 15 x 10^6 cells). Western blot confirmed that as the amount of bead-bound BJAB TRAIL DISC used in the assays increased so too did the amount of DISC present (Figure 5.9 A). In correlation with the greater amount of DISC formed in BJAB cell line, DISC from fewer BJAB cells (5 to 10 x 10^6 cells) contained equivalent amounts of DISC-bound caspase-8 (Figure 5.9 A) and/or an equivalent catalytic activity (Figure 5.9 B) to the TRAIL DISC from 50 x 10^6 Jurkat cells. Thus, when DISC-bound caspase-8 is present at similar levels, the Type I and II DISCs exhibit a similar catalytic activity against substrates \textit{in vitro}. Therefore, the extent of substrate cleavage observed in the two cell types is likely related to the absolute amount of TRAIL DISC formed.

Throughout these experiments (Figure 5.9) it appeared that the cleavage of Bid by either Type I or Type II TRAIL DISC (as judged by the ratio of cleaved fragment to full length protein) was much less efficient than that of procaspase-3 C163A. Thus, procaspase-3 C163A seemed to be the preferred substrate for both Type I and II DISC-bound caspase-8. Furthermore, decreasing the amount of BJAB DISC did not induce a change in the substrate preference (Figure 5.9). This suggested that, contrary to the literature, the low level of DISC in a Type II cell line does not preferentially cleave Bid. However, I reasoned that it was possible that cleaved caspase-8 released from the DISC might have a different substrate preference. Importantly, this was found not to be the case since Bid was also inefficiently cleaved by recombinant p182/p102 heterotetrameric caspase-8 (Figure 5.9 B lane 12). Thus, compared to Bid, procaspase-3 C163A appears to be a better substrate for both DISC-bound and fully cleaved heterotetrameric caspase-8. These experiments strongly suggest that there is no difference in the substrate preference of a Type I and Type TRAIL II DISC, with both able to cleave procaspase-3 C163A and Bid. Therefore the suggestion that a Type II DISC is not sufficiently active to cleave procaspase-3 and instead preferentially cleaves Bid (Barnhart et al, 2003, Scaffidi et al, 1998) appears to be unfounded. Essentially, lower TRAIL DISC formation in a Type II cell leads to an overall reduction in total
Comparison of TRAIL DISC from Type I and Type II Cells

A - DISC

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B - Substrate Cleavage

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Figure 5.9: Assay of bead-bound TRAIL DISC illustrates that the catalytic activity of a Type I Bjab DISC correlates with the amount of DISC present.

BJAB and Jurkat E6.1 cells were either left untreated or treated with 500 ng/ml of bTRAIL. Cells were incubated for 1 hr at 4°C followed by 25 min at 37°C, lysed in DISC lysis buffer and streptavidin beads added to cleared lysates for overnight capture of DISC. The percentage apoptosis (% PS positive cells) induced in the cell lines, as measured by annexin V/PI staining, is shown in A. Cellular lysate supernatants (A, right panel) were probed to ensure an equal cell numbers were used. DISC isolated from Jurkat E6.1 and unstimulated BJAB cells was split into 4 samples representing that from 50 x 10^6 cells. Bead-bound DISC from TRAIL treated BJAB cells was split into 28 samples, 4 replicates of DISC from an increasing number of cells (0.5 – 15 x 10^6). One set of the replicates was used to investigate the amount of DISC present by Western blot (A, left panel, asterisk indicates residual signal caused by reprobe of the blot). The other replicates were used to assay the cleavage of recombinant procaspase-3 C163A, Bid or IETD.AFC (B). DISC-bound beads, as well as recombinant active caspase-8, were incubated with either procaspase-3 or Bid for 3 hr at 37°C and cleavage determined through Western blot of assay supernatants (B Western blots). Cleavage of IETD.AFC, expressed as IETDase activity, was assayed through measuring the increase in the fluorogenic cleavage product (B graph).
substrate cleavage, rather than a specific loss of procaspase-3 cleavage, which delays the onset of apoptosis.

5.6 Type I BJAB cell line expresses less Bid and more procaspase-3 than a Type II Jurkat E6.1 cell line

The previous section had shown that there was no difference in the endogenous substrate preference of a Type I and Type II TRAIL DISC. If the type of DISC formed did not determine which protein substrates were cleaved, then it was possible that the expression level of potential DISC substrates within the cell lines might be important. To investigate this, whole cell pellets were separated by SDS-PAGE and probed to ascertain the expression levels of Bid, caspase-3 and other key apoptotic proteins (Figure 5.10). Interestingly, it was discovered that the Type II Jurkat cell line expressed significantly more Bid, whilst the Type I BJAB cell expressed significantly more caspase-3 (Figure 5.10 A). The greater abundance of Bid in the Type II Jurkat cell line may cause it to be cleaved more readily than procaspase-3, and thus substrate availability may in part explain the dependency of this cell line on the intrinsic pathway for the induction of apoptosis.

In line with previous data (Scaffidi et al, 1998) there was no marked difference in the expression of either FADD or caspase-8, therefore this could not be a contributing factor to the lower level of TRAIL DISC formed in Type II cells. However, there were marked differences in the expression levels of XIAP, caspase-9, Bcl-2, Bcl-XL and Bax. The difference in Bax expression was anticipated since Jurkat cells have been previously shown not to express this protein (Brimmell et al, 1998, Karpinich et al, 2006). The lower Bcl-2 expression in the BJAB cell line was most likely substituted for by high expression of Bcl-XL and vice versa in the Jurkat cell line. The higher expression of XIAP in the BJAB cell line was interesting as increased XIAP expression, relative to caspase-3 and Smac, has been suggested to contribute to the Type II phenotype through preventing the activity of caspase-3 (Jost et al, 2009, Sun et al, 2002).

In terms of c-FLIP expression, the Jurkat E6.1 cell line expressed more c-FLIP_{S}, whilst the BJAB cell line expressed a greater amount of c-FLIP_{L}. The greater expression of c-FLIP_{S} in the Jurkat TRAIL DISC could contribute to reduced DISC activity. However, only the cleaved form of c-FLIP_{L} was detected within the Jurkat TRAIL DISC.
Comparison of TRAIL DISC from Type I and Type II Cells

Figure 5.10: Type I BJAB cells express less Bid and more caspase-3 than the Type II Jurkat E6.1 cells.

Cell pellets were solubilised in 1 x sample buffer and an equal number of cells separated by SDS-PAGE. The expression of several key proteins involved in apoptosis was investigated by Western blot. Blots shown are representative of the results obtained from 3 independent sets of cell pellets. The three sections of the c-FLIP blot are from the same exposure, which was cropped to fit all three bands in the figure. The approximately 36 kDa band detected by the c-FLIP antibody may represent an alternative isoform of c-FLIP as discussed in Figure 4.11 and main text.
Comparison of TRAIL DISC from Type I and Type II Cells

(Figure 4.12), indicating that increased expression of c-FLIP\textsubscript{s} did not lead to its recruitment to the DISC. A band of approximately 36 kDa was detected by the c-FLIP antibody in the BJAB cell line (Figure 5.10) and was also detected in the supernatant samples from BJAB and Jurkat E6.1 TRAIL DISCs (Figure 4.11 and Figure 4.12). This may represent an alternative isoform of c-FLIP that, based on the molecular weight of the isoforms deposited in the UniProt database (www.uniprot.org), may either be FLAME-1 beta (isoform 9, 30 kDa), FLAME-1 delta (isoform 10, 34 kDa) or Usurpin gamma (isoform 12, 33 kDa).

5.7 Procaspase-3 is cleaved prior to Bid in Type I BJAB and Type II Jurkat cell lines

The assay experiments had shown that procaspase-3 and Bid were cleaved by both Type I and Type II TRAIL DISC (Figure 5.8 and Figure 5.9). However, it was possible that differences in the availability of substrates (high procaspase-3 expression in BJAB cells, high Bid expression in Jurkat cells, Figure 5.10) could affect which protein was cleaved by the DISC. To investigate this, BJAB and Jurkat cells were treated with TRAIL to investigate the kinetics of procaspase-3 and Bid cleavage. TRAIL treatment of BJAB cells rapidly induced cleavage of caspase-8, -3 and -9 at 30 min, whereas the Type II Jurkat cell line caspase cleavage, which was overall much lower, was not observed until 2 hr post TRAIL addition (Figure 5.11).

The majority of procaspase-9 cleavage observed in the BJAB cells was to p37, which is the subunit produced through cleavage by caspase-3. The p35 subunit, which is generated through the activation of the intrinsic pathway and is an Apaf-1-dependent event, was detected but in lower amounts and later than the p37 subunit (Figure 5.11). Together these observations illustrate the predominance of the extrinsic signalling pathway in the BJAB cells following TRAIL treatment. In the Jurkat cell line similar levels of caspase-9 p35 and p37 were detected following TRAIL treatment suggesting delayed apoptosis induction through both the extrinsic and intrinsic pathways (Figure 5.11).

Interestingly, despite the high level of XIAP (Figure 5.10), TRAIL treatment of the BJAB cell line induced rapid activation of caspase-3. Caspase-3 was cleaved into p20 and rapidly underwent autocatalysis into p19 and p17 subunits. Furthermore, fast activation of caspase-3 was demonstrated by the almost immediate processing of procaspase-9 into the p37 subunit (Figure 5.11). In Jurkat cells, procaspase-3
Figure 5.11: Procaspase-3 is cleaved before Bid in TRAIL treated Type I BJAB and Type II Jurkat cell lines.

BJAB and Jurkat E6.1 cells were treated with 500 ng/ml TRAIL for between 0.5 and 6 hr. Following treatment, cell pellets from were collected and the percentage apoptosis (% PS positive) for each sample was measured by annexin V/PI staining. Cell pellets for each time point were solubilised and an equal number of cells separated by SDS-PAGE. The cleavage of procaspases-8/-3/-9 and Bid was assessed by Western blot.
cleavage was dramatically reduced compared to that in the BJAB cell line.

Caspase-3 was mainly processed to the p19 subunit in the Jurkat E6.1 cell line and the mature p17 subunit was not detected until 4 hr post TRAIL addition (Figure 5.11 lane 11). The inefficient generation of caspase-3 p17 and cleavage of procaspase-9 to p37 in the Jurkat cell line suggested that (at the time points examined here) caspase-3 had not been very efficiently activated by caspase-8 or caspase-9.

The appearance of cleaved Bid was not readily detectable by Western blot of whole cell pellets (data not shown). However, the level of full length Bid rapidly decreased in the BJAB cell line with increasing treatment time and had completely disappeared by the 2 hr time point. The disappearance of full length Bid coincided with increased detection of caspase-9 p35, illustrating that the intrinsic pathway had likely been activated (Figure 5.11 lane 4). In the Type II Jurkat cell line, Bid cleavage was not easily visualised, although there did seem to be a slight decrease in full length Bid after 6 hr of TRAIL treatment (Figure 5.11 lane 12). The detection of some p35 caspase-9 in the Jurkat cell line 2 hr after TRAIL addition (Figure 5.11 lane 10) implies a low level activation of the intrinsic pathway by undetectable levels of cleaved Bid.

When comparing the kinetics of procaspase-3 and Bid cleavage, it appeared that procaspase-3 was cleaved prior to Bid in both the Type I BJAB and Type II Jurkat cell lines (Figure 5.11). Thus, the substrate preference of Type I and II DISC did not appear to be altered by the overall level of procaspase-3 or Bid expression. In the BJAB cell line, procaspase-3 cleavage was first detected at 30 min whilst Bid cleavage was not detected until 1 hr. In the Jurkat cell line procaspase-3 cleavage was first visualised at the 1 hr time point, whilst no clear Bid cleavage occurred at the time points investigated here. However, the low level of caspase-3 activity in the Jurkat cell line may provide a sufficient amount of time for cleaved Bid to accumulate (at later time points) and further activate the intrinsic pathway, thus potentially creating some dependency on this amplification loop. Therefore, it is likely that, as also suggested by the data in Section 5.5, there is no particular difference in the substrate preference of a Type I versus a Type II DISC. The Type II DISC has an overall lower catalytic activity, which results in reduced substrate cleavage (compared to the Type I cell line) in the Type II cell line.
Comparison of TRAIL DISC from Type I and Type II Cells

5.8 Discussion
The experiments completed in this chapter have investigated the differences between the TRAIL DISC formed in Type I and Type II cell lines. These experiments have shown that:

1. Type I BJAB cells express more Fas and TRAIL-R1/-R2 on their surface than the Type II Jurkat cell line, which does not express TRAIL-R1.
2. Type I BJAB cells form more DISC in response to both Fas and TRAIL. In the case of TRAIL, this may be related to the expression of TRAIL-R1 and higher expression of TRAIL-R2 at the BJAB cell surface.
3. TRAIL DISC forms faster in a Type I BJAB compared to a Type II Jurkat cell line.
4. TRAIL DISC exists as a high molecular weight complex in both Type I BJAB and Type II Jurkat cell lines. However, the TRAIL-R2 DISC is more highly aggregated than a TRAIL-R1-containing DISC.
5. Type I BJAB and Type II Jurkat TRAIL DISCs are capable of cleaving procaspase-3 and Bid; although procaspase-3 appears to be a better substrate.
6. In Type I BJAB and Type II Jurkat cells exposed to TRAIL procaspase-3 cleavage appears to occur before detectable Bid cleavage.
7. It seems likely that, in a Type II cell line, less DISC formation results in overall less substrate cleavage rather than the previously suggested inability to cleave procaspase-3 (Barnhart et al, 2003, Scaffidi et al, 1998).

5.8.1 The apoptotic TRAIL signalling pathway progresses slower in the Type II Jurkat cell line than the Type I BJAB cell line
Treatment of Type II cells with TRAIL results in a slow rate of apoptosis; DISC formation is both reduced (Figure 5.4) and delayed (Figure 5.5), as is the cleavage of the downstream substrates procaspase-3 and Bid (Figure 5.11). The faster TRAIL-induced cleavage of procaspases-8, -9, -3 as well as Bid in the Type I BJAB cell compared to the Type II Jurkat cell line shown here (Figure 5.11) contrasts with a previous study, which suggested procaspase-3 and Bid were cleaved faster in a Type II cell line than a Type I cell line (Ozoren & El-Deiry, 2002). However, in the latter study the cell line designated as Type I (SW480 cell line) has subsequently, on the basis of Bcl-XL overexpression experiments, been shown to be a Type II cell line (Hasegawa et al, 2007).
Therefore, my experiments, where prototypic Type I and II cell lines have been used, demonstrate that TRAIL (like Fas (Scaffidi et al, 1998)) induces rapid substrate cleavage in a Type I cell line and slower substrate cleavage in a Type II cell line.

5.8.2 Type I and Type II TRAIL DISCs exhibit the same substrate preference

Purified Type I and Type II TRAIL DISC were enzymatically active and cleaved procaspase-3, Bid and IETD.AFC. When TRAIL DISC was isolated from an equal number of cells, the activity of the Jurkat TRAIL DISC against all substrates was lower than that of the BJAB TRAIL DISC (Figure 5.8). However, assay of equivalent amounts of DISC-bound caspase-8 (rather than equivalent cell number) produced similar levels of substrate (procaspase-3 C163A, Bid and IETD.AFC) cleavage (Figure 5.9). Thus, in contrast to previous suggestions (regarding Fas (Barnhart et al, 2003, Scaffidi et al, 1998)) the Type I and Type II TRAIL DISCs do not appear to have a different substrate preference. Instead Type I and Type II DISCs appear to be more active against procaspase-3 than Bid both in vitro (Figure 5.9) and in intact cells (Figure 5.11). Since the pattern of procaspase-3 and Bid cleavage was the same in the two cell lines, despite different expression levels of these proteins (Figure 5.10), it appears as though substrate availability may not be a significant determinant of pathway predominance. The more efficient cleavage of procaspase-3 by all forms of active caspase-8 could potentially be caused by the accessibility of the substrate cleavage site or the specific recognition motif present (IETD in procaspase-3 (Cohen, 1997, Han et al, 1997), LQTD or IEAD in Bid (Li et al, 1998)). The ability of the Type II TRAIL DISC to cleave caspase-3 in the presence of Bid was clearly demonstrated in the TRAIL treated cells. However, in future this could be further confirmed in vitro through simultaneous incubation of purified Type II DISC with procaspase-3 C163A and Bid.

The data in this chapter have shown that the amount of DISC formed determines whether a cell is Type I or Type II, not through altering DISC substrate preference, but by determining the overall level of substrate cleavage (Figure 5.12). Therefore, in a Type I cell substantial DISC formation results in extensive cleavage of procaspase-3, which overcomes the potential for XIAP inhibition (Sun et al, 2002). Furthermore, there is rapid and extensive cleavage of Bid, leading to the activation of the intrinsic pathway, which further aids caspase-3 activation. However, due to the time taken for the intrinsic pathway to activate caspase-9 and the extensive direct cleavage of procaspase-3 by caspase-8, the intrinsic pathway is not required for apoptosis to
Comparison of TRAIL DISC from Type I and Type II Cells

Figure 5.12: A model for Type I versus Type II signalling.

In Type I cells ligation of death receptors causes the formation of a large amount of DISC, which results in abundant caspase-8/-10 activation at the DISC and/or in the cytosol. This results in direct cleavage and activation of a large amount of caspase-3 that, due to the high quantity, cannot be inhibited by XIAP and thus initiates the caspase cascade resulting in apoptosis. Bid is also cleaved in the Type I cell and initiates activation of caspase-9 through the intrinsic pathway. However, due to the time delay in its activation, and the high activity of caspase-8, caspase-9 activated through this pathway aids caspase-3 activation but is not required for apoptosis to proceed. In Type II cells, a lower level of DISC is formed upon death receptor ligation, resulting in reduced activation of caspase-8/-10 and thus reduced caspase-3 and Bid cleavage. The low level of processed caspase-3 in the Type II cell line may be inhibited by XIAP causing apoptosis to progress slowly. During this time cleaved Bid accumulates resulting in activation of the intrinsic pathway, which causes activation of caspase-9 and the release of Smac. This aids caspase-8-mediated caspase-3 activation, resulting in the dependency of Type II cells on the intrinsic pathway.
progress. In a Type II cell line, low DISC formation results in a low level of procaspase-3 and Bid cleavage (Figure 5.12). It has been suggested that the reduced caspase-3 activation in Type II cells makes it vulnerable to inhibition by XIAP and this may partially explain the requirement for the intrinsic pathway (Jost et al., 2009, Sun et al., 2002). The low level of caspase-3 activity (caused by poor activation by DISC activated caspase-8 and possible inhibition by XIAP) delays apoptosis, potentially allowing time for cleaved Bid to accumulate and initiate the intrinsic pathway. The intrinsic pathway is then able to boost caspase-3 activation by releasing mitochondrial localised Smac and cytochrome c, which negate the inhibitory effect of XIAP and activate the apoptosome complex, respectively. Without the activation of the intrinsic pathway apoptosis would still likely progress in a Type II cell but at an even slower rate. This may explain the suggestion that blockade of the intrinsic pathway (via overexpression of Bcl-2/Bcl-XL or loss of caspase-9) only delays, rather than prevents, apoptosis in Type II cells (Belka et al., 2000, Itoh et al., 1993, Suliman et al., 2001).

At the time points investigated here (up to 6 hr) detectable intrinsic pathway activation, as assessed by Bid cleavage and Apaf-1-dependent activation of caspase-9, was still quite low. Therefore, in future it could be worthwhile to treat the Type II Jurkat cells with TRAIL for longer than 6 hr to establish if and when major activation of caspase-9 via the intrinsic pathway occurs. It could also be established whether this, along with the release of Smac, results in increased caspase-3 activity and thus increased apoptosis in Type II cells.

5.8.3 Low TRAIL DISC formation in Type II Jurkat cell line may be the result of the which TRAIL receptor subtypes are expressed

It has been suggested that in a Type II cell line there is a lower level of Fas DISC clustering (Algeciras-Schimnich et al., 2002, Soderstrom et al., 2005), which may affect DISC formation (Figure 5.1). However, upon stimulation with TRAIL, both the Type I BJAB and Type II Jurkat cell lines formed high molecular weight DISC (Figure 5.6). Since the Jurkat cell line forms much less TRAIL DISC than the BJAB cell line (Figure 5.4), it appears as though the amount of TRAIL DISC does not determine the extent of high molecular weight DISC formation or vice versa. The Fas DISC has been estimated to be between 1 and 7 MDa (Feig et al., 2007). However, it is difficult to calculate the size of a complex that migrates outside of known and accurate molecular weight standards (Figure 5.6 and Figure 5.7). Irrespective of this, the sucrose density gradient data did demonstrate that the TRAIL DISC is greater than 700 kDa. This finding concurs with published data that demonstrated a shift in DISC components to
molecular weights of greater than 700 kDa upon fractionation of TRAIL treated cell lysates (Wagner et al, 2007). However, the obvious difference, detected here by sucrose density gradient, in the peak of TRAIL-R1 and TRAIL-R2 within in the BJAB TRAIL DISC (Figure 5.6 A and Figure 5.7 A) was not observed by Wagner et al. This was most likely because the Superdex™ 200 column used in their study can only resolve proteins up to 600 kDa.

The detection of some ligand-bound receptor in the low molecular weight fractions of the BJAB cell line (Figure 5.6 A and Figure 5.7 A) supports the suggestion in Chapter 4 that the stoichiometry of the TRAIL receptor in the BJAB DISC was likely to be skewed. Therefore, sucrose density separation of the high molecular weight TRAIL DISC from the ligand-bound receptor before mass spectrometry analysis would aid a more accurate calculation of DISC stoichiometry and enable the model I proposed for DISC assembly (Figure 4.15) to be refined. Furthermore, purification of TRAIL DISC from high molecular weight fractions could reduce the extent of non-specific protein purification (Figure 4.4 and Figure 4.8) resulting in more confident mass spectrometry identifications of all TRAIL DISC components in the Type II Jurkat cell line.

Jurkat TRAIL DISC was found to be more highly aggregated than BJAB TRAIL DISC (Figure 5.6). However, this appeared to be a property of TRAIL-R2, rather than of the Jurkat cell line, since TRAIL-R2 in the BJAB DISC also peaked in higher molecular weight fractions than TRAIL-R1 (Figure 5.6 A and Figure 5.7 A). Furthermore, specific stimulation of TRAIL-R2 in the BJAB cell line produced a DISC of higher molecular weight than that formed upon stimulation with wildtype TRAIL (Figure 5.7). The discovery that TRAIL-R2 DISC is more aggregated fits with published data that suggested TRAIL-R2 requires more extensive cross-linking for efficient activation, whilst TRAIL-R1 is similarly activated by crosslinked or non-crosslinked TRAIL (Berg et al, 2007, Kelley et al, 2005, Muhlenbeck et al, 2000, Natoni et al, 2007, Wajant et al, 2001). My studies have utilised soluble trimeric TRAIL (Figure 4.1) that, unlike antibody or crosslinked ligand, may not readily facilitate the extensive aggregation of TRAIL-R2 suggested to be required for maximal signalling. Therefore, this could be a contributing factor to the lower DISC formation observed upon stimulation of TRAIL-R2 in both Jurkat and BJAB cell lines (Figure 5.4). In the future it would therefore be interesting to investigate the effect of cross-linked TRAIL on the formation and molecular weight of the Jurkat TRAIL DISC. In addition, sucrose density gradients could be used to determine the aggregation of TRAIL DISC formed in BJAB cells upon
stimulation with TRAIL-R1 specific ligand or in Jurkat cells treated with TRAIL-R2 specific ligand.

A common theme throughout this thesis has been the low level of FADD recruitment to the TRAIL DISC. Only a small proportion of total cellular FADD-TAP was likely associated with the TRAIL DISC (Chapter 3), whilst mass spectrometry could not confidently identify FADD in the Type II Jurkat TRAIL DISC (Chapter 4). In this chapter, TRAIL-R2 (when activated on the surface of Jurkat and BJAB cells) has been shown to be less efficient than TRAIL-R1 at recruiting FADD and thus forming active DISC (Figure 5.4, Figure 5.6 B and Figure 5.7 B). Other studies have also demonstrated that selective ligation of TRAIL-R2 results in reduced TRAIL DISC formation (Sprick et al, 2000, Sprick et al, 2002). Therefore, it is possible that the absence of TRAIL-R1 expression in the Type II Jurkat cell line (Figure 5.3) is responsible for the low formation of DISC upon TRAIL treatment. This could be investigated by inducing significant cell surface expression of TRAIL-R1 in the Jurkat E6.1 cell line and then analysing DISC formation upon TRAIL treatment. However, it should be noted that absence of TRAIL-R1 expression is unlikely to be universally responsible for lower TRAIL DISC formation in Type II cell lines as another prototypic Type II cell line (CEM) has been reported to express both TRAIL-R1 and TRAIL-R2 at the cell surface (Sprick et al, 2000).

The absence of TRAIL-R1 cell surface expression may be a contributing factor to the lower DISC formation of the Jurkat cell line, however the functionality of TRAIL-R2 may also be important. Fractionation of the TRAIL DISC by sucrose density gradient demonstrated that, compared to BJAB, ligand-bound Jurkat TRAIL-R2 was less efficient at aggregating and/or recruiting FADD to form a high molecular weight complex. This was demonstrated by the detection of an extensive amount of ligand-bound TRAIL-R2 in the low molecular weight fractions of the Jurkat cell line (Figure 5.6 B) compared to that detected in the same low molecular weight fractions of the BJAB cell line (Figure 5.6 A and 5.7). This observation may explain why TRAIL DISC forms more slowly in the Jurkat as compared to the BJAB cell line (Figure 5.5) and also why mass spectrometry could not identify a significant association of FADD-TAP with the Jurkat TRAIL DISC (Chapter 3). In this context, it has been suggested that FADD recruitment to the Fas DISC may be reduced in a Type II cell line due to an increased sequestration of FADD by phosphorylated PEA15 (Peacock et al, 2009). While this could potentially explain the reduced recruitment of FADD to Jurkat TRAIL-R2, mass
Comparison of TRAIL DISC from Type I and Type II Cells

spectrometry analysis of purified FADD-TAP (Chapter 3) did not provide evidence to support this suggestion, as no stable interaction with PEA15 was identified.

An alternative explanation for the lower DISC formation in the Jurkat cell line may be related to its lower expression of TRAIL-R2 compared to the BJAB cell line (Figure 5.3). Ionising radiation has been reported to increase the recruitment of FADD to the Jurkat TRAIL DISC. However, the authors concluded this was not solely the result of ionising radiation-induced increases in TRAIL-R2 expression (Verbrugge et al, 2008). Therefore, although the overall availability of cell surface TRAIL-R2 may contribute to the level of TRAIL DISC formed, it may not represent the sole cause of the decreased capacity of Jurkat TRAIL-R2 to recruit FADD. For example, others have suggested that DNA-damaging agents facilitate TRAIL receptor aggregation, possibly involving lipid rafts, to result in the increased DISC formation observed upon treatment with TRAIL (Lacour et al, 2003). Therefore, lipid rafts may play a role in the increased recruitment of FADD to the Jurkat TRAIL DISC observed upon exposure to ionising radiation and TRAIL (Verbrugge et al, 2008).

In addition to the increased FADD recruitment, the TRAIL DISC from irradiated Jurkat cells also contained more caspase-8 and -10. This resulted in increased caspase activation and independence from the mitochondrial amplification loop (Verbrugge et al, 2008). Therefore, in future it would be interesting to investigate the effect of ionising radiation on Jurkat cells to determine how it increases DISC formation. Initially, TRAIL DISC isolated from both control and irradiated cells treated with wildtype or TRAIL-R2 specific ligand could be analysed by sucrose density gradients and mass spectrometry. Together these approaches would allow investigation of the effect of ionising radiation on the formation of high molecular weight DISC as well as alterations in DISC composition and/or stoichiometry. This may help identify factors that regulate the recruitment of FADD to TRAIL-R2 and therefore elucidate why this process is especially inefficient in the Jurkat cell line.

In summary, this chapter has demonstrated that in the Type II Jurkat cell line the absence of TRAIL-R1 and a decreased ability of TRAIL-R2 to aggregate and/or recruit FADD likely combine to result in low TRAIL DISC formation. This causes reduced Bid and procaspase-3 cleavage, slow progression of apoptosis, and ultimately the reported reliance on the intrinsic pathway for maximal caspase-3 activation.
Chapter 6: Final Overview
6 Final Overview

The aim of this thesis was to characterise the initial phase of TRAIL signalling. This has been achieved through purification and mass spectrometry of FADD-TAP as well as TRAIL DISC from Type I and Type II cells. Furthermore, the Type I and Type II TRAIL DISC has been characterised in terms of its formation and functionality. The major findings from this work (Figure 6.1) are discussed below.

The key findings from Chapter 3 were the identification, by mass spectrometry, of the interaction between FADD-TAP and calpain S1 (under control and TRAIL treated conditions) or HSC70 (under TRAIL treated conditions). It was speculated that an interaction between FADD and calpain S1 could result in the modulation of calpain activity and the ability of FADD to perform its apoptotic role. The interaction of FADD-TAP with HSC70 suggests that this protein may be involved in the subcellular redistribution of FADD upon TRAIL stimulation.

In Chapter 4, all known components of the BJAB TRAIL DISC were identified through mass spectrometry. Furthermore, a potential new interaction between TRAIL-R1/-R2 and transferrin receptor was identified in the BJAB (by mass spectrometry and Western blot) and Jurkat E6.1 (by Western blot) cell lines. It was hypothesised that this association may in some way aid the targeting of TRAIL receptors and DISC for internalisation. The mass spectrometry data obtained from the BJAB TRAIL DISC also enabled the calculation of DISC component stoichiometry. This led me to propose a new model for the recruitment of procaspase-8/-10 and/or c-FLIP to the TRAIL DISC (Figure 4.15). In this model, DED protein dimerisation occurs via the small catalytic subunit and the DEDs, resulting in the recruitment of four molecules of procaspase-8/-10/c-FLIP to each molecule of FADD. In other studies, two molecules of FADD have been proposed to self-associate to form a bridge between adjacent complexes (Figure 6.1 DISC 2) to which caspase-8/other DED proteins are recruited (Carrington et al, 2006, Sandu et al, 2006). Based on my proposed model of four DED proteins per FADD (Figure 6.1 DISC 3), eight molecules of caspase-8/other DED proteins would be recruited to each FADD bridge (Figure 6.1 DISC 4).

The data shown in Chapter 5 strongly argued that the level of TRAIL DISC formation is a key determinant of whether a cell exhibits a Type I or Type II phenotype. The Type I/II phenotype does not appear to be the result of preferential cleavage of Bid, and thus preferential activation of the intrinsic pathway, by a Type II DISC but instead is caused
Figure 6.1: Schematic of the major findings of my thesis.

Chapter 3 (orange text) identified a TRAIL-induced association between FADD-TAP and HSC70, which I postulated may aid redistribution of FADD. Furthermore, a constitutive association between FADD-TAP and calpain S1 was identified, which may be involved in as yet identified signalling pathways. Chapter 4 (purple text) identified a potential interaction between transferrin receptor and TRAIL-R1/-R2 that persisted even upon formation of DISC in response to TRAIL treatment. Furthermore, using the mass spectrometry data in a quantitative fashion it was possible to determine that following TRAIL ligation (1), each molecule of FADD, which forms bridges between adjacent complexes with Fas DD (2), likely recruits approximately four DED proteins (3). If correct this would mean that eight DED molecules are recruited to each FADD bridge (4). Chapter 5 (green text) demonstrated that Type I and Type II DISC are both able to cleave procaspsae-3 and Bid and that the dependency of Type II cells on the intrinsic pathway is caused by the lower amount of DISC formed. In the Jurkat Type II cell line, it appeared as though lack of TRAIL-R1 expression and the inefficient recruitment of FADD to TRAIL-R2 may be responsible for lower DISC formation upon TRAIL treatment.
by an overall reduction in substrate cleavage. The result is a slow activation of apoptosis in Type II cells, allowing time for the intrinsic pathway to be activated. This amplification loop aids caspase-3 activation through the activation of caspase-9 and the relief of XIAP inhibition by the release of Smac. In the Type II Jurkat cell line, the lower level of DISC formation may be related to the absence of TRAIL-R1 expression and/or the inefficient recruitment of FADD to TRAIL-R2.

As has been discussed throughout this thesis, the data shown here have opened new avenues for future research. However, I will now summarise some of the key experiments that should be completed in the near future.

1. The presence of calpain S1 and HSC70 in TAP purified samples should be confirmed by Western blot. This would increase confidence in the validity of the interactions identified by mass spectrometry and justify further investigation into ascertaining the nature of these interactions.

2. It is crucial to confirm a direct interaction between transferrin receptor and TRAIL-R1/-R2. This is to ensure that the interaction observed was not the result of co-purification of these molecules from cellular lysates. This could be achieved through immunostaining the receptors for confocal imaging and/or through the expression of fluorescent tagged transferrin and TRAIL receptors for fluorescence resonance energy transfer (FRET) studies.

3. My proposed model for DED protein recruitment to the DISC could be tested using the in vitro DISC reconstitution method (Figure 3.3 and Hughes et al, 2009). Mutations could be made in recombinant procaspase-8/-10/c-FLIP to prevent their dimerisation through the small catalytic subunit and/or DEDs. The recruitment of these mutant DED proteins to the in vitro DISC could then be assessed and if found to be reduced would support my suggestion that three additional DED proteins are recruited to the DISC through self-association (Figure 4.15).

4. Characterisation of the TRAIL DISC formed in a TRAIL-R1 transfected Jurkat E6.1 cell line could confirm whether its absence reduces DISC formation in this Type II cell line. If my suggestion is correct then expression of TRAIL-R1 in the Jurkat cell line should increase DISC formation and result in a more Type I-like phenotype.

5. Characterisation of the DISC formed in irradiated Jurkat E6.1 cells upon treatment with TRAIL (wildtype or TRAIL-R2 specific) could help identify the reason for the inefficient recruitment of FADD to TRAIL-R2.
Appendices
Appendix 1: FADD-TAP expression did not alter the expression of TRAIL receptors in the FADD-TAP D10 cell line.

Wildtype, FADD null and FADD-TAP (FT) D10 cells were incubated with either goat serum alone (no antibody) or with goat serum containing PE-conjugated isotype control or TRAIL receptor specific antibody. After washing with PBS the fluorescence intensity was analysed by flow cytometry. The numbers represent the mean fluorescence intensity (geometric mean) with the SEM (n=3).
Appendix 2: The 22 proteins identified through analysis of the mass spectrometry data from large scale Jurkat E6.1 TRAIL DISC purifications.

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Proteins were identified by mass spectrometry and analysed as described in the Materials and Methods. Data represents best identification from the 2 untreated controls or 3 bTRAIL treated samples. For those proteins identified by multiple peptides the number of spectra, the protein probability, the cleavage status and number of unique peptides are given. For those proteins identified by a single peptide additional information on the peptide sequence and Mascot ion score are provided. UD, unable to determine position in SDS-PAGE gel; TH, protein higher than expected molecular weight in SDS-PAGE gel.
### Appendix 3: The 52 proteins identified through analysis of the mass spectrometry data from large scale BJAB TRAIL DISC purifications.

<table>
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<th>Spot</th>
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Proteins were identified by mass spectrometry and analysed as described in the Materials and Methods. Data represents best identification from the 3 untreated controls or 3 bTRAIL treated samples. For those proteins identified by multiple peptides the number of spectra, the protein probability, the cleavage status and number of unique peptides are given. For those proteins identified by a single peptide additional information on the peptide sequence and Mascot ion score are provided. UD, unable to determine position in SDS-PAGE gel; TH, protein higher than expected molecular weight in SDS-PAGE gel.
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