# Characterisation of the Role of the NEDD8 E3 Ligase DCNL5 in the Apoptosis Response

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# Abstract

Defective in cullin neddylation-like (DCNL) proteins are known to coordinate the addition of NEDD8 to the cullin subunit of the largest family of ubiquitin E3 ligases, the Cullin-RING ligases (CRLs), in a process known as neddylation. The human genome encodes five DCNL proteins which are thought to exhibit a large degree of overlap in function, with only a few neddylation processes having been definitively ascribed to a single DCNL homologue. It currently remains unclear whether these DCNL proteins have functions that extend beyond their roles in cullin neddylation. In the present study we now describe a novel role for one of the family members, DCNL5, in the programmed cell death response known as apoptosis. We have shown that cells lacking DCNL5 function fail to promote caspase 8 cleavage – an important early activation step - in response to various inducers of the extrinsic apoptosis pathway.

Caspase 8 cleavage and activation requires polyubiquitination which is known to be mediated by cullin 3 in coordination with the dual ubiquitin and NEDD8 ligase RBX1. This process is thought to occur in lipid rafts at the plasma membrane and in the cytosol. In the present work, we provide the first indication that DCNL5 is able to translocate out of the nucleus where it was previously thought to be exclusively located, and this occurs in response to TNFα-related apoptosis-inducing ligand (TRAIL) stimulation. In addition, we present evidence for the first known interaction between DCNL5 and cullin 3 in U2OS cells under endogenous conditions. The DCNL5 KO cells demonstrated a lack of a polyubiquitination event that occurs in WT cells; unmodified caspase 8 was shown to associate with a polyubiquitinated protein (the identity of which we were unable to determine) in response to TRAIL and this interaction was absent in KO cells, perhaps representing the key mechanism underlying DCNL5 involvement.

This emerging function for DCNL5 in promoting caspase 8 cleavage was confirmed in multiple cancer cell lines including U2OS, H460 and HeLa cells. Importantly, we demonstrated that siRNA-mediated silencing of DCNL5 prevented CASP8 cleavage. A lot of our work suggested that DCNL5's role in CASP8 activation is mediated by the cullin CUL3. However, treatment with the neddylation inhibitor MLN4924

caused a reduction, but not a total loss of caspase 8 cleavage, suggesting that if cullin 3 is involved, it may be independent of its neddylation status. This hints at the surprising possibility that a CRL complex exists that that does not require neddylation for some of its function. Furthermore, while our data suggests that DCNL5 may regulate apoptosis via cullin 3, we were unable to exclude a cullin neddylation-independent role for DCNL5 in this process. Future work will need to answer this question by identifying and characterising the molecular target of DCNL5 in apoptosis signalling to ascertain the precise mechanism underlying DCNL5 regulation of this clinically important signalling event.

# Table of Contents

# List of Tables

# List of Figures

# Acknowledgements

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Thank you all,

Ollie

# Author's Declaration

I declare that the work presented in this thesis is my own, unless otherwise cited or acknowledged. It is entirely of my own composition and has not, in whole or in part, been submitted for any other degree.

Oliver Hsia

May 2020

# Abbreviations

| | |
|---|---|
| α | Alpha |
| ß | Beta |
| κ | Kilo |
| m | Milli |
| µ | Micro |
| n | Nano |
| ° | Degrees |
| APAF-1 | Apoptosis protease activating factor 1 |
| APC | Anaphase promoting complex |
| ATG8 | Autophagy-related protein 8 |
| ATP | Adenosine triphosphate |
| BID | BH3-interacting death domain agonist |
| CAND1 | Cullin-associated NEDD8-dissociated protein 1 |
| CARD | Caspase recruitment domain |
| Cdc53 | Cell division control protein 53 |
| cFLIP | Cellular FLICE-like inhibitory protein |
| CHD | Cullin homology domain |

CHX Cycloheximide

cIAP Cellular inhibitor of apoptosis

COP9 Constitutive photomorphogenesis 9

CRL Cullin RING ligase

CSN COP9 signalosome

Dcn1 Defective in cullin neddylation

DcR Decoy receptor

DCUN1D1/DCNL1 Defective in cullin neddylation-like protein

DED Death effector domain

dIP Denaturing immunoprecipitation

DISC Death inducing signalling complex

DMEM Dulbecco's modified Eagle's medium

DMSO Dimethyl sulphoxide

DNA Deoxyribonucleic acid

Dox Doxycycline

DR Death receptor

FACS Fluorescence activated cell sorting

FADD Fas-associated death domain protein

FBS Foetal bovine serum

GFP Green fluorescent protein

H460 Lung carcinoma cells

HECT Homologous to the E6-AP carboxyl terminus

IAP Inhibitor of Apoptosis

IL- Interleukin

IP Immunoprecipitation

ISG15 Interferon-stimulated gene 15

IκBα Inhibitor of κB

JNK c-Jun N-terminal kinase

KO Knock out

MAPK Mitogen activated protein kinases

MDM2 Mouse double minute 2 homologue

NAE NEDD8 activating enzyme

NEDD8 Neural precursor cell expressed developmentally down-regulated protein 8

NEDP1 (DEN1) De-neddylase 1

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells

P/S Penicillin streptomycin

PARP1 Poly-(ADP ribose) polymerase 1

PBS Phosphate buffered saline

PI Proteasome Inhibitor

PKC Protein kinase C

PONY Potentiating neddylation domain

RBR RING between RING finger

RING Really interesting new gene

RIP Receptor-interacting protein kinase

RNA Ribonucleic acid

SB Sample buffer

SCCRO Squamous cell carcinoma-related oncogene

SCF Skp-cullin-F-box

siRNA Small interfering ribonucleic acid

SMURF1 SMAD- ubiquitin regulatory factor 1

SUMO Small ubiquitin-like modifier

TBS-T Tris buffered saline + Tween 20

TBS-T Tris buffered saline

TEMEDN, N, N', N'-tetramethylenediamine

Tet Tetracycline

TGF-β Transforming growth factor beta

TNFR TNF receptor associated factor

TNFα Tumour necrosis factor alpha

TRADD TNFR-associated death domain protein

TRAF TNF receptor associated factor

TRAIL TNFα-related apoptosis inducing ligand

TRIM13 Tripartite motif containing 13

TUBE Tandem ubiquitin binding entities

U2OS Bone osteosarcoma cell line

UAE Ubiquitin activating enzyme

Ub Ubiquitin

UBA Ubiquitin associated domain

UBD Ubiquitin binding domain

UCHL3 Ubiquitin C-terminal hydrolase isozyme 3

UIM Ubiquitin interacting motif

UPS Ubiquitin proteasome system

WT Wild type

XIAP X-linked inhibitor of apoptosis

ß-TrCP Beta-transducing repeat containing E3 ubiquitin protein ligase

# 1 Introduction

## 1.1 Ubiquitin

Ubiquitin is a small signalling protein that was first discovered in 1975 and consists of 76 amino acids (Schlesinger, Goldstein and Niall, 1975) and is pivotal in a variety of cellular processes including the universal degradation pathway of living organisms which is its best studied role. Ubiquitin was initially described as a modifier of proteins which targeted them for degradation by the 26S proteasome (Hershko and Ciechanover, 1998) but has since been shown to have a role in a wide array of processes. Ubiquitin is highly conserved and expressed in most tissues in eukaryotes and acts predominantly through its association with other proteins via specific isopeptide linkages on lysine residues. The small protein possesses a C-terminal di-glycine motif which is exposed prior to conjugation by processing enzymes: these enzymes modify inactive ubiquitin precursors which is required for subsequent addition of ubiquitin to target proteins (Amerik and Hochstrasser, 2004). Ubiquitin is added to target lysine (K) residues in substrates or to one of 7 lysine's present internally within ubiquitin molecules (Peng *et al.*, 2003; Haglund and Dikic, 2005) to form polyubiquitin chains and these chains are summarised in Figure 1-1 (Rudolph *et al.*, 2001; Pickart and Fushman, 2004).

The lysine residue and the number of ubiquitin molecules added to each residue on a target protein determines its fate, which can vary from degradation by the 26S proteasome via polyubiquitin chains on K48 (Chau *et al.*, 1989; Thrower, 2000) and K11 (Meyer and Rape, 2014) to endocytosis-mediating sorting through the use of multi mono-ubiquitination on various lysines (Nakatsu *et al.*, 2000; Raiborg *et al.*, 2002). Another more recently discovered type of ubiquitin chain is the branched polyubiquitin modification. These chains were discovered by proteomic analyses which found that ubiquitin molecules within a polyubiquitin chain were capable of being modified at two or more lysine residues (Hyoung *et al.*, 2007). Physiologically, these branched chains have been shown to play roles in enhancing protein degradation by K11 branched chains (Meyer and Rape, 2014) and K48/K63 branched chains (Liang *et al.*, 2018) as well as roles in regulating NF-κB signalling (Ohtake *et al.*, 2016). Some of these types of ubiquitin modifications are shown below in Figure 1-1.

An additional level of regulation in ubiquitin biology includes the post-translational modifications of ubiquitin molecules themselves. Ubiquitin has been shown to undergo acetylation at lysine residues K6 and K48 which inhibited K11, K48 and K63-mediated polyubiquitination while separately stabilising the monoubiquitination of the histone H2B which was identified as an endogenous substrate for acetylated ubiquitin (Ohtake *et al.*, 2015). Ubiquitin and the ubiquitination process is also regulated phosphorylation. Phosphorylation at serine 65 arises due to oxidative stress and this modification of ubiquitin causes an accumulation of polyubiquitinated proteins and a global reduction in protein turnover rates in *Saccharomyces cerevisiae* (Swaney, Rodríguez-Mias and Villén, 2015). The mitochondrial serine/threonine kinase PINK1 also mediates the phosphorylation of ubiquitin at the same serine residue mentioned above, as well as the ubiquitin-like domain of the E3 ubiquitin ligase parkin, which serves to relieve its autoinhibition via an allosteric mechanism which allows parkin to promote degradation of depolarized mitochondria (Okatsu *et al.*, 2018). Overall, there exists significant complexity in ubiquitin biology and thus many layers of regulation for the process of ubiquitination.

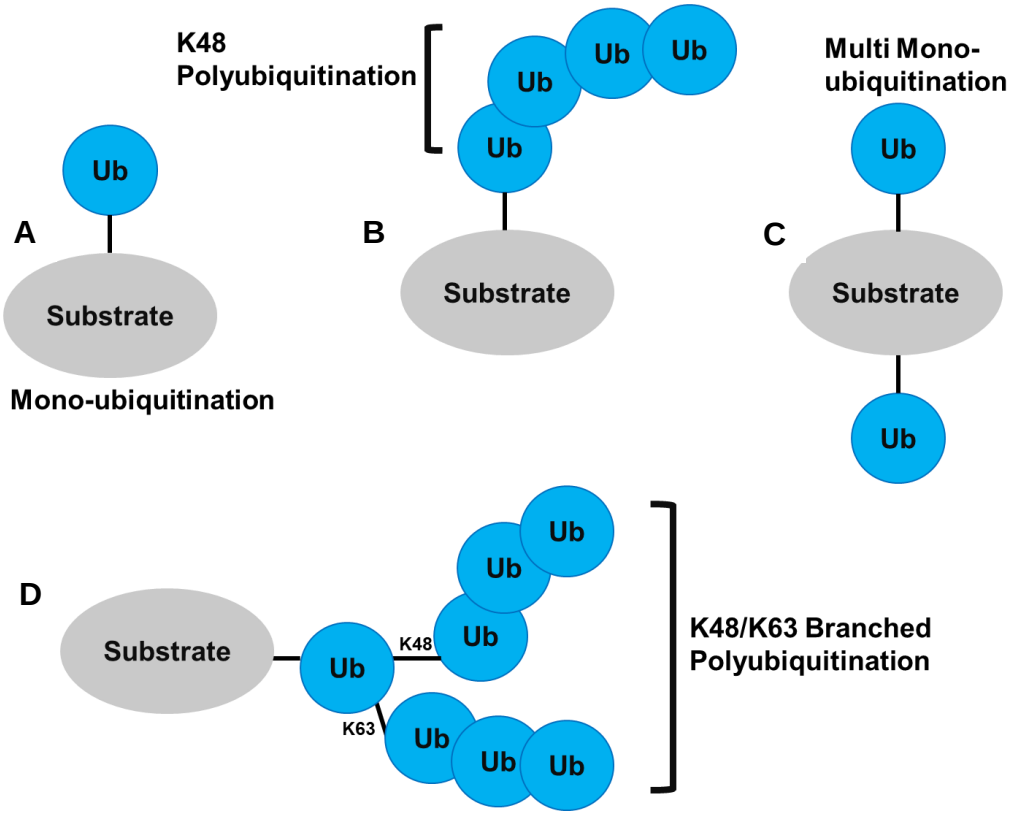



**Figure 1-1 Types of Ubiquitination.**

(A) Mono-ubiquitination involves the covalent linkage of a single ubiquitin molecule to a target lysine residue of a substrate via an iso-peptide bond. (B) Polyubiquitination involves the addition of multiple ubiquitin molecules which are linked internally via their N-terminal lysine residues. The length of the chain can vary from 2 molecules to more than 10, and these residues can be homogenous in the lysine linkage or heterogenous. (C) Multi mono-ubiquitination involves the addition of individual ubiquitin molecules at 2 or more different residues on a target protein. (D) Branched polyubiquitination involves the formation of chains stemming from multiple lysine residues within a ubiquitin molecule and can be homogenous or heterogenous in the lysine linkages.

### 1.1.1 The Ubiquitin Proteome System (UPS)

As already mentioned, one of the key roles of ubiquitination is to target proteins for degradation via the 26S proteasome. The ubiquitin proteasome system (UPS) is vital in maintaining the correct balance of proteins within a cell which in turn controls signalling pathways, feedback loops and other regulatory mechanisms. The UPS begins with the addition of ubiquitin to target proteins using the E1/E2/E3 enzymes described in section 1.1.2, and in cases where proteins have been targeted for degradation, this culminates in recognition of the ubiquitin signals which has shown to require at least 4 ubiquitin molecules in a chain (Thrower, 2000) and the subsequent degradation by a large intra-cellular protease called 26S

proteasome. Proteins are broken down into peptides in the proteasome before these are then degraded down further into the constituent amino acids by peptidases in the cytosol. Addition of ubiquitin is an ATP dependent process as is the breakdown of proteins within the 26S proteasome. An overview of the UPS is shown in Figure 1-2.

The 26S proteasome consists of a central 20S complex which is flanked on either end by two 19S caps which recognize ubiquitinated proteins and process them for degradation by the 20S core complex (Baumeister *et al.*, 1998). The core of the complex is a cylindrically shaped structure possessing caspase, trypsin and chymotrypsin-like proteolytic activity (Bedford *et al.*, 2010) and consists of two outer α rings and two inner ß rings, with each one containing three individual proteolytic subunits (ß5, ß2 and ß1) (Ciechanover, 2005). The 19S regulatory particle can be divided into two further sub-complexes consisting of the lid and the base which cap the ends of the 20S core particle. The lid contains 9 proteins including the de-ubiquitinating enzyme Rpn11 (Lander *et al.*, 2012) while the base consists of a ring of AAA-ATPase family members (Baumeister *et al.*, 1998). These ATPases generate mechanical force through cycles of ATP binding and hydrolysis to unfold proteins, open the gated chamber of the 20S core and translocate the polypeptides to the catalytic core for further degradation (Bar-nun and Glickman, 2012).

Proteasome inhibitors (PIs) have become an effective therapeutic strategy for treatment of diseases, most notably multiple myeloma (MM) and other lymphomas, as well as being predicted to be useful in treating conditions such as atherosclerosis and diabetes given their reliance on finely-tuned proteasome function (Thibaudeau and Smith, 2019). PIs represent a key axiom of MM treatment in all phases (Moreau *et al.*, 2012) and there are a few key drugs that are used routinely, including the reversibly binding peptide-like molecule bortezimib, the second generation irreversible binding peptide-like molecule carfilzomib and the orally available ixazomib (Merin and Kelly, 2014; Besse *et al.*, 2019).

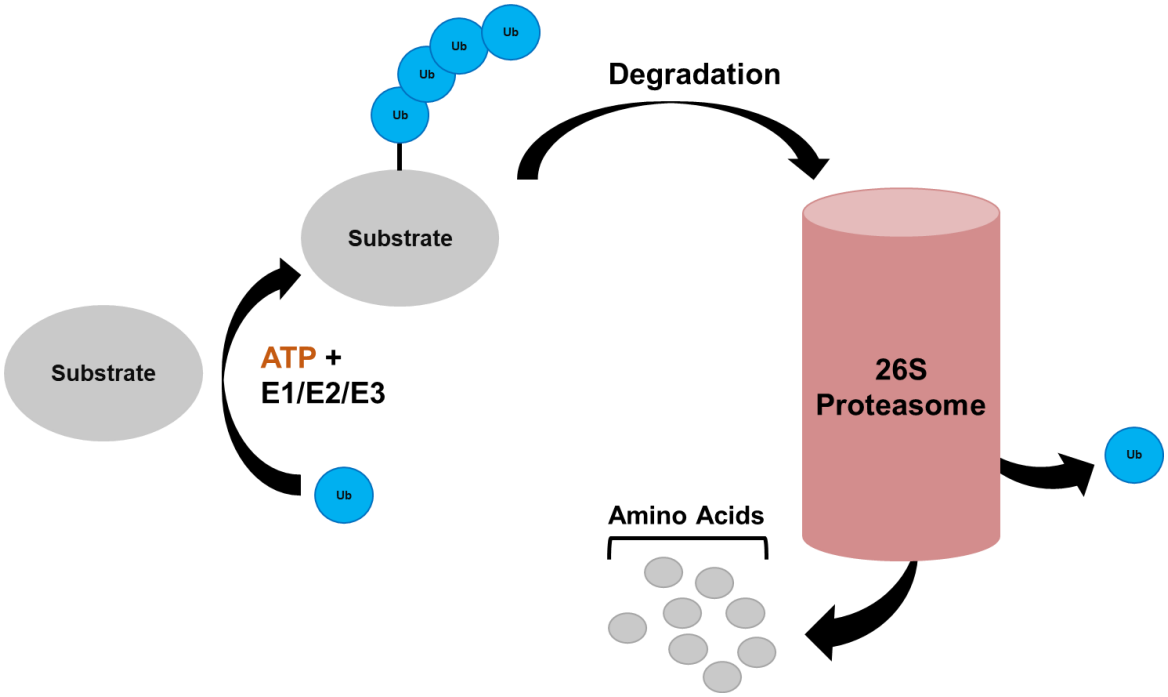



**Figure 1-2 The Ubiquitin Proteasome System Overview.**
Proteins are targeted for degradation in an ATP-dependent manner using the sequential actions of E1, E2 and E3 enzymes. Proteins that are targeted for degradation, typically via polyubiquitin chains on lysines 11 or 48, are then transported to the 26S proteasome by chaperone proteins and degraded in a multistep and ATP-dependent process in the caps and core cylinder of the proteasome. Proteins are broken down into peptides in the proteasome and are then further degraded in the cytosol to individual amino acids.

### 1.1.2 Ubiquitination Machinery

The process of ubiquitination is mediated by 3 classes of proteins which act sequentially and cooperatively following initial priming by processing enzymes (Larsen, Krantz and Wilkinson, 1998): E1 (ubiquitin activating), E2 (ubiquitin conjugating) and E3 (ubiquitin ligase) enzymes. The E3 enzymes are the most numerous (600 proteins) and thus convey a higher degree of specificity and accuracy for a target than E2 or E1 enzymes. There are approximately 40 E2 enzymes while there are only two E1 enzymes in cells called UBA1 and UBA6 (Hershko and Ciechanover, 1998; Pelzer *et al.*, 2007; Jin *et al.*, 2007; Schulman and Wade Harper, 2009). The E1 enzyme uses ATP to adenylate ubiquitin rendering it highly reactive (Haas and Rose, 1982), before transferring ubiquitin to the sulfhydryl group of an E2 enzyme (Stewart *et al.*, 2016) in a transthiolation reaction. An E3 ligase enzyme then mediates the binding of the C-terminal amide moiety of ubiquitin to the ε-amino group of the target protein's lysine residue(s)

via an isopeptide bond (Hershko and Ciechanover, 1998; Soucy *et al.*, 2010). Repeated rounds of this process can occur in order to form polyubiquitin chains via one of the 7 lysine residues contained within the ubiquitin amino acid sequence or via the N-terminus (Ciechanover and Ben-Saadon, 2004).

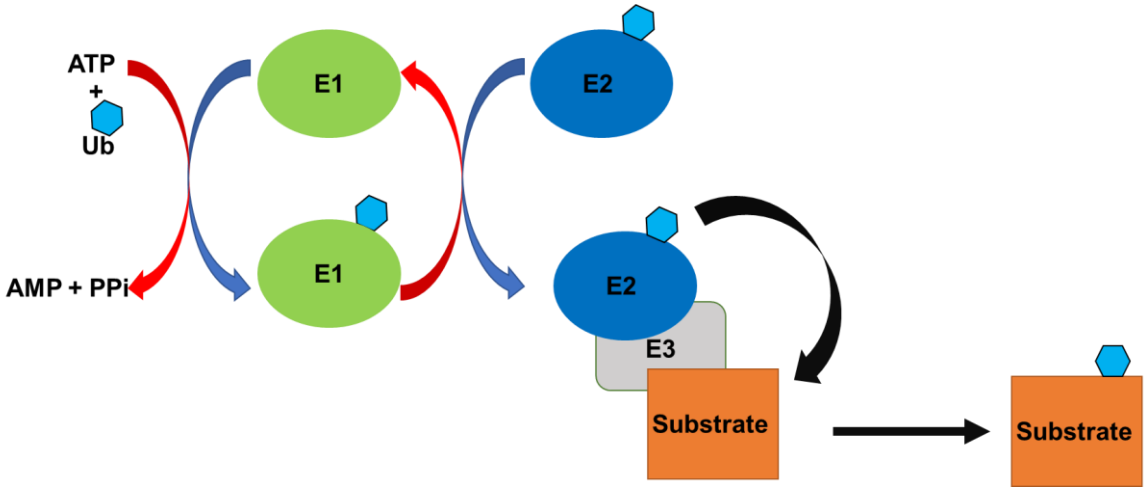



**Figure 1-3 The Ubiquitination Process.**

This diagram outlines the core components and steps required in the ubiquitination of a target substrate. Briefly, ubiquitin is activated in an ATP dependent fashion by the ubiquitin activating enzyme (E1) before being transferred to the sulfhydryl group of an E2 enzyme by a thioester linkage. The mechanism of transfer of ubiquitin to a target substrate with the assistance of an E3 enzyme depends on the family of E3 enzyme in question. In all cases, the E3 enzyme, which is highly specific, binds to the E2 enzyme laden with ubiquitin and mediates the transfer of the molecule to the target protein in either a scaffold capacity (RING E3s) or as an active enzyme catalysing the process (HECT E3s).

### 1.1.3 Ubiquitin E3 Enzymes

Ubiquitin E3 enzymes are the most abundant of the enzymes responsible for the ubiquitination process; they can interact with both the target protein and the ubiquitin-charged E2 enzyme. E3 enzymes are critical in the targeting of specific proteins for ubiquitination and the assembly of structures which allow this process. There are 2 major and well characterized categories of E3 enzymes, along with a third, more recently discovered variant. The 2 more prominent categories are characterized by their core protein domains: Homologous to the E6-AP Carboxyl Terminus (HECT) (Rotin and Kumar, 2009) and Really Interesting New Gene (RING)-based E3 ligases (Petroski and Deshaies, 2005). While their differences are numerous, the fundamental mechanistic contrast between the two

families is the HECT domain has catalytic activity and forms a thioester bond with ubiquitin before transferring it to the target substrate (Scheffner *et al.*, 1993), while the RING domain proteins act as a platform and recruit the E2 enzyme to a target substrate to directly mediate the transfer of ubiquitin from the E2 (Jackson *et al.*, 2000).

The third category is known as RING between RING fingers (RBRs), and these complexes have both RING domains as well as an active site-containing cysteine, and includes the E3 ligase Parkin (Eisenhaber *et al.*, 2007; Riley *et al.*, 2013). Briefly, RBR E3s were initially defined based on a predicted tripartite motif of three zinc-binding domains consisting of two RING domains linked via an in-between RING (IBR) domain (Morett and Bork, 1999; Van Der Reijden *et al.*, 1999). RBRs were later shown to feature an active site cysteine within the second RING domain which differentiated them from RING E3 ligases (Wenzel *et al.*, 2011). The first RING domain mediates the interaction with the ubiquitin-charged E2 enzyme, which then transfers the ubiquitin molecule to the catalytic cysteine of the second RING domain and from there ubiquitin is added to a target substrate (Walden and Rittinger, 2018).

### 1.1.4 Cullin RING Ligases

The most abundant variant of the ubiquitin E3 enzymes is a family of proteins known as cullin-RING ligases (CRLs). CRLs are a protein complex constructed around a small RING-finger protein (RBX1/2) and a cullin protein (of which there are 8 in mammalian cells). Cullin proteins are described in more detail in section 1.2.3. An example of a CRLs is the SCF complex (Skp1–Cullin–F-box) which is involved in ubiquitination of proteins involved in the regulation of the cell cycle while the anaphase promoting complex (APC) is a cullin-like RING-ligase complex which also regulates the cell cycle in a collaborative manner with the SCF complex (Peters, 1998). The core components of the SCF complex include the RING protein Rbx1, Cullin 1, Skp1 and an F-box protein, the latter being involved in recruiting and mediating substrate specificity (Zheng *et al.*, 2002). The structural arrangement of these components to form the SCF complex is shown below in Figure 1-4. The cullin protein and RING protein form the cores of CRLs, while the substrate receptor is formed of the protein known as F-BOX and this interacts with

SKP1 which is an adaptor protein. Substrate receptors vary for different cullin proteins and therefore for different CRL complexes (Lydeard, Schulman and Harper, 2013). CRL complexes thus are structurally similar but have different functions which is dictated by the substrate adaptors that in turn impart the ability of these complexes to regulate numerous intracellular targets.

CRL complexes require modification by the ubiquitin-like protein NEDD8 (neural precursor cell expressed, developmentally downregulated 8), which is added in a reversible reaction to a highly conserved lysine residue in all cullin proteins (Hori *et al.*, 1999). While the APC mentioned above is technically a CRL, it is unique in not being regulated by neddylation since the core of the APC, the APC2 subunit is homologous to cullin 1 (Zheng *et al.*, 2002) but is not neddylated (Pan *et al.*, 2004). Neddylation allows a structural rearrangement of the RING protein which favours the addition of ubiquitin to target substrates, while inhibiting the interaction with CAND1, an exchange factor required for recruitment of new substrate adaptors to the CRL core (Duda *et al.*, 2008; Baek *et al.*, 2020). This modification of the cullin core of CRLs is essential for their activity and ability to mediate ubiquitination. Further details about the neddylation process and pathway are described in section 1.2.

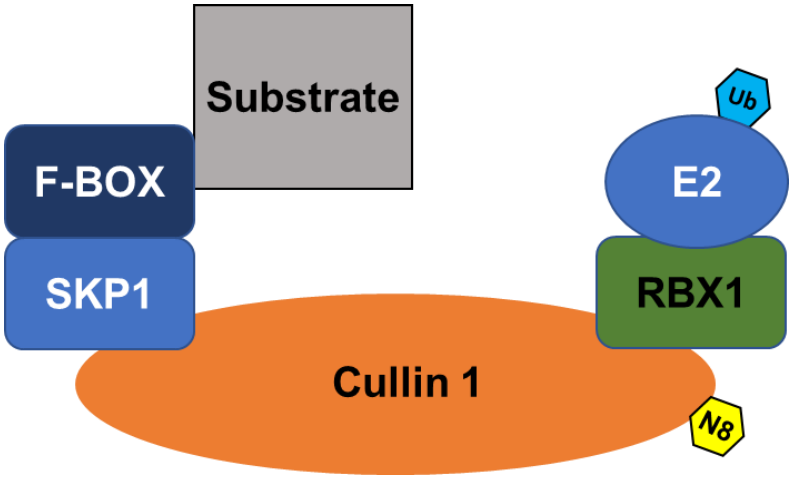



**Figure 1-4 Components of the SCF Complex.**

The SCF complex is an example of a cullin-RING ligase which is the largest family of ubiquitin E3 ligases. Shown in this schematic diagram is the arrangement of the core components. Cullin 1 is bound at its C-terminus to the RING protein RBX 1, which interacts with a ubiquitin-laden E2 enzyme via its C terminal domain. The N-terminal domain of cullin 1 interacts with the substrate receptor SKP1 which in turn is bound to the substrate receptor-binding protein F-BOX. Cullin 1 is neddylated which is required for its catalytic activity as cores of CRL complexes.

## 1.2 NEDD8

### 1.2.1 Structure and Function

There are other proteins which are structurally related to ubiquitin and have similar functions and are known as ubiquitin-like proteins. These include SUMO (small ubiquitin-like modifier), NEDD8 (neural precursor cell expressed, developmentally downregulated 8) interferon-stimulated gene 15 (ISG15), FAT10 and autophagy-related protein 8 (ATG8) (van der Veen and Ploegh, 2012) and many more; there are roughly 20 ubiquitin-like proteins. The focus of this thesis however is on NEDD8. NEDD8 was discovered in the early 1990's and was later characterized and shown to be 60% identical and 80% homologous to ubiquitin (Kamitani *et al.*, 1997). Both Ubiquitin and NEDD8 require processing to expose a di-glycine motif which ultimately becomes covalently linked to the specific lysine(s) in a target protein (Gong and Yeh, 1999a; Walden, Podgorski and Schulman, 2003; Shen *et al.*, 2005; Reverter *et al.*, 2005). The process of addition of NEDD8 to target proteins is known as neddylation and bears significant similarity to the process of ubiquitination. NEDD8 is added to lysine residues in target proteins via an iso-

peptide bond (Enchev, Schulman and Peter, 2015), like with ubiquitin, only the addition of NEDD8 does not target proteins for degradation. Another difference is that neddylation typically occurs as monomers rather than as chains like with ubiquitin, however recent data has shown that there may be a role for NEDD8 outside of its monomeric addition to lysines. Unanchored trimeric, acetylated NEDD8 has been recently shown to attenuate PARP1 activation following the initiation of oxidative stress, which likely delays the initiation of PARP1-dependent cell death (Keuss *et al.*, 2019).

The key and best characterised role of neddylation is to induce a structural rearrangement of cullin proteins (Duda *et al.*, 2008; Baek *et al.*, 2020), which as previously described, form the cores of the ubiquitin E3 ligases family of CRLs. Thus, NEDD8 is a key regulator of the processes including cell cycle progression through the activation of complexes such as the SCF. Cullins and their role as NEDD8 targets are described in 1.2.3. NEDD8 is thought to predominantly act through mono-neddylation (for reasons presently not fully understood) of one or multiple lysine residues in a target protein (which are predominantly cullin proteins), however there are *in vitro* instances of hyper-neddylation (which includes formation of NEDD8 chains) of cullins (Ohki *et al.*, 2009) in addition to the aforementioned role of free, trimeric NEDD8. Neddylation has been implicated in diseases such as Alzheimer's for its role in regulating CRL activity (Chen, Neve and Liu, 2012) and cardiac biology (Kandala, Kim and Su, 2014) and more recently has been identified as a potential therapy target in small intestinal neuroendocrine tumours (Fotouhi *et al.*, 2019) highlighting the physiological importance of this process and the overall impact understanding the role of NEDD8 is in therapy. Currently, the physiological role of NEDD8 in disease is its regulation of the largest family of ubiquitin E3 ligases and this underlines the importance of studying the neddylation process.

### 1.2.2 NEDD8 Machinery

Like ubiquitin, NEDD8 is added to its target via 3 proteins, known as NEDD8 E1, E2 and E3 enzymes (Gong and Yeh, 1999a; Kurz *et al.*, 2005b). While this subset of enzymes is specific for NEDD8, the E3 enzymes also catalyse the ligation of ubiquitin and are thus also ubiquitin E3 ligases (Enchev, Schulman and Peter,

2015). The process of neddylation (outlined in Figure 1-5) involves NEDD8 activating enzyme (NAE), one of two E2 enzymes (UBC12/UBE2M and UBE2F), one of either RING-box proteins 1 or 2 (RBX1/2) which are NEDD8 E3s as well as ubiquitin E3s. This process is aided by proteins known as defective in cullin neddylation 1 domain containing protein (DCUN1D1-5 but also known as DCNL proteins 1-5, which they will be referred to henceforward) (Enchev, Schulman and Peter, 2015; Gong and Yeh, 1999b; Soucy *et al.*, 2009; Walden, Podgorski and Schulman, 2003; Gong and Yeh, 1999a).

NEDD8 requires processing by de-neddylase enzymes including de-neddylase 1 (DEN1 but also referred to as NEDP1) which is a NEDD8-specific cysteine protease that cleaves 5 amino acid residues from its C-terminal end to form what is known as mature NEDD8 (Wu *et al.*, 2003) and ubiquitin C-terminal hydrolase isozyme 3 (UCHL3) which is a dual ubiquitin and NEDD8 protease (Wada *et al.*, 1998). NEDP1 was initially described as de-neddylating SCF complexes (Mendoza *et al.*, 2003) but has since been shown to be responsible for the de-neddylation of non-cullin proteins in cells but capable of cullin de-neddylation *in vitro* (Chan *et al.*, 2008). UCHL3 and NEDP1 have been shown to demonstrate overlap in function *in vivo* since lack of either protein does not result in neddylation defects (Kurihara *et al.*, 2000; Chan *et al.*, 2008). The COP9 signalosome complex subunit 5 (CSN5) is the major enzyme responsible for cullin de-neddylation in cells (Cope *et al.*, 2002) and is discussed in more detail later in this report.

The E1 enzyme NAE consists of two proteins which form a hetero-dimer, APPBP1, the regulatory subunit and UBA3, the catalytic subunit (Osaka *et al.*, 1998; Gong and Yeh, 1999a; Walden, Podgorski and Schulman, 2003) and NAE is structurally and functionally related to the ubiquitin E1 UAE (Soucy *et al.*, 2010) and the fact that NAE consist of two proteins while UAE exists as a single protein may indicate that the NEDD8 E1 may precede the ubiquitin E1 in evolutionary terms. NAE adenylates NEDD8 and then forms a thioester bond with adenylated NEDD8 before the ubiquitin-like protein is transferred to an active site cysteine of one of the two E2 enzymes (Gong and Yeh, 1999a; Walden, Podgorski and Schulman, 2003). Specificity in the NEDD8 conjugation pathway is achieved by the unique interaction between the E2 enzymes and a UBA3-specific groove formed by loops

which are unique to the NAE complex and not present in any other UBL E1 enzymes (Huang *et al.*, 2004). The dual NEDD8 and ubiquitin E3 enzymes RBX1 and RBX2 mediate the addition of NEDD8 to cullin proteins (Scott *et al.*, 2014). RBX1 forms an integral part of CRL1, CRL2, CRL3 and CRL4 while RBX2 forms a part of CRL5 (Enchev, Schulman and Peter, 2015). RBX proteins thus promote the ubiquitination of substrates as ubiquitin E3s while also serving to promote the neddylation of the cullin proteins to which they are bound. It has been shown by crystal structure that NEDD8 itself guides the multifunctional RING E3 via a linker region which acts as a lever to position the E2 active site close to a specific acceptor lysine target in the cullin protein with the assistance and coordination from accessory proteins including the DCNL proteins (Scott *et al.*, 2010; Scott *et al.*, 2014) which are discussed in detail in section 1.2.4. It is worth noting that RBX1 is able to interact with both UBC12 (UBE2M) and UBE2F, while RBX2 is specific for UBE2F (Huang *et al.*, 2009).

NEDD8 is cleaved from CRLs by the COP9 signalosome (CSN) (not shown in the diagram) which is thus a key regulator of E3 ligase activity (Wolf, Zhou and Wee, 2003). The CSN is a highly conserved multi-protein complex which a major regulator of de-neddylation and is the cullin de-neddylase enzyme in cells. It is incredibly selective for neddylated CRL complexes and posses no de-ubiquitination or de-neddylation activity (Fischer *et al.*, 2011; Lingaraju *et al.*, 2014). The CSN is required for the efficient assembly and maintenance of active CRL complexes through its ability to remove NEDD8 from cullin proteins to promote CRL disassembly (Cope *et al.*, 2002), which in turn is required for the formation of new CRL complexes with different substrate adaptors (Wolf, Zhou and Wee, 2003). More recently, the CSN has been shown to associate tightly with around 10-20% of CRL complexes (Lydeard, Schulman and Harper, 2013) and this association often occurs at the substrate receptor region of CRLs which is intriguing given the diversity in structure of these modules. Furthermore, this interaction occurs independently of cullin neddylation since MLN4924 treatment has little effect on global organisation of the CRL network (Bennett *et al.*, 2010). The variable SR module-CSN interaction likely explains the differing association of the CSN with different cullins, for example association with cullin 4B is close to 40% whereas association with cullin 3 is less than 5% (Bennett *et al.*, 2010). It is

thought that the balance between substrate availability and CSN abundance *in vivo* dictates the CRL landscape and provides significant and precise control over cellular processes (Emberley, Mosadeghi and Deshaies, 2012; Enchev *et al.*, 2012). The CSN subunit 5 (CSN5) possesses a JAMM $Zn^{2+}$-metalloprotease motif which forms the catalytic core of the CSN complex and is critical to its de-neddylase activity (Cope *et al.*, 2002; Ambroggio, Rees and Deshaies, 2004). CSN5 requires assembly with the other subunits of the CSN for it catalytic activity *in vivo* (Sharon *et al.*, 2009; Birol *et al.*, 2014).

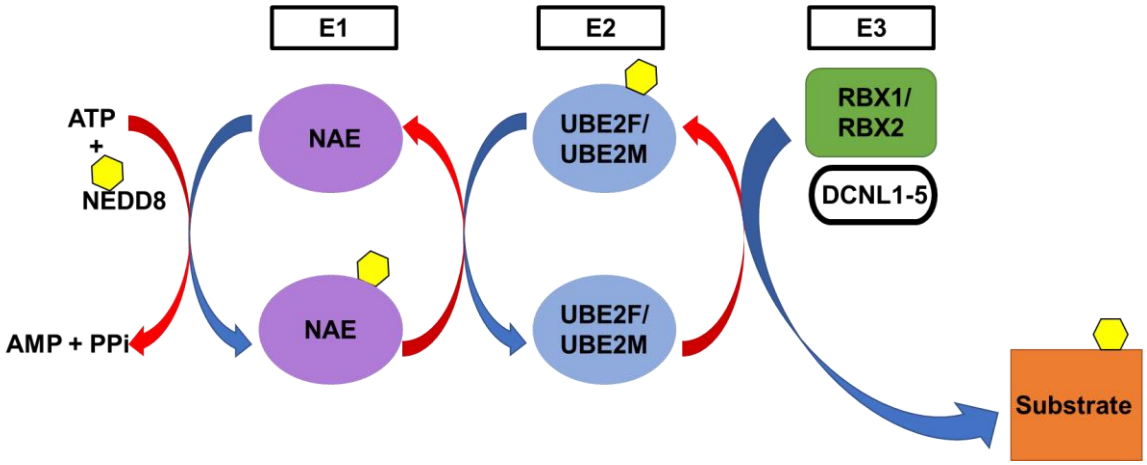



**Figure 1-5 The Neddylation Process.**

This schematic diagram provides a simplistic overview of the process of NEDD8 conjugation to a target substrate (which in most known cases is a cullin protein). Following the processing of NEDD8 by NEDP1 (not shown), it is conjugated to the E1 NAE in an ATP dependent process and forms a thioester bond with a cysteine residue. Next, NEDD8 is transferred to one of the two E2 enzymes to an active site cysteine residue, before the E3 enzymes RBX1 or RBX2 mediate the conjugation of NEDD8 to the cullin protein to which the E3 is already bound as the core of a CRL complex. DCNL family members facilitate the addition of NEDD8 to cullin proteins and can bind both the E2 enzyme and the cullin protein.

### 1.2.3 Cullin Proteins

Cullin proteins were briefly mentioned in section 1.1.4 for their role in forming the cores of the ubiquitin E3 ligase family known as cullin-RING ligases. Cullin proteins are molecular scaffold proteins which were originally discovered in nematodes (Kipreos *et al.*, 1996) for their role in cell cycle regulation, while also independently being discovered in budding yeast (Mathias *et al.*, 1996) for their role in ubiquitin-dependent proteolysis of cell cycle regulators (which gave rise to

their name in yeast as Cdc or cell division control proteins). In humans there exist 7 homologues: cullins 1 through 3, cullin 4A and cullin 4B, and cullins 5 and 7 (Sarikas, Hartmann and Pan, 2011). There also exist other cullin-related proteins such as PARC (also known as cullin 9) which was found to play a role in p53 regulation but not through ubiquitination activity (Nikolaev *et al.*, 2003) and has been shown not to form CRL complexes unlike all other cullin proteins (Cardozo and Pagano, 2004). Another protein called APC2 which is a subunit of the highly complex APC has been shown to show some cullin-homology (Yu *et al.*, 1998; Zachariae *et al.*, 1998; Tang *et al.*, 2001). Cullin protein structure has largely been elucidated in the context of its role in forming CRL complexes and significant biochemical data exists confirming the high degree of conservation between different organisms from yeast to humans in the functionality of the protein family as a scaffold protein (Liu and Nussinov, 2011; Lydeard, Schulman and Harper, 2013; Baek *et al.*, 2020).

Most cullin protein family members share core structural similarity; cullins 1 to 5 have 3 cullin repeats in their N-terminal domain which is structured like a stalk. Their globular C-terminal domain consists of a cullin homology domain (CHD) as well as neddylation site in a subdomain known as the WHB (winged helix-B) domain, which is conserved across all cullins. Cullin 7 is not only larger in size than cullins 1-5 but is also thought to be structurally different (Sarikas, Hartmann and Pan, 2011; Lydeard, Schulman and Harper, 2013). Little structural data is available for cullin 7, but it is known to contain a domain known as the CPH domain that is conserved between cullin 7, PARC (also known as cullin 9) and HERC2 (a ubiquitin E3 ligase). This domain has been shown to be responsible for protein-protein interactions with p53 (Kaustov *et al.*, 2007). The cullin repeat number 1 (CR1) is responsible for the recognition of adaptor proteins and substrate binding proteins to mediate binding of different substrates in cullins 1-5. The C-terminal domain of cullin proteins, specifically the α/β subdomain, is responsible for binding to RBX proteins which in turn bind to the E2 enzyme laden with ubiquitin (Sarikas, Hartmann and Pan, 2011; Liu and Nussinov, 2011). The WHB subdomain of the C-terminal region of cullins interacts with RBX RING domain in unmodified CRLs (Zheng *et al.*, 2002). The basic structure and organization of cullin protein domains is shown below in Figure 1-6.

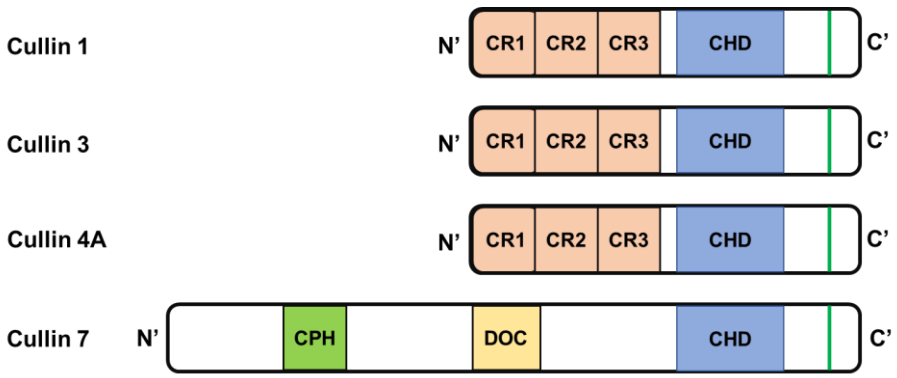



**Figure 1-6 Cullin Proteins Domains.**

Shown in this diagram is the domain and general structure of cullins 1,3 4A and 7. The first 3 are similar in size (around 750 amino acids) and consist of a stalk-like N-terminal domain which consists of 3 cullin repeat regions (CR1-3). CR1 is responsible for the recognition of adaptor proteins. The C-terminal domains of cullins 1-5 is globular and contains a cullin homology domain (CHD) and a neddylation site (shown by the green line), both of which are present in cullin 7. Cullin 7 is larger than the other cullin proteins and contains a domain (CPH) which is conserved between cullin 7 and the protein known as PARC (cullin 9) as well as a DOC1-like domain which is also seen in complexes such as the anaphase promoting complex. Cullin 7 structure is largely unknown beyond this.

Cullin proteins bind to their RING domain-containing partners RBX1 or RBX2 via a conserved intermolecular ß sheet interface (Zheng *et al.*, 2002). Cullin proteins have long been known to require neddylation to promote CRL-catalysed ubiquitination of target substrates. NEDD8 is conjugated to the cullin C-terminal WHB domain (Zheng *et al.*, 2002) which contributes to the relative repositioning of the cullin C and N-terminal regions which is thought to promote ubiquitination activity of the CRL complex (Duda *et al.*, 2008). It is only recently that the full role of neddylation has been uncovered as a result of the determination of the crystal structure of neddylated $CRL1^{\beta\text{-}TRCP}$ in complex with the E2 UBE2D and its substrate phospho-IκBα (Baek *et al.*, 2020). NEDD8 functions as a catalyst for a series of dynamic structural rearrangements that promote cullin WHB domain and RING rearrangement which in turn allows for the positioning of the Ub-laden E2 enzyme close to the substrate to promote ubiquitination (Baek *et al.*, 2020). In essence, NEDD8 brings into proximity the normally distal elements of a CRL-E2 complex to facilitate the transfer of ubiquitin from the E2 active site to the target substrate.

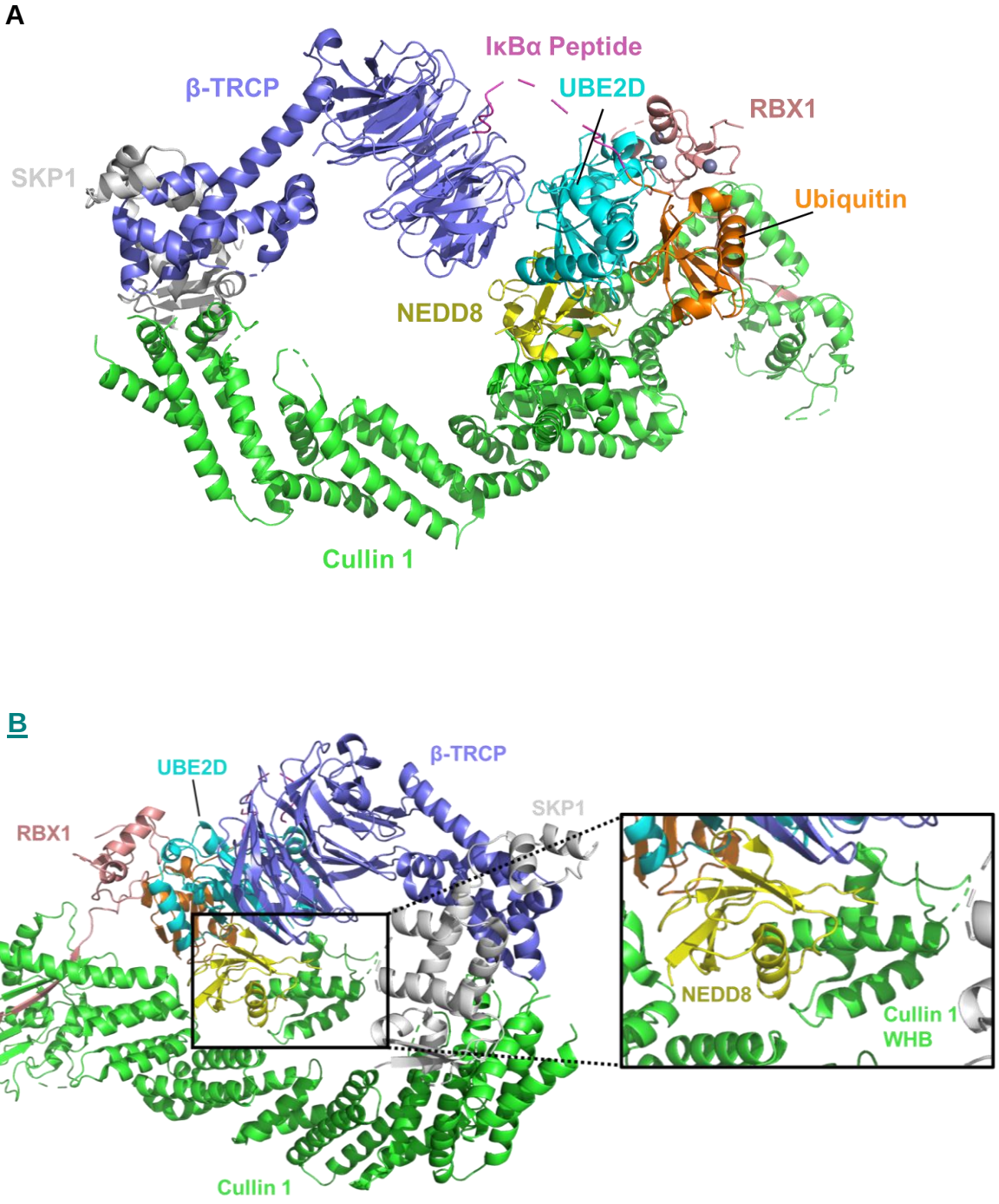



**Figure 1-7 Structure of CRL1$^{\beta\text{-TRCP}}$ Complex**

(A) provides an overview of all the proteins involved in the CRL1$^{\beta\text{-TRCP}}$ complex assembly. Cullin 1 (green) binds to SKP1 (grey) via its N terminal domain while SKP1 in turn binds to the F-Box protein β-TRCP (purple). The substrate IκBα is shown in pink, while the E2 enzyme UBE2D is shown in cyan. Cullin 1 interacts with the RING protein RBX1 via a conserved β-sheet. Ubiquitin is shown in orange and NEDD8 is shown in yellow. (B) shows the structure from an opposite angle and provides a clearer picture of the cullin WHB subdomain interacting with NEDD8. *This Cryo-EM structure was accessed from the protein data bank (accession code* 6TTU*) and EMDataResource database* (*accession code EMD-10585)* (Baek *et al.*, 2020) *and processed using Pymol 2.4 software*.

Non-neddylated cullin can interact with a protein called CAND1. CAND1 blocks the C-terminal cullin neddylation site and the CAND1 C-terminus inhibits cullin-adaptor interaction mediated by the cullin N-terminus (Helmstaedt *et al.*, 2011). CAND1 protein serves to sequester inactive cullin proteins in complexes with DCNL proteins (Keuss *et al.*, 2016; Liu *et al.*, 2018) and acts as an regulator of CRL formation (Liu *et al.*, 2002). CAND1 functions as a substrate receptor exchange factor, it facilitates the assembly of new CRL complexes through recruitment and exchange of F-BOX proteins (Pierce *et al.*, 2013). It is likely that substrate adaptor availability drives the displacement of CAND1 – then the cullin gets neddylated. A simplified outline of the neddylation of a cullin protein and assembly of a ubiquitin E3 ligases is shown in Figure 1-8.

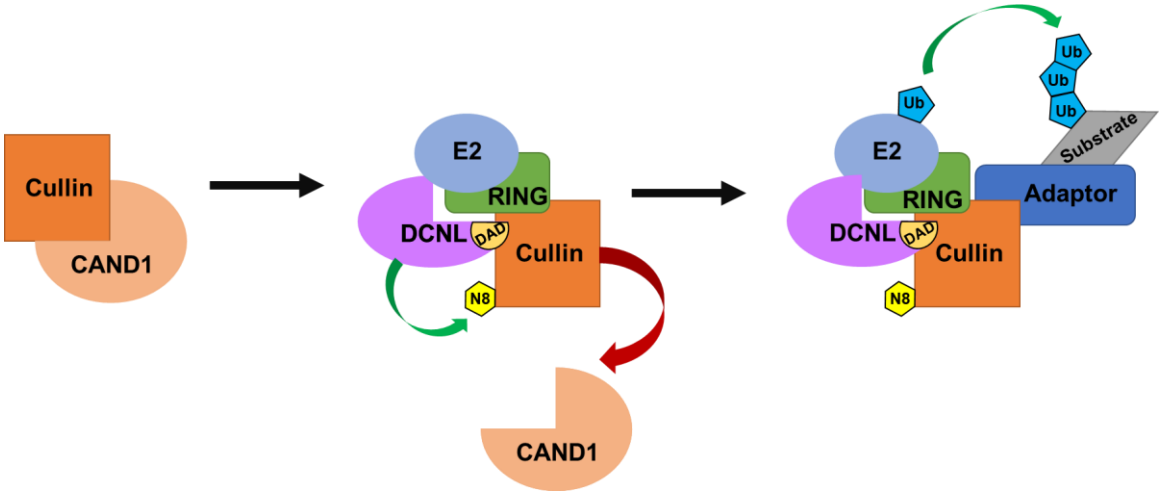



**Figure 1-8 Regulation of Cullin RING Ligases Assembly by CAND1 and NEDD8.**
This simplified diagram shows the basic stages involved in activation of CRL complexes. The DCNL family of proteins aid in releasing CAND1 binding from inactive cullin proteins and are responsible for coordinating the binding of the RING protein to the cullin in order to mediate neddylation.The DCNL proteins bind to cullin proteins via an interaction surface that includes 3 highly conserved and essential amino acids known as the 'DAD' patch. Neddylation is required for conformational changes needed to facilitate the transfer of ubiqutin to a target substrate.

### 1.2.4 Defective in Cullin Neddylation-Like (DCNL) Protein Family

DCNL proteins were initially discovered and studied in budding yeast where the single homologue is known as Dcn1p (defective in cullin neddylation 1) and in *C. elegans* where it is known as Dcn1. This protein was found to greatly increase the rate of the neddylation reaction in both organisms (Kurz *et al.*, 2005a). It was later demonstrated that the human variant of the protein, DCNL1, functions to recruit NEDD8-laden E2 (Kim *et al.*, 2008) and is also pivotal in the release of the effects of CAND1 on CRL complexes. Un-neddylated cullins exist in complex with CAND1 and DCNL proteins (Keuss *et al.*, 2016; Liu *et al.*, 2018) while neddylated complexes exist in active ubiquitination E3 complexes (Kim *et al.*, 2008).

Human cells express 5 DCNL proteins (Sarkaria *et al.*, 2006; Kim *et al.*, 2008; Meyer-Schaller *et al.*, 2009; Bommeljé *et al.*, 2014; Huang *et al.*, 2014; Keuss *et al.*, 2016) which each have distinct N-terminal domains which are thought to dictate their subcellular localisation (Keuss *et al.*, 2016). DCNL3 is unique in having a myristoylation site and in localising to the plasma membrane (Meyer-Schaller *et al.*, 2009), DCNL1 and 2 contain N-terminal UBA domains and localise

to the nucleus and cytoplasm (Wu *et al.*, 2011) while DCNL4 and 5 posses nuclear localisation sequences and thus localise almost exclusively to the nucleus (Huang *et al.*, 2011; Keuss *et al.*, 2016). The basic domain structure of the DCNL family of proteins are shown in Figure 1-9. DCNL1 is the most ubiquitously expressed of the DCNL family of proteins and is also the best studied homologue. Further evidence for the role of the N-terminal domain of these proteins in their localisation includes the fact that mutations in the nuclear localization sequence of DCNL5 resulted in its diffusion into the cytoplasm (Keuss *et al.*, 2016).

A key region in the DCNL proteins is the 'DAD' patch, which is part of the conserved C-terminal region called the potentiating neddylation domain (PONY) (Kurz *et al.*, 2008). The DAD patch is so-named due to consisting of D226, A253, and D259 residues in yeast Dcn1 (Kurz *et al.*, 2008). Mutations in these key residues of the cullin-interaction subsurface lead to a loss of neddylation and were shown to be sufficient and essential for the binding to cullins (Kurz *et al.*, 2008). The subcellular localisation of DCNL family members has been shown to occur independently of their ability to bind cullin proteins (Keuss *et al.*, 2016). Whether the DAD-patch region is responsible for binding to other proteins remains unknown.

DCNL proteins are all expressed in tissue culture cells and differ in their subcellular localisation as mentioned previously. Expression of these proteins is more varied in animal tissues: DCNL1 is widely expressed in cancer cell lines as well as most types of animal tissue (specifically mouse tissue) including testis, brain, lung and spleen. DCNL2 expression in tissues is limited to brain, liver and kidney tissues and expression is good in cancer cell lines in tissue culture. DCNL3 expression is strong in only testis and brain and its expression in cancer cell lines is less pronounced. DCNL4 is expressed in most tissues and expression in cancer cell lines is similar. DCNL5 is expressed the strongest in cancer cell lines compared to all the other DCNL proteins, while its expression is limited to the testis and thymus in mouse tissue (Keuss *et al.*, 2016). DCNL proteins have been shown to show no preference for any particular cullin *in vitro* (Keuss *et al.*, 2016) and their function and role *in vivo* is thought to be regulated by the subcellular localisation of both DCNL proteins and cullin proteins (Keuss *et al.*, 2016). Below, we will

discuss the roles of DCNL1, DCNL3 and DCNL5 *in vivo*. DCNL2 and DCNL4 lack experimental characterisation and their physiological roles are largely undetermined, however structurally DCNL2 is most related to DCNL1 while DCNL4 is most related to DCNL5 so it is possible that there is overlap of function in cells.

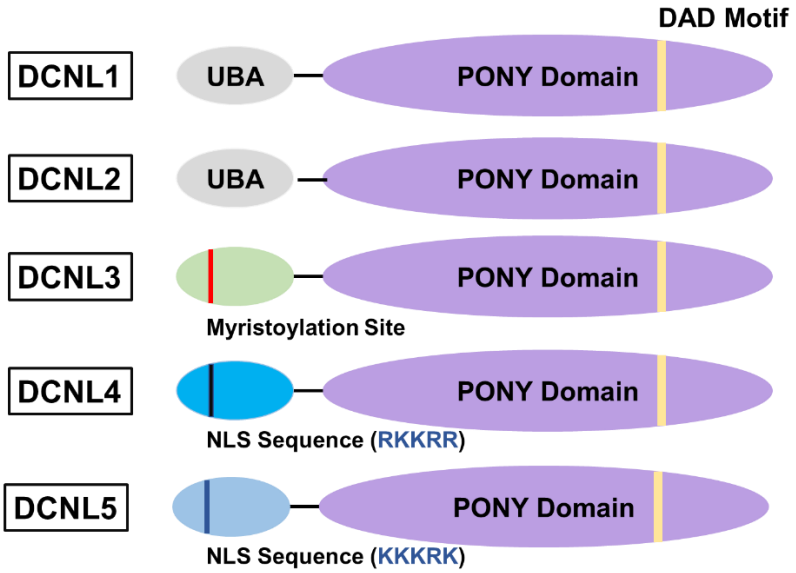



**Figure 1-9 Key Domains of DCNL Proteins.**

Shown in this figure are shematic diagrmas representing the 5 DCNL protein family members in humans. The proteins are structurally very similar and are known to overlap significantly in function *in vitro*, however their key differences lie in the N-terminal domains. DCNL1 and DCNL2 contain a UBA domain and DCNL1 is the most ubiquitously expressed of these proteins, localising to the cytosol and to the nucleus. DCNL3 contains a myristoylation site in its N-terminal region which allows it to localise to the plasma membrane, as well as to the cytosol and the nucleus. DCNL4 and DCNL5 contain a nuclear localization sequence in their N-terminal regions (RKRR and KKKRK respectively) which allow them to localise predominantly to the nucleus. The C-terminal region of all DCNL proteins consists of a potentiating neddylation domain (PONY) which is responsible for binding to cullin proteins via the highly conserved amino acid residue motif known as the 'DAD' patch.

### 1.2.4.1 Mechanism of DCNL Proteins

The structure of yeast Dcn1 was solved in 2008 which revealed the existence of an N-terminal UBA domain and a C-terminal domain which was termed the potentiating of neddylation (PONY) domain (shown in Figure 1-10) and contained within this domain were three key residues responsible for mediating the

interaction of Dcn1 with cullin proteins as mentioned previously. These residues are located in close proximity to the neddylated lysine in yeast cullin homologue Cdc53 (Kurz *et al.*, 2008). Dcn1 was shown to interact directly with the NEDD8 E2 UBC12, similar to RBX proteins which are NEDD8 E3s, while not participating directly in the catalytic transfer of NEDD8 to the cullin protein. In order to determine whether Dcn1 plays a catalytic role in the transfer of NEDD8 via transthiolation reaction using cysteine residues, the five cysteine residues in Dcn1 were mutated individually. None of the resulting mutants lacked the ability to promote neddylation of Cdc53 *in vivo* and thus it was concluded that Dcn1 functions as a scaffold-like protein that lacks a catalytic role in the transfer of NEDD8 but plays an essential role in promoting neddylation (Kurz *et al.*, 2008).

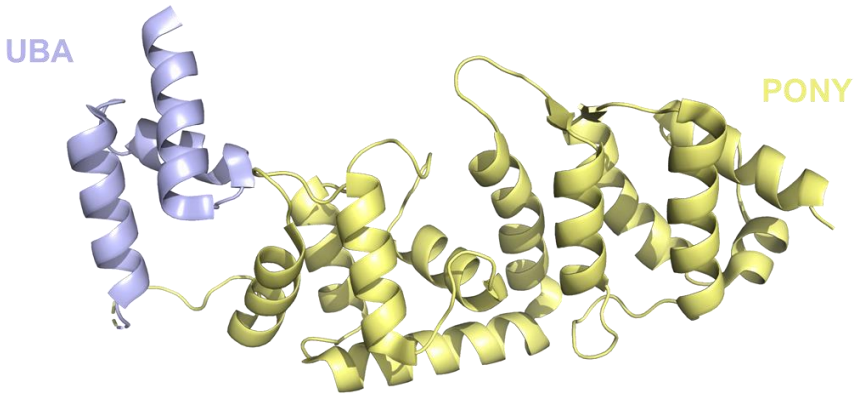



**Figure 1-10 Structure of *S. cerevisiae* DCN1**

This ribbon representation of budding yeast DCN1 shows the N-terminal UBA domain (residues 12-56) in light purple and the C-terminal PONY domain (residues 70-269) in yellow. *This structure was accessed from the protein data bank (accession code 3BQ3)* (Kurz et al, 2008) *and processed using Pymol 2.4 software*.

The DCNL protein family have been described both as scaffold-type E3 ligases and as co-E3 ligases which play an auxiliary role in neddylation. Given that they have been characterised as playing an essential role in promoting neddylation (discussed more below) and the yeast DCNL homologue Dcn1 has been shown to be sufficient to promote neddylation in a purified reconstituted system while also interacting directly with the E2 enzymes, (Kurz *et al.*, 2008), they are considered to be true NEDD8 E3s rather than auxiliary factors in this thesis. Mechanistically,

the role of yeast Dcn1 was suggested to restrict the normally flexible E3 RING domain-bound - NEDD8-laden UBC12 complex to promote a catalytically favourable arrangement to allow the transfer of Rub1 (yeast homologue of NEDD8) to Cdc53 (Scott *et al.*, 2010). Thus, in yeast, the two E3 enzymes Dcn1 and the RBX1 homologue Hrt1 function cooperatively to promote the transfer of NEDD8 to a target cullin protein; both are required in the process and loss of either one significantly impairs neddylation.

More recently, human DCNL proteins have been shown to interact with the NEDD8 E2 enzymes UBC12 and UBE2F via N-terminal acetylated regions of the E2s and this interaction was shown to vary between DCNL proteins, representing potential therapeutic targets (Monda *et al.*, 2013). It has been shown that yeast Dcn1 recognised the acetylated N-termini of UBC12 (Scott *et al.*, 2011) and this was later expanded to show in humans that all DCNL proteins interact with UBC12 or UBE2F – and this acetylation was shown to neutralize a positive charge at the N-terminus of NEDD8 E2 enzymes (which would normally obstruct the interaction), as well as making positive interactions upon burial in a deep pocket within the DCNL PONY domain (Scott *et al.*, 2011; Monda *et al.*, 2013). A visual summary of the role of DCNL proteins can be seen in Figure 1-11. The critical acetylated residue was a methionine therefore this represents a key acetylation-specific interaction of the PONY domain. The researchers elucidated specificity for UBC12 and UBE2F peptides to inhibit different DCNL proteins which suggested that it may be possible to develop small molecules to specifically target N-acetyl-methionine-dependent protein interactions between different DCNL proteins (Monda *et al.*, 2013).

Inhibitors have been developed which target this DCNL-acetylated-E2 interaction and are described in more detail below, but briefly, these compounds elucidate new non-redundant roles for the DCNL proteins since one of these compounds called DI-591 specifically reduces cullin 3 neddylation *in vivo* by targeting DCNL1-UBC12 interaction preferentially (Zhou *et al.*, 2017). In addition, the affinity of this compound for DCNL1 and DCNL2 over the other DCNL proteins suggests that there are structural differences within the PONY domains of the DCNL proteins which may provide insight into their potential roles in cell, which is perhaps

unsurprising since the sequences of DCNL1 and DCNL2 are 82% identical, while DCNL1 and DCNL3 are only 40% identical (Monda *et al.*, 2013).

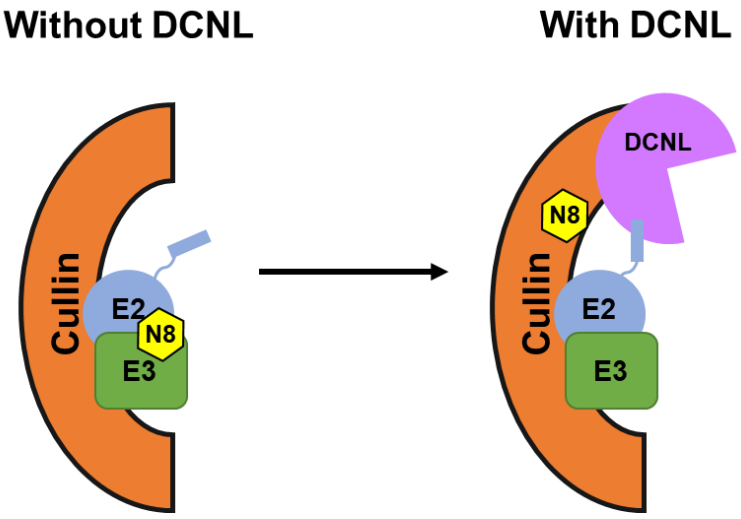



**Figure 1-11 DCNL Mechanism Outline**

This simplified diagram illustrates the role DCNL proteins play in promoting neddylation. DCNL proteins bind to the E2 enzyme via the E2 acetylated N-terminus which buries into a deep pocket within the DCNL PONY domain, and bind to cullin proteins via their DAD-patch. DCNLs stabilise the flexible linker of the E2 to promote the favourable catalytic transfer of NEDD8 to the cullin protein.

### 1.2.4.2 DCNL1

As previously mentioned, DCNL1 is the most studied of the DCNL family members, and part of this stems from the fact that it is the oldest of the DCNL proteins from an evolutionary standpoint and is the most closely related DCNL protein to the single homologs found in lower organisms (Kurz *et al.*, 2008). DCNL1 is not solely responsible for cullin neddylation in mammalian cells (in contrast to in lower organisms) because KO mice are viable which would likely not be the case if all cullins were affected by the absence of DCNL1; all cullins are still mostly neddylated (Huang *et al.*, 2011). DCNL1 has been shown to play an essential role in cullin 1 neddylation via its ability to promote nuclear localisation of neddylation components and recruitment of the E2 enzyme UBC12 (Huang *et al.*, 2011). The UBA domain in the N-terminus of DCNL1 has not been shown to be required for its neddylation activity *in vitro* or *in vivo* and its precise role has until recently remained elusive (Kurz *et al.*, 2008; Wu *et al.*, 2011). Research has now shown that the UBA domain undergoes monoubiquitination by the Ariadne RBR ligases TRIAD1 and HHARI and this depends on CRL association (Kelsall *et al.*, 2019) of all

the proteins. This monoubiquitination was shown to be required for CRL activity and was suggested to promote structural remodelling and substrate switching (Kelsall *et al.*, 2019). It has also been shown that polyubiquitin chain binding to the DCNL1 UBA domain inhibits its ability to promote neddylation, therefore the UBA domain may function as a sensor to regulate DCNL1 activity (Huang *et al.*, 2015). It was suggested that the binding of polyubiquitinated proteins to the UBA domain regulated the subcellular localisation of DCNL1. Binding of polyubiquitinated proteins to the UBA domain was shown to promote monoubiquitination and nuclear export of the protein. This finding was consistent with previous research which showed that monoubiquitination of DCNL1 drives its nuclear export (Wu *et al.*, 2011). Thus, there exist multiple roles for the UBA domain of DCNL1 however further work is required to better elucidate these.

DCNL1 is also known as squamous cell carcinoma-related oncogene (SCCRO1) and has been shown to be overexpressed in squamous cell carcinomas which correlates with poor clinical outcome (Sarkaria *et al.*, 2006). DCNL1 has also been demonstrated to function as an oncogene through its ability to facilitate malignant transformation of cells in culture and xenograft formation in nude mice and has been implicated in the pathogenic progression of platelet-derived growth factor (PDGF)-dependent gliomas (Broderick *et al.*, 2010). The UBA domain in DCNL1 has been shown to contain mutations in human cancers and this is thought to reduce the ability to regulate DCNL1 activity in cells and increase its transforming activity (Huang *et al.*, 2015).

#### 1.2.4.3 DCNL3

As stated above, DCNL3 contains a myristoylation site in its N-terminus which accounts for its localisation at the plasma membrane (Meyer-Schaller *et al.*, 2009; Huang *et al.*, 2011; Keuss *et al.*, 2016). DCNL3 has been shown to act in a non-redundant manner to mediate the neddylation of cullin 3 specifically at the plasma membrane of mammalian cells (Meyer-Schaller *et al.*, 2009). This was an interesting finding since it represented the first piece of evidence for a unique role of DCNL protein in cells, which is at least in part dictated by their subcellular localisation. DCNL3 has more recently been suggested to lack E3 ligase activity and actually function as a tumour suppressor by sequestering cullin proteins at the

plasma membrane, preventing them from translocating to the nucleus and inhibiting their DCNL1-mediated neddylation (Huang *et al.*, 2014). This finding was supported by the discovery that DCNL3 knockdown in cells increased the neddylation of cullins 1, 3, 4A and 4B (Keuss *et al.*, 2016), suggesting that DCNL3 may actually inhibit cullin neddylation. This is however in contradiction with the compelling previous finding that ectopically expressed human DCNL3 is able to promote cullin (Cdc53) neddylation in yeast DCNL1 KO cells in a heterologous assay, which supports the assertion that DCNL3 possesses E3 ligase activity (Meyer-Schaller *et al.*, 2009). To date, the precise role of DCNL3 is ambiguous and requires further investigation, but if its inhibitory role is real, it may stem from its ability to sequester cullins away from neddylation machinery (Keuss *et al.*, 2016; Huang *et al.*, 2014). This data suggests that the E3 ligase activity of DCNL proteins may not be the same *in vivo* and raises the possibility that this protein family have roles outside of promoting cullin neddylation.

#### 1.2.4.4 DCNL5

DCNL5 has been implicated as an oncogene and is found to be overexpressed in oral and lung squamous cell carcinomas (Bommeljé *et al.*, 2014). Researchers showed that its neddylation ability *in vitro* relied only on its PONY domain through the use of various mutants including whole N-terminal deletion constructs, while *in vivo* its neddylation activity required both its PONY domain and its N-terminal nuclear localisation sequence (Bommeljé *et al.*, 2014; Keuss *et al.*, 2016). siRNA-mediated knockdown of DCNL5 in cancer cell lines resulted in a decrease in viability of cancer cells with high endogenous DCNL5 expression (Bommeljé *et al.*, 2014). DCNL5 has also been implicated in immune signalling because in addition to high expression in immune tissues, DCNL5 undergoes phosphorylation at a specific N-terminal serine reside in response to toll-like receptor signalling by IKKα (Thomas *et al.*, 2018). This phosphorylation was found not to affect the kinetics of cullin neddylation reactions mediated by DCNL5, and IKK-mediated signalling events such as NF-κB signalling were unaffected in the absence of DCNL5 (Thomas *et al.*, 2018). This finding provides some insight into the potential post-translational regulation of DCNL proteins but the specific role of phosphorylation of the N-terminus of DCNL5 was not able to be determined. In the present research, we describe a role for DCNL5 in the apoptosis response and there may

therefore exist a level of crosstalk between immune signalling described above and apoptosis at the level of DCNL5 – possibly involving phosphorylation of the protein.

It has also been shown that DCNL5 and DCNL1 independently contribute to cullin 4A neddylation in cells (Keuss *et al.*, 2016) indicating a novel function for DCNL5. Keuss and colleagues then went on to show that DCNL5 is involved in the DNA damage response (while DCNL1 is not) by demonstrating that DCNL5 co-localises with cullin 4A at sites of DNA damage (Keuss *et al.*, 2016).The localisation of cullin 4A to sites of DNA damage was in keeping with previous research (Meir *et al.*, 2015). What was even more interesting was that this response was the same in a DAD-patch mutant of DCNL5 which is unable to bind cullins. It was shown that the cell death following irradiation (sensitivity) in the DCNL5 KO cells can be rescued by expression of WT DCNL5 as well as the DAD-patch mutant DCNL5, suggesting that this role of DCNL5 is cullin-independent (Keuss *et al.*, 2016). This data supported a role for DCNL5 in the DNA damage response *in vivo* but more intriguingly this finding appears to be one of the first likely non-cullin roles for any DCNL member described.

### 1.2.5 Non-Cullin Roles for NEDD8

There are several other non-cullin targets that have been described in the literature, however to date, cullin proteins are the only fully verified substrates for NEDD8. Under normal conditions, the roles of ubiquitin and NEDD8 are well defined and well separated and this is in part due to a single amino acid difference in the C-termini of the proteins (alanine in NEDD8 and arginine in ubiquitin) which prevents ubiquitin being selected by neddylation machinery (Whitby *et al.*, 1998). When neddylation is studied in vivo, cancer cells or cancer-derived cell lines often exhibit un-physiologically high NEDD8 expression while overexpression of NEDD8 specifically is often used to discover and analyse potential novel targets for the ubiquitin-like protein. This perturbs the ratio of ubiquitin to NEDD8 and results in artificial neddylation of targets through the use of ubiquitin machinery (Hjerpe *et al.*, 2012). The ubiquitin E1 UBE1 was shown to be able to promote neddylation under conditions in which the normally equal ratio of NEDD8:ubiquitin is artificially perturbed by NEDD8 overexpression, however how UBE1 engages with

NEDD8 machinery to actually promote aberrant neddylation was not evaluated (Hjerpe *et al.*, 2012). In spite of this important discovery, some non-cullin targets have been described for NEDD8 and these can be (if they haven't been already) validated along the lines of the criteria laid out by Enchev and colleagues to determine whether a candidate protein is a true physiological neddylation target. This criteria includes whether the modification occurs under physiological levels of NEDD8 and NEDD8 substrate expression levels and whether this neddylation can be inhibited by MLN4924 (Enchev, Schulman and Peter, 2015). The examples of non-cullin targets presented below require a careful degree of scrutiny as in almost all cases, full verification of the substrates under physiological conditions as well as inhibition with MLN4924 is often lacking.

NEDD8 has been reported to be critical for the activation of the HECT-domain E3 ligase (Scheffner and Kumar, 2014) SMAD ubiquitination regulatory factor 1 (SMURF1); NEDD8 and the NEDD8 E2 enzyme UBC12 (UBE2M) were shown to interact directly with SMURF1 and SMURF1 was found to auto-neddylate itself on multiple lysine residues via an active site cysteine unrelated to the HECT active site (Xie *et al.*, 2014). This finding was expanded upon more recently to show that both SMURF1 and SMURF2 contain non-covalent NEDD8 binding sites within their catalytic HECT domains in addition to covalent residues described previously. In SMURF proteins, mutations in the five residues in the conserved NEDD8-binding sequence resulted in reduced neddylation and reduced auto-ubiquitination (therefore increased stability) (He *et al.*, 2017). Loss of non-covalent interactions with NEDD8 reduced ubiquitination of SMURF substrates which play roles in cell migration and TGFβ signalling pathways (He *et al.*, 2017). Thus, NEDD8 seems to play covalent and non-covalent roles in the regulation of HECT-domain-containing E3 ligases in addition to its well studied role in regulating CRL activity. The fact that researchers used endogenous expression-level yeast constructs when analysing Rsp5 (yeast SMURF1 homologue) neddylation (Xie *et al.*, 2014) lends some credence to the assertion that this non-cullin E3 ligase may be a true target for neddylation.

Other reported non-cullin targets for NEDD8 are caspase proteins. These proteases are responsible for mediating the programmed cell death response known as

apoptosis. In *D. melanogaster*, drICE is an effector caspase (Hay and Guo, 2006), and a systematic *in vivo* RNAi analysis coupled with genetic validation identified drICE to be neddylated under endogenous conditions by the RING E3 ligase inhibitor of apoptosis 1 (IAP1) and de-neddylated by DEN1 (Broemer *et al.*, 2010). Neddylation of drICE was shown to inhibit its ability to cleave a downstream target (PARP1) in vitro which was suggested to account for the anti-apoptotic effects of NEDD8 *in vivo* (Broemer *et al.*, 2010). As well as identifying a role for NEDD8 in the apoptosis response, this work also revealed the role for IAP proteins as NEDD8 E3 ligases. The human orthologue of IAP1, XIAP, was shown to posses NEDD8 E3 ligases activity but it was not demonstrated to function as a ligase for the human orthologue of drICE, caspase 7, questioning the role for NEDD8 in human caspase regulation as well as the validity of caspases as NEDD8 targets (Nagano *et al.*, 2012).

Other research has independently identified human caspase 1, which is involved in inflammatory signalling, as a target for neddylation. NEDD8 was shown to be required for the self-cleavage of the inactive pro-caspase 1 zymogen to generate its catalytically active subunits (Segovia *et al.*, 2015). Inhibition of neddylation with the NAE inhibitor MLN4924 resulted in a reduction in caspase 1 processing (perhaps due to cullin involvement upstream), while increased auto-catalytic activity was seen with overexpression of NEDD8 (although this should be considered carefully given the previously described limitations of studying NEDD8 overexpression) (Segovia *et al.*, 2015). The role of caspases as potential targets for NEDD8 will be briefly explored in the present research, however cullin proteins remain the only true and fully verified substrates for NEDD8 and great caution needs to be advised when determining whether a substrate is a real target for NEDD8.

## 1.2.6 Neddylation Inhibitors

### 1.2.6.1 MLN4924 – NAE Inhibitor

Given the importance of neddylation in the regulation of CRL activity, NEDD8 has relatively recently become a therapeutic target and an inhibitor of neddylation has been developed. This inhibitor, known as MLN4924, specifically targets the

NEDD8 E1 enzyme NAE to inhibit its ability to bind to NEDD8 and thus inhibit the neddylation process. MLN4924 creates a covalent NEDD8-MLN4924 adduct which structurally resembles the adenylated NEDD8 (NEDD8-AMP) but cannot be processed further to form the high energy NEDD8-NAE thioester. The adduct formed by MLN4924 which sits within the NAE active site is highly stable and blocks enzymatic activity and therefore results in potent ablation of neddylation (Brownell *et al.*, 2010). MLN4924 has shown promise in treating various cancers in primary research settings. The drug has been shown to supress colon cancer cell growth specifically through the inhibition of cullin 1 neddylation which culminates in the activation of autophagy via suppression of the P13K/AKT/mTOR pathway (Lv *et al.*, 2018). MLN4924 has also been shown to induce cell cycle arrest at G2 phase as well as induce DNA damage and sensitise oesophageal squamous cell carcinoma cells to cisplatin by enhancing apoptosis (Lin *et al.*, 2018). Mechanistically, the inhibitor has been shown to prevent the growth of human gastric cancer cells through stabilisation of CDT1 (a replication licensing factor) as well as other CRL substrates via inhibition of cullin 1 neddylation preferentially. Stabilization of CDT1 promotes DNA re-replication and G2-phase cell cycle arrest (Lan *et al.*, 2016). MLN4924 has also shown promise in clinical trials for the treatment of acute myeloid leukaemia and myelodysplastic syndromes; the drug was successfully administered with minimal off-target effects and modest clinical activity was observed (Swords *et al.*, 2015). The drug is also being used currently in other clinical trials targeting the same conditions as well as non-small cell lung cancer and the role of the drug in combination with chemotherapy for the treatment of bile duct cancer of the liver.

#### 1.2.6.2 COP9 Signalosome Inhibitor

A potent, selective and orally bioavailable inhibitor of CSN5 has been developed, called CSN5i-3, which traps CRLs in the neddylated state, prompting subsequent inactivation of a small subset of CRLs by inducing degradation of their substrate recognition module and stabilisation of the CRL substrates (Schlierf *et al.*, 2016). The researchers showed that the results of treatment with this inhibitor resembled the effects of treatment with MLN4924, and they showed that the inhibitor differentially affected the viability of cancer cell lines. Overall, this inhibitor may show even more specificity towards certain CRLs than MLN4924 which is largely

general in its action, and CSN5i-3 has shown promise in pre-clinical studies. A key question that remains regarding the mechanism of this inhibitor is how does trapping CRLs in their neddylated (and thus active) state inhibit their ubiquitination activity? It is currently thought that trapping CRLs in their active state promotes substrate receptor molecule degradation by auto-ubiquitination of the CRL. This hypothesis was confirmed when CRL substrate receptor degradation was shown to be inhibited by pre-treatment with proteasome inhibitors prior to CSNi-3 treatment, indicating that CSNi-3 promotes proteasome-mediated degradation of substrate receptor molecules (Schlierf *et al.*, 2016). It is currently unclear why CSN inhibitors appear to show more specificity for a subset of CRLs when compared to MLN4924.

#### 1.2.6.3 DCNL Inhibitors

Since the advancement in the understanding of the interaction between DCNL proteins and their respective NEDD8 E2 enzymes, UBC12 and UBE2F, progress has been made in targeting this interaction. A few inhibitors targeting the interaction between DCNL proteins and the NEDD8 E2s (described in detail in section 1.2.4.1 above) have been the developed, one of which was called DI-591. This cell-permeable compound was shown to bind to DCNL1 and DCNL2 with KD values of 21.9nM and 11.2nM respectively, however even at concentrations of up to 10μM there was no detectable binding to DCNL3, 4 or 5 (Zhou *et al.*, 2017). What was also interesting is that the compound specifically reduced the neddylation of cullin 3 only while having minimal effect on the neddylation of other cullin proteins. This specific reduction in cullin neddylation promoted accumulation of NRF2 protein and its transcriptional activation (Zhou *et al.*, 2017), which is the case because NRF2 is a cullin 3 CRL substrate, thus, inhibiting cullin 3 neddylation inhibits the degradation of NRF2 (Cullinan *et al.*, 2004; Kobayashi *et al.*, 2004). Interestingly, another research group developed a class of inhibitors that target this interaction interface between DCNL proteins and NEDD8 E2 enzymes and their inhibitors specifically targeted DCNL1 and DCNL2, *in vitro*, however *in vivo* they found that DCNL1-UBC12 interactions were inhibited preferentially and this also resulted in specific reduction in cullin 3 neddylation (Scott *et al.*, 2017). This work highlights the potential therapeutic benefit of these types of inhibitors in the

targeting of DCNL1 as an oncogene in cancer therapy, as well as regulating specific cullin neddylation in cells.

Another class of inhibitors have been discovered which contain a pyrazolo-pyridine core and target the N-terminal acetyl group-binding site within UBC12 through the optimisation of an initial lead compound through the addition of hydrophobic interactions to mimic the endogenous acetylated N-terminus of UBE2M (Kim *et al.*, 2019). Interestingly, the researchers showed that these compounds engage DCNL1 in cells and selectively reduce the steady state levels of neddylated cullin 1 and cullin 3 in two squamous carcinoma cell lines which harboured DCNL1 amplification. They also showed that the compounds also target DCNL2-mediated neddylation *in vitro* (Kim *et al.*, 2019). The emergence of new compounds that can specifically target individual DCNL proteins as well as specific cullins in cells represents a key therapeutic avenue given the higher degree of control over the target and given the role of DCNL1 as an oncogene these compounds may represent useful bases for drug development in cancer therapy.

## 1.3 Apoptosis

Cell death can occur by a variety of mechanisms, including programmed cell death by the apoptosis pathway. This tightly regulated process results in a range of hallmark events including DNA fragmentation, chromatin condensation and cell lysis and is mediated by a key family of enzymes known as caspases which are cysteine-aspartic proteases (casp-ase). The apoptosis pathway can be broken down into two key branches: the extrinsic pathway and the intrinsic pathway (Fulda and Debatin, 2006), these differ in their triggers and in some of the caspases involved and both are discussed in more depth in the subsequent sections. Apoptosis is a highly regulated and tightly controlled process, involving many steps, and is often altered in cancer cells. Diseased cells can have mutation in the p53 gene, resulting in a dysregulation of the cell cycle, an impairment in the apoptosis pathways and can often lead to the formation of tumours. Tumorous cells often have mechanisms in place to evade cell death and significant work has been undertaken to better understand the pathways involved in mediating cell death. As well as the two main branches of the pathway, there also exist other

mechanisms that can cause death, including necrosis as well as caspase-independent apoptosis (Tait and Green, 2008).

The focus of the present work, however, is on the extrinsic and intrinsic apoptosis pathways. We focus on the extrinsic branch of the pathway in particular and more specifically on the role of caspase 8 which is one of the key initiator caspases and a core component of signalling complexes required for propagating the apoptosis stimulus via this branch of the pathway. The apoptosis pathways, as well as the various proteins and complexes involved in this process are discussed in the next sections of this report. A general overview of the two branches of the apoptosis pathway are shown in Figure 1-12. The extrinsic pathway is activated by a variety of ligands acting at a variety of receptors but shown below is only the binding of TNFα to a trimeric TNF receptor 1 molecule. (TNF-related apoptosis inducing ligand, TRAIL, also acts through the same basic pathway). This causes recruitment of adaptor proteins and can ultimately result in the cleavage and activation of caspase 8. The intrinsic branch of the pathway involves the release of cytochrome c from the mitochondrion and activation of caspase 9 which mediates downstream cleavage of effector caspases, similar to the role of caspase 8. Both pathways converge on the activation of executioner caspases which mediate cleavage of downstream proteins including poly(ADP ribose) polymerase (PARP) which is a repair enzyme preferentially cleaved by caspases 3 and 7 (Lazebnik *et al.*, 1994).

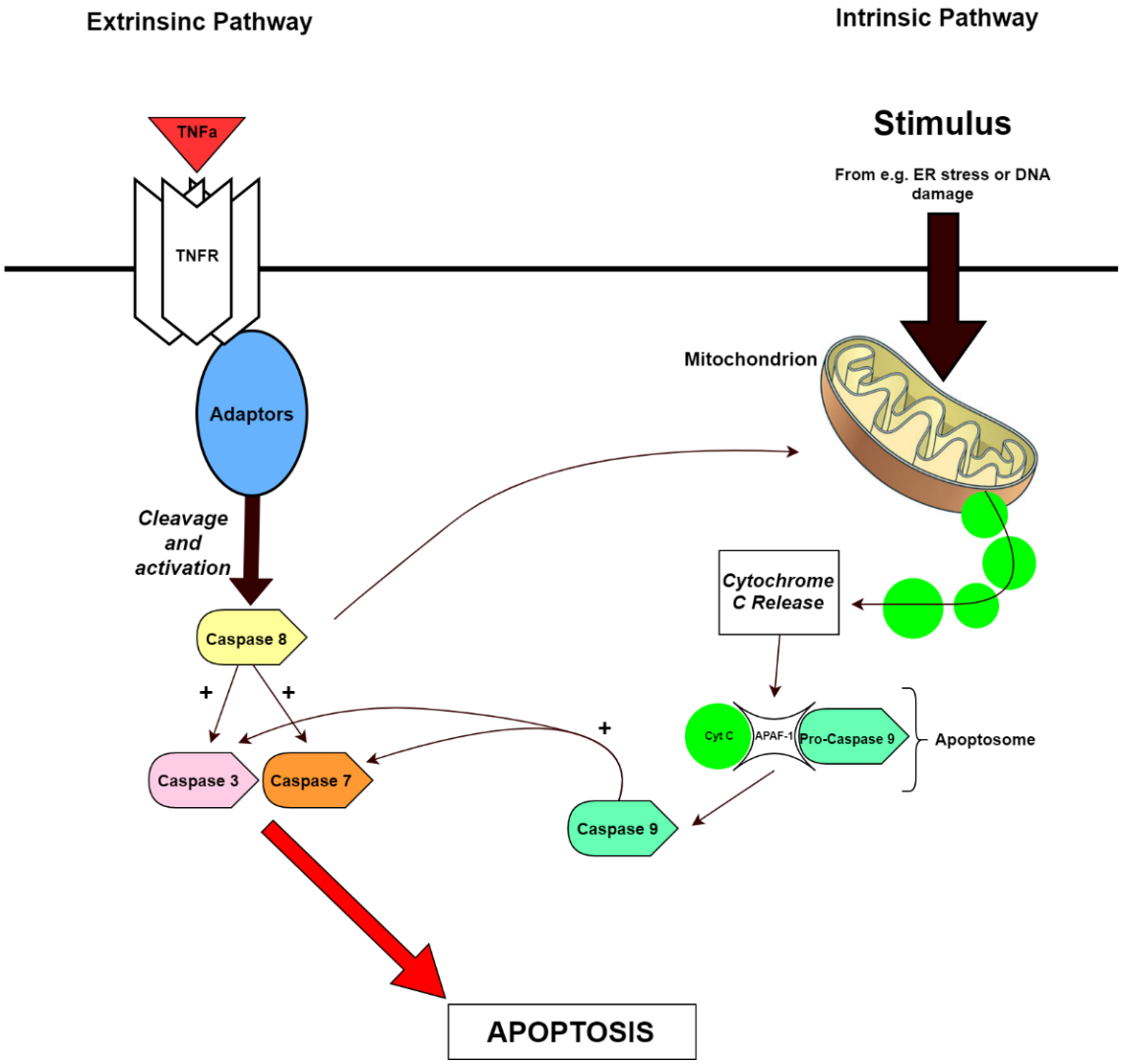



**Figure 1-12 A General Overview of the Two Branches of the Apoptosis Pathway.**

This simplified diagram shows a broad overview of the extrinsic and the intrinsic branches of the apoptosis response. The extrinsic branch of the pathway is the receptor-mediated branch and can be triggered by TNFα or TNFα-related family of ligands. Binding of the ligand to TNFα-superfamily receptors results in trimerization of the receptor and formation of a receptor-bound complex of death-domain containing proteins. This leads to the cleavage and activation of the key initiator caspase, caspase 8, which is then able to activate the effector caspases 3 and 7. The intrinsic branch is triggered by internal stimuli including DNA damage and ER stress, and results in the release of cytochrome c from the mitochondrion. Cytochrome c forms the apoptosome along with pro-caspase 9 and APAF-1 which results in the cleavage and activation of caspase 9. Like caspase 8, caspase 9 is an initiator caspase and can activate the downstream effector caspases. Caspase 8 can also activate the intrinsic branch of the pathway to amplify the apoptosis signal, via a protein called BID.

### 1.3.1 Caspase Proteins

The caspase family of proteins are proteases which cleave target substrates at specific sites after an aspartic acid residue via an active cysteine residue in their active sites. Caspases are the key mediators of apoptosis and can be grouped by their function and role in apoptosis. In humans, caspases 2, 8, 9 and 10 are known as initiator caspases, caspases 3, 6 and 7 are known as executioner caspases and caspases 1, 4, 5 and 12(L) (long form) are inflammatory caspases. The short form of caspase 12(S) is truncated and functionally redundant while the long form is specific to individuals of African descent and plays a role in attenuating the inflammatory and innate immune responses to endotoxins (Saleh *et al.*, 2004). Caspase 14 also exists but its function is less well characterised. We will focus on the initiator caspases and executioner caspases in this report. Caspases differ in their structure but also in the sequences within peptides or proteins that they recognise for cleavage (Julien and Wells, 2017).

All caspases except for caspase 9 (Shi, 2002) exist as inactive pro-enzymes (pro-caspase 9 possesses some activity) consisting in their simplest form of a pro-domain and small and large subunits. Initiator caspases as well as inflammatory caspases contain domains known as death effector domains (DED) and caspase recruitment domains (CARD) within their pro-domains which serve to mediate homophilic interactions between pro-caspases and their adaptors (Chang and Yang, 2000). Executioner caspases contain much shorter pro-domains (~30 amino acids compared to over 100 amino acids for initiator caspases) which are thought to actually inhibit caspase activation. Active executioner caspases have been shown to consist of hetero-dimers of both subunits which are required for their proteolytic activity (Thornberry *et al.*, 1992). An overview of the domains within the different caspase family members can be seen in Figure 1-13.

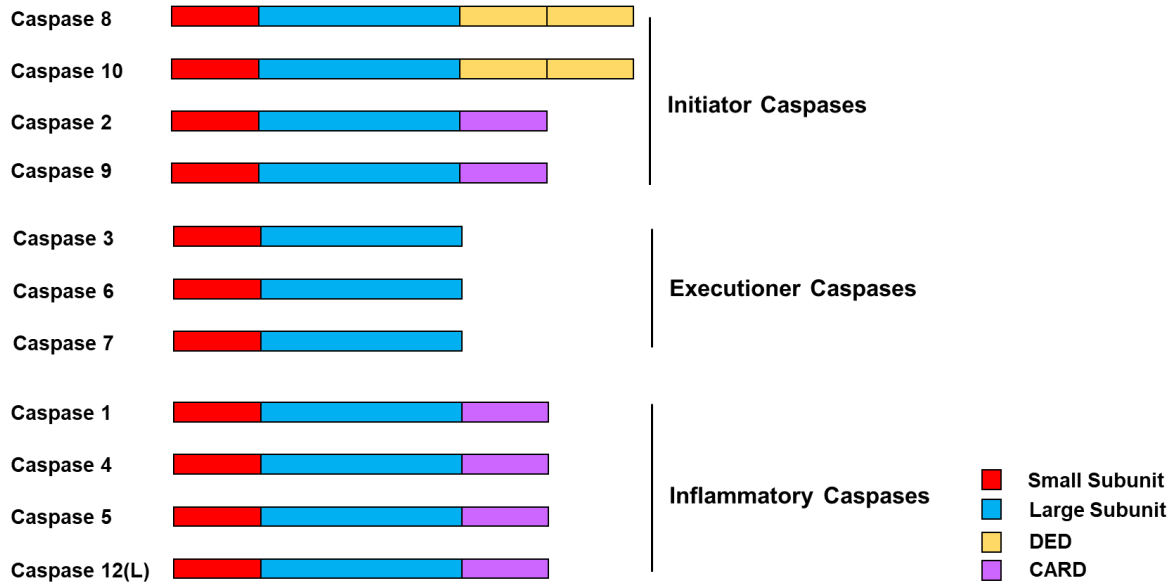



**Figure 1-13 Caspase Family Domains.**

Caspases can be grouped into three families: initiator, executioner and inflammatory caspases. The fundamental make up of all caspases is similar, they consist of small and large subunits as well as a pro-domain region consisting of death effector domains (DED) in initiator caspases 8 and 10 or caspase recruitment domains in initiator caspases 2 and 9 as well as all inflammatory caspases. These regions mediate inter and intra-caspase molecule interactions as well as interactions with adaptor proteins. Executioner caspases contain very short pro-domains (not shown in the figure) which serve to inhibit their activation.

## 1.3.2 Intrinsic Apoptosis

The intrinsic apoptosis pathway is also known as the mitochondrial apoptosis pathway since it involves the release of factors from the mitochondria. This pathway is activated by internal stimuli including ER stress and DNA damage and the key caspase responsible for propagating the signal and mediating downstream events is caspase 9, one of the initiator caspases. Apoptotic stimuli cause the release of a protein called cytochrome c from the intermembrane space. This protein functions as an electron shuttle in the respiratory chain and is an essential protein for normal functioning of cells. Cytochrome c is released into the cytosol via a two-step process involving solubilization of the protein followed by the permeabilization of the outer mitochondrial membrane by a protein called Bax (Ott *et al.*, 2002).

Cytosolic cytochrome c is then able to mediate the allosteric activation and oligomerization (in the presence of deoxy ATP) of the adaptor molecule apoptosis-

protease activating factor 1 (APAF-1) to form what is known as the apoptosome (Cain, Bratton and Cohen, 2002). This complex is able to recruit seven molecules of pro-caspase 9 via the caspase recruitment domains (CARD) of each APAF-1 molecule which allows for the formation of dimers between the two proteins through binding by two highly charged and complementary surfaces (Qin *et al.*, 1999). Recruitment of pro-caspase 9 to the apoptosome complexes allows for activation and cleavage of the initiator via self-processing mechanisms. It was demonstrated that cleaved caspase 9 is not required to form an active cell death complex, only pro-caspase 9 is required (Acehan *et al.*, 2002). Cleaved and activated caspase 9 is then able to mediate activation of executioner caspases resulting in the downstream consequences of apoptosis.

When studying the intrinsic apoptosis, chemical agents can be used to specifically trigger this branch of the pathway. One such agent is staurosporine which is a potent inhibitor of protein kinase C (PKC) in addition to two thirds of all kinases making it very promiscuous, and it is derived from *Streptomyces staurosporeus*. Staurosporine has been used extensively as an inducer of apoptosis and it is understood to cause, amongst other events, cytochrome C release from mitochondria to activate downstream caspases and the characteristic events underlying apoptosis (Zhang *et al.*, 2005; Manns *et al.*, 2011; Yadav *et al.*, 2015; Malsy *et al.*, 2019). Staurosporine has been shown to require several hours to mediate cytochrome C release (Bossy-Wetzel, Newmeyer and Green, 1998) which is important when studying the timing of apoptosis events. Staurosporine has also been shown to induce necroptosis (a type of programmed necrosis, which itself is a form of premature cell death mediated by autolysis) in instances where caspase activity is compromised, including via the use of pan-caspase inhibitors (Dunai *et al.*, 2012).

### 1.3.3 Extrinsic Apoptosis

The extrinsic branch of the apoptosis pathway differs from the intrinsic pathway both in terms of the mechanism of activating executioner caspases but also in the triggering of the pathway. Extrinsic apoptosis is mediated by death receptors which are members of the tumour necrosis factor receptor (TNFR) superfamily. Some of the key receptors involved in extrinsic apoptosis include Fas/Apo1,

TNFR1, death receptor 4 (DR4) and death receptor 5 (DR5) with the last two being involved in TNF-related apoptosis-inducing ligand (TRAIL) signalling. These receptors all contain intracellular death domains which recruit death domain-containing adaptor proteins required to mediate downstream signalling events required for apoptosis. TNFR family members consist of type I transmembrane proteins possessing 2 to 4 cysteine-rich domains in the extracellular domain, and most receptors trimerize upon ligand binding. Ligand binding leads to the recruitment of the aforementioned death domain-containing proteins TNFR-associated death domain protein (TRADD) or Fas-associated death domain protein (FADD) (Locksley, Killeen and Lenardo, 2001). TRADD is recruited to TNFR1 following binding of the cytokine TNFα while FADD is recruited to the Fas receptor or DR4/DR5 following Fas or TNFα-related apoptosis inducing ligand (TRAIL) binding respectively (Walczak and Krammer, 2000). Extrinsic apoptosis involves the activation of the initiator caspase 8 or 10 to mediate the cleavage and activation of effector caspases which are common to both the extrinsic and intrinsic branches of the apoptosis pathway. It is important to note that there exists crosstalk between the two pathways which was initially discovered in Fas and TNFα signalling: caspase 8 can mediate the cleavage of a protein called BID to form truncated BID (tBID) which then translocates to the mitochondria and promotes cytochrome C release (Li *et al.*, 1998).

#### 1.3.3.1 TNFα-Mediated Apoptosis

TNFα is a cytokine which induces a variety of cellular processes including inflammatory cytokine production and cell survival as well as cell death. TNF is produced as a type II transmembrane protein and is cleaved by a metalloprotease called TNF alpha converting enzyme (TACE) (Black *et al.*, 1997). TNF signals through two key receptors, TNFR1 and TNFR2 with soluble TNFα showing a preference for TNFR1 and membrane-bound TNFα showing a preference for TNFR2 (Wajant, Pfizenmaier and Scheurich, 2003). Furthermore, TNFR1 expression is constitutive while TNFR2 expression is highly regulated and this receptor lacks an intracellular death domain. TNF receptors trigger multiple intracellular signalling pathways including I-κB kinase (IKK) and c-Jun N-terminal kinase (JNK) to mediate gene expression regulation via transcription factors including NF-κB and AP-1 (Varfolomeev and Ashkenazi, 2004).

TNFα binds to pre-trimerized TNFR1 receptors (Chan *et al.*, 2000) which promotes binding of the death domain-containing protein TRADD (Locksley, Killeen and Lenardo, 2001). Following binding of TRADD, additional adaptors are recruited to the receptor bound complex (known as complex I) including receptor-interacting protein (kinase) 1 (RIP1) and TNFR-associated factor 2 (TRAF2). These then activate the IKK complex which stimulates NF-κB activity by promoting the phosphorylation and subsequent ubiquitination and degradation of IκBα, which allows for the nuclear translocation of NF-κB and subsequent transcriptional activities which promote survival (Miyamoto *et al.*, 1994) via mechanisms including increased expression of cellular inhibitors of apoptosis (cIAPs). cIAPs are also recruited to complex I by TRAF2 (Chen and Goeddel, 2002).

It has long been known that association of FADD is required to mediate apoptosis as this adaptor is responsible for the recruitment of pro-caspase 8 to form what is known as the death-inducing signalling complex (DISC), which was initially discovered and detailed in Fas signalling (Peter and Krammer, 2003) and also plays an essential role in TRAIL-mediated apoptosis. In the case of TNFα signalling, it was discovered that a second complex (complex II) forms downstream of the receptor-bound complex which is responsible for pro-death signalling (Micheau and Tschopp, 2003). Complex II thus forms following dissociation of the core components of complex I into the cytosol and consists of FADD, pro-caspase 8 and RIP3. The transition between complex I and complex II hinges on the association between TRADD and FADD (Hsu *et al.*, 1996).

The precise components of complex II are thought to vary, but another key component of this complex is a protein called cellular FLICE-like inhibitory protein (cFLIP) of which there are two isoforms: short ($cFLIP_S$) and long ($cFLIP_L$). cFLIP is structurally related to caspase 8 but each isoform has a different mechanism for inhibiting caspase 8 activation. $cFLIP_L$ contains two DED domains and an inactive caspase-like domain while $cFLIP_S$ consists only of two DED domains (Krueger *et al.*, 2001). Both isoforms are recruited to the DISC via their DED domains while $cFLIP_S$ blocks caspase 8 cleavage by preventing the initial cleavage step of the pro-enzyme while $cFLIP_L$ allows for this step to occur but prevents full maturation of

caspase 8 by forming a hetero-dimer with caspase 8 as an intermediary fragment (Krueger *et al.*, 2001).

For TNFα signalling to induce apoptosis, this requires the inhibition of NF-κB activation, the co-treatment with an inhibitor of protein translation such as cycloheximide, or the depletion of endogenous cellular inhibitors of apoptosis (cIAPs). In addition, down regulation of cFLIP is a key factor in promoting apoptosis following TNFα signalling and this can be achieved experimentally through the use of the protein synthesis inhibitor cycloheximide which specifically reduces levels of cFLIP but not of the other components of the DISC/complex II (Pająk, Gajkowska and Orzechowski, 2005). An outline of the two main physiological outcomes of TNFα signalling as well as the key components involved is shown below in Figure 1-14.

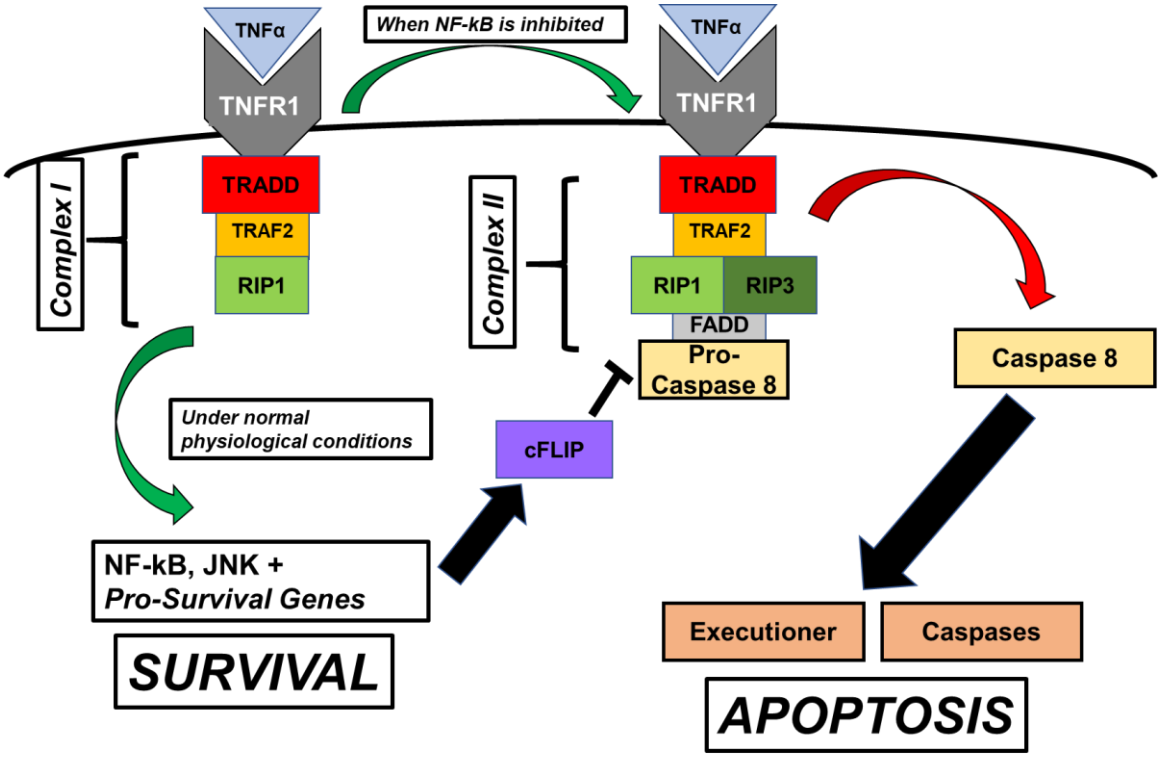



**Figure 1-14 An Overview of TNFα Signalling in Apoptosis.**

TNFα binds to trimerized TNFR1 which causes the recruitment of the death domain-containing protein TRADD. This in turn recruits the secondary adaptors TRAF2 and RIP1 to form the receptor-bound complex I, which promotes the phosphorylation and degradation of the inhibitor of NF-κB known as IκBα which allows for nuclear translocation of NF-κB and upregulation of genes related to inflammation and survival. This pathway is the default pathway following TNFα binding to its receptor. In circumstances where NF-κB signalling is inhibited or the inhibitor of apoptosis cFLIP is downregulated, a second complex forms which is cytosolic (complex II) which involves the recruitment of FADD which in turn recruits the initiator caspase 8 (shown here) or caspase 10 as ell as RIP3. FADD and pro-caspase 8 form what is known as death-inducing signalling complex (DISC) which is common to various death-inducing pathways from, different TNFα-like ligands. The DISC promotes caspase 8 cleavage and activation which then activates downstream effector caspases to mediate the various processes involved in apoptosis and cell death.

Further complicating our understanding of this signalling pathway, TNFα has been shown to induce two distinct pathways of caspase 8 activation and this has been shown to hinge on the role of the kinase RIP1. In the RIP1-independent pathway, caspase 8 cleavage is regulated by cFLIP and this can be overcome using cycloheximide as mentioned above to specifically reduce the levels of this endogenous inhibitor. In a RIP1 dependent manner, caspase 8 can also be activated using a Smac mimetic which targets inhibitor of apoptosis (IAP) proteins in the same way as the endogenous Smac protein. This promotes the release of

RIP1 from the activated complex I to form a complex consisting of RIP1, FADD and caspase 8 (Wang, Du and Wang, 2008). This complex was later named the Ripoptosome and shown to also consist of the other initiator caspase , caspase 10, as well as $cFLIP_L$ (Feoktistova *et al.*, 2011). The complexity of TNFα-mediated apoptosis makes studying the effect of the cytokine as an inducer of cell death challenging, and we therefore additionally employ the use of TRAIL in our experimental work, which has a better characterised and more streamlined mechanism.

#### 1.3.3.2 TRAIL-Mediated Apoptosis

TNF-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein that is related to both TNFα and Fas. TRAIL can be proteolytically cleaved from the cell surface to release its extracellular C-terminal portion while pre-formed TRAIL can also be stored in the cytoplasm and released during the activation-induced cell death (AICD) process which serves as a negative regulator of T lymphocytes (Monleón *et al.*, 2001). TRAIL binds to trimeric receptors in a similar fashion to TNFα as described in the previous section, and in the initial stages of research into TRAIL it was shown that TRAIL is selective in inducing apoptosis in tumour cells compared to normal cells (Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999), however, it quickly emerged that there were likely species specific differences in sensitivity to TRAIL (Jo *et al.*, 2000) as well as organ and tissue specific differences (Seki *et al.*, 2003). Another potential indication that TRAIL may represent a useful therapeutic agent was the high expression of its receptors DR4 and DR5 in various malignancies (Sträter *et al.*, 2002; Kurbanov *et al.*, 2005). This is also supported by the finding that TRAIL resistant MCF-7 cells undergo constitutive endocytosis of DR4 and DR5 which is thought to confer this resistance (Zhang and Zhang, 2008).

TRAIL can bind to one of 4 homologous receptors in humans which include DR4 and DR5 as mentioned already, as well as decoy receptor 1 (DcR1) and decoy receptor 2 (DcR2). DR4 and DR5 are involved in mediating apoptosis (thus their name ‘Death Receptor’) and have conserved intracellular death domain motifs that are involved in recruitment of the death domain containing protein FADD to mediate apoptosis (Pan *et al.*, 1997a; Walczak *et al.*, 1997; Pan *et al.*, 1997b). DcR1 and DcR2 were

shown to be non-functional homologues of DR4 and DR5 that actually protected cells from apoptosis by different mechanisms (which are discussed in 1.3.3.3) (Pan *et al.*, 1997a; Degli-Esposti *et al.*, 1997b; Degli-Esposti *et al.*, 1997a; Sheridan *et al.*, 1997; Marsters *et al.*, 1997).

FADD interacts with the intracellular death domains of death receptors via homophilic interactions (Walczak and Krammer, 2000). In TRAIL signalling, FADD is the key adaptor molecule and as in TNFα signalling, FADD then recruits pro-caspase 8 via DED domain interaction between the two proteins (see Figure 1-13 for more information about caspase domain structure), this is an event which occurs at the receptor-bound complex in TRAIL signalling but not in TNFα signalling, where this occurs at the cytosolic complex II (Harper *et al.*, 2003). FADD and pro-caspase 8 form the death-inducing signalling complex (DISC) which serves as a platform for caspase 8 cleavage and activation (Kischkel *et al.*, 2000; Sprick *et al.*, 2000). The mechanism underlying caspase 8 cleavage is discussed in depth in section 1.3.4, but very briefly it involves its ubiquitination and auto-processing to produce catalytically active clusters of caspase 8 which are maintained in aggregates in the cytosol to mediate downstream cleavage events.

There exist two mechanisms by which caspase 8 can activate downstream effector caspases and this is categorized into type I and type II cells (Scaffidi *et al.*, 1998). In type I cells, caspase 8 cleavage at the DISC occurs in sufficient quantities to induce caspase 3 cleavage and activation within 30 minutes. In type II cells, little caspase 8 is produced at the DISC so mitochondrial outer membrane permeabilization is required to activate the apoptosome to assist in the activation of downstream effector caspase activation. It was shown that mitochondrial involvement is present in both cell types however it is only essential for type II cells, Furthermore it was shown that overexpression of the protein Bcl-2, which is an inhibitor of apoptosis, was able to inhibit TRAIL-induced apoptosis in only the type II cells (Scaffidi *et al.*, 1998). This finding was taken further when it was discovered that caspase 8 cleavage can occur both upstream and downstream of mitochondria in a feedback loop which was shown by overexpressing Bcl-2 and finding that caspase 8 and BID cleavage were reduced resulting in resistance to TRAIL. This supported a finding whereby executioner caspases are able to promote

caspase 8 cleavage and thus serve as a feedback loop to amplify the apoptotic process (Fulda, Meyer and Debatin, 2002).

DISC formation occurs at the receptor bound complex II in TRAIL signalling as opposed to the cytosolic complex II in TNFα signalling. While less common and less well understood, TRAIL signalling can also promote inflammatory signalling via a second complex that forms after the DISC which involves the recruitment of proteins including TRAF2 and RIP1 (which are all involved in pro-survival signalling from complex I during TNFα signalling) as well as the regulatory subunit of the IKK kinase complex NEMO (Varfolomeev *et al.*, 2005). This second complex has been shown to activate JNK, p38 and NF-κB signalling pathways, and activation of these pathways and the formation of this second complex was shown to be increased by caspase activation which was postulated to act regulatory mechanism (Varfolomeev *et al.*, 2005). Consensus seems to be that activation of pro-inflammatory responses occurs downstream of caspase 8 recruitment to the DISC and thus also requires FADD, however the involvement of RIP1 and FLIP may not be essential (Grunert *et al.*, 2012). A summary of the TRAIL signalling pathways can be seen in Figure 1-14. It is interesting that TNFα and TRAIL can induce pro-survival and pro-apoptosis signalling pathways but do so from different complexes with similar components.

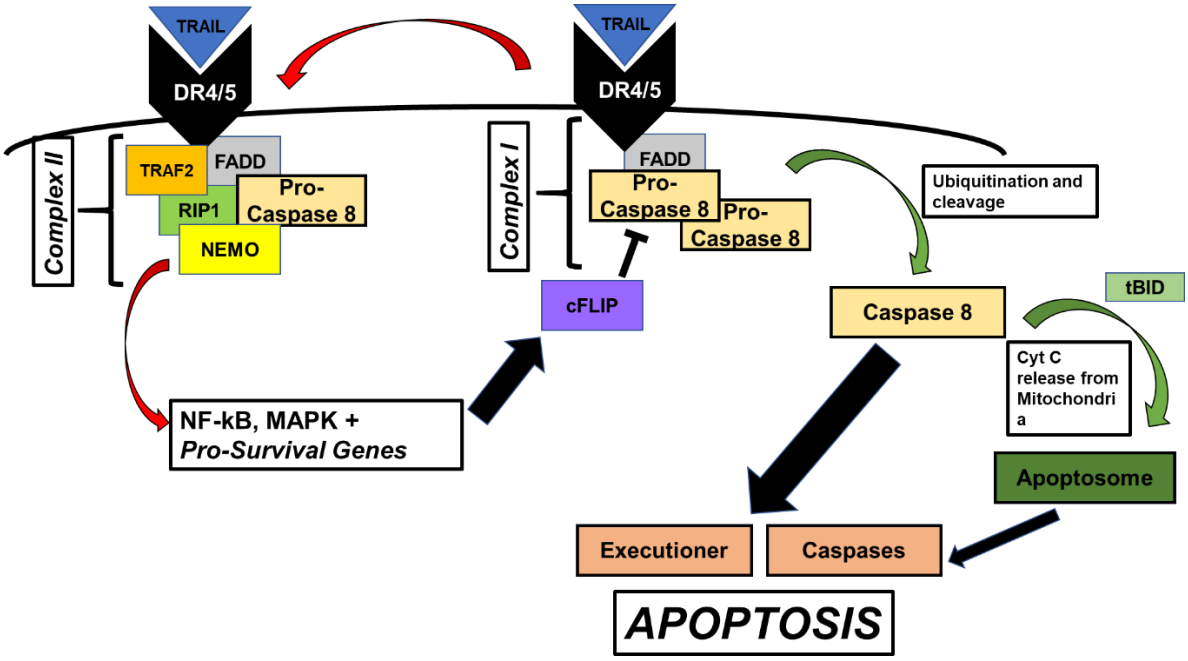



**Figure 1-15 An Overview of TRAIL Signalling in Apoptosis.**

TRAIL binding to its receptors DR4 or DR5 leads to the recruitment of FADD which interacts with the intracellular death domains of the trimerized receptors. FADD in turn recruits pro-caspase 8 via homophilic interactions between their DED domains, to form the DISC (complex I). At the DISC, caspase 8 is ubiquitinated and undergoes auto-catalytic processing to form active pools of caspase 8 which are released from the DISC. In type I cells this is sufficient to induce downstream caspase activity, in type II cells amplification of the signal is required which is mediated by cleaved BID (tBID) which can promote cytochrome C release from mitochondria to ultimately activate caspase 9 via the apoptosome and promote effector caspase activation. Under specific circumstances, and possibly in a feedback loop, a second complex can form (complex II) which consists of FADD and pro-caspase 8 as well as RIP1, TRAF2 and NEMO. This complex can promote inflammatory signalling via pathways such as MAPK and NF-κB which can upregulate inhibitors such as cFLIP which shut off the apoptosis induction pathway. The inflammatory signalling is also thought to play a role in signalling for phagocytic engulfment of apoptotic cells.

### 1.3.3.3 Regulation of TRAIL signalling

TRAIL signalling is tightly regulated by various proteins including cFLIP which was mentioned previously for its role in TNFα signalling, as well as by proteins called inhibitors of apoptosis (IAPs) which were also mentioned briefly. cFLIP plays a key role in DISC-mediated apoptotic signalling in TRAIL signalling as well as in TNFα signalling – cFLIP is structurally similar to caspase 8 but is lacks catalytic activity The short form of cFLIP is truncated and contains DED domains only, while the long form closely resemble the full-length pro caspase 8 but lacks catalytic

activity. Expression of cFLIP was found to be up-regulated in gallbladder carcinoma and down-regulation of the protein sensitised the cells to TRAIL-induced apoptosis (Zong *et al.*, 2009). cFLIP has also been shown to become up-regulated in response to TRAIL treatment in non-small-cell lung carcinoma cells that evaded TRAIL-mediated apoptosis indicating a mechanism by which cancer cells are able to evade cell death. This resistance was inhibited both by co-stimulation with the protein synthesis inhibitor cycloheximide (which specifically interacts with cFLIP in an inhibitory fashion) as well as by inhibition of $Ca^{2+}$/calmodulin signalling (Kaminskyy *et al.*, 2013). cFLIP expression has also been shown to correlate with sensitivity to TRAIL in human melanoma cells, where TRAIL resistance was found to be conveyed as a result of high cFLIP expression in cells that were not sensitive to TRAIL stimulation, and resistance to TRAIL was able to be induced by ectopic expression of cFLIP in cells that were highly sensitive to the drug (Geserick *et al.*, 2008).

TRAIL signalling is also regulated by expression of its receptors, with decoy receptors acting as crucial negative regulators of TRAIL signalling. DcR1 lacks a cytoplasmic domain (Pan *et al.*, 1997a) and transient overexpression of this receptor did not induce apoptosis which contrasts from overexpression of DR4 and DR5 which significantly increased apoptosis (Degli-Esposti *et al.*, 1997b), while DcR1 also appears to inhibit TRAIL signalling due to sequestering of TRAIL ligand away from the functional receptors (Sheridan *et al.*, 1997). It was suggested that the lower expression of DcR1 in cancer cells accounted for their increased sensitivity to TRAIL compared to normal cells. DcR2 contains a truncated intracellular death domain and is able to induce NF-κB activation (Degli-Esposti *et al.*, 1997a) similarly to DR4/5 but is unable to induce apoptosis. Furthermore, it was shown by two groups independently that overexpression of DcR2 protected normally sensitive cells to TRAIL stimulation and confirmed the inability of the receptor to induce apoptosis (Degli-Esposti *et al.*, 1997a; Marsters *et al.*, 1997).

TRAIL receptor variation and the existence of decoy receptors has made using TRAIL in therapy challenging. There is however a large body of data on the significance on the ratio of decoy receptors to functional death receptors in tumour cells, with one group reporting that high DcR1 expression on leukemic

blasts is associated with poor outcome and causes apoptosis resistance and another group reporting that reduction in levels of an additional decoy receptor more recently discovered called DcR3 enhances TRAIL-mediated apoptosis in pancreatic cancer (Wang *et al.*, 2013). DcR2 has also been shown to be a p53 target gene that regulates chemosensitivity (Liu *et al.*, 2005), thus understanding the distribution and expression of TRAIL receptors is key in developing new cancer therapy strategies.

## 1.3.4 Caspase 8

Caspase 8 is the critical initiator caspase involved in the extrinsic branch of the apoptosis pathway. It is also involved in amplification of the apoptosis signal via cleavage of BID and activation of the intrinsic branch under certain circumstances, as mentioned previously. Caspase 8 is cleaved and activated following stimulation of TNFα, TRAIL and Fas and its recruitment to death signalling complexes as well as its activation and cleavage has been well studied and well characterised.

### 1.3.4.1 Activation of Caspase 8

Pro-caspase 8 consists of a pro-domain which contains two death effector domains (DEDs) as well as two enzymatic domains known as p18 and p10 sub-domains. The full-length monomeric caspase 8 molecule is recruited to the DISC (we will focus on DISC assembly following TRAIL stimulation) via its DED domains which interact with the DED domains of FADD via homophilic interactions. Recruitment to the DISC and DED interactions result in the dimerization of pro-caspase 8 which is a required step in promoting its activation (Muzio *et al.*, 1998; Boatright *et al.*, 2003; Pop *et al.*, 2007). Research has shown that dimerization-incompetent processed caspase 8 as well as monomeric-procaspase 8 exhibit a lack of catalytic activity (Keller *et al.*, 2009) and that cleaved caspase 8 shows specificity towards pro-caspase 8 and dimerization enhances intramolecular cleavage due to structural rearrangements, suggesting a model whereby different types of cleavage within caspase 8 populations can occur (Keller, Grütter and Zerbe, 2010).

There exist two pro-caspase 8 isoforms: 8a (p55) and 8b (p53) which are collectively known as p55/53 caspase 8. This full-length form of caspase 8

undergoes cleavage at aspartic acid residues contained within linkers either between the pro-domain and the catalytic domains, or between the two catalytic sub-domains, thus various intermediate cleaved forms of caspase 8 are produced during activation. An outline of the cleavage products of caspase 8 can be seen in Figure 1-16. Pro-caspase 8 can be cleaved at aspartic acid residues 374 and 384 between the two enzymatic sub-domains to form what is known as the p43/41 fragment (a and b isoforms respectively) as well as free p10 catalytic domain – these cleavage products typically appear first after activation (Chang *et al.*, 2003). The p43/41 fragment has been shown to be catalytically active, stable, and capable of activating some downstream events including caspase 3 cleavage and BID cleavage and represents a key stage in apoptosis signal transduction (Hughes *et al.*, 2009). When studying caspase 8 cleavage, the stability of the p43/41 fragment makes it a good marker for caspase 8 cleavage while detection of other fragments can be more challenging.

Cleavage can also occur at aspartic acid residues in the linker region between the pro-domain and the catalytic domain ($Asp^{210}$ and $Asp^{216}$) to form the p26/24 fragment (a and b isoforms respectively) as well as the p30 fragment which consist of the two catalytic sub-domains. This p30 fragment has been shown to play a role in sensitising cells towards death receptor-induced apoptosis (Hoffmann *et al.*, 2009). Further processing of caspase 8 occurs via cleavage at the remaining aspartic acid residues yet to be cleaved in each fragment, ultimately producing more of the p26/24 fragment, as well as p10 and p18 fragments. p10 and p18 fragments combine to form a hetero-tetramer which is referred to for simplicity in this report as p18 (caspase 8), and this form is catalytically active and responsible for the majority of downstream cleavage events mediated by caspase 8 and is formed at the DISC and released into the cytosol (Lavrik *et al.*, 2003) which is regarded as the most up to date view and contrasts with earlier evidence that the p18 hetero-tetramer forms in the cytosol rather than at the DISC itself (Medema *et al.*, 1997).

Caspase 8 cleavage has been shown to occur by three different methods: intramolecular processing within the same pro-caspase 8 molecule, intermolecular processing between pro-caspase 8 molecules associating with the same DISC

(intradimer processing) and intermolecular processing between pro-caspase 8 molecules associated with different DISCs (interdimeric) (Kallenberger *et al.*, 2014). Furthermore, mathematical modelling which was supported by experimental work demonstrated that the cleavage that occurs between the two catalytic sub-domains p18 and p10 occurs as a result of interdimeric processing, while the cleavage between the pro-domain and the p18 domain occurs as a result of intradimeric processing (Kallenberger *et al.*, 2014). Overall, their model suggests that this dual mechanism of caspase 8 activation ensures that low TRAIL concentrations trigger weak signalling while higher concentrations of the ligand promoted activation which provides a significant level of precision to the apoptosis response. This study also provided insight into the role of the FLIP proteins which are able to form hetero-dimers with pro-caspase 8 in a manner which does not promote apoptosis signalling due to the restrictions on the ability for caspase 8 to be cleaved by interdimer processing (Kallenberger *et al.*, 2014; Riley *et al.*, 2015).

More recently it has been discovered that cFLIP may also play a cooperative role in caspase 8 activation rather than simply an antagonist role. This was shown to depend on the isoform of cFLIP, with the short variant ($cFLIP_S$) acting only in an antagonistic fashion while the long form ($cFLIP_L$) was able to promote and inhibit caspase 8 activation (Hughes *et al.*, 2016). By reconstituting the DISC and using structure-guided mutagenesis, Hughes and colleagues discovered that pro-caspase 8 hetero-dimerizes with cFLIP and the activation of pro-caspase 8 was controlled by the ratio of unbound $cFLIP_{L/S}$ to pro-caspase 8 which in turn regulates the composition of the pro-caspase 8:cFLIP heterodimer. Formation of pro-caspase 8:$cFLIP_L$ demonstrated enzymatic activity and was able to promote pro-caspase 8 activation while pro-caspase 8:$cFLIP_S$ lacked activity and readily blocked pro-caspase 8 activation (Hughes *et al.*, 2016). Thus, the levels of the different isoforms act as a regulator of pro-caspase 8 oligomerisation and cleavage and targeting either isoform may represent key therapeutic targets.

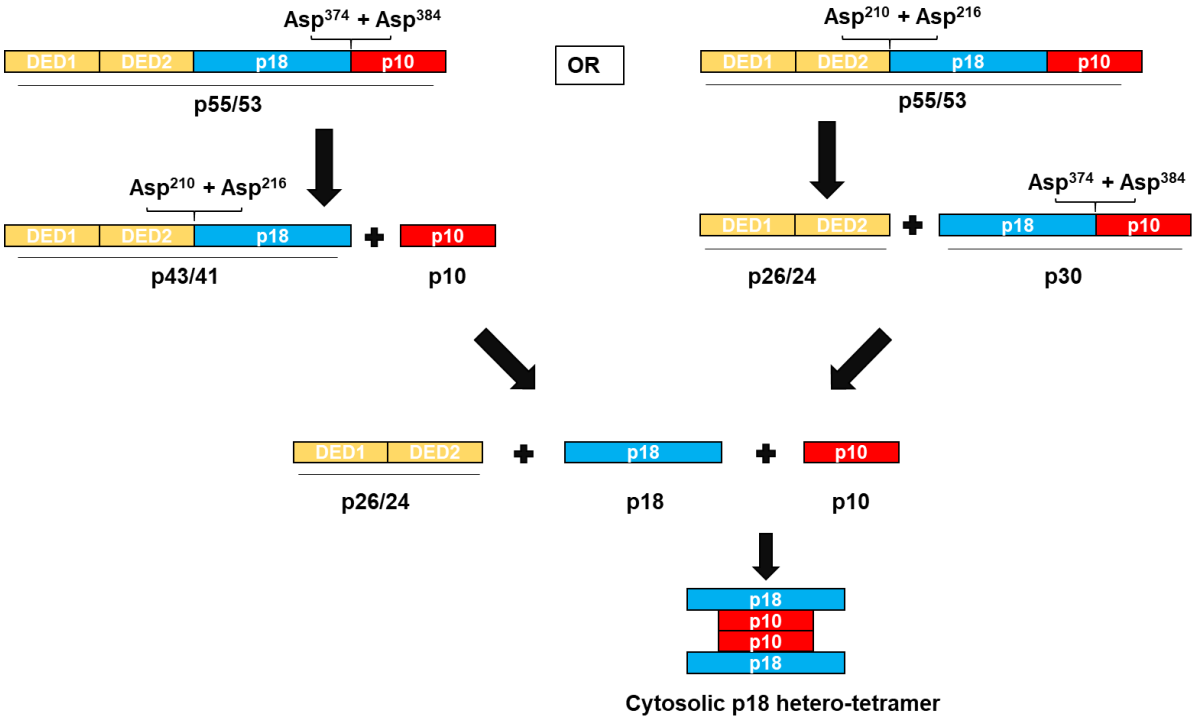



**Figure 1-16 Caspase 8 Cleavage Products.**

This diagram shows the key fragments of caspase 8 that form following its cleavage at specific aspartic acid residues within its amino acid sequence. Pro-caspase 8 consists of two DED domains as well as a catalytic domain which is composed of two subdomains, p18 and p10. Cleavage of caspase 8 can occur by two pathways and both result in the formation of active p18 caspase 8 which consists of a hetero-tetramer of p10 and p18 subunits. Cleavage of the p55/53 (isoform a and b respectively) full-length caspase 8 can occur at aspartic acid residues in the linker between the two catalytic sub-domains to produce p43/41 and p10 fragments, or between the pro-domain and the catalytic domain to produce p26/24 and p30 fragments. p43/41 ad p26/24 have been shown to possess catalytic activity and an ability to mediate cleavage of some downstream components. Cleavage of the other linker region at aspartic acid residues of the processed fragments results in the formation p26/24, p18 and p10 fragments, with the latter two fragments combining to form the active form known as p18, which is responsible for the majority of downstream cleavage events. p18 caspase 8 is released from the DISC into the cytosol.

### 1.3.4.2 Caspase 8 Ubiquitination by Cullin 3

Caspase 8 was shown in 2009 to be regulated by ubiquitination by cullin 3 in what was demonstrated to be a critical modification that occurs at the DISC. This ubiquitination was shown to be polyubiquitin via K63 and K48 linkages on a lysine within the C-terminal domain of caspase 8, more specifically, within the p10 catalytic sub-domain (Jin *et al.*, 2009). The work by Jin and colleagues was extensive in elucidating the importance of cullin 3 in caspase 8 activation, showing

that cullin 3 interacts with the DISC and demonstrating that reduction in cullin 3 expression resulted in a reduction in caspase 8 ubiquitination, while overexpression of cullin 3 increased caspase 8 modification (Jin *et al.*, 2009). The interaction of cullin 3 was dependent on its interaction with RBX1, and the de-ubiquitinating enzyme A20 was shown to reverse this modification. The role of A20 as a regulator of this pathway was further investigated more recently and the polyubiquitination by K48 and K63 linkages were shown to be counteracted by A20 thus providing another regulator of caspase 8 activity (Lim *et al.*, 2017).

In addition to the role of cullin 3 in caspase 8 ubiquitination, Jin and colleagues also provided insight into a role for the scaffold protein p62 (Jin *et al.*, 2009). p62 is a ubiquitin-binding protein, which binds polyubiquitinated proteins via its UBA domain and has been shown to play a role in interactions with protein aggregates as well as targeting proteins for degradation (Seibenhener *et al.*, 2004). It was shown that in the context of caspase 8 activation, p62 interacts with the DISC and promotes aggregation of cullin 3-modified caspase 8 in ubiquitin-rich foci in the cytosol. This aggregation enhances activity and promotes the auto-proteolytic release of caspase 8 into the cytosol (Jin *et al.*, 2009). The role of p62 was shown to enhance interaction with A20 to mediate the de-ubiquitination of pro-caspase 8 suggesting that p62 may play a role as an adaptor between pro-caspase 8 and A20 (Lim *et al.*, 2017). As yet, no research has been undertaken into determining the substrate adaptor for the cullin-3/RBX1-based CRL complex, and no work has been done in studying the effect of neddylation inhibition or the role of the DCNL protein family in the ubiquitination of caspase 8 – the present research aims to investigate the link between cullin 3 polyubiquitination of caspase 8 and the neddylation pathway.

#### 1.3.4.3 Caspase 8 In Disease

The role of caspase 8 in the apoptosis pathway has been described in detail, however its importance in normal physiology and role in disease requires more analysis. Caspase 8 is often dysregulated in human cancers, given its role in promoting cell death it is perhaps unsurprising that a characteristic of many cancers is an inactivation of the protein through a variety of mechanisms including epigenetic modulation and posttranslational modifications. While the frequency

of mutations in caspase 8 in cancers is low, there have been instances of mutated caspase 8 which is unable to transduce death signals by acting as a dominant negative inhibitor of death in colorectal carcinomas (ref Kim et al, 2003) and in head and neck carcinomas, in this latter case due to a lengthening of the protein by 88 amino acids (Mandruzzato *et al.*, 1997). In hepatocellular carcinomas, the caspase 8 gene was found to be frequently inactivated due to a frameshift somatic mutation which caused a two base-pair deletion resulting in premature termination of translation in the p10 catalytic subunit and thus a loss of caspase 8 and therefore cell death function (Soung *et al.*, 2005).

There exist various caspase inhibitors, many of which are used routinely in studying caspase cleavage in cell, and some which are available for use clinically, for example emricasan which was developed to protect liver cells from excessive apoptosis. Caspase 8 inhibitors have been shown to work cooperatively with other drugs that target the apoptosis pathway to induce necroptosis in acute myeloid leukaemia cells which are often resistant in the absence of caspase 8 inhibition (Brumatti *et al.*, 2016) indicating therapeutic benefits to targeting caspase 8. Caspase 8 inhibitors have also been used to reduce the outgrowth of lung tumours which was in part caused by a reduction in the release of pro-inflammatory cytokines including IL-6, IL-1α and TNFα (Terlizzi *et al.*, 2015). Given the complexity of the pathway governing caspase 8 activation, and the many different regulators and interactions that occur in apoptosis, developing specific drugs to inhibit caspase 8 activation or drugs to promote its activation represent a key challenge. This thesis aims to elucidate additional regulation in the caspase 8 activation pathway by DCNL5 which may in future represent a therapeutic target.

## 1.4 Thesis Aims

The overall aim of this thesis was to characterise the role of DCNL5 in the apoptosis response. While the initial work planned to validate and further investigate the recent discovery that DCNL5 has a unique function amongst the DCNL protein family in the DNA damage response, it became clear during this study

that DCNL5 plays additional roles *in vitro* including in the extrinsic branch of the apoptosis pathway. To date, few unique functions for the DCNL proteins have been described and definitively characterised and the present work aimed to comprehensively detail the role DCNL5 plays in cell death signalling, in what appeared to be a cullin-dependant manner.

The role of DCNL5 in the apoptosis response was investigated using an array of activators of the pathway, and apoptosis induction was detected using an ATP-based luciferase assay. The cleavage of caspase 8, which is a key marker for apoptosis induction, was analysed by western blotting and we specifically aimed to optimise for the detection of the p18 form of cleaved caspase 8. It has already been comprehensively shown that caspase 8 cleavage impairment results in diminished apoptosis induction and thus cell death, and the importance of DCNL5 in the signalling mechanism was investigated through siRNA-mediated manipulation of DCNL5 levels.

Cullin 3 has been shown to be required for the successful cleavage of caspase 8 in death receptor signalling, so we wanted to confirm this finding in U2OS cells and a variety of other cancer cell lines. Finally, the role of DCNL5 was characterised in a variety of cancer cell lines by western blotting following siRNA-mediated gene silencing, as well as by establishing a stable CRISPR/Cas9 KO cell line using H460 cells and validating these cells by genome DNA sequencing in addition to testing their ability to undergo caspase 8 cleavage.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Reagents and Kits

Catalogue number preceded by '#'.

**Abcam, Cambridge, UK**
QVD-OPh, caspase inhibitor (# ab141421
Recombinant human TRAIL protein (# ab9960)
Staurosporine (#ab120056)

**Active Biochem, Kowloon, Hong Kong**
MLN-4924 (Pevonedistat) (#A-1139)

**Bio-Rad Laboratories Ltd., Hertfordshire, UK**
40% Acrylamide/Bis Solution, 37.5:1 (#1610148EDU)
Precision Plus Protein All Blue Pre-Stained Protein Standards (# 1610373)

**Corning Inc., Maine, USA**
6 well polystyrene TC-treated cell culture plate (# CLS3516)
12 well polystyrene TC-treated cell culture plate (# CLS3513)
24 well TC-treated cell culture plate (# 3526)
75cm$^2$ Polystyrene Cell Culture Flask (# CLS430641)
150cm$^2$ Polystyrene Cell Culture Flask (# CLS430825)
96 well clear flat bottom polystyrene TC-treated microplates (# CLS3596)

**Fisher Scientific, ThermoFisher Scientific, Leicestershire, UK**
Ammonium Persulfate (#A/6160/60)
Bovine Serum Albumin (#BP9702-100)
Glycerol (#G/0650/17)
Tris Base (#BP152-1)

**GE Dharmacon, Little Chalfont, UK**
ON-TARGETplus Non-targeting siRNA #1, 5 nmol (#D-001810-01-05)

Individual ON-TARGETplus DCUN1D5 siRNA (number 1)
SMARTpool: ON-TARGETplus CUL3 siRNA

**Gibco by ThermoFisher Scientific, Paisley, UK**
Dulbecco's Modified Eagle Medium (DMEM) (#11965084)
Fetal Bovine Serum (#10100147)
L-Glutamine (#25030081)
Opti-MEM Reduced Serum Medium (#31985062)
Phosphate Buffered Saline (PBS) (#10010023)
Penicillin-Streptomycin (#15140130)
RPMI (#21875034)
0.05% Trypsin-EDTA (#25300054)

**Formedium, Norfolk, UK**
Tryptone (#TRP02)
Yeast Extract Powder (#YEA02)

**Invitrogen by ThermoFisher Scientific, Leicestershire, UK**
Dynabeads™ Protein A (#10001D)
NuPAGE™ MES SDS Running Buffer (20X) (#NP0002)
NuPAGE™ MOPS SDS Running Buffer (20X) (#NP0001)
Invivogen, California, USA

Recombinant Human TNFα (#rcyc-htnfa)

**Melford Laboratories Ltd., Suffolk, UK**
Dithiothreitol (DTT) (#MB1015)

**Merck, Darmstadt, Germany**
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (#11836170001)
Immobilon Western Chemiluminescent HRP Substrate (#WBKLS0500)
PhosSTOP™ (#4906845001)

**New England Biolabs, Massachusetts, USA**
BamHI-HF (#R3136S)

Gel Loading Dye, Purple (6X), no SDS (#B7025S)
NotI-HF (#R3189S)
Quick-Load® Purple 1 kb Plus DNA Ladder (#N0550S)
Q5® High-Fidelity DNA Polymerase (#M0491S)
T4 DNA Ligase (#M0202S)

**Promega, Wisconsin, USA**
Magne® HaloTag® Beads (#G7281)
QuantiLum® Recombinant Luciferase (#E1701)
Wizard® Genomic DNA Purification Kit (#A1120)
Wizard® Plus SV Minipreps DNA Purification Systems (#A1330)

**Qiagen, Hilden, Germany**
Blood & Cell Culture DNA Mini Kit (#13323)
QIAquick Gel Extraction Kit (#28704)
QIAquick PCR Purification Kit (#28104)

**Sartorius, Gottingen, Germany**
Minisart 0.2 um pore size syringe filter (#16534K)

**Sigma-Aldrich, Dorset, UK**
Ammonium Persulfate (#A3678)
Ampicillin Sodium Salt (#A0166)
B-Mercaptoethanol (#M6250)
BIS-TRIS (#B9754)
Bromophenol Blue (#B0126)
Calcium Chloride Solution (1M) (#21115
Cycloheximide (#01810)
Doxorubicin Hydrochloride (#D1515)
Doxycycline Hyclate (#D9891) (
Ethidium Bromide Solution (#E1510)
Iodoacetamide (#l1149)
Magnesium Chloride Solution (#M1028)
Neocarzinostatin (from Streptomyces carzinostaticus) (#N9162)
N,N,N',N'-Tetramethylethylenediamine (TEMED) (#T9281)

NP-40 Substitute (#74385)
Poly-D-Lysine Hydrobromide (#P6407)
Sodium Pyrophosphate (#221368)
Staurosporine (from Streptomyces) (#S5921)
Tetracycline (#87128)
Triton X-100 (#T9284)
TWEEN 20 (#P7949)
1,10 Phenanthroline (OPT) (#131377)

**STARLAB, Milton Keynes, UK**
StarTub Reagent Reservoir 55ml (#E2310-1010)

**Synchem, Felsberg, Germany**
D-Luciferin free acid (#s039)

**Stratech, Cambridge, UK**
Bleomycin Sulphate, (#S1214-SEL)
Etoposide (#S1225-SEL)

**ThermoFisher Scientific, Illinois, USA**
Lipofectamine 2000 Transfection Reagent (#52887)
Lipofectamine™ RNAiMAX Transfection Reagent (#13778100)
Mr Frosty™ Freezing Container (#5100-0001)
Pierce BCA Protein Assay Reagent A (#23228)
Pierce BCA Protein Assay, Reagent B (#23224)
Pierce™ Protein A/G Magnetic Beads (#88802)
Pierce™ Protein G Magnetic Beads (#88847)
Thermo Scientific™ Shandon™ Immu-Mount™ (#10622689)
UltraPure Agarose (#16500500)

**VWR Chemicals, Leicestershire, UK**
Sodium Chloride (#27810.295P)
Sodium Dodecyl Sulphate (442444H)

## 2.1.2 Specialist Equipment

**Bio-Rad Laboratories Ltd., Hertfordshire, UK**

Protein gel casting and Western blotting equipment (Mini Protean III)

Agarose gel casting equipment (Mini-Sub/Wide Mini-Sub Cell GT gel system)

**BMG-Labtech, Offenburg, Germany**

FLUOstar Omega microplate reader

**Carl Ziess Ltd, Cambridge, UK**

LSM Exciter laser scanning microscope

AxioVision light microscope

**Sartorius, Gottigen, Germany**

IncuCyte® SX1 live-cell analysis system and software

**ThermoFisher Scientific, Illinois, USA**

Mr Frosty™ Freezing Container (#5100-0001)

Nanodrop™ spectrophotometer

XCell SureLock Mini-Cell apparatus

## 2.1.3 Commercially Derived Cell Lines

| Name | Disease/Origin | Supplier |
|---|---|---|
| A375 | Skin, Malignant Melanoma | A gift from Professor George Baillie's Laboratory |
| DU-145 | Prostate; derived from metastatic site: brain | A gift from Professor George Baillie's Laboratory |

| | | |
|---|---|---|
| HeLa | Cervix | A gift from Professor Kostas Tokatlidis's Laboratory |
| HEK 293 | Human Embryonic Kidney Cells | ATCC, Virginia, USA |
| H460 | Lung Carcinoma | ATCC, American Tissue Culture Collection (ATCC), Virginia, USA Virginia, USA |
| MCF-7 | Mammary gland tissue derived from metastatic site; Adenocarcinoma | A gift from Professor Neil Bulleid's Laboratory |
| U2OS | Osteosarcoma | ATCC, Virginia, USA |
| 22Rv1 | Prostate Carcinoma | A gift from Professor George Baillie's Laboratory |

**Table 1 List of Commercially Obtained Cell Lines**

### 2.1.4 Plasmids

| **Identity** | **Description** | **Source** |
|---|---|---|
| PCDNA3.1(+)-Empty | Empty pcDNA3.1(+) mammalian expression plasmid | Made by Dr. Matthew Keuss |
| PCDNA3.1(+)-DCUN1D5- | pcDNA3.1(+) mammalian expression plasmid containing un-tagged | Made by Dr. Matthew Keuss |

| | | |
|---|---|---|
| | functional DCNU1D5 gene sequence | |
| PCDNA3.1(+)-DCUN1D5(D195A A219R D225A) | pcDNA3.1(+) mammalian expression plasmid containing un-tagged functional DCNU1D5 (DAD mutant) gene sequence | Made by Dr. Matthew Keuss |
| pcDNA5 FT/TO GFP Puro | pcdna5-FRT/to-GFP with a puromycin cassette 45825 | MRC-PPU Reagents, University of Dundee |
| pcDNA5 FT/TO GFP Puro DCUN1D5 | DCUN1D5 gene cloned into pcdna5-FRT/to-GFP with a puromycin cassette 45825 | MRC-PPU Reagents, University of Dundee |
| pcDNA5 FT/TO GFP Puro DCUN1D5 (D195A A219R D225A) | DCUN1D5 gene (DAD mutant) cloned into FRT/TO GFP with puromycin cassette. 45825 DAD Patch Mutant | MRC-PPU Reagents, University of Dundee |
| pCAG -CAS9-2A-GFP WT | | A gift from Professor Neil Bulleid's Laboratory – originally sourced from Dr. Adam West |

**Table 2 List of Plasmids**

## 2.1.5 Antibodies

### 2.1.5.1 Primary Antibodies

Antibody dilutions described below are for western blotting unless otherwise stated.

| Epitope | Host Species | Dilution (in TBS-T) | Catalogue # | Supplier |
|---|---|---|---|---|
| Actin (Clone C4) | Mouse | 1:5000 in 5% Milk | MAB1501 | Merck Millipore |
| α-Tubulin | Rabbit | 1:1000 in 5% Milk | 2125 | Cell Signalling Technology |
| Caspase 3 | Mouse | 1:5000 in 5% Milk | 9662 | Cell Signalling Technology |
| Caspase 8 (1C12) | Mouse | 1:5000 in 5% BSA | 9746 | Cell Signalling Technology |
| Caspase 8 p18 (d7) | Mouse | 2µg/ml of protein lysate?? | sc-393776 | Santa Cruz Biotechnology |
| Cullin 3 | Rabbit | 1µg per immunoprecipitation | NB100-58788 | Novus Biologicals |
| Cullin 3 (Clone C3) | Mouse | 1:1000 in 5% Milk or 1µg per immunoprecipitation | 611848 | BD Bioscience |
| Cullin 3 (N Terminal) | Sheep | 1:1000 in 5% Milk | N/A | UofDundee MRC PPU |
| Cullin 4A | Rabbit | 1:1000 in 5% Milk | 2699 | Cell Signalling Technology |
| DCNL5 | Sheep | 1:2000 in 5% Milk or 1µg per immunoprecipitation | N/A | UofDundee MRC PPU |

| | | | | |
|---|---|---|---|---|
| FADD | Rabbit | 1:1000 in 5% Milk | ab124812 | Abcam |
| GAPDH | Mouse | 1:1000 in 5% Milk | Ab110305 | Abcam |
| GFP | Chicken | 1:5000 in 3% BSA | Ab13970 | Abcam |
| IκBα (N Terminal) | Mouse | 1:1000 in 5% Milk | 4814 | Cell Signalling Technology |
| PARP1 | Rabbit | 1:1000 in 5% Milk | 9532 | Cell Signalling Technology |
| TNFR1 (C25C1) | Rabbit | 1:1000 in 5% Milk | 3736 | Cell Signalling Technology |
| TRADD (Clone 37) | Mouse | 1:1000 in 5% Milk | 610572 | BD Bioscience |
| Ubiquitin (Clone Ubi-1) | Mouse | 1:1000 in 5% Milk | MAB1510 | Merck Millipore |

**Table 3 List of Primary Antibodies**

### 2.1.5.2 Secondary Antibodies

| **Antibody** | **Catalogue #** | **Supplier** |
|---|---|---|
| Anti-mouse IgG HRP | 1705047 | Bio-Rad Laboratories |
| Anti-protein G HRP | 1706425 | Bio-Rad Laboratories |
| Anti-rabbit IgG HRP | 1705046 | Bio-Rad Laboratories |
| Anti-sheep IgG HRP | A3415 | Sigma-Aldrich |

**Table 4 List of Secondary Antibodies**

## 2.1.6 Standard Solutions

(v/v) denotes volume per volume, (w/v) denotes weight per volume.

All solutions were made up using distilled water unless specified otherwise.

**Denaturing Immunoprecipitation Lysis Buffer**

30mM Tris HCl pH 7.5

150mM NaCl

2mM $CaCl_2$

2mM $MgCl_2$

10mM Iodoacetamide

1% SDS (w/v)

1% Triton-X 100 (v/v)

0.5% NP40 (v/v)

Protease Inhibitor Cocktail (Roche)

**4x Laemmli Sample Buffer (LSB)**

250mM Tris pH 7.5

8% SDS (w/v)

10% Glycerol (w/v)

0.5nM EDTA

0.2% Bromophenol Blue (w/v)

**Ponceau S**

0.1 % Ponceau (w/v)

5% acetic acid (v/v)

**TAE Buffer**

40 mM Tris Acetate

1 mM EDTA

**TN Lysis Buffer**

20mM Tris HCl pH 7.5

150mM Na Cl
1mM MgCl2
0.5% Triton X-100 (v/v)
1% NP40 (v/v)
10mM Iodoacetamide
10µM OPT
Phospho-stop Inhibitor Tablet (Roche)
Protease Inhibitor Cocktail (Roche)

**Transfer Buffer (Tris-Glycine)**
25mM Tris
192mM Glycine
20% Methanol (v/v)

**Tris Buffered Saline (TBS)**
20mM Tris pH 7.5
150mM NaCl
*pH Adjusted to 7.6 prior to use*

**Tris Buffered Saline with Tween 20 (TBS-T)**
TBS with 0.1% Tween 20 (v/v)

**2YT Medium**
1.6% (w/v) Tryptone
1% (w/v) Yeast Extract
0.5% (w/v) NaCl
*Autoclaved prior to use*

### 2.1.7 Computer Software

GraphPad Prism (versions 5 and 7) were used to make all graphs as well as perform statistical analysis. Error bars were plotted using the standard error of the mean and 1 or 2-way ANOVA tests were used for statistical analysis.

# 2.2 Methods

## 2.2.1 Cell Culture Methods

### 2.2.1.1 Cell Growth Media

Immortalised cancer cell lines were all grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%v/v foetal bovine serum (FBS) and 1% v/v penicillin-streptomycin (P/S).

### 2.2.1.2 Growth and Subculturing of Cancer Cells

U2OS, H460, HeLa, 22rv1, DU-145, A375 and MCF7 cells were grown in T-75 flasks and kept in a sterile and humidified incubator at 5% $CO_2$, 37°C. The cells were grown until they reached 80-90% confluency, at which point they were washed with pre-warmed phospho-buffered saline (PBS), detached using 3ml of trypsin-EDTA while incubating in the aforementioned incubator for 4 minutes (for all cell lines except H460 and MCF-7, which required 6-8 minutes). DMEM was used to quench the trypsin activity and cells were either passaged at ratios of between 1:10 and 1:3, per ATCC protocols, or plated for use in experiments. Media was not changed between passages except for A375 cells which were monitored and changed when required.

### 2.2.1.3 Plating of Cells

Following trypsinization of cells and inactivation of the trypsin, as described in 2.2.1.2, cells were either counted by hand using a haemocytometer or counted using an automated cell counter (Countess II, ThermoFisher). Cells were plated at densities of approximately 65000 cells/ml to 800000 cells/ml, depending on treatment and when the cells were to be harvested. For seeding using 96-well plates, multichannel pipettes were used to transfer cells from a reservoir to the wells. Cells were always plated at least 24 hours prior to harvesting. In the case of the inducible rescue cell lines, 5ng/ml of doxycycline was added 24 hours prior to any drug treatments.

#### 2.2.1.4 Resurrection and Cell Thawing

Cryogenic vials of cells were thawed in a 37°C water bath for 1 minute before transferring the contents to 50ml Falcon tubes containing 10ml of pre-warmed culture media. Cells were spun at 500g for 5 minutes before removing the media, re-suspending the cell pellet in 15ml of cell culture media and transferring this to T75 flasks (or T25 flasks for DCNL1 WT and KO cell lines which struggled to grow at low densities). Thawed cells were typically passed within 48 hours, in the cases where cells were not confluent in that time, the media was changed to remove dead cell debris.

#### 2.2.1.5 Cryopreservation of Cells

Following trypsinization of cells and inactivation of the trypsin, as described in 2.2.1.2, the cells were pelleted at 1000RPM for 5 minutes in 15ml or 50ml Falcon tubes. Following aspiration of the media/trypsin solution, the cell pellet was re-suspended in freeze media (DMEM with 5% DMSO v/v) and 1ml was aliquoted for each cryogenic vial (typically 3 vials per confluent T75 flask of cells). These vials were placed in a Mr Frosty™ freezing container (topped up with isopropyl alcohol) and stored at -80°C overnight or for up to a few weeks, before transferring the vials to liquid nitrogen storage.

### 2.2.2 Molecular Biology Methods

#### 2.2.2.1 Genomic DNA Extraction

Approximately $5x10^6$ cells were grown in 10cm dishes and harvested for each cell that line required sequencing. A genomic DNA extraction kit (Promega) was used according to the manufacturer instructions (eluting at the final stage in a 100µl volume) and the concentration of each sample of genomic DNA was measured using a nanodrop.

#### 2.2.2.2 Polymerase Chain Reaction (PCR) and Agarose Gel Analysis

PCR reactions were set up using the different genomic DNA samples as follows:

| Volume (µl) | Reagent |
|---|---|
| 10 | 10x Reaction Buffer |
| 1 | dNTPs (25mM) |
| 2.5 | Primers (10µM solution of forward and reverse) |
| 0.5 | Q5 polymerase |
| 2 (volume required for 100ng) | cDNA template |
| 34 | Nuclease-free water |

**Table 5 List of PCR Components**

PCR reactions were carried out in a thermocycler by performing 12 cycles touchdown PCR at 72°C followed by conventional PCR for 25 cycles at 62°C.

DCNL5 Forward Sequence: 5'-CTGTCTCTGGGAACTGGGTG

DCNL5 Reverse Sequence: 5'-GCAGGGCACGTAGACTCTTA

The PCR products were analysed by 1% agarose gel electrophoresis; gels were cast using ethidium bromide and resolved for 40 minutes at 100V in a Mini-Sub Cell GT gel system (Bio-Rad). Agarose gel bands for the PCR products were excised and processed using a QIAquick extraction kit (QIAgen) according to the manufacturer instructions, using a final volume of 45µl for elution of the product. The DNA was then cleaned up using a QIAquick PCR purification kit (QIAgen) according to the manufacturer instructions.

#### 2.2.2.3 Restriction Digest and Ligation

pcDNA3.1 vector as well as the cleaned-up PCR products were digested using BamHI and NotI enzymes (NEB) using approximately 1µg of vector and 500ng of insert in each reaction. Band intensities were calculated to allow for the accurate

measurement of vector:insert rations for the subsequent ligation reaction. We used a 3:1 vector to insert ratio and used T4 ligase (NEB) to catalyse the ligase reactions overnight. Ligated DNA was stored at -20°C when not in use.

#### 2.2.2.4 Transformation, Expression and Mini-Prep

Competent cells (XL1-blue) were thawed on ice for approximately 15 minutes, after which 5µl of DNA was added to each 50µl tube of competent cells and allowed to incubate on ice for a further 10 minutes. The cells were then heat-shocked in a 42°C water bath for 45 seconds before incubating the cells on ice for another two minutes. SOC media (200µl) was then added to the tubes and these were transferred to a 37°C tube rack and shaken at 600RPM for 1 hour. Following this incubation, the mixture was poured onto agar plates which contained ampicillin for selection pressure, and spread using a sterile spreader, before incubating the plates (upside down) at 37°C overnight.

4 single colonies from each plate were picked and used to inoculate separate vials of 2YT media (5ml volume) containing 0.1mg/ml ampicillin. These universal tubes were then incubated overnight at 37°C while shaking to allow for growth of bacteria expressing the vector. DNA was purified from these colonies using a kit (Promega Wizard® Plus SV miniprep) per the manufacturer's instruction, eluting the DNA with 50 rather than 100µl of nuclease free water. and the concentration was measured using a Nanodrop spectrophotometer. The eluted DNA was sent for sequencing at the MRC PPU Sequencing centre in Dundee, the rest was stored at -20°C for future use.

### 2.2.3 Plasmid Transfection of Cells

U2OS, H460 and HeLa cells were plated in 6 well plates at a density of 75000 cells/ml 24 hours prior to transfection. The normal cell culture media was aspirated from each well and replaced with 1.5ml of Opti-MEM prior to addition of any reagents. 100µl of Opti-MEM was combined with 2µl of Lipofectamine 2000 in an Eppendorf tube, while 100µl of Opti-MEM was combined with 1µg of DNA in a separate tube. If multiple samples were needed these were scaled appropriately These separate Eppendorf tubes were allowed to incubate for 5 minutes at room

temperature before they were combined, mixed gently and allowed incubated for a further 15 minutes. The Lipofectamine/DNA solution was added to the relevant well of cells and mixed up and down. A control reaction was always performed using Lipofectamine with 1µg of empty DNA vector per experiment. The transfections were performed for 4-5 hours before the media in each well was changed back to normal cell culture media. 48 hours later, cells were treated and harvested for analysis.

### 2.2.4 RNA Interference

For siRNA-mediated knockdowns, SMARTpool siRNA oligos were used against human DCNL5 and cullin 3 or a non-targeting control (Dharmacon). The transfections were performed using RNAiMAX (ThermoFisher) similar to the method described in 2.2.3, except cells were plated at 100000 cell/ml the day before transfection, 3µL of siRNA (at a stock concentration of 10µM) was used per reaction and the cells were allowed to grow for 72 hours rather than 48 hours after transfection, before treating cells and harvesting for analysis.

### 2.2.5 CRISPR/Cas9

3µg of guide RNA vector for exon 1 of DCNL5 which was generated previously by mutagenesis PCR of pEsgRNA (refs Munoz et al, 2014 and Keuss et al, 2016) with the target sequence 5'-GCAGCAGTAGCGGAAGACGGAGG-3' (plus strand) was pre-incubated with 3µg of GFP-tagged Cas9 (pCAG -CAS9-2A-GFP WT) in 500µl of Opti-MEM. This solution was then combined with 500µL of Opti-MEM containing 10µl of lipofectamine 2000 and incubated together for 15 minutes. This solution was then added to a 10cm dish of 70% confluent H460 cells in 5ml of Opti-MEM for 5 hours at 37°C. Following this, the media was changed to normal culture media and the dish was cultured at 37°C for a further 48 hours. The cells were then trypsinized, with emphasis on breaking up any clumps of cells, counted, and then plated at densities of 10 cells/ml and 100 cells/ml in 96 well plates. Single colonies were selected for after 2 weeks, transferred to and grown in T-25 flasks before analysing DCNL5 expression by western blot from cell lysates, ensuring the selected cell colonies continued to grow in flasks. We found that we only had partial KO of DCNL5 in our selected clones, so we repeated the process with one

of the potential KO clones, re-plating in 96 well plates and re-selecting for single colonies and validating them by western blot. We further validated the success of the process by sequencing the genomic DNA from the successful clones.

### 2.2.6 DNA Damage Induction

Stocks of the DNA damage-inducing agents were made up as follows:

| Drug | Stock Concentration | Working Concentrations | Treatment Time |
|---|---|---|---|
| Bleomycin Sulphate | 10mg/ml | 50-1000µg/ml | 24-72 Hours |
| Doxorubicin Hydrochloride | 10mg/ml | 1-10µg/ml | 24 Hours |
| Etoposide | 100mM | 0.25-1mM | 24 Hours |
| Neocarzinostatin | 0.5mg/ml | 0.6-2.5µg/ml | 24 Hours |

**Table 6 List of DNA Damage-Inducing Agents**

The drugs in Table 6 were used for the indicated times and diluted to working concentrations using DMEM culture media. The diluted drugs were added directly to wells containing attached cells plated at the required density.

### 2.2.7 Neddylation and Proteasome Inhibition

The NEDD8 activating enzyme inhibitor MLN4924 and the proteasome inhibitor MG-132 were used at concentrations of 3µM and 25µM respectively, for 1-hour pre-treatments prior to addition of apoptosis-inducing drugs to plated cells.

## 2.2.8 Apoptosis Induction

### 2.2.8.1 Cycloheximide and TNFα

Cycloheximide stock was prepared using PBS at a concentration of 4mg/ml, while TNFα was made up to a 100µg/ml stock using distilled water. Subsequent dilutions into working concentrations were performed using DMEM culture media. Cycloheximide was used at concentrations of either 5 or 10µg/ml and was added to cells 1 hour before TNFα was added, in the cases of combination treatment. TNFα was used at concentrations ranging from 12.5-200mg/ml and treatment times ranged from half an hour up to 24 hours depending on the assay. Cells were harvested at the end of the desired treatment time.

### 2.2.8.2 TRAIL

Recombinant human TRAIL was prepared by re-suspending the lyophilised protein in 0.5ml of distilled water to a final concentration of 0.5mg/ml. Working dilutions were performed using DMEM culture media. The drug was added to cells for treatment times of 0.5 to 24 hours depending on the assay.

### 2.2.8.3 Staurosporine

Lyophilised staurosporine was dissolved in DMSO to make a 2mM stock solution, which was then diluted to working concentrations using DMEM. The drug was added to cells at concentrations of 100-200nM.

## 2.2.9 Lysate Preparation

### 2.2.9.1 Preparation of Cell Lysates for Western Blotting

Cells were grown in 10cm dishes, 6cm dishes, 6-well plates or 12-well plates depending on the application. Following treatments, cells were harvested in either TN lysis buffer or directly in 2x sample buffer. In the case of TN lysis buffer, cells were initially washed with ice cold PBS before adding the desired volume of TN buffer and incubating on ice or at 4°C for 15 minutes, rotating the dish half way through to ensure complete lysis of cells. The cells were subsequently scraped using cell lifters and transferred to 2ml Eppendorf tubes and incubated on ice for

a further 10 minutes. The lysates were then clarified by centrifugation at 13000g and supernatants were transferred to fresh 1.5ml Eppendorf tubes and then were stored at -80°C or the protein concentration was measured by BCA. For cells harvested in sample buffer, they were scraped either using cell lifters or using a pipette tip directly, transferred to Eppendorf tubes, 10% v/v mercapto-ethanol was added and the samples were boiled and shaken at 1000rpm before storing at -20°C for use in SDS-PAGE gels at a later time.

#### 2.2.9.2 Preparation of Cell Lysates for Immunoprecipitation

Cells that had been cultured and treated in 10cm dishes, were removed from incubators and washed twice with ice cold PBS before adding TN lysis buffer (typically 200µl) and incubating on ice or at 4°C for 15 minutes, rotating the plates half way through. Cells were then scraped using cell lifters and transferred to 2ml Eppendorf tubes (typically 2 or 3 10cm plates were combined for each cell type and each reaction) and lysed on ice for a further 10 minutes. The cell lysates were then clarified by centrifugation at 13000g and supernatants were transferred to 1.5 ml Eppendorf tubes and stored at -80°C or used immediately for protein concentration determination and immunoprecipitation.

#### 2.2.9.3 Preparation of Cell Lysates for Immunoprecipitation Under Denaturing Conditions

Cells that had been cultured and treated in 10cm dishes, were removed from incubators and washed once with ice cold PBS before adding 300µl of PBS to the dishes. The cells were scraped using cell lifters and transferred to 2ml Eppendorf tubes (as before, multiple 10cm dishes were used per treatment) before pelleting the cells at 600g for 15 minutes. The supernatant was removed and discarded, and the pellet was re-suspended in denaturing IP lysis buffer containing 1% SDS. The cell suspension was then boiled at 95°C while under constant agitation of 750RPM. The supernatant was then transferred to fresh tubes and either stored at -80°C or used immediately for protein concentration determination and immunoprecipitation.

## 2.2.10 BCA Protein Assay

Cell lysates were diluted 1:5 in distilled $H_2O$ and 25µl of each sample was added to a well of a clear 96-well plate. Samples were analysed in duplicate. Standards of bovine serum albumin (BSA) at concentrations of 2, 1, 0.5, 0.25 and 0 mg/ml were made up and diluted using distilled $H_2O$. 25µl of each standard was added to a well of the same 96-well plate, also in duplicate. Reagent A and reagent B (ThermoFisher) were mixed at a ratio of 1:50 in a sufficient volume to add 200µl to each well of protein sample and protein standards. The 96-well plate was then incubated at 37°C for 30 minutes before measuring the absorbance at 570nm (due to technical constraints of the machine) using a pre-defined BCA protocol (which included a 10 second shake prior to reading the absorbance). The dilution of the samples was automatically taken into account and the protein concentration was calculated by the Optima software.

## 2.2.11 Immunoprecipitation

### 2.2.11.1 Non-denaturing Immunoprecipitation

Protein lysates prepared were prepared as described in 2.2.9.2. Where possible, 2mg of protein lysate was used for each immunoprecipitation reaction. A pre-determined amount of lysate was transferred to separate tubes for use as whole-cell lysate and combined with 4x sample buffer (containing 10% ß-mercapto-ethanol) and subsequently boiled at 95°C and spun down and stored at -20°C. Antibody was added to the lysate in 2ml tubes (the amounts are summarised in Table 3 List of Primary Antibodies which were then rotated overnight at 4°C. The following day, magnetic beads (protein A or G depending on which antibody had been used) were prepared by washing twice with PBS and twice with lysis buffer (absent of inhibitors) with the aid of a magnetic rack. 50µl of beads were required to be added per immunoprecipitation reaction therefore a sufficient volume of bead-slurry was washed and prepared, ultimately resuspending in the same volume as initially removed from the vial of beads. Once the re-suspended beads were added to the IP reactions, the tubes were returned to 4°C and rotated for a further hour. In this time the antibody should have bound to the magnetic beads, however washes are required to remove any non-specific binding. The beads were

washed once with distilled water, 3 times with lysis buffer (absent of inhibitors) and 3 times with PBS. The beads were spun briefly to ensure all the beads were at the bottom of the tubes, before removing the last of any PBS present. The beads were then eluted by re-suspending them in 2x sample buffer (containing 10% ß-mercapto-ethanol) gently, ensuring no air bubbles were introduced. The bead-sample buffer suspension was left at room temperature for 20 minutes for the protein complex to be eluted, before boiling the samples at 95°C and spinning down, ready for loading onto SDS-PAGE gels or to store at -80°C.

#### 2.2.11.2 Denaturing Immunoprecipitation

Protein lysates prepared were prepared as described in 2.2.9.3. Generally, 1mg of protein lysate was used for the immunoprecipitation. Prior to adding antibody, a pre-determined amount of lysate was transferred to separate tubes for use as whole-cell lysate and combined with 4x sample buffer (containing 10% ß-mercapto-ethanol) and subsequently boiled at 95°C and spun down. Unlike with a normal immunoprecipitation, due to the relatively high concentration of SDS present in the protein lysate (1% w/v), a 10-fold dilution was required to be performed in order to enable the antibody to bind to the protein in the lysate. Each sample of lysate was diluted using 10-times more denaturing IP lysis buffer (absent of inhibitors) in 10ml Falcon tubes. Antibody was then added to the diluted lysates (per amounts shown in Table 3) and the tubes were then rotated at 4°C overnight. The following day, magnetic beads (protein A or G depending on which antibody had been used) were prepared by washing twice with PBS and twice with lysis buffer (absent of inhibitors) with the aid of a magnetic rack. 50µl of beads were required to be added per immunoprecipitation reaction therefore a sufficient volume of bead-slurry was washed and prepared, ultimately resuspending in the same volume as initially removed from the vial of beads. Once the re-suspended beads were added to the IP reactions, the tubes were returned to 4°C and rotated for a further hour. Due to the large volume of protein lysate (diluted in buffer), the supernatant-bead solution was removed and transferred to 2ml tubes in rounds until all the beads had bound to the side of the tube. The beads were then washed once with distilled water, 3 times with lysis buffer (absent of inhibitors) and 3 times with PBS. The beads were spun briefly to ensure all the beads were at the bottom of the tubes, before removing the last of any

PBS present. The beads were then eluted by re-suspending them in 2x sample buffer (containing 10% ß-mercapto-ethanol) gently, ensuring no air bubbles were introduced. The bead-sample buffer suspension was left at room temperature for 20 minutes for the protein complex to be eluted, before boiling the samples at 95°C and spinning down, ready for loading onto SDS-PAGE gels or to store at -80°C.

### 2.2.12 HALO-TUBE Pulldowns

Similarly to normal immunoprecipitation reactions, protein lysates were prepared as described in 2.2.9.2. Typically, 0.75-1.5 mg of protein lysate was used per reaction, and as with immunoprecipitation reactions, a sample of lysate was removed initially for use as whole-cell lysate to run on SDS-PAGE gels. The magnetic HALO-TUBE beads, as well as control HALO beads were washed and prepared in the same fashion as for immunoprecipitation reactions detailed in 2.2.11.1. The beads were then added to the protein lysates and rotated at 4°C for 2 hours. The protein-bound beads were then washed and eluted the same way as described in 2.2.11.1.

### 2.2.13 SDS-Polyacrylamide Gel Electrophoresis Analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 1mm thick gels cast by hand using Bio-Rad Mini-Protean III gel units. 1M Bis-Tris solution was made by dissolving 20.9g of Bis-Tris in 100ml of distilled water and setting the pH to between 6.5-6.7. Ammonium persulfate was dissolved in distilled water to make a 10%w/v solution. Stacking and resolving gels were made up as 100ml solutions and stored at 4°C:

| | 14% | 10% | 8% | Stacking Gel |
|---|---|---|---|---|
| 1M Bis-Tris pH 6.5-6.7 | 30ml | 30ml | 30ml | 30ml |

| | | | | |
|---|---|---|---|---|
| 40% 37.5:1 Acrylamide (5%) | 31.5ml | 22.5ml | 18ml | 11.2ml |
| Distilled Water | 28.5ml | 37.5ml | 42ml | 48.8ml |

**Table 7 Components of SDS-PAGE Gels**

For 2 gels, 10ml of the required resolving gel was mixed with 60µl APS (of a 10% w/v stock in distilled water) followed by 0.25% (v/v) tetramethylethylenediamine (TEMED). The solution was vortexed before pouring approximately 4.5 ml of the solution into the casting plates and covering with 70% ethanol to allow for the polymerisation to occur. Once the gel had set, the ethanol was poured off and the gels were washed with distilled water, ensuring all water was removed using blocking paper before processing to the next step. 5ml of stacking gel solution was mixed with 30µl of APS (10% w/v stock) followed by 0.2% (v/v) of TEMED, vortexed, then poured on top of the resolving gel to the top of the short plate. The combs were then immediately inserted between the plates. Once the stacking gel had set, the gels were either loaded into Bio-Rad gel tanks for electrophoresis or stored at 4°C between moist blue roll and wrapped in clingfilm for up to a week.

Pre-cast 4-12% gradient gels were also used (NuPAGE®), and preparation for loading samples simply involved washing the gels in water, removing the comb, filling the wells with running buffer and inserting the gels into XCell SureLock Mini-Cell apparatus (ThermoFisher). The non-gradient gels run in the Bio-Rad apparatus were ran at a constant voltage of 120V, either until the dye front at reached the bottom of the gel and achieved good separation, or until the dye front had run off the gel for proteins that required better separation. The gradient gels were run at a constant voltage of 200V for 35 minutes.

## 2.2.14 Western Blotting

### 2.2.14.1 Transfer of Proteins onto Membranes

Following SDS-PAGE, described in the previous section, proteins required the transfer to a membrane to allow for the detection of specific proteins using

antibodies. Assembly of the transfer 'sandwich' involved the use of a cassette, 2 sponges, 3 layers of filter paper for each side of the gel and a membrane. 2 types of membrane were used throughout this project, nitrocellulose and PVDF, and both were of pore size 0.45µm. PVDF requires activation, so the membrane was submerged in methanol for 30 seconds prior to its use. 6.5cm x 9cm rectangles of membrane were used in combination with 7cm x 9cm rectangle of Whatman filter paper. The filter paper and sponges used in the transfer process were pre-wetted in transfer buffer and stored at 4°C prior to the transfer process. Transfer buffer was made fresh and chilled on ice prior to use.

The gel was removed from its assembly and laid out on top of the pre-wetted filter papers which themselves were on top of one of the sponges. Next, the membrane (activated, in the case of PVDF) was laid on top of the gel and bubbles were removed using a roller. Finally, 3 more layers of filter paper were added on top, as well as the second sponge, and the cassette was closed and inserted into a Bio-Rad transfer tank. Transfers were run on ice using pre-chilled transfer buffer, and typical transfer times were 60-100 minutes at 100V. For gradient gels, these were always run at 100V for 100 minutes. For nitrocellulose membranes, efficiency of the transfer as well as the loading was verified by submerging the membrane in ponceau solution for 1 minute and de-staining with water to remove non-specific staining.

#### 2.2.14.2 Blocking of Membranes and Incubation with Primary Antibodies

Following the transfer process and optional ponceau staining, membranes were washing in TBST twice for 10 minutes while rocking gently. To reduce non-specific binding, membranes were then incubated in 5% milk (w/v) TBST for 1 hour at room temperature while shaking gently. Following the blocking step, appropriate dilutions of primary antibody were made up in a falcon tube using either 5% milk TBST or 5% BSA TBST, as detailed in Table 3 . The membrane was cut into portions using the molecular weight marker as a guide in cases where the same membrane was probed for different proteins. Membranes were incubated at 4°C overnight in the falcon tubes while rotating on a roller.

#### 2.2.14.3 Incubation of Membranes with Secondary Antibodies and Immunodetection of Proteins

Following incubation with primary antibody overnight, membranes were washed twice for 10 minutes in TBST while shaking gently. Secondary anybody was prepared in the same way as the primary, according to the dilutions summarised in Table 4. Membranes were incubated with secondary antibodies in 5% milk TBST for 1 hour at room temperature while rotating, after which the membranes were washed 3 times in TBST for 10 minutes. HRP-conjugated secondary antibodies were imaged using a chemiluminescent reaction induced by exposing the membranes to an equal parts mixture of Luminol Reagent and Peroxide Solution (Millipore). This was achieved by adding approximately 1ml of the combined solutions to the membrane (which can be dried using paper towel to increase absorption of the solution). This solution was allowed to absorb into the membrane for anywhere from a few seconds to up to 5 minutes for weaker signals. Membranes were then transferred to transparent sheets and held in place inside an imaging cassette using tape. The membranes were developed on X-ray film using an X-OMAT imager. Film exposure time varied depending on the strength of the signal anticipated. All western blotting experiments were performed 3 times using biologically independent samples (n = 3) unless otherwise stated in figure legends. Representative western blots are shown in all cases.

### 2.2.15 Luciferase Assay

2x Lysis and ATP Assay Buffer was made up as follows:

50mM Tris/Phosphate pH 7.8
16mM MgCl2
2mM DTT
2% v/vTriton-X-100
30% v/v Glycerol
1% w/v BSA

BSA was dissolved fully before proceeding to the next reagents.

0.25mM D-Luciferin

8μM Sodium Pyrophosphate Tetra-basic Decahydrate
500ng/ml Luciferase.

This viability assay was always performed using a 96-well format (using black, clear bottom plates), and cells were grown in media containing phenol red. Phenol red-free media can be used when plating cells for treatments and measurement of viability which should increase the signal detected, however this was not an issue in this project. Cells were plated at a volume of 100μl per well at the required density for the required duration of time. Treatments were added directly to the wells, ensuring the volume of drug added to each well was previously removed in order to ensure the volume remained a constant 100μl. Following drug treatments, the plates were removed from the humidified incubators and 100μl of 2x ATP lysis buffer was added to each well using a multi-channel pipette, before shaking the plate at 900RPM for 10 minutes at room temperature to ensure full lysis of the cells. The plate was imaged using FLUOstar microplate reader set to measure the absorbance from above the wells. Cell viability was measured in relation to vehicle control treatments in duplicate or triplicate.

### 2.2.16 Immunofluorescence Microscopy

U2OS cells were plated on coverslips which had been coated with 10μg/ml Poly-d-lysine and after 24 hours, coverslips were treated with TRAIL for different amounts of time at 37°C. Cells were then washed once in ice-cold PBS then fixed with ice-cold methanol for 1 minute. The coverslips were then washed 3 times with PBS and then blocked for 1 hour with 3% BSA in PBS at room temperature. The coverslips were then washed 3 times in PBS before incubating the coverslips with GFP antibody (1:5000 dilution) overnight in 3% BSA. The coverslips were then washed 3 times in PBS, before incubating them with anti-chicken antibody (1:1000 in 3% BSA) for 30 minutes at room temperature. The coverslips were then washed 3 times with PBS before counter-staining with Red Dot (1:200 in $H_2O$) for 10 minutes at room temperature and washing twice with PBS. The coverslips were then mounted onto slides with Shandon™ Immu-Mount™ (Thermo Scientific) and imaged on a Zeiss LSM 710 confocal microscope. Images were processed using ImageJ.

### 2.2.17 FACS/Fluorescence Viability Analysis

WT and KO DCNL5 cells were seeded on 96 well plates, and 24 hours later recombinant TRAIL was added to the relevant wells at the relevant concentrations. A 3-hour staurosporine treatment was started to coincide with the end of the 24-hour TRAIL treatment, at which point the plates were processed for annexin V and propidium iodide staining per manufacturer instructions (MabTag AnxF100PI). Briefly, this involved removing the culture medium from the plates, resuspending the cells in 90µl of annexin-V buffer and then adding 5µl of annexin-V conjugate and 5µl of propidium iodide solution. The plates were then incubated in the dark for 20 minutes, before imaging the plates with an IncuCyte® S3 live cell analysis system (Sartorius). The proprietary software was then used to generate images of representative wells showing the fluorescence of WT and KO cells in response to the apoptosis-inducing agents.

### 2.2.18 Statistical Analysis

Results presented are expressed as mean + SEM. Statistically significant differences were calculated using one or two-way ANOVA and a $p$ value of $<0.05$ was considered significant. Prism ® software version 7 was used to perform the statistical analysis as well as generate the graphs presented in this study. N (n) numbers are indicated in the figure legends.

# 3 The Role of DCNL5 in the Apoptosis Response

## 3.1 Introduction

### 3.1.1 DCNL5 Involvement in the DNA Damage Response

Previous data from Keuss and colleagues (Keuss *et al.*, 2016), highlighted in Figure 3-1 below, demonstrated a novel role for DCNL proteins in the DNA damage response. Figure 3-1A shows that the DCNL5 KO cells are significantly more sensitive to the gamma irradiation and that expression of WT or DAD-mutant DCNL5 in inducible cell lines rescues that sensitivity such that they respond the same, if not slightly less than WT cells to the treatment. Additionally, DCNL5 was shown to be recruited to sites of DNA damage following UV irradiation in what is known as a laser stripe experiment. Figure 3-1B shows bands of GFP-tagged DCNL5 appearing within 5 minutes of UV treatment and importantly both WT DCNL5 as well as the cullin non-binder DAD-mutant DCNL5 are recruited to the sites of UV irradiation, which causes dsDNA breaks and damage, further confirming the role for DCNL5 in the DNA damage response, independently of its ability to bind its canonical binding partner: cullin proteins. The significance of this finding was that perhaps there exist other targets for the DCNL protein family. DNA damage-induction has been shown to trigger the programmed cell death response known as apoptosis, and in studying DNA damage we will also analyse whether apoptosis is occurring and if DCNL5 plays a role here.

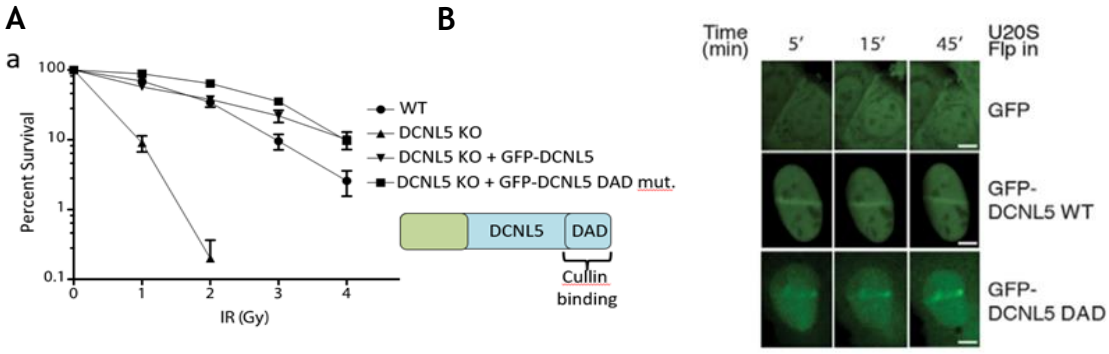



**Figure 3-1 DCNL5 involvement in DNA damage is independent of cullin binding.**
(A) This graph (Kurz, unpublished) shows the survival rates in different cell lines following gamma irradiation. The DCNL5 KO cells are highly sensitive to the irradiation, however the survival rate is recovered with the WT and DAD patch DCNL5 cells. (B) This fluorescence microscopy image shows that both WT and DAD patch mutated DCNL5 are recruited to DNA damage sites following micro-irradiation with a laser (Keuss *et al.*, 2016).

The finding that DCNL5 involvement in the DNA damage response may occur independently of its interaction with cullin proteins is novel, however it is important to remember that cullin proteins are known to play roles in the DNA damage response. Cullin proteins interact with RING proteins to form the cores of cullin-RING-ligase (CRL) complexes which are the largest family of ubiquitin E3 ligases. CRLs are responsible for 20% of the global ubiquitination of intracellular proteins via the ubiquitin proteasome system (UPS) (Zhao and Sun, 2013) and cullin proteins themselves are often found to be overexpressed in tumours. Cullin 4A has been implicated in the DNA damage response and is found to show higher expression in cancer cells.

Cullin 4A interacts with the adaptor protein DNA domain binding protein DDB1 as well as the RING protein RBX1 to mediate the ubiquitination and degradation of numerous DNA-damage responsive proteins as well as proteins that mediate DNA methylation (Zhao *et al.*, 2010). Cullin 4A is also found to be overexpressed in various cancers including liver cancer (Chen *et al.*, 2018) and breast cancer (Chen *et al.*, 1998), and therefore represents an important therapeutic target. Cullin 4A knockdown has been shown to increase DNA damage repair following induction of damage which results in reduction in cancerous mutations (Liu *et al.*, 2009). It can therefore be inferred that high levels of cullin 4A may be a hallmark of some cancerous cells. In the context of DNA damage induction and DNA repair cullin 4A

represents a key protein to focus on and in the present study cullin 4 levels may correlate with apoptosis induction. Tumour cells are known to be resistant to cell death via pathways including apoptosis and thus studying and analysing cullin proteins in the context of DNA damage and apoptosis is an important consideration in understanding cancer signalling pathways. Some cullin proteins can be targets of caspases and degraded during the apoptosis response, which will be an important marker in the present work.

### 3.1.2 Aims:

Initially we wanted to further elucidate the role of DCNL5 in the DNA damage response by using chemical inducers of damage. In doing so we discovered that DCNL5 KO cells showed a defect in the apoptosis response and we then aimed to establish and characterise the role of DCNL5 in this pathway.

## 3.2 Results

### 3.2.1 DCNL5 Appears to have no Effect on Sensitivity to dsDNA Damage-Inducing Agents

In order to investigate the finding by Keuss and colleagues that DCNL5 was required in the DNA damage response following radiation treatment (Keuss *et al.*, 2016), we initially wanted to induce double-stranded DNA breaks using chemical agents. From the outset we anticipated that we may not be able to achieve the same results using chemical inducers of DNA damage given the different treatment durations and mechanisms underlying DNA damage induction. DNA damage inducting agents can induce both single stranded and double-stranded DNA breaks, the latter of which can lead to deleterious effects on a cells genetic make-up, resulting in a global DNA damage response which is complex and multifaceted and leads to DNA repair mechanisms followed eventually by apoptosis if repair is unsuccessful (Roos and Kaina, 2006).

In order to measure the effect of the DNA-damage inducing drugs, we opted to use an ATP-based cell viability assay which can be measured using a spectrophotometer (see Figure 3-2). 24 hour treatments with 3 different drugs, doxorubicin, etoposide and neocarzinostatin, were performed in 96-well plates using U2OS cell lines either stably expressing GFP (with WT DCNL5, referred to herein as 'WT') or lacking DCNL5 expression (CRISPR-Cas9 knock out cells referred to as 'KO'). As highlighted in Figure 3-3, KO cells show no increase in sensitivity to any of the drugs, in fact in the case of etoposide the KO cells appear slightly less sensitive to the treatment. This suggests that at least over a 24-hour period, DCNL5 expression has no major effect on cell viability following treatment with these DNA-damage inducing drugs. This contrasts with the results from Keuss and colleagues who showed that DCNL5 was required in the DNA damage response using the same U2OS cell lines. In our data, we did not include a positive control to indicate DNA damage induction, so we are unable to confirm whether DNA damage was successfully induced, however the loss of viability does appear to indicate that cell death is occurring which is typically a direct consequence of damage to the DNA backbone.

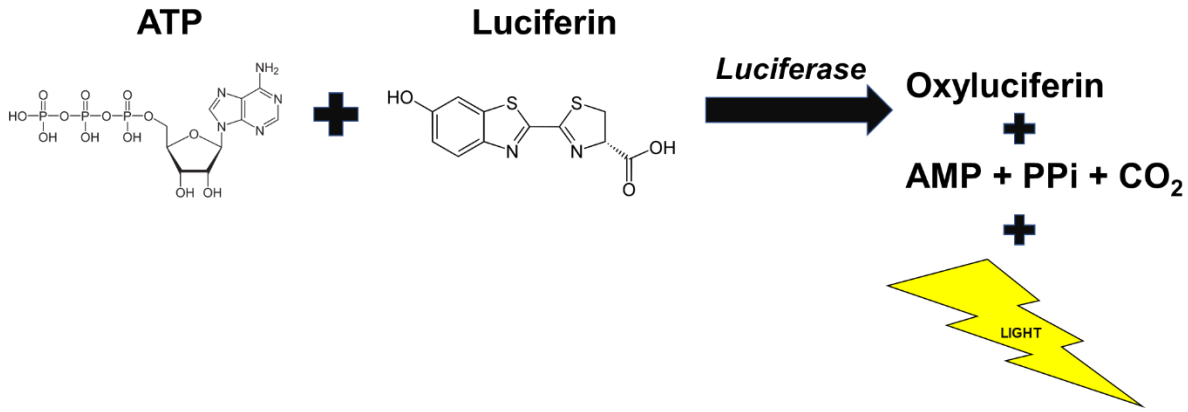
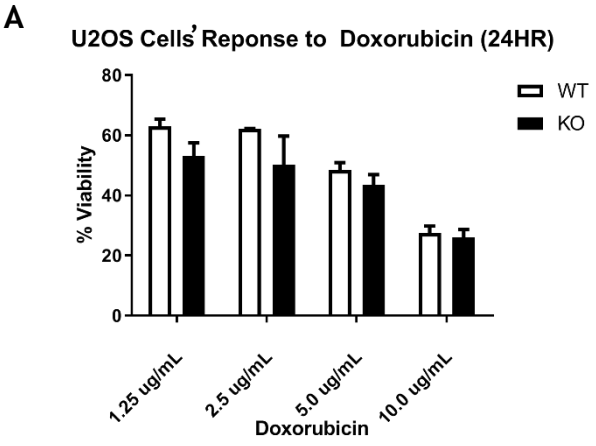
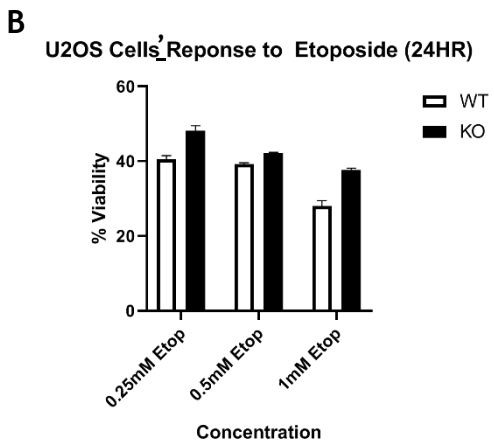



**Figure 3-2 Luciferase ATP Viability Assay.**

This simple diagram summarises the key principle behind the viability assay employed in this chapter. Viable cells produce ATP, and this can react with luciferin present in the lysis buffer to produce oxy-luciferin, AMP, inorganic phosphate, carbon dioxide and most importantly, light. The reaction in catalysed by the luciferase enzyme which originates from fireflies (Lampyridae family) which is also present in the lysis buffer. The amount of light emitted correlates with the number of cells that are alive in each well of a 96-well plate, and this can be quantified using a spectrophotometer.



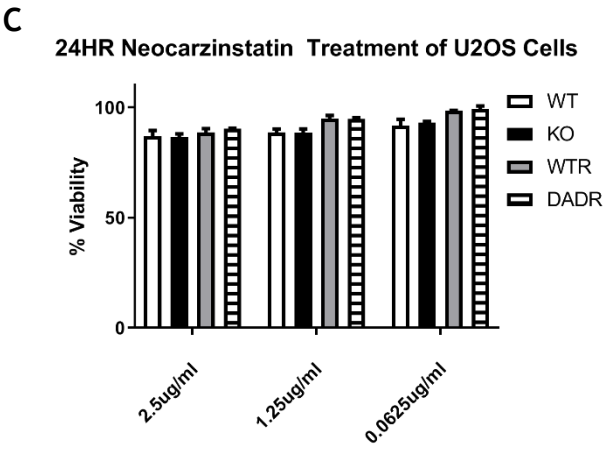



**Figure 3-3 WT and DCNL5 KO Cells are Similarly Sensitive to dsDNA Damage-Inducing Agents.**

ATP-based luciferase viability assays performed using doxorubicin (A), etoposide (B) and neocarzinostatin (C) for 24 hours showed no major difference in response of WT or KO DCNL5 cells to the treatments. DCNL5 KO cells appear to be slightly less sensitive to etoposide treatment, more-so at the highest concentration tested. *Data shown represent the mean + SEM from 3 independent experiments.*

### 3.2.2 DCNL5 KO Cells are Resistant to Cell Death in Response to Bleomycin Sulphate Treatment

Using the same luciferase assay mentioned in the previous section, we subjected U2OS cells to treatment with bleomycin sulphate which is a known inducer of dsDNA breaks (Nagai *et al.*, 1969; Stubbe and Kozarich, 1987; Claussen and Long, 1999) with a mechanism that is still not fully understood. Treatment for 72 hours with bleomycin, as shown in Figure 3-4A, was able to induce a clear response in cells through the significant detectable loss of cell viability. Unexpectedly, only the WT cells responded to the drug, resulting in less than 20% of cells remaining viable at the highest concentration of the drug (800µg/ml) while the KO cells were almost entirely resistant at over 80% viability at the same concentration. The response of the WT cells to bleomycin was dose-dependent, increasing in a linear fashion with the increase in drug concentration. At all drug concentrations the difference between WT and KO cells was statistically significant ($p<0.001$).

As well as the WT and KO DCNL5 cells, we also used inducible Flp-In™ DCNL5 KO cells which can express either GFP-tagged WT DCNL5 (referred to moving forward as WT-rescue or 'WT-R' cells) or a GFP-tagged version of the cullin non-binding mutant DAD-DCNL5 (referred to as DAD-rescue or 'DAD-R' cells) following induction with doxycycline. These cells allow for the re-introduction of DCNL5 expression in a controlled and simple to use manner without the need for transfection. We subjected WT, KO, WT-rescue and DAD-rescue cells to 72 hour bleomycin treatment for the same viability assay and the results are shown in Figure 3-4B. Two concentrations of bleomycin sulphate were used, 1 and 0.5 mg/ml, and in both cases the response of each cell line was similar. As before, WT cells were highly sensitive (<25% viable) to the treatment while the KO cells were significantly ($p<0.001$) more resistant (~75% viable). The WT-rescue cells were almost as sensitive as the WT cells (the difference was statistically insignificant) while the DAD-rescue cells showed the same resistance as the KO cells and this resistance was statistically significant when compared to the WT cells ($p<0,001$). Overall, this data provides compelling evidence that DCNL5 plays a role in mediating cell death. This data suggests a role for DCNL5 that involves its DAD-patch which is in contrast to the results from Keuss and colleagues, where they described a DAD-independent role for DCNL5 in the DNA damage response

(Keuss *et al.*, 2016) which is likely a different mechanism to what we see here. The mechanism of cell death is unclear at this point, but it may include apoptosis. It is also possible that the KO cells fail to metabolise bleomycin which may explain the lack of response. What is evident from this data is that the role of DCNL5 requires its DAD-patch and thus cullin-binding properties.

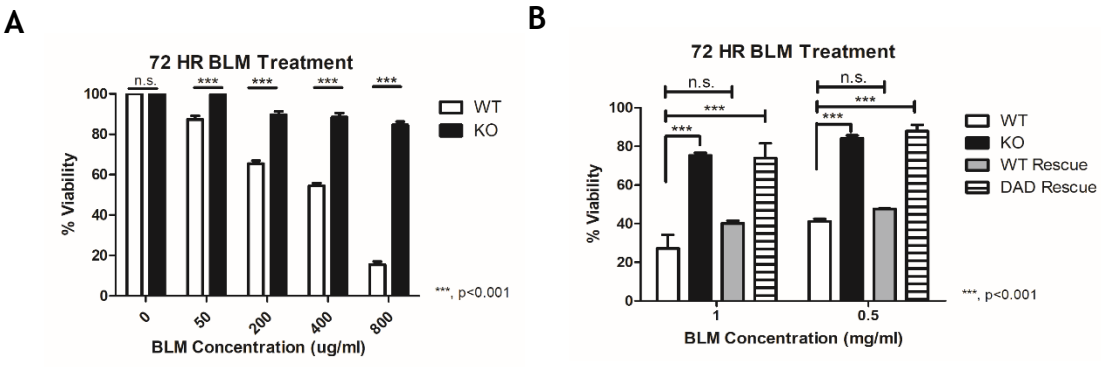



**Figure 3-4 Bleomycin sulphate was able to induce cell death in WT but not DCNL5 KO cells, in a cullin-dependent manner.**

(A) ATP-based viability assays showed that WT cells were sensitive to the bleomycin treatment compared to vehicle treatment, in a dose-dependent manner. KO Cells, in contrast, were relatively unaffected even at the highest concentrations. To further investigate this response, WT and DAD mutant rescue cell lines (induced with doxycycline) were treated with bleomycin alongside the WT and KO cell lines. (B) This viability data shows that the WT Rescue, but not the DAD-mutant rescue cells, were sensitive to the treatment. *Data shown represent the mean + SEM from 3 independent experiments, analysed by 2-way ANOVA comparing WT to KO, WTR or DADR cells for indicated treatments and statistical significance of p<0.001 was achieved*.

### 3.2.3 Bleomycin Treatment Causes a Reduction in Cullin 4 Protein levels and Induces Apoptosis

Cullin proteins have many roles within the cell but they are often known for their functions in controlling and regulating progression through the cell cycle as well as their roles in the DNA damage response. Given the duration of the bleomycin sulphate treatment described previously, we suspected initially that there may be some dysregulation of the cell cycle and therefore wanted to establish whether the neddylation state of the cullin proteins were affected in the KO cells or by the treatment. We also wanted to focus in particular on cullin 4, given its well-known role in the DNA damage response, which is likely to be involved at least to a certain extent in this situation. Given that DCNL proteins regulate cullins by neddylation, we wanted to determine whether the neddylation status of this family proteins was affected in the KO cells, which may in turn influence cell cycle progression.

We found that the neddylation of the cullin proteins 3, 4 and 5 were largely unaffected in the WT and KO cells, as shown in Figure 3-5A and as previously reported (Keuss *et al.*, 2016). Bleomycin treatment had no effect on the protein levels of cullin 3 following treatment, however cullin 4A and 4B showed a reduction in protein amount in WT but not KO cells (see Figure 3-5A and B), indicating that DCNL5 is required for this loss of CUL4A. Interestingly, cullin 5 levels appear to increase in response to the treatment but this finding was not further investigated. The data presented in Figure 3-5A lacks independent experimental repeats and a loading control so interpretation requires caution, furthermore cells were harvested directly in sample buffer for these experiments therefore protein concentration determination was not possible and therefore even loading of the SDS-PAGE was diffucult.

Bleomycin has been shown to induce the apoptosis response (Vernole *et al.*, 1998; Mungunsukh *et al.*, 2010) so it was possible that the levels of cullin 4 varied between the cell lines due to differing induction of apoptosis. Apoptosis involves the cleavage of a variety of proteins within the cell by a family of proteases known as caspases. While cullin 4A has a well-characterised role in the DNA damage response, we also suspected that cullin 4A and 4B may be degraded via the apoptosis pathway, likely by one of more of the caspase proteins.

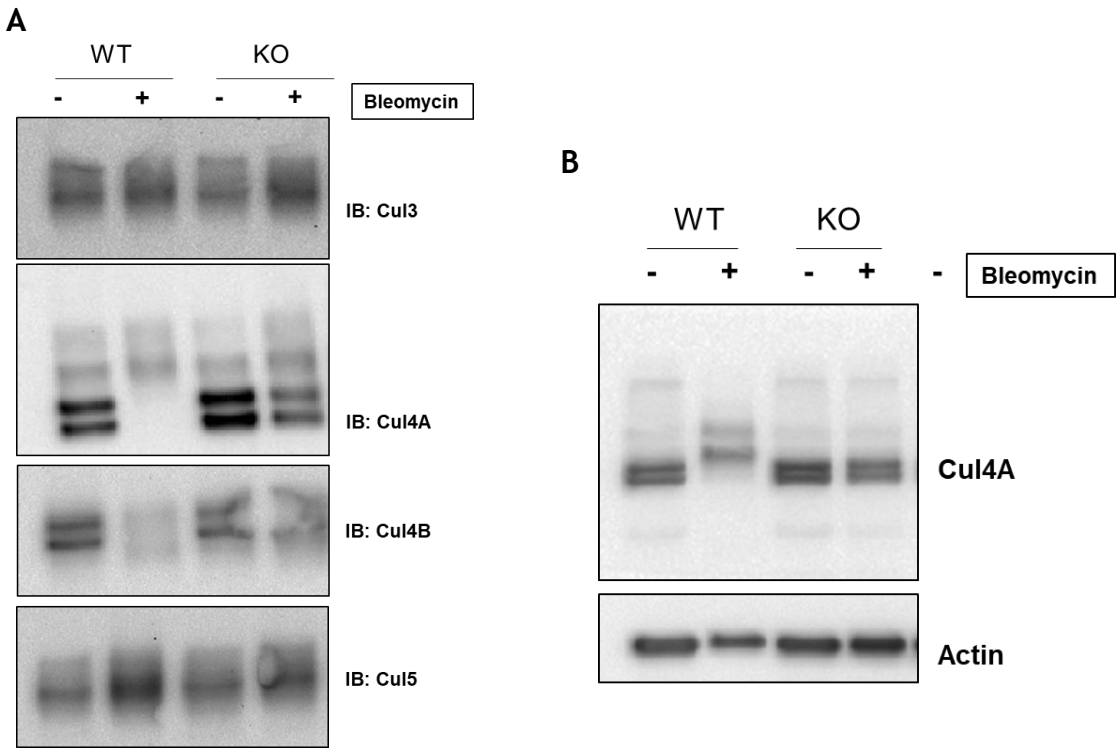



**Figure 3-5 Cullin 4 Protein Levels are Reduced Following Bleomycin Treatment in WT but not DCNL5 KO Cells**

(A) Immunoblot showing 72 hour bleomycin treatment at a concentration of 0.5mg/ml lead to a reduction in cullin 4A and 4B levels in DCNL5 WT but not KO cells, while the other cullin protein levels were largely unchanged, however there is an indication that cullin 5 levels may increase with treatment. This experiment lacks an appropriate loading control as well as independent repeats therefore interpretation is difficult. (B) This immunoblot is representative of 2 independent experiments (referred to henceforward as n = 2), and shows that cullin 4A levels appear to be lower in WT cells than KO cells following 72-hour bleomycin treatment. KO cells appear to undergo almost no cullin 4A degradation in response to bleomycin. There appears to be slightly less protein in the WT treated cells based on the reduction in band size of the actin loading control. *IB = immunoblot*

The possibility that cullin 4A and 4B were being degraded by the apoptosis pathway was investigated over two ways. First, we checked for any caspase 3 cleavage sites (since this is one of the key executioner caspases) within the amino acid sequences using a predictive programme called ScreenCap3 (Fu *et al.*, 2014). We found that both cullin 4A and cullin 4B contained multiple cleavage sites predicted with relatively high degrees of certainty (0.98 to 0.72) while for reference, cullin 3 contained no predicted caspase 3 cleavage sites. This data indicates that the reduction in cullin 4 levels may occur as a result of cleavage by caspase 3 (and possibly other caspases) due to the bleomycin treatment and is not

as a result of lack of DCNL5 expression. Lack of DCNL5 may prevent cullin 4 cleavage by caspases and DCNL5 could therefore be important in caspase-mediated cleavage or caspase cleavage directly which is a required step in caspase activity. In addition, lack of DCNL5 does not significantly affect the neddylation of cullins 3 or 5 *in vivo* which is consistent with existing data.

**Cullin 4A**

**Name**

>sp|Q13619|CUL4A_HUMAN Cullin-4A OS=Homo sapiens OX=9606 GN=CUL4A PE=1 SV=3

**Cleavage sites**

Probability: 0.987731 Site:REDSLD|S (D127)

Probability: 0.835933 Site:VQDLLD|F (D367)

Probability: 0.775639 Site:FKDKVD|H (D373)

Probability: 0.748045 Site:KSASVD|A (D470)

**Cleveage sites in your query**

MADEAPRKGSFSALVGRTNGLTKPAALAAAPAKPGGAGGSKKLVIKNFRDRPRLPDNYTQ
DTWRKLHEAVRAVQSSTSIRYNLEELYQAVENLCSHKVSPMLYKQLRQACEDHVQAQILP
FREDSLD|SVLFLKKINTCWQDHCRQMIMIRSIFLFLDRTYVLQNSTLPSIWDMGLELFRT
HIISDKMVQSKTIDGILLLIERERSGEAVDRSLLRSLLGMLSDLQVYKDSFELKFLEETN
CLYAAEGQRLMQEREVPEYLNHVSKRLEEEGDRVITYLDHSTQKPLIACVEKQLLGEHLT
AILQKGLDHLLDENRVPDLAQMYQLFSRVRGGQQALLQHWSEYIKTFGTAIVINPEKDKD
MVQDLLD|FKDKVD|HVIEVCFQKNERFVNLMKESFETFINKRPNKPAELIAKHVDSKLRAG
NKEATDEELERTLDKIMILFRFIHGKDVFEAFYKKDLAKRLLVGKSASVD|AEKSMLSKLK
HECGAAFTSKLEGMFKDMELSKDIMVHFKQHMQNQSDSGPIDLTVNILTMGYWPTYTPME
VHLTPEMIKLQEVFKAFYLGKHSGRKLQWQTTLGHAVLKAEFKEGKKEFQVSLFQTLVLL
MFNEGDGFSFEEIKMATGIEDSELRRTLQSLACGKARVLIKSPKGKEVEDGDKFIFNGEF
KHKLFRIKINQIQMKETVEEQVSTTERVFQDRQYQIDAAIVRIMKMRKTLGHNLLVSELY
NQLKFPVKPGDLKKRIESLIDRDYMERDKDNPNQYHYVA

**Cullin 4B**

**Name**

>sp|Q13620|CUL4B_HUMAN Cullin-4B OS=Homo sapiens OX=9606 GN=CUL4B PE=1 SV=4

**Cleavage sites**

Probability: 0.987731 Site:REDSLD|S (D281)

Probability: 0.775639 Site:FKDKVD|H (D527)

Probability: 0.748045 Site:KSASVD|A (D624)

Probability: 0.747286 Site:SSGSGD|G (D10)

Probability: 0.729584 Site:VRSATD|G (D43)

**Cleveage sites in your query**

MMSQSSGSGD|GNDDEATTSKDGGFSSPSPSAAAAAQEVRSATD|GNTSTTPPTSAKKRKLN
SSSSSSSNSSNEREDFDSTSSSSSTPPLQPRDSASPSTSSFCLGVSVAASSHVPIQKKLR
FEDTLEFVGFDAKMAEESSSSSSSSSPTAATSQQQQLKNKSILISSVASVHHANGLAKSS
TTVSSFANSKPGSAKKLVIKNFKDKPKLPENYTDETWQKLKEAVEAIQNSTSIKYNLEEL
YQAVENLCSYKISANLYKQLRQICEDHIKAQIHQFREDSLD|SVLFLKKIDRCWQNHCRQM
IMIRSIFLFLDRTYVLQNSMLPSIWDMGLELFRAHIISDQKVQNKTIDGILLLIERERNG
EAIDRSLLRSLLSMLSDLQIYQDSFEQRFLEETNRLYAAEGQKLMQEREVPEYLHHVNKR
LEEEADRLITYLDQTTQKSLIATVEKQLLGEHLTAILQKGLNNLLDENRIQDLSLLYQLF
SRVRGGVQVLLQQWIEYIKAFGSTIVINPEKDKTMVQELLDFKDKVD|HIIDICFLKNEKF
INAMKEAFETFINKRPNKPAELIAKYVDSKLRAGNKEATDEELEKMLDKIMIIFRFIYGK
DVFEAFYKKDLAKRLLVGKSASVD|AEKSMLSKLKHECGAAFTSKLEGMFKDMELSKDIMI
QFKQYMQNQNVPGNIELTVNILTMGYWPTYVPMEVHLPPEMVKLQEIFKTFYLGKHSGRK
LQWQSTLGHCVLKAEFKEGKKELQVSLFQTLVLLMFNEGEEFSLEEIKQATGIEDGELRR
TLQSLACGKARVLAKNPKGKDIEDGDKFICNDDFKHKLFRIKINQIQMKETVEEQASTTE
RVFQDRQYQIDAAIVRIMKMRKTLSHNLLVSEVYNQLKFPVKPADLKKRIESLIDRDYME
RDKENPNQYNYIA

**Cullin 3**

**Name**

>sp|Q13618|CUL3_HUMAN Cullin-3 OS=Homo sapiens OX=9606 GN=CUL3 PE=1 SV=2

**Cleavage sites**

None

**Figure 3-6 Cullin 4A and B Contain Predicted Caspase 3 Cleavage Sites.**

ScreenCap3 software (ref Fu et al 2014) indicates multiple predicted cleavage sites within cullin4A and 4B amino acid sequences while none are present in cullin 3. This indicates that cullin 4 is degraded as a result of apoptosis.

The data presented thus far indicates that bleomycin appears to initiate apoptosis as determined by loss of cell viability which we detected using the ATP assay, and by potential caspase cleavage in occurring WT but not KO cells (based on the predicted caspase cleavage sites for cullin 4). We wanted to confirm that other caspase substrates are cleaved in response to bleomycin treatment to interrogate the hypothesis that DCNL5 KO cells lack caspase activity. We therefore decided to track the levels of two proteins that are cleaved following the induction of the apoptosis pathway: caspase 3 and PARP1. Caspase 3 is known as an 'executioner' caspase and is the last in a series of caspase activation reactions and is a key mediator of the downstream effects of apoptosis. PARP1 is cleaved by caspases and has downstream apoptotic effects and is also used as a marker for apoptosis.

In order to determine whether bleomycin induces apoptosis, we used only the WT cells which we have shown are responsive to treatment (in terms of loss of viability), to analyse caspase 3 and PARP1 cleavage by western blotting. Figure 3-7 shows that following bleomycin treatment of WT cells, cullin 4A levels drop – this reduction is visible after 48 hours but after 72 hours the protein levels are almost entirely depleted. Interestingly, we can observe an increase in molecular weight of cullin 4A after 48 hours, possibly indicating an increase in neddylation following DNA damage induction. Pro-caspase 3 and full length PARP1 levels are entirely gone following 72-hour bleomycin treatment, along with cullin 4A, supporting the notion that treatment is causing the cells to undergo apoptosis. As a further experimental control, cycloheximide and TNFα were used in combination to induce apoptosis (this is a well-described control for the pathway) and this treatment caused reductions in pro-caspase 3 and to a lesser extent PARP1, while also causing a reduction in cullin 4A levels. This is a further indication that bleomycin is inducing apoptosis in WT U2OS cells and that cullin 4A is degraded as a result of this process – perhaps directly by caspase proteins.

MLN4924 was shown to inhibit neddylation by the presence of only the lower molecular weight, un-neddylated form of cullin 4A being present following

treatment (comparing lanes 1 and 5 in Figure 3-7). It is interesting to note that in the figure, the neddylation inhibitor MLN4924 appears to protect cullin 4A from being degraded, since the higher molecular weight portion (likely neddylated cullin 4A) is still present. MLN4924 treatment also appears to reduce caspase 3 cleavage but not PARP1 cleavage. The overall significance of this is unclear, it is possible that MLN4924 may inhibit caspase cleavage and activation which thus has a protective effect on cullin 4A. The fact that we observe what may be neddylated cullin 4A with treatment using MLN4924 is counterintuitive but may be explained by the inhibitor preventing interaction between caspases and cullin 4A. Given the duration of bleomycin treatment (72 hours), it is perhaps unsurprising that cullin 4A can be neddylated since MLN4924 is likely not effective for that duration – perhaps it simply delays caspase cleavage and thus cullin 4 degradation. This finding was not investigated further.

The data presented in this section provides an indication that bleomycin is inducing the apoptosis response in cells after prolonged treatment, as we see similar reductions in full length caspase 3 and PARP1 which are known to be downstream cleavage products of this pathway. Furthermore, it is likely that cullin 4A and 4B are being degraded by caspase 3 (or other caspases) as a result of apoptosis induction, based on the predicted caspase 3 cleavage sites within cullin 4 amino acid sequences. The lack of cullin degradation in KO cells likely occurs as a result of a lack of apoptosis occurring rather than due to a specific interaction of cullins with DCNL5. It seems plausible therefore that DCNL5 plays a role in the apoptosis response, and this hypothesis is probed in the following sections.

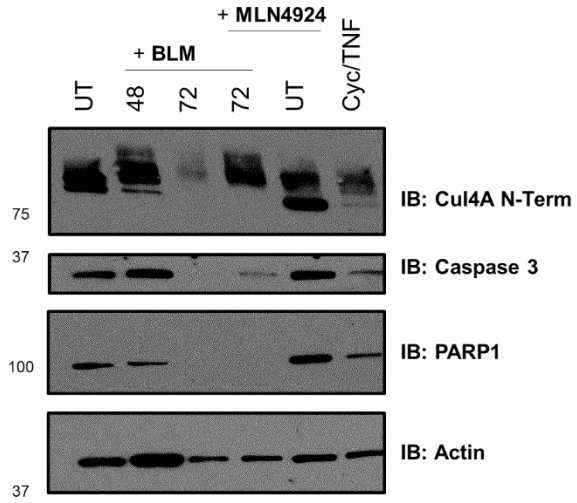



**Figure 3-7 Bleomycin Treatment of WT U2OS Cells Causes Apoptosis.**

In this representative western blot (n = 2), WT cells are shown and cullin 4A levels decrease as bleomycin (0.5mg/ml) treatment time increases, with almost no protein present after 72 hours. In a similar fashion, pro-caspase 3 levels as well as PARP1 levels are entirely gone following 72-hour bleomycin treatment. Cycloheximide (10µg/ml) in combination with TNFα (50ng/ml) is used to induce apoptosis , and this treatment causes reduction in caspase 3 and PARP1 levels while also causing a reduction in cullin 4A levels, indicating that loss of cullin 4A likely occurs as a result of apoptosis. The NEDD8-activating enzyme inhibitor MLN4924 was used in order to quickly investigate whether neddylation had a role in cullin 4A degradation and it appears as though MLN made no difference. In cells not treated with bleomycin (UT) but treated with MLN4924 (3µM) we see a larger lower molecular weight band representing un-neddylated cullin 4A which confirms the drug worked.

### 3.2.4 DCNL5 is Required for Caspase Activity in Extrinsic but not Intrinsic Apoptosis

We next tested whether DCNL5 is required for apoptosis - there are two main branches of the apoptosis pathway, the extrinsic branch and the intrinsic branch. We wanted to establish whether both branches were affected in the DCNL5 KO cells or if the defect in the pathway was restricted to a single branch. The translational inhibitor cycloheximide is used in combination with the cytokine TNFα to induce the extrinsic branch of the pathway specifically due to cycloheximide specifically inhibiting an endogenous inhibitor of apoptosis called cFLIP. There is some overlap and crosstalk between extrinsic and intrinsic branches however (which are discussed in section 3.3), which is very difficult to fully overcome. 24-hour treatment with CHX and TNFα, as measured by luciferase assay, caused 80% loss of viability for WT cells while only about 40% loss in the KO cells. A variety of TNFα concentrations were tested with some data shown in Figure 3-8A, however overall the KO cells were on average 3-times as resistant to the treatment . Figure 3-8B shows optimisation of the concentration of CHX to be used in subsequent experiments; 10μg/ml of CHX and 50ng/ml of TNFα were generally used in further experiments, unless stated otherwise.

Unlike with CHX/TNFα treatment, staurosporine activates the intrinsic branch of the apoptosis pathway. Staurosporine is a protein kinase inhibitor that causes cytochrome C release from the mitochondria in cells and caspase 9 activation, resulting in downstream caspase cleavage which can be detected by western blotting. Its precise mechanism and specificity towards activating the intrinsic pathway remains unclear however (Stepczynska *et al.*, 2001). 24 hour staurosporine treatment caused a drop in viability in both WT and KO cells (drops of 50% and 60% respectively) at 100nM concentration and the difference was statistically significant ($p<0.01$), while 200nM caused an even larger, statistically significant response in the KO cells (which were only 20% viable) than the WT cells (which were 40% viable). This indicates that the DCNL5 KO cells are not resistant to intrinsic apoptosis stimulation.

In order to establish whether the defect in KO cells was related to a lack of caspase activity, we monitored the cleavage of caspase 3 and PARP1 by western blot

following stimulation of both extrinsic and intrinsic branches of the apoptosis pathway using the same drugs as above, only for shorter time periods (24 hours was found to be too long to detect cleavage due to degradation of the cleavage products). CHX/TNFα treatment for 6 hours led to cleavage of both caspase 3 and PARP1 in WT cells but not KO cells (Figure 3-8D and Figure 3-8E), which was consistent with the WT cells undergoing more apoptosis than the KO cells as shown in Figure 3-8A. As shown in Figure 3-8E, Staurosporine treatment for 6 hours caused cleavage of caspase 3 and PARP1 in both WT and KO cell lines, which was again consistent with the viability data from Figure 3-8C which indicates that DCNL5 cells are resistant to extrinsic apoptosis only.

The role of the DAD-patch and thus potential cullin binding was confirmed with CHX/TNFα treatment in addition to the bleomycin data already shown. Figure 3-9 shows that DAD-mutant DCNL5 rescue cells are equally resistant to the induction of extrinsic apoptosis as KO cells, while both WT and WT-rescue cells are more sensitive. Overall, this data indicates that the resistance of DCNL5 KO cells to apoptosis is restricted to extrinsic branch of the pathway, and that this resistance is likely conferred due to a lack of caspase activity given that caspase 3 and PARP1 fail to undergo cleavage and are therefore unable to mediate some of the downstream effects of apoptosis. As with bleomycin, DAD-rescue cells respond the same as KO cells, in this case to CHX/TNFα treatment, while WT-rescue cells are as sensitive to the treatment as WT cells (the difference between the two is statistically insignificant). This further supports a role for cullin binding by DCNL5 in its role in mediating cell death, and this role is limited to the extrinsic branch of the apoptosis pathway which is induced by both CHX/TNFα treatment and bleomycin treatment.

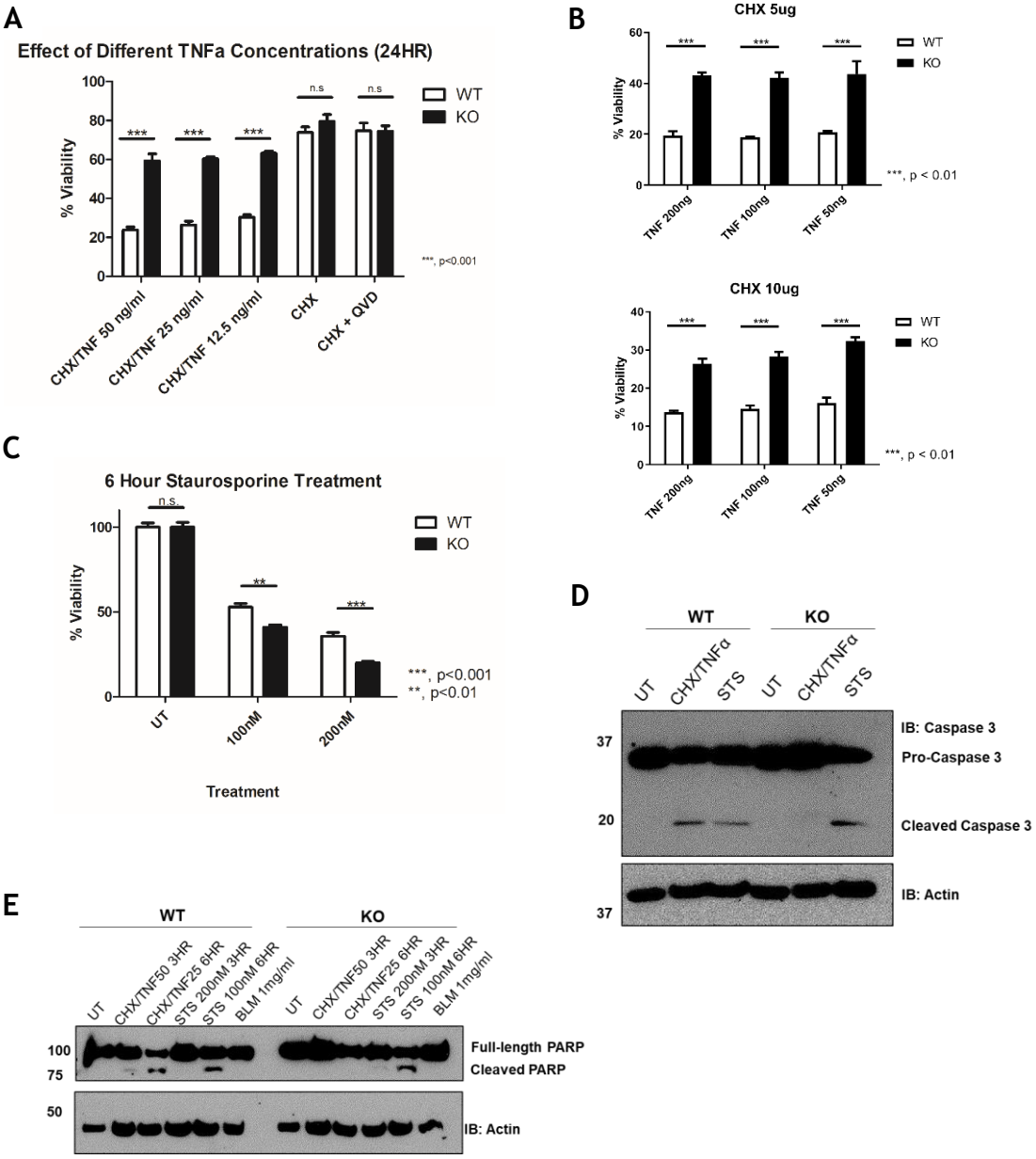



**Figure 3-8 WT Cells are Sensitive to Extrinsic and Intrinsic Apoptosis While DCNL5 KO Cells Are Only Sensitive to Intrinsic Apoptosis.**

(A) Luciferase viability assay shows that DCNL5 KO cells are more resistant to combination CHX/TNFα treatment than WT cells (extrinsic apoptosis). A concentration of 50ng/ml TNFα was deemed optimal for subsequent experiments due to the largest difference in response between the two cell lines. The pan-caspase inhibitor QVD-OPh was used as a control to ensure that it had no effects on viability of the cells for use in later experiments. (B) CHX concentration was also optimised in combination with TNFα concentration – unless stated otherwise CHX was used at 10µg/ml and TNFα was used at 50ng/ml. (C) Staurosporine (intrinsic apoptosis) cased a reduction in viability of both WT and KO cells, with the KO cells appearing slightly more sensitive at the highest concentration. (D) This representative western blot shows that CHX (10µg/ml) and TNFα (50ng/ml for 6 hours) combination treatment causes caspase 3 cleavage in WT but not KO cells. Staurosporine (100nM, 6 hours) on the other hand causes cleavage in both WT and KO cells. (E) This representative western blot, with drug treatments and treatment times indicated, shows that as with caspase 3, CHX/TNFα treatment causes cleavage of PARP1 in WT cells but not KO cells. This cleavage appears stronger after 6 hours rather than 3, despite the lower concentration of TNFα. Staurosporine causes PARP1 cleavage in both cell lines, again this cleavage is seen most after 6 hours with only a small amount being visible after 3. Bleomycin treatment for 72 hours is likely too long to detect the cleaved product which will be degraded in that time. *Data shown in (A), (B) and (C) represent the mean + SEM from 3 independent experiments, analysed by 2-way ANOVA comparing WT to KO cells for indicated and statistical significances of p<0.001 and p<0.01 were achieved, as indicated on the graphs*. *IB = immunoblot*

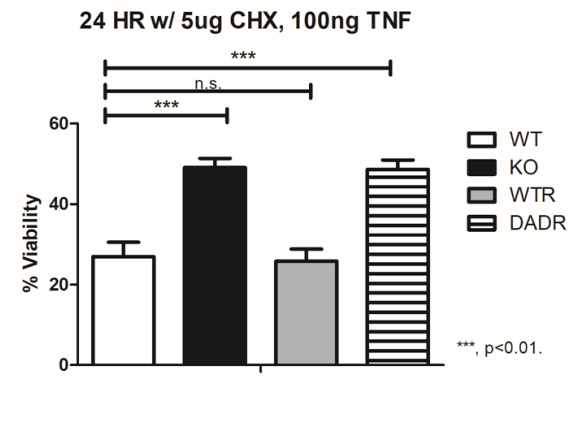



**Figure 3-9 DAD-Patch Interaction is Required for DCNL5 Role in Apoptosis.**

As with bleomycin, DAD-mutant cells behave the same as KO cells and are resistant to CHX/TNFα treatment while expression of WT DCNL5 rescues the sensitivity of the KO cells. This suggests that the DAD-patch is required for this function of DCNL5 which may represent cullin interaction. *Data shown represent the mean + SEM from 2 independent experiments, analysed by 1-way ANOVA comparing WT to KO, WTR and DADR cells for indicated treatments and statistical significances of p <0.01 were achieved*. n.s = not significant

In order to further confirm the finding that lack of caspase cleavage is the mechanism underlying the DCNL5 KO cells' resistance to extrinsic apoptosis induction, we used a pan-caspase inhibitor QVD-OPh (Abcam) to inhibit the action of caspases in the cells. 1 hour pre-treatment at a concentration of 40μM was shown to reduce the sensitivity of WT cells to CHX/TNFα treatment (Figure 3-10A) and Figure 3-10B and C show the effect of the inhibitor on WT and KO cells following stimulation with CHX and TNFα for 24 hours. The WT cells are rendered almost 200% less sensitive to CHX/TNFα treatment with the use of the inhibitor while KO cells are about 50% less sensitive. Interestingly, WT cells pre-treated with the caspase inhibitor respond the same as KO cells without the inhibitor, strongly indicating that the KO cells have significant caspase activity impairment in the extrinsic branch of the pathway.

QVD-OPh also has an effect on WT cells that have been stimulated with staurosporine, but to a lesser extent. Figure 3-10D shows that this effect is more visible and more statistically significant ($p<0.001$ vs $p<0.01$) at the highest concentration of the drug where cells are almost 100% more viable. Figure 3-10D also shows that KO cells exhibit almost no change in response to staurosporine with caspase inhibition at 100nM and 200nM staurosporine concentrations not statistically significant for both), suggesting perhaps that these cells are dying via caspase-independent pathways. It is possible that KO cells undergo caspase-independent cell death due to their compromised caspase activity and thus inhibiting caspases with QVD has little effect. This result requires further investigation.

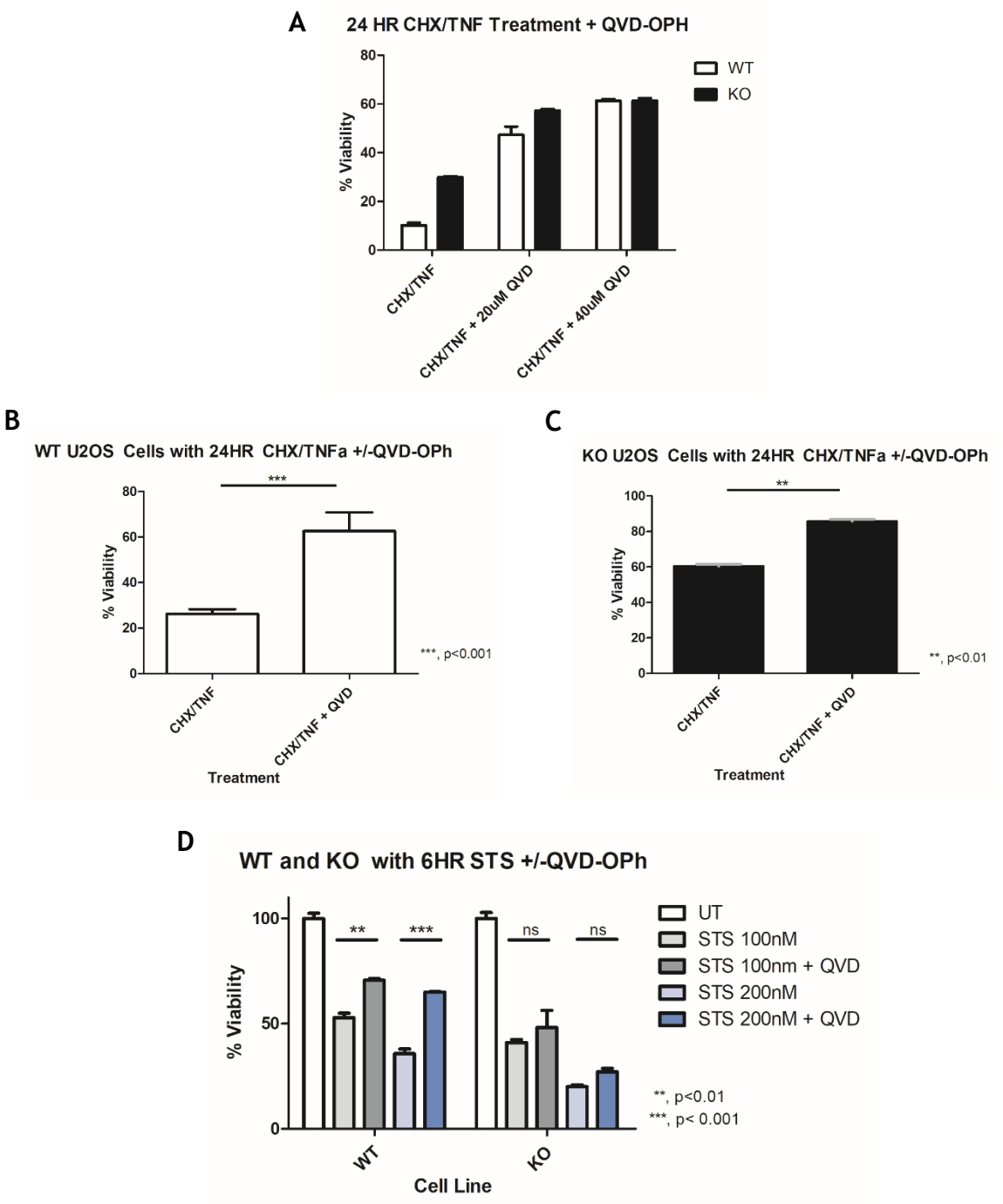



**Figure 3-10 The Pan-Caspase Inhibitor QVD-OPh Increases Resistance of WT Cells to Apoptosis More Than DCNL5 KO Cells, in Relative Fold-Change Terms.**

(A) We tested the broad-spectrum caspase inhibitor QVD-OPh (Abcam) at a concentration of 20μM and 40μM for 1 hour prior to apoptosis induction with CHX/TNFα and found that 40μM was the better concentration to use given its increased effectiveness. (B) QVD treatment increased the viability of WT cells from just over 20% to over 60% relative to untreated (-QVD) cells. (C) QVD increased the viability of KO cells from 60% to just over 80% relative to untreated (-QVD) cells. This indicates that inhibition of caspase activity in WT cells renders their sensitivity the same as KO cells without the use of the inhibitor. (D) QVD reduces the sensitivity of WT cells to in a statistically significant manner, with the effect more noticeable at higher concentrations. QVD has much less of an effect on KO cells, with no statistically significant effect on KO cell viability with the inhibitor at either STS concentration). *Data shown represent the mean + SEM from 3 independent experiments analysed by 2-way ANOVA comparing -QVD to +QVD and statistical significances of p<0.001 and p<0.01 were achieved, as indicated on the graphs*. *ns = not significant.*

### 3.2.5 DCNL5 knockout cells are not impaired in their response to CHX or TNFα

Having established that DCNL5 KO cells have an impairment in the extrinsic branch of the apoptosis pathway and that this impairment is likely caused by a defect in caspase activation, we wanted to verify that the KO cells responded the same as the WT cells to cycloheximide and TNFα separately. The combination treatment of the two drugs induces extrinsic apoptosis but we wanted to ensure that the lack of response by KO cells was due only to the lack of apoptosis signalling and not due to a lack of response to the cytokine TNFα and/or to the protein synthesis inhibitor cycloheximide. Cycloheximide causes protein translation arrest, which results in a global reduction in protein synthesis and allows for monitoring of steady state protein levels. We used a cycloheximide chase experiment to quantify the steady state decay of TNF receptor I (responsible for interacting with TNFα) in order to verify that WT and KO cells exhibit a similar response, and that turnover of the receptor isn't a factor in response of KO cells to TNFα signalling, as well as confirming that global expression of TNF receptor is similar between the two cell lines.

Figure 3-11A shows a western blot tracking levels of TNF-receptor 1 (TNFR1) during the 1-hour cycloheximide chase experiment. This western blot appears to indicate globally lower TNFR expression in KO cells, however the half life was estimated based on the reduction in relative protein amount following CHX stimulation, and this was calculated to be 48 and 39 minutes for WT and KO cells respectively. While there is a difference, the values are similar enough to suggest that the difference in sensitivity of KO cells is likely not due to a different half life of the receptor, however further work is required to substantiate this finding and it is possible that this discrepancy may account for some of the difference in response. Figure 3-11C from a separate experiment which was performed twice independently (also shown in Figure 3-13D a few pages later) shows that the TNFR levels in WT and KO cells are broadly similar in response to 1-hour CHX treatment further supporting the finding that the receptor levels and half life are similar in both cell lines and likely have no impact on the lack of apoptosis in KO cells. This half life experiment was only done once and therefore requires repeating and further optimising to draw more significant conclusions.

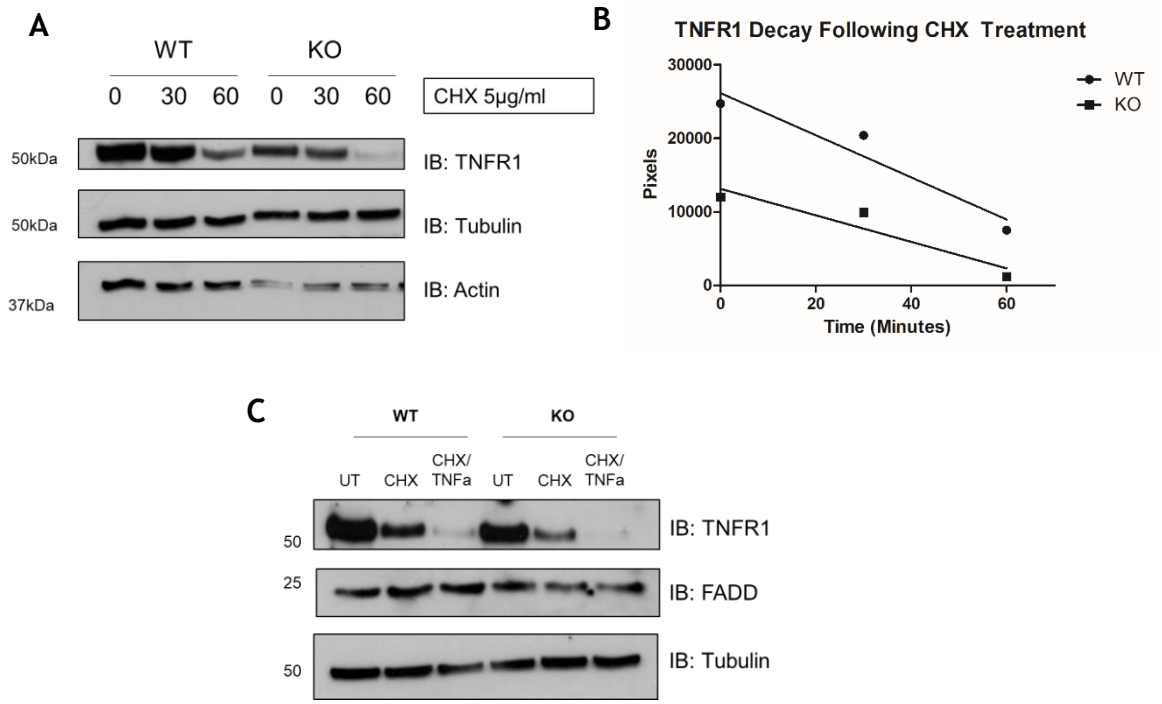



**Figure 3-11 TNFR1 Steady State Level Decay is Largely the Same in WT and DCNL5 KO Cells Following CHX Chase.**

(A) This western blot (n = 1) shows the steady state levels of TNFR1 following a CHX chase, globally less receptor was detected in the KO cells but the reduction in levels appears similar. The difference in actin levels in the KO cells appears to be a result of incomplete staining with the antibody since tubulin levels are consistent between cell lines. (B) Quantification of TNFR half life based on the decrease in protein (pixel) levels following CHX stimulation. The equations of WT and KO trendlines were y = -290 + 26000 and y = -180 + 13000 for WT and KO cells, respectively. The half life of TNFR in WT cells was calculated to be 48 minutes while the half life in KO cells was 39 minutes. (C) This representative western blot (n = 2) shows that after 1-hour cycloheximide treatment, the reduction in TNFR1 level appears to be similar in WT and KO cell lines.

In addition to observing the effect of cycloheximide on TNFR levels and half life in WT and KO cells (which appear to be largely similar), we wanted to establish whether the KO cells were able to respond the same as the WT cells to TNFα ligand. TNFα stimulation causes the phosphorylation and subsequent ubiquitination and degradation of IκBα which allows for the nuclear translocation of NF-κB which normally exists in complex with IκBα. This degradation thus allows for transcription of genes involved in immune signalling by NF-κB (Miyamoto *et al.*, 1994). We tested for TNFα sensitivity by tracking the protein levels of IκBα by western blot for 1 hour after stimulation with recombinant TNFα. A normal response involves the phosphorylation and subsequent ubiquitination and 26S

proteasome-mediated degradation of IκBα (summarised in Figure 3-12B) which allows for NF-κB nuclear translocation and gene expression regulation as mentioned previously. As can be seen in Figure 3-12A, in both cell lines we saw an increase in molecular weight of IκBα after 10 minutes, representing the phosphorylation of the protein, followed by strong reduction in levels after 30 minutes with levels beginning to return after 60 minutes as new protein is made. Importantly, DCNL5 WT and KO cells exhibit the same response to the TNFα signal which indicates that the KO possess functional receptor and have no impairment in recognising the ligand. Additionally, this confirms that this particular ubiquitin E3 ligase activity is unaffected by the absence of DCNL5. $SCF^{\beta\text{-}TrCP}$ is the ligase responsible for the degradation of IκBα (Kanarek and Ben-Neriah, 2012) and DCNL5 KO cells exhibit normal processing of the protein. The cullin core of SCF requires neddylation which is mediated by one of the 5 DCNL proteins in humans and likely depends on the sub-cellular localisation of the CRL complex. Overall, this data suggests that the fault in downstream caspase activity and apoptosis response lies downstream of the TNFα/TNFR ligand-receptor interface and upstream of caspase 3 cleavage.

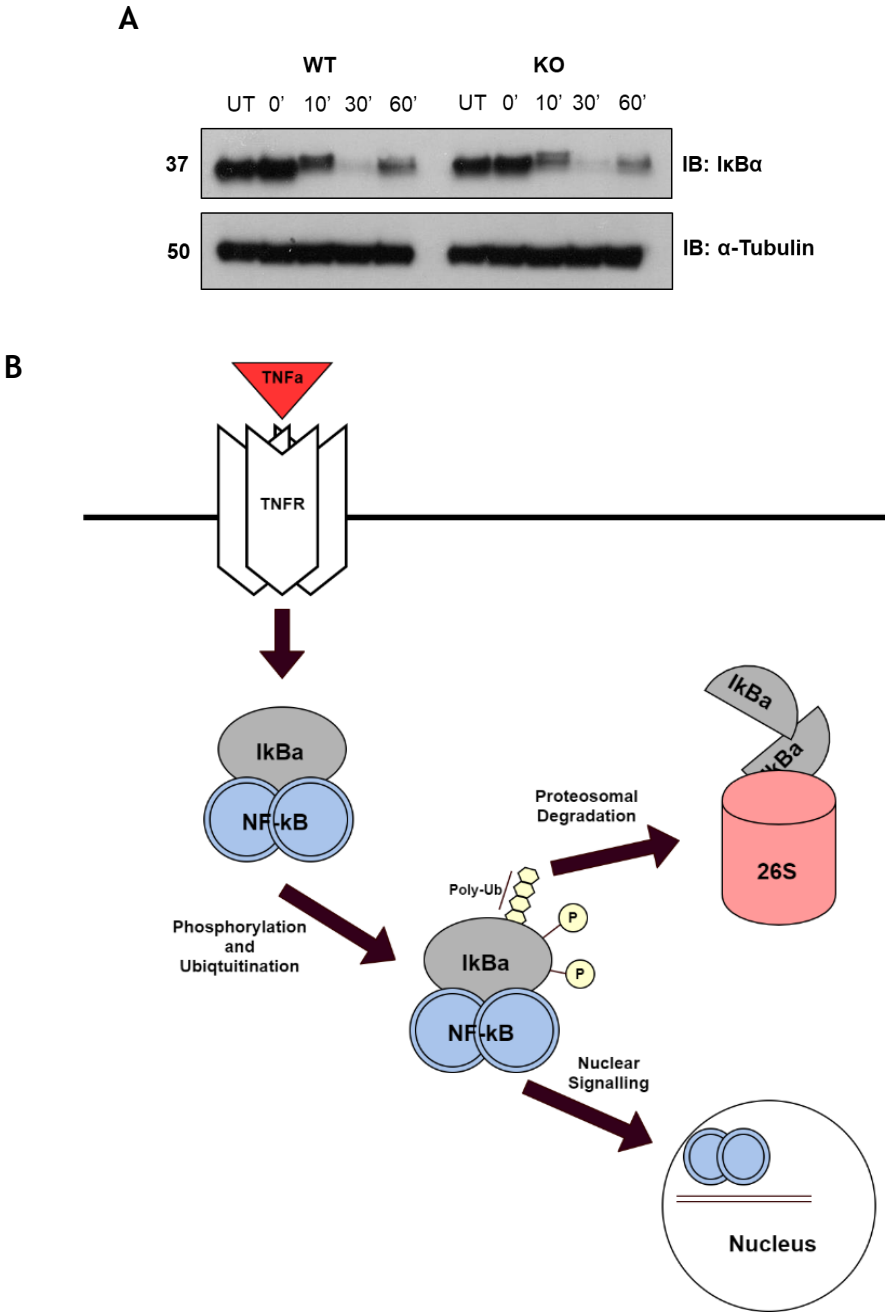



**Figure 3-12 WT and DCNL5 KO Cells Undergo Normal IκBα Degradation and Respond the Same to TNFα Stimulation.**

(A) This representative western blot shows that both WT and KO cells undergo phosphorylation and proteosomal degradation of IκBα following TNFα stimulation, indicating normal and succesful reponse to the ligand. (B) This outline visualises the phosphorylation, ubquitination and subsequent 26 proteosome-mediated degradation of IκBα which allows for nuclear translocation of NF-κB and pro-inflammatory gene signalling.

### 3.2.6 Caspase 8 is not Fully Activated in DCNL5 KO Cells

Having established that the defect in downstream apoptosis events occurs upstream of caspase 3 cleavage but downstream of the TNFα receptor-ligand interface, we wanted to determine whether caspase 8, one of the initiator caspases and the one which is activated immediately downstream of the TNFα receptor, undergoes cleavage in the DCNL5 KO cells. TNFα signalling initially leads to the formation of a receptor-bound complex known as complex I consisting of the death domain containing protein TRADD as well as proteins including TRAF2 and RIPK1. For apoptotic signalling, a second complex, known as complex II, is formed in the cytosol and the core consists of TRADD, FADD and pro-caspase 8 (summarised in Figure 3-13B). This complex mediates the activation of caspase 8 through proteolytic cleavage into different intermediates ultimately leading to formation of its active form, known as p18, which is a hetero-tetramer of two subunits (p18 and p10).

We stimulated WT and KO cells using the combination treatment of cycloheximide and TNFα and used the neddylation inhibitor MLN4924 as well as the caspase inhibitor QVD-OPh to investigate their effects on caspase 8 cleavage. Figure 3-13A shows the cleavage of caspase 8 detected via western blot, and it is clear that the WT cells undergo cleavage to form both p43/41 and the active p18 forms of caspase 8 while KO cells appear to undergo no processing of the protein. Interestingly, MLN pre-treatment appears to inhibit formation of the p18 fragment of caspase 8 in WT cells, while the pan-caspase inhibitor QVD has a similar effect. This suggests a role for NEDD8 in the full processing of caspase 8 but perhaps not in the initial cleavage to the p43/41 fragment. Caspase inhibitors such as QVD-OPh work by binding to the catalytic site of caspases to inhibit their activity, and the data here indicates that caspase 8 is able to be processed to its p43/41 form but not the catalytically crucial form p18, suggesting that initial processing of caspase 8 occurs independently of the catalytic activity of the protein. This western blot lacks independent repeats as well as appropriate loading controls, thus the conclusions drawn here require caution and these data provide only an indication of what may be happening.

Given that caspase 8 is just one of the components of complex II in TNFα signalling, we wanted to determine whether any of the other proteins in the complex where affected by the absence of DCNL5 expression. Global levels of TRADD (the first and death domain-containing protein recruited to the TNFR1) as well as FADD (the death domain-containing protein recruited to the second complex along with pro-caspase 8) were unaffected by CHX/TNFα treatment (Figure 3-13C and D) indicating that at the very least these proteins are expressed and present in both WT and KO cells. Co-IP experiments would have been able to determine whether recruitment of these proteins to complex II are affected in the DCNL5 KO cells compared to the WT cells, and would thus be useful experiments to do in future work. Figure 3-13D also shows that TNFR1 levels drop significantly after TNFα treatment which, as well as being due to an increase in the steady-state decay of the receptor, arises due to the rapid internalisation of the receptor following signalling.

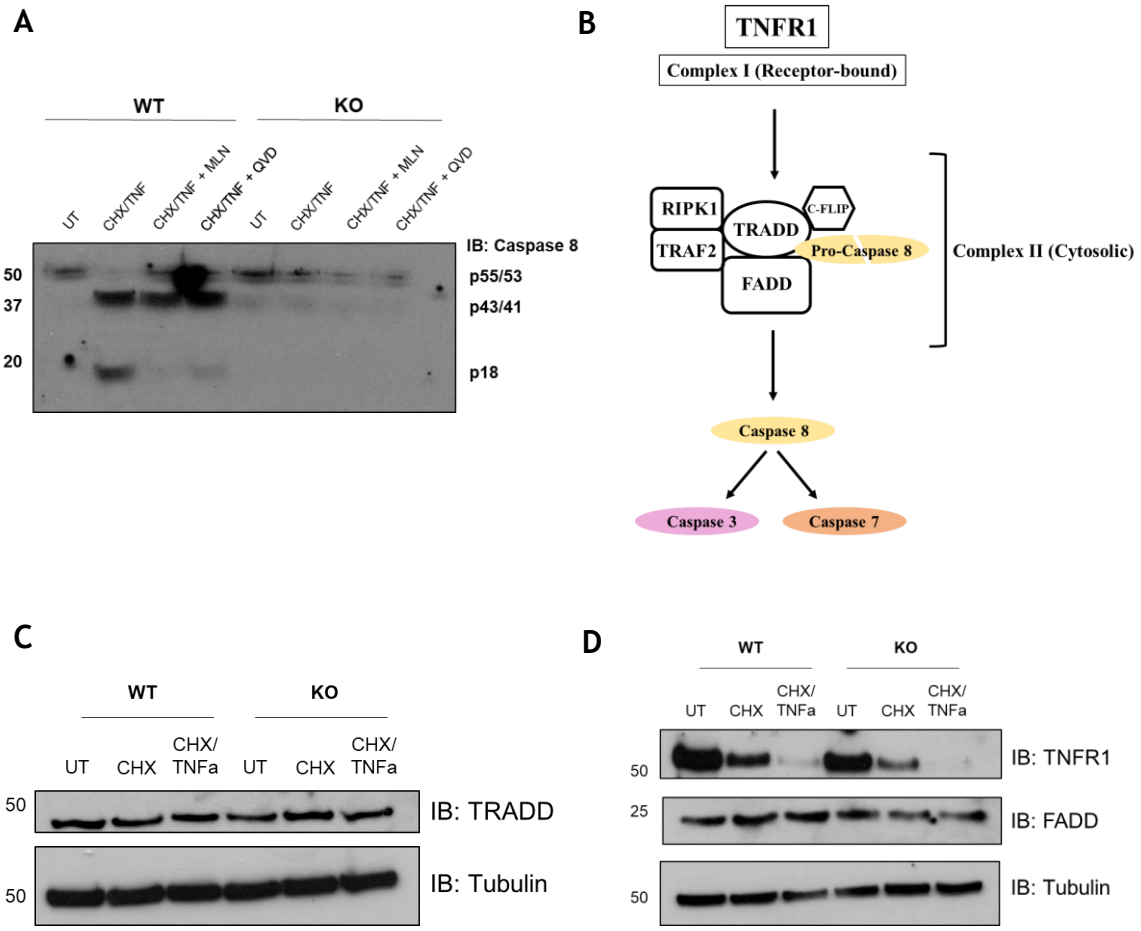



**Figure 3-13 Caspase 8 Cleavage is Impaired in DCNL5 KO Cells While TRADD and FADD Levels are Unaffected by CHX/TNFα Treatment**

CHX was used at 5μg/ml while TNFα was used at 50ng/ml. (A) This western blot lacks loading controls or independent experimental repeats (n = 1), but it appears to show that CHX/TNFα induces caspase 8 cleavage to p43/41 and p18 fragments in WT but not KO cells. MLN and QVD treatments inhibit formation of the p18 fragment in the WT cells. (B) This diagram illustrates the formation of a receptor-bound complex (I) followed by formation of a cytosolic complex (II) containing proteins including TRADD, FADD and importantly pro-caspase 8. It is this complex that is responsible for the cleavage and activation of caspase 8. (C) Representative western blot (n = 2) showing that TRADD protein levels are the same in WT and KO cells and unchanged by CHX/TNFα treatment. (D) Representative western blot (n = 2) showing that FADD protein levels are also unaffected by treatment and the same in both cell lines. TNFR1 levels drop significantly after TNFα stimulation due in part to the internalisation and degradation of the receptor.

### 3.2.7 DCNL5 KO Cells are Resistant to TNF-Related Apoptosis-Inducing Ligand (TRAIL) Stimulation

In order to confirm that the extrinsic branch of the apoptosis pathway is compromised in the absence of DCNL5 expression in U2OS cells, we used another inducer of the pathway, TNF-related apoptosis inducing ligand (TRAIL). As before, we used an ATP-based viability assay and 24 hour treatment times to induce apoptosis in the U2OS cells, and the result is shown in Figure 3-14A. 200ng/ml was deemed to be an appropriate concentration moving forward. DCNL5 KO cells are more resistant in viability terms to the treatment with respect to WT cells, as shown in panel B. This finding is very important – KO cells are strongly resistant to TRAIL-induced apoptosis and corroborates the findings with CHX/TNFα. Figure 3-14B also shows that the resistance of DCNL5 KO cells can be rescued by re-expression of WT DCNL5 but not DAD-mutant DCNL5 which is consistent with the data for CHX/TNFα treatment as well as bleomycin treatment. Interestingly, the DAD-mutant cells appear to be even more resistant to TRAIL than the KO cells, while WT-rescue cells appear more sensitive than the WT cells. This result held up during repeats of the experiment and indicated that expression of DAD-mutant DCNL5 may have more of an effect on viability than merely the absence of DCNL5 – perhaps DCNL5 interaction via the DAD-patch binds and sequesters proteins that are required for apoptosis and thus renders these cells even less sensitive to cells simply lacking DCNL5 expression. Figure 3-14C shows the expression of DCNL5 and GFP-DCNL5 in the rescue cell lines – DCNL5 levels are slightly higher in the rescue cell lines than the WT cells, which may account for the higher sensitivity of the WT-rescue cells to TRAIL with respect to the WT cells.

While not entirely identical the TNFα and TRAIL signalling pathways share many common aspects and mechanisms - one of these is caspase 8 cleavage, with both pathways converging on this key protein. We treated the U2OS cell lines with 100ng/ml of TRAIL for 1 and 3 hours and analysed these samples by immuno-blotting for caspase 8 as well as probing for cullin 3 and caspase 3. Cullin 3 has been shown to be a key regulator of caspase 8 activity and is known to ubiquitinate and promote cleavage and thus warranted investigation in our work (Jin *et al.*, 2009). The resulting western blot is shown in Figure 3-14D. Cleavage of p41/41 forms of caspase 8 are reduced significantly in DCNL5 KO and DAD-mutant cells

compared to WT and WT-rescue ones. Catalytically active p18 caspase 8, while harder to detect, shows the same reduction in protein levels in the KO and DAD-mutant cells. This data is consistent with the TNFα data in 3.2.6, indicating that DCNL5 is required for caspase 8 cleavage in TRAIL signalling as well. Global levels of cullin 3 and pro-caspase 3 were largely similar between cell lines. We did not probe for cleaved caspase 3 so cannot say whether this was affected but we anticipate that we would have seen a similar reduction in cleavage that we observed for TNFα signalling.

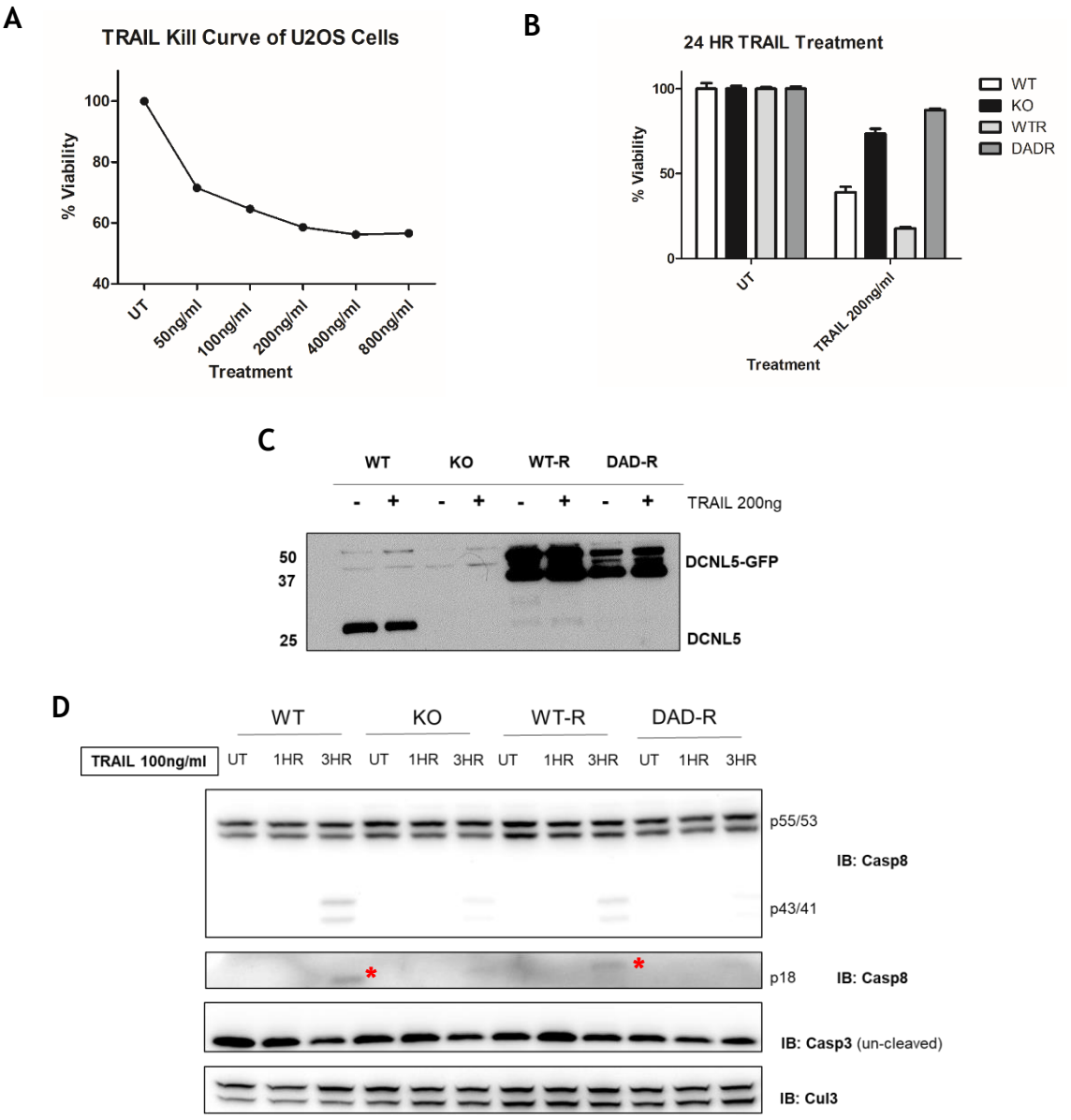



**Figure 3-14 DCNL5 KO Cells are Resistant to TRAIL and Exhibit a Reduction in Caspase 8 Cleavage.**

(A) This kill curve using a range of TRAIL concentrations indicates that U2OS cells are sensitive to treatment and that 200ng/ml was the optimal concentration to use moving forward. *Data shown represent the mean + SEM of 2 independent experiments*. (B). Viability assay shows that DCNL5 KO and DAD-mutant cells are resistant to TRAIL. DAD-mutant cells are even less sensitive to the drug than KO cells. WT-rescue cells are more sensitive than WT cells suggesting perhaps that increased DCNL5 expression leads to increased sensitivity to the ligand. *Data shown represent the mean + SEM from 3 independent experiments*. (C) This representative western blot confirms expression of DCNL5 in WT cells, lack of expression in the KO cells and expression of GFP-tagged DCNL5 in the rescue cell lines. The rescue cells overall express slightly more DCNL5 than the WT cells and may indicate that DAD-DCNL5 has a dominant negative effect. (D) This representative western blot shows that caspase 8 cleavage is reduced in DCNL5 KO and DAD-mutant cells, for both p41/41 and p18 fragments. p18 is harder to detect and is highlighted (*) in the WT and WT-rescue cells. Global levels of pro-caspase 3 and cullin 3 appear largely the same between the cell lines. *IB = immunoblot*

### 3.2.8 DCNL5 KO Cells Demonstrate Resistance to Apoptosis by TRAIL Using Annexin V and Propidium Iodide Staining

Another method to detect apoptosis in cells is to use annexin V and propidium iodide staining, in combination with flow cytometry or fluorescence imaging to detect the level of each marker following treatment with a drug that causes cell death. Annexin V is a marker for early-stage apoptosis; it binds to phosphatidylserine on the cytoplasmic side of the plasma membrane, which is translocated to the extracellular side of the membrane during early-stage apoptosis and therefore detectable using fluorescence imagery. Propidium iodide (PI) is a nuclear marker and its signal can be detected once the nuclear envelope has been degraded and the contents of the nucleus have been released from the cell during late-stage apoptosis and necrosis and therefore typically represents dead or dying cells.

We incubated cells plated in 96-well plates with annexin V and PI following 24-hour TRAIL stimulation and imaged the plates using a Sartorius InuCyte® live cell analysis system. Individual wells of the 96-well plate were imaged, and representative examples are shown in Figure 3-15. The WT cells show a dramatic increase in signal of both FITC-conjugated annexin V as well as propidium iodide, indicating that TRAIL causes apoptosis in these cells and is sufficient to cause death. The KO cells, however, show almost no signal for either marker indicating that these cells are entirely unaffected by the treatment and are unable to

undergo apoptosis or necrosis as a result of the TRAIL treatment over a 24-hour period. This data represents an additional read-out and experimental determination of the level of apoptosis and further highlights quite how significant the role of DCNL5 is in this response.

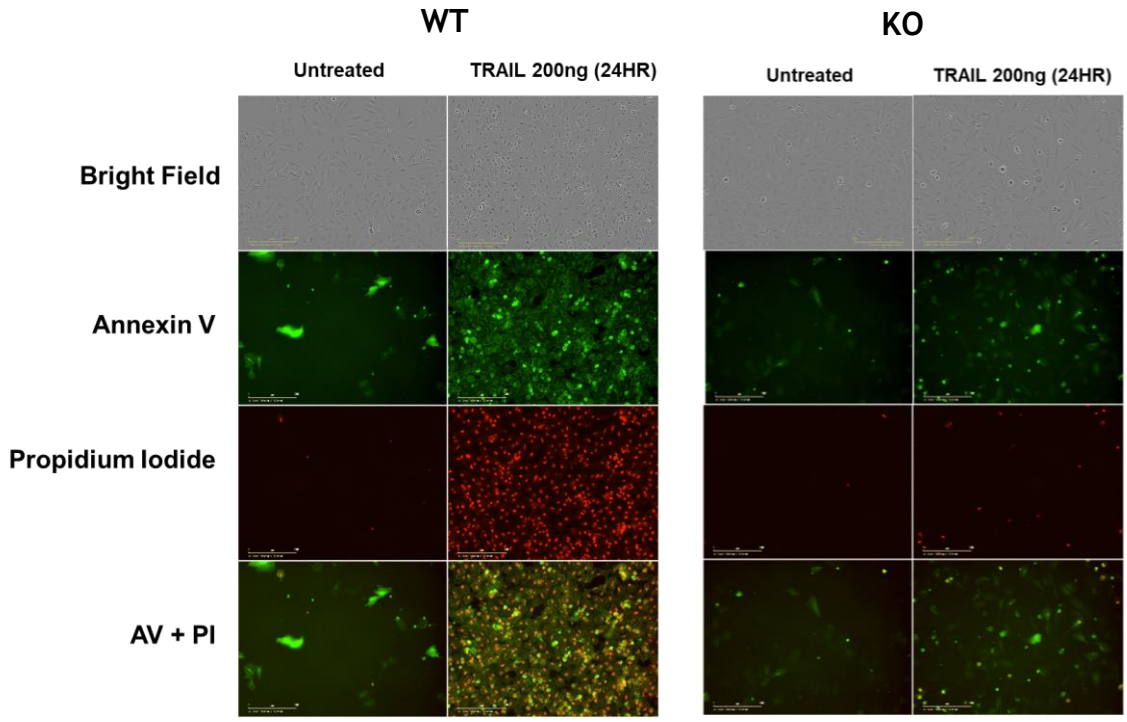



**Figure 3-15 DCNL5 KO Cells are AV and PI Negative After 24 Hour TRAIL Stimulation.**
These representative fluorescent images, taken from individual wells of a 96-well plate, show bright field images along with annexin V (conjugated to FITC) and PI fluorescence in cells following 24-hour treatment with 200ng/ml of TRAIL. TRAIL treatment causes a dramatic increase in annexin V positive cells which show a characteristic 'bubbling of their membranes due to the translocation of annexin V allowing for the fluorescence to be detected. The fluorescence of propidium iodide is also very strong in WT cells indicating that both early and late stage apoptosis and cell death is occurring. The KO cells, in stark contrast, show almost no AV or PI signal which suggests that these cells are not undergoing apoptosis or dying at all in response to the TRAIL treatment.

### 3.2.9 DCNL1 KO Cells Undergo Apoptosis in Response to TRAIL and CHX/TNFα Treatments

Given the functional complementation of the DCNL family of proteins, at least *in vitro*, where it has been shown that loss of individual DCNL expression has little effect on global cullin neddylation and that each DCNL protein can neddylate each cullin protein (Keuss *et al.*, 2016), we anticipated that the phenotype we observed in the DCNL5 KO cells is due to a unique role for DCNL5 in caspase 8 activation. This role likely cannot be performed by other DCNL proteins since we anticipate that if this were not the case, we would not see such a severe phenotype. In order to investigate this, we used DCNL1 KO cells to test for sensitivity to TNFα and TRAIL. DCNL1 is the most widely expressed of the DCNL family members in terms of tissue distribution as well as subcellular localisation and is also the best studied and the best characterised DCNL protein. It is localised in both the nucleus and the cytoplasm with generally high levels of expression across tissue culture cell lines and tissues. To test whether DCNL1 has similar apoptosis requirements as DCNL5, we subjected DCNL1 KO and WT cells (which were generated in parallel during the CRISPR/Cas9 process) to CHX/TNFα and TRAIL treatments in the same way as for DCNL5 cell lines.

Figure 3-16A shows the result of the 24-hour TNFα and TRAIL treatments on DCNL1 cells. The combination treatment of cycloheximide and TNFα appears to induce more death in the DCNL1 KO cells than the WT cells, while TRAIL is equally potent to both cell lines. Figure 3-16B shows a viability assay following 24-hour TRAIL treatment of all the DCNL5 cell lines along with DCNL1 WT and KO cells. As before DCNL5 KO and DAD-mutant cells are almost entirely resistant to the treatment while WT and WT-rescue cells are highly sensitive. DCNL1 WT and KO cells are even more sensitive to the treatment than DCNL5 cells, confirming that DCNL1 does not play the same role in the apoptosis pathway as DCNL5. Had we seen the same resistance to treatments, it would have indicated a more general role for DCNL proteins as a whole, however this process is likely dependent on DCNL5 specifically.

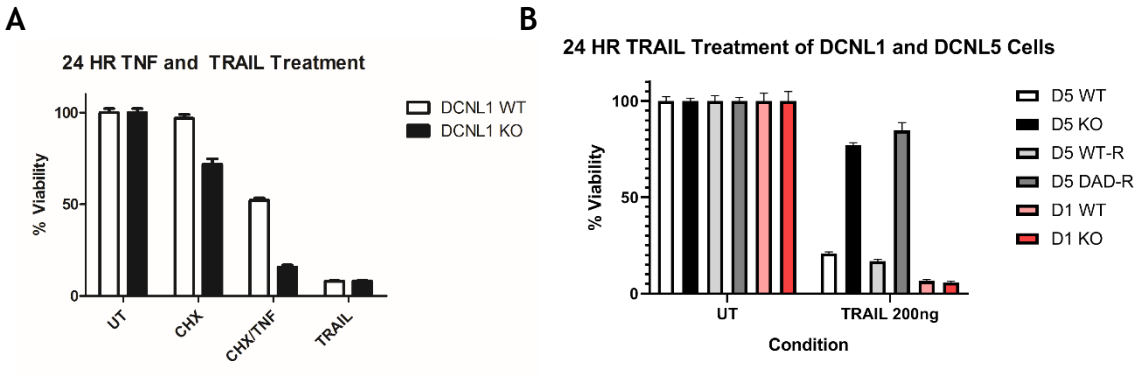



**Figure 3-16 DCNL1 KO Cells Are Sensitive to Both CHX/TNFα and TRAIL-Mediated Apoptosis.** (A) This viability assay shows the effect of both CHX/TNFα and TRAIL treatments on DCNL1 WT and KO cells. The KO cells appear more sensitive to the TNFα treatment than the WT cells while TRAIL kills both WT and KO cells entirely.(B) This viability data shows the effect of TRAIL on all of the DCNL5 cell lines used thus far as well as WT and KO DCNL1 cells. As before, DCNL5 KO cells are much more resistant to the treatment than WT cells, the DAD-mutant even more-so. The DCNL5 WT-rescue cells respond the same as WT cells. The same treatment completely kills both DCNL1 WT and DCNL1 KO cells, confirming that DCNL1 does not have the same effect as DCNL5 in this pathway. *Data shown in (A) and (B) represent the mean + SEM from three independent experiments.*

## 3.3 Discussion

### 3.3.1 Summary

The results presented in this chapter provide evidence for a novel role for DCNL5 in the apoptosis pathway. We have shown that DCNL5 is required in the extrinsic but not intrinsic branch of the apoptosis pathway and that this role of DCNL5 does depend on its DAD-patch region and thus potentially cullin protein interaction. We have demonstrated that the role of DCNL5 lies downstream of the ligand-receptor complex and upstream of the executioner caspase activities and provide evidence for the strong reduction in caspase 8 cleavage in the apoptosis resistant cell lines. DCNL1 KO cells are no less sensitive to the same treatments, suggesting that the role of DCNL5 is unique amongst the DCNL protein family in mediating this function, which would represent a potentially novel function of DCNL5.

### 3.3.2 Efficacy of Chemical Inducers of DNA Damage Was Largely Unaffected by DCNL5 Expression

The initial hypothesis that we were investigating in this chapter was the potential role of DCNL5 in the DNA damage response, which was thought to occur independently of its ability to interact with its usual binding partners, members the cullin protein family (Keuss *et al.*, 2016). Initial experimental work aimed to induce dsDNA damage in order to replicate the UV and gamma irradiation experiments performed by Keuss and colleagues. Neocarzinostatin is known for being an ionising radiation mimetic and induces dsDNA breaks through binding to the minor groove of DNA (Povirk, 1996; Smith, Bauer and Povirk, 1994) and thus represented a good drug to use to attempt to mimic the DNA damage those researchers were able to induce. Etoposide is able to induce dsDNA breaks through its interaction with DNA topoisomerase II by causing a covalent interaction between the enzyme and DNA, which inhibits the re-ligation step in the transient repair of DSBs and locks the enzyme to the DNA backbone (Burden and Osheroff, 1998). Doxorubicin also induces dsDNA damage through its interaction with topoisomerase II through a similar but less well understood mechanism to etoposide (Pommier *et al.*, 2010).

We used the same U2OS cells used in previous research (Keuss *et al.*, 2016), and immediately found that WT cells were similarly sensitive (as determined by viability assay) to these 3 different DNA-damage inducing agents compared to DCNL5 KO cells. This was unexpected and in clear contrast to the results reported by Keuss and colleagues who had shown that in the absence of DCNL5 expression, cells were much more sensitive to DNA damage induction. They used gamma and UV irradiation to induce damage and showed that the role of DCNL5 occurred independently of its ability to bind cullin proteins. Our data here using chemical anti-cancer agents indicated that DCNL5 may not play a significant role however a clear distinction needs to be made here between short pulses of treatment with ionising radiation and 24 hour-long treatments with these anti-cancer drugs. The increased treatment duration we employed in this research coupled with the diverse range of effects caused by these drugs over longer periods of time make it likely that dsDNA damage is not the only process occurring in the cells following these treatments. Furthermore, we did not include a positive control to confirm successful induction of DNA damage.

The experimental limitation for this research was a lack of access to UV and gamma irradiation sources which is why we employed the use of chemical inducers of DNA damage. Further work would need to be undertaken to better optimise the chemical dsDNA treatments and to validate the findings by Keuss and colleagues that DCNL5 is an essential protein in the DNA damage response. This would be achieved through the employment of UV and gamma irradiation of WT and KO cells and subsequent detection of DNA damage could be analysed using a variety of assays (Collins and Azqueta, 2012). Our preliminary data suggests that DCNL5 may not be absolutely required in the DNA damage response however the treatments we used, and methods of analysing DNA damage were different to previous research and proper controls are required to draw stronger conclusions.

### 3.3.3 DCNL5 is Required for Extrinsic Apoptosis

Another drug we used to attempt to induce DNA damage was bleomycin sulphate, which is used clinically in the treatment of cancer including squamous cell carcinomas and testicular cancers (Hecht, 2000). Bleomycin is able to induce both ssDNA breaks and dsDNA breaks in the presence of the required cofactors (Nagai

*et al.*, 1969; Stubbe and Kozarich, 1987; Claussen and Long, 1999), and while its mechanism has been studied, it is not fully clear how the drug functions at a molecular level (Chen *et al.*, 2008). We found that 72-hour treatment with the drug caused a statistically significant ($p<0.001$) reduction in viability in WT cells and inducible WT-rescue cells compared to DCNL5 KO cells as well as DAD-mutant DCNL5 cells. We suspected that given the relatively long duration of the bleomycin treatment we were using, it was likely that this response was not due directly to the DNA damage induction and therefore a different mechanism to the reported role of DCNL5 in the DNA damage response. Bleomycin is also known to affect progression through the cell cycle (Twenytman, 1983) and more relevantly to the present study, is known to activate the apoptosis pathway, specifically the extrinsic branch (Vernole *et al.*, 1998; Mungunsukh *et al.*, 2010). It seemed possible then that bleomycin was inducing apoptosis in the cells, and the DCNL5 KO and DAD-mutant cells were unable to undergo this process. Our findings indicate a potential mechanism of chemotherapy resistance in bleomycin treated cancers mediated by DCNL5 which would be an avenue worth investigating in future study.

DCNL proteins have been shown to exhibit a significant overlap in function in cells, siRNA-mediated knockdown of individual DCNL proteins had little effect on the neddylation of all cullin proteins (Keuss *et al.*, 2016). This was also shown to be the case for DCNL1 and DCNL5 KO cell lines. We showed in WT but not DCNL5 KO U2OS cells that bleomycin treatment specifically caused reductions in cullin 4 protein levels. Crucially, we were able to show that the loss of cullin 4A and 4B is likely due to apoptosis and is mediated by the cleavage of caspase 3, since we detected cleavage sequences in cullin 4A and 4B but not cullin 3, which was unaffected by the bleomycin treatment. This data was supported by the combination treatment of CHX and TNFα, used as a control for apoptosis induction (Pająk, Gajkowska and Orzechowski, 2005; Wang, Du and Wang, 2008), which, like bleomycin, caused reductions in the levels of un-cleaved caspase 3 and PARP1 which are activated during apoptosis. This apoptosis response was largely absent in the KO cells and the DAD-mutant cells. There is a small chance that the reduction in cullin 4A levels may be a cause rather than a result of apoptosis since overexpression of the protein is associated with tumour cells and downregulation

of expression has been associated with reduced cell invasion and an increase in apoptosis (Chen *et al.*, 2018). The DCNL5 KO cells here show resistance to the drug and exhibit unchanged cullin 4A levels indicating that this resistance may be conferred by the higher cullin expression. It seems much more likely, especially given the supporting CHX/TNFα data, that the KO cells are lacking the ability to undergo apoptosis and caspase cleavage which is what we have shown in this work.

We determined that DCNL5 KO cells are deficient only in extrinsic apoptosis, lacking caspase 3 cleavage and cell death. This phenotype was mirrored in DAD-mutant cells, indicating a requirement for the DAD-patch and thus potential cullin binding for this role. It remains a possibility that the DAD-patch may confer binding to a protein other than a cullin family member. Co-immunoprecipitation experiments with WT cells would be able to tell us whether DCNL5 shows an increased interaction with a cullin protein in response to the CHX/TNFα treatment and if so, which one. This would provide confirmation that DCNL5 interacts with a cullin in order to mediate its role in the extrinsic apoptosis response and support the DAD-mutant data we have obtained in the present study.

TNFα stimulation on its own typically promotes the phosphorylation of IκBα, which is normally bound to the transcription factor NF-κB. This phosphorylation then leads to the ubiquitination of IκBα which is mediated by the E3 ligase $SCF^{\beta\text{-}TrCP}$ (Kanarek and Ben-Neriah, 2012). This promotes its proteasome-mediated degradation which allows for the nuclear translocation of NF-κB and the transcription of genes involved in immune signalling (Miyamoto *et al.*, 1994). We showed that WT and KO cells undergo IκBα phosphorylation and subsequent degradation which serves to upregulate pro-inflammatory genes (Karin, 1999). This is important in not only confirming that the KO cells are able to recognise the TNFα ligand and transduce the signal, but it also seems to support the finding that lack of DCNL5 does not affect the activity of most CRL complexes including $SCF^{\beta\text{-}TrCP}$ which is the ligase responsible for ubiquitinating IκBα in response to TNFα stimulation, however we did not independently validate this assertion. SCF requires neddylation for its activity (Amir, Iwai and Ciechanover, 2002) and DCNL5 KO cells therefore do not lack neddylation of cullin 1 in this context. Other DCNL proteins mediate this neddylation event.

CHX has been known for decades to inhibit the elongation phase of eukaryotic translation through direct binding to the ribosome (Obrig *et al.*, 1971). We showed that CHX stimulation alone in a CHX-chase experiment had a similar effect on TNF-receptor homeostasis in both cell lines, however this finding needs to be validated by more independent repeats of the experiment. Importantly, we demonstrated that the half life of TNF receptor 1 was similar in both cell lines indicating that the sensitivity difference to CHX/TNFα between the cell lines was not caused by different responses to either CHX or TNFα. Overall, this data strongly supports the hypothesis that DCNL5 plays a role unique to DCNL proteins in the caspase enzyme cascade resulting from extrinsic apoptosis induction and the absence of caspase activity confers resistance to treatments inducing the extrinsic apoptosis pathway. We used TRAIL as another independent inducer of the extrinsic apoptosis pathway (which is more commonly used in studying this pathway) and found that DCNL5 KO and DAD-mutant cells were even less sensitive to the treatment compared to WT cells than with the CHX/TNFα combination treatment.

We found that DCNL5 was required for caspase 8 cleavage following both TNFα and TRAIL stimulation, and the role of DCNL5 was dependent on cullin binding since DAD-mutant cells showed the same and arguably even less cleavage than DCNL5 KO cells. The lack of caspase 8 activity is likely to account for the lack of downstream cleavage of caspase 3 and PARP1 observed during TNFα stimulation (Stennicke *et al.*, 1998). We have managed to locate the level at which extrinsic apoptosis is compromised in the KO cells and thus discovered a novel role for DCNL5 in promoting caspase 8 activity.

The finding that DCNL5 KO and DAD mutant cells were more resistant to TRAIL than to TNFα-induced apoptosis was interesting. While both ligands are inducers of the extrinsic apoptosis pathway, they differ in the downstream proteins recruited and complexes formed. TNFα recruits the death domain-containing protein TRADD (Locksley, Killeen and Lenardo, 2001) as part of the receptor-bound complex (complex I) which in turn is responsible for recruiting RIP, TRAF2 and FADD. A second complex (complex II) is formed in the cytosol, which consists of TRADD, RIP, FADD and caspase 8, and this complex is responsible for the activation of caspase 8 to mediate the downstream apoptosis events and is known as the

death inducing signalling complex (DISC) (Micheau and Tschopp, 2003; Peter and Krammer, 2003). TRAIL signalling differs in that FADD and caspase 8 are recruited directly to the receptor-bound complex at one of 2 death receptors (DR4 or DR5) to form the DISC, and this complex is sufficient for caspase 8 activation (Walczak and Krammer, 2000). Furthermore, TRADD has been shown to be indispensable for TNFα-mediated apoptotic signalling while FADD is not, whereas FADD is absolutely required for TRAIL-mediated apoptosis.

TRAIL signalling often leads to higher levels of apoptosis in cells compared to TNFα (80% vs 60% in multiple myeloma cell lines, (Gazitt, Shaughnessy and Montgomery, 1999)) and in the present work we found that DCNL5 KO cells undergo 40-60% apoptosis (depending on concentrations used) following TNFα treatment and closer to only 20% apoptosis following TRAIL stimulation. The reason our KO cells are more sensitive to CHX/TNFα treatment compared to TRAIL treatment, to which they are almost entirely resistant, may be in part due to the order of DISC formation. Caspase 8 is recruited and activated at the DISC and in TNFα signalling this occurs at the second, cytosolic complex. We have shown that caspase 8 cleavage is lacking in KO cells therefore it is likely that the role of DCNL5 is in promoting caspase 8 cleavage and since this occurs at the second complex, there are preceding events that may contribute to cell death in TNFα signaling or rather events that may dilute the importance of DCNL5 in caspase 8 activation. In TRAIL signalling, caspase 8 is recruited to TRAIL receptors directly by FADD to form the DISC and our data suggests that the role of DCNL5 is immediate and essential in this response and therefore results in a higher degree of resistance when DCNL5 is absent compared to TRAIL compared to TNFα stimulation.

To further investigate the importance of caspase activity in mediating cell death following induction of the extrinsic apoptosis pathway we used a pan-caspase inhibitor called QVD-OPh. QVD-OPh uses an N-terminal quinoline group which is conjugated to the amino acids valine (V) and aspartate (D) as well as a C-terminal ester attached to a phenoxy ring (Caserta *et al.*, 2003). This peptide can covalently and irreversibly bind to caspase protein's active sites and thus QVD-OPh is a broad-range caspase inhibitor capable of inhibiting caspase 3 and caspase 8, amongst others. Existing research indicated that concentration of 10μM was

required to inhibit PARP1 cleavage (Kuželová, Grebeňová and Brodská, 2011) however we found that 40μM was an optimal concentration to use using U2OS cells. Our data indicated that inhibition of caspase cleavage with this drug does effectively reduce sensitivity to extrinsic apoptosis, and that KO cells respond less to QVD-OPh than WT cells, presumably because caspase activity in these cells is already significantly reduced.

### 3.3.4 DCNL5 is not Required for Intrinsic Apoptosis

Staurosporine is widely accepted as a specific inducer of the intrinsic apoptosis pathway (Gescher, 2000) and we found that the drug caused cell death in WT and KO cells as measured by viability assay and that KO cells were in fact statistically more sensitive to the drug at a concentration of 200μM ($p<0.001$). Our data support a model whereby DCNL5 plays a role in extrinsic apoptosis, specifically at the level of caspase 8 activation, which is not a required step for intrinsic apoptosis. The intrinsic pathway activates downstream caspases via the apoptosome which is a caspase 9-activating complex, following mitochondrial outer membrane permeabilization and release of cytochrome C (Cain, Bratton and Cohen, 2002).

Caspase 8 can mediate the cleavage of a protein called BID to form truncated BID (tBID) which then translocates to the mitochondria and promotes cytochrome C release (Li *et al.*, 1998) and this therefore represents a level of crosstalk between the extrinsic and the intrinsic pathways. This crosstalk is known to amplify the apoptosis induction, however we have shown that the KO cells are specifically defective in caspase 8 activation. As a result, there is a lack of both extrinsic apoptosis and caspase 9 cleavage and activation via the intrinsic/mitochondrial branch of the pathway. Staurosporine specifically activates the intrinsic branch and culminates in caspase 3 cleavage and this data reinforces our finding that DCNL5 is required for caspase 8 activation, and not for any other caspase proteins.

### 3.3.5 The Role of DCNL5 in Extrinsic Apoptosis Appears Unique Within the DCNL Family

Another key finding presented in this chapter is the discovery that the most ubiquitously expressed DCNL protein, DCNL1, which is known to localise to the cytosol (Keuss *et al.*, 2016), does not play an essential role in the extrinsic apoptosis pathway. We found that the DCNL1 KO cells were more sensitive to the WT cells following CHX/TNFα treatment and equally sensitive to TRAIL. This is in obvious contrast the finding that DCNL5 KO cells were less sensitive to WT cells to the same treatments and indicates that DCNL1 does not have the same role in this pathway as DCNL5. This finding is interesting because DCNL proteins have been shown to overlap significantly in function with regards to cullin interaction, with cullins being the main binding partners of DCNL proteins. It has been shown that in cells, all DCNL proteins are able to neddylate any cullin protein family member (Keuss *et al.*, 2016). siRNA-mediated loss of each individual DCNL proteins had minimal impact on the neddylation of any of the cullin proteins and the same DCNL1 and DCNL5 KO cell lines used in the present research were previously used to demonstrate that total loss of either protein largely had no effect on the neddylation state of cullin proteins, showing that there is significant redundancy in function of this family of proteins in cells (Keuss *et al.*, 2016).

There is precedent for specific roles of DCNL family members *in* vivo, which supports our assertion here that the role for DCNL5 in apoptosis may be specific to this family member. DCNL3 has been studied and shown to posses a unique role in the neddylation of cullin 3 at the plasma membrane (Meyer-Schaller *et al.*, 2009). We did not investigate the effect of DCNL3 knockdown, however it would be interesting to see if this family member is also unable to compensate for the loss of DCNL5 expression since caspase 8 processing at the DISC occurs at the plasma membrane following TRAIL signaling (Jin et al, 2009). There is also literature supporting a unique role for DCNL1 in the neddylation of cullin 3 to promote midbody localization and activity of a cullin 3-based CRL during abscission (Huang *et al.*, 2017).

The phenotype we have characterised in this chapter is severe: lack of DCNL5 protein expression results in a significant resistance to Bleomycin, TNFα and

TRAIL-mediated cell death while exhibiting a clear reduction in caspase 8 cleavage. It remains a possibility that the N-terminus of DCNL5, which is predicted to be unstructured, provides a function which is specific to that family member. We have shown that the DAD-patch is essential for DCNL5 involvement in caspase 8 cleavage, but the DAD-patch is a highly conserved motif within a highly conserved C-terminal region known as the PONY domain which is conserved across all DCNL proteins (Monda *et al.*, 2013). Therefore, why can't the other DCNL proteins mediate this function via their respective DAD-patches? DCNL1 and DCNL2 are cytosolic and DCNL3 can localise to the plasma membrane, where the initial stages of apoptosis occur, so it is curious as to why DCNL5 has such a significant and unique role in this pathway since DCNL5 is also understood to be a predominantly nuclear protein (Keuss *et al.*, 2016; Thomas *et al.*, 2018). It is likely that the N-terminal domain of DCNL5 confers the protein with specific binding properties that are required for its interaction with as yet unknown proteins to promote caspase 8 activity. This feature is likely lacking in other DCNL proteins, however in order to critically evaluate this hypothesis, more information about the structure of DCNL5 is required.

# 4 The Role of DCNL5 Interaction in Caspase 8 Activation

## 4.1 Introduction

### 4.1.1 Caspase 8

There is some debate as to where in the cell pro-caspase 8 is stored. It has been shown that pro-caspase 8 localises to the cytosol (Zhivotovsky *et al.*, 1999) and separately it has been shown to localise only to the mitochondria, specifically the inner membrane space, the intermembrane and the matrix (Qin *et al.*, 2001). More recently, it was shown that pro-caspase 8 does not actually localise to mitochondria as had been previously reported; the researchers supported the finding that pro-caspase 8 as well as caspases 2, 3, 6,7 and 9 were located in the cytosol in 3 different models of apoptosis (van Loo *et al.*, 2002). They concluded that separate compartmentalization of pro-apoptotic co-factors such as cytochrome c and AIF from silent precursor caspases in the cytosol provides an additional regulatory step in the control of the apoptosis response.

Following TRAIL binding to its receptors (death receptor 4 or 5, DR4/5) which are located on the cell surface, cytoplasmic death domain regions of the receptor are able to recruit the death domain containing protein FADD which then leads to the recruitment of the initiator caspase pro-caspase 8 to form the death induced signalling complex (DISC). The DISC is located at the plasma membrane and translocates to regions of the plasma membrane termed 'lipid rafts'. Lipid rafts consist of dynamic assemblies of cholesterol and sphingolipid-rich regions of the plasma membrane (Simons and Van Meer, 1988; Simons and Ikonen, 1997) which are known to be important in promoting and amplifying signal transduction (Simons and Toomre, 2000). Lipid rafts have been demonstrated to play key roles in Fas and TNFα-related cell death and inflammatory signalling events (Muppidi and Siegel, 2004; Legler *et al.*, 2003; Muppidi, Tschopp and Siegel, 2004) as well as key roles in TRAIL-mediated apoptosis.

DISC association with lipid rafts is an essential step in caspase 8 cleavage and activation, while association of the DISC in non-rafts is associated with pro-survival signalling via NF-κB. cFLIP an the kinase RIP1 are associated with non-rafts and

knockdown of cFLIP increases association of the DISC with lipid rafts and increases caspase 8 activation (Song *et al.*, 2007). The importance of the association of the DISC with lipid rafts is further highlighted by the fact that a TRAIL-sensitive lung cancer cell line (H460) was used to generate a resistant sub-population of cells and this resistance was shown to be in part conferred by a decrease in translocation of DR4/DR5 into lipid rafts compared to the parental cell line (Ouyang *et al.*, 2013). Thus, pro-caspase 8 association with the DISC at lipid rafts is a crucial step in caspase 8 cleavage. Following multiple processing events including ubiquitination by cullin 3, active caspase 8 is released into the cytosol where it is able to cleave downstream targets required for successful induction of apoptosis. A simple summary of caspase localisation through TRAIL signalling is shown in Figure 4-1 below.

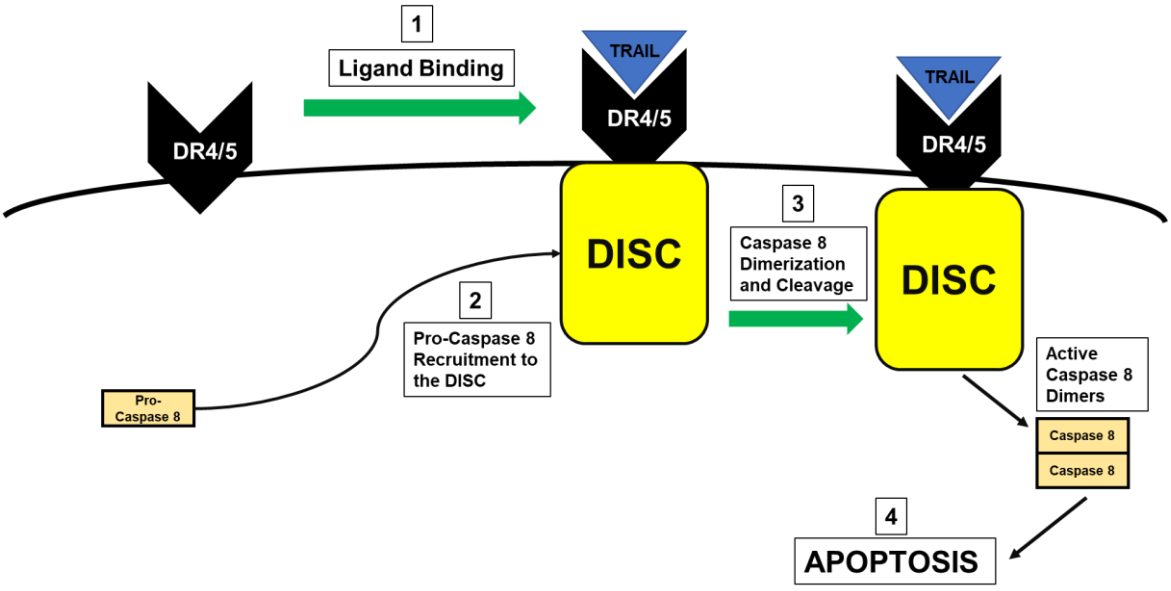



**Figure 4-1 Subcellular Localisation of Caspase 8.**

This schematic diagram shows the subcellular localisation of caspase 8 prior to and during TRAIL-mediated apoptotic signalling. Pro-caspase 8 is understood to localise to the mitochondrion, specifically in the inner membrane and the intermembrane space, as well as the cytosol, in the absence of an apoptotic signal. TRAIL binding to death receptor 4 or 5 (1) leads to assembly of the death-induced signalling complex (DISC) which includes pro-caspase 8 (2). DISC assembly occurs at the plasma membrane in lipid rafts, and following ubiquitination and cleavage (3), active caspase 8 hetero-tetramers are released from the DISC into the cytosol (4) to mediate downstream cleavage events required for apoptosis.

### 4.1.2 Caspase 8 Cleavage Requires Ubiquitination Mediated by Cullin 3

We have discussed the importance of the association of the DISC with lipid rafts, however another key regulator of caspase 8 activation is also thought to localise to these regions: cullin 3. Cullin 3 has been shown to be uniquely responsible for the only known direct and positive regulation of caspase 8 activity, in seminal work by Jin and colleagues (Jin *et al.*, 2009). They showed that neddylated cullin 3, in combination with RBX1, mediates the poly-ubiquitination of caspase 8 at lysine residue 461 which is located in the C-terminal region of caspase 8, within its p10 subunit (see Figure 4-2). They also showed that modified caspase 8 is then recognised by the ubiquitin binding protein p62 which translocates ubiquitinated caspase 8 to higher molecular weight structures corresponding to ubiquitin-rich aggregates. This association with p62 is thought to drive the processing and auto-proteolytic cleavage of caspase 8 and subsequent release into the cytosol (Jin *et*

*al.*, 2009). They demonstrated that cullin 3 knockdown reduced caspase 8 ubiquitination however caspase 8 was still able to be recruited to the DISC. Overall this work emphasised the importance of this poly-ubiquitination event mediated by cullin 3 in promoting caspase 8 cleavage and activation.

More recent research has shown that the de-ubiquitinating enzyme A20 reversed the poly-ubiquitination of caspase 8 mediated by cullin 3 which has the effect of impeding caspase 8-dependent cell death (Lim *et al.*, 2017), further reinforcing the importance of this ubiquitination event in the activation of caspase 8. Caspase 8 is ubiquitinated at other residues by different E3 ligases which tend to target the protein for degradation and therefore have anti-apoptotic effects, which makes studying the activation of caspase 8 complicated. We hypothesize that DCNL5 may play a role in the neddylation of cullin 3 which is required for its activity as the core of a CRL complex to mediate the poly-ubiquitination of caspase 8.

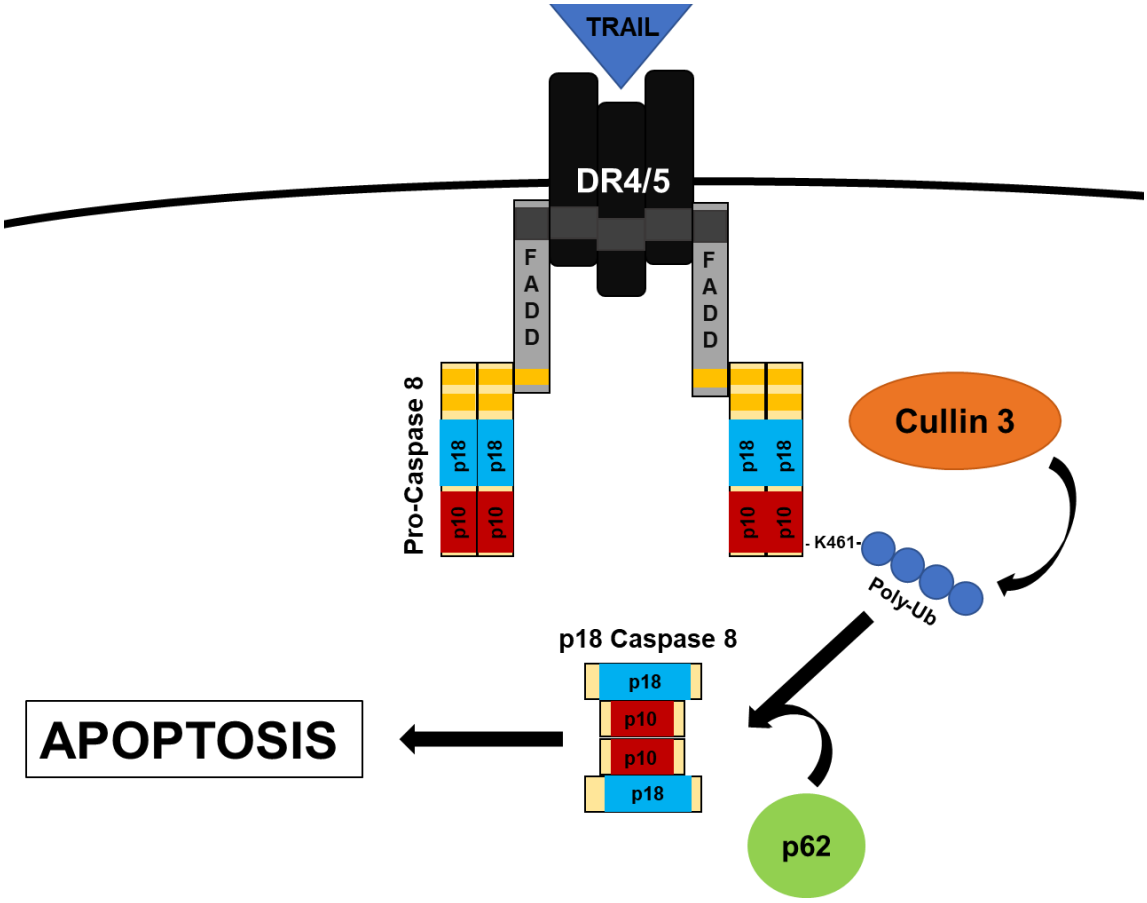



**Figure 4-2 Cullin 3-Mediated Ubiquitination of Caspase 8 Following TRAIL Signalling.**
This schematic overview summarises some of the key stages and interactions involved in caspase 8 cleavage. FADD associates with death domains present on the intracellular regions of death receptors 4 or 5 (DR4/5), these are shown in dark grey. Pro-caspase 8 is then recruited to form the DISC and interacts with FADD via death effector domains (DED) shown in orange. The DISC translocates to lipid rafts and there cullin 3 mediates the poly-ubiquitination of caspase 8 at lysine 461 in the C-terminal region of the p10 subunit of caspase 8. Ubiquitination at this residue is essential for p62 association which in turn allows for translocation of caspase 8 to ubiquitin-rich aggregates which allow for the auto-proteolytic cleavage of caspase 8 to form the hetero-tetramer of p10 and p18 subunits (known simply as p18) which is catalytically active and able to mediate downstream cleavage events required for apoptosis.

### 4.1.3 Aims

The aims for this chapter were initially to examine the temporal and spatial distribution of DCNL5 in response to TRAIL stimulation, to determine whether DCNL5 is located in the cytosol and whether the protein can interact with cullin 3 *in vivo*. Following this, we wanted to determine whether caspase 8 ubiquitination was affected in the absence of DCNL5 expression, or whether ubiquitination as a whole was affected. Finally, we wanted to investigate whether neddylation plays a role in the process of caspase 8 cleavage and activation.

## 4.2 Results

### 4.2.1 DCNL5 Translocates to the Cytosol Following TRAIL Stimulation

Caspase 8 is a key component of the death inducing signalling complex (DISC) and its cleavage occurs at the plasma membrane as well as in the cytoplasm. DCNL5 has been shown to be a predominately nuclear protein and contains a nuclear localisation sequence in its N-terminus, however we suspected that given the results thus far, DCNL5 must travel from the nucleus to the cytoplasm in response to TRAIL stimulation. In order to determine whether this was the case, we used fluorescence microscopy to track the localisation of GPF-tagged DCNL5 following treatment with TRAIL. We used treatment times of up to 6 hours to follow the movement of DCNL5 and found that the DCNL5 appeared in the cytoplasm within 1 hour, at which point the levels appeared to be the highest, and as treatment time increased the amount of DCNL5 in the cytoplasm decreased. This suggested that DCNL5 may be shuttled out of the nucleus in response to the TRAIL stimulation and either shuttled back into the nucleus or degraded once the signal was no longer being transduced.

While the levels of fluorescence haven't been calculated, the images shown in Figure 4-3 are fairly clear and representative of what was visible under the microscope. GFP-tagged DCNL5 co-localises with the red dot marker as they are both nuclear. It can be seen clearly that after 1 hour of TRAIL treatment there is green fluorescence outside the nucleus which was not there in the absence of ligand stimulation. This experiment lacks a useful GFP-only negative control to support the finding that DCNL5 is specifically responsible for the localisation of the detectable fluorescence. Despite this, our findings here are the first clear indication that DCNL5 can localise outside the nucleus and supports its role in the cleavage and activation of caspase 8 which occurs at the plasma membrane and in the cytosol. It appears that it is only a small proportion of DCNL5 that localises to the cytosol, however it likely only requires a small amount of the protein to mediate activation of caspase 8.

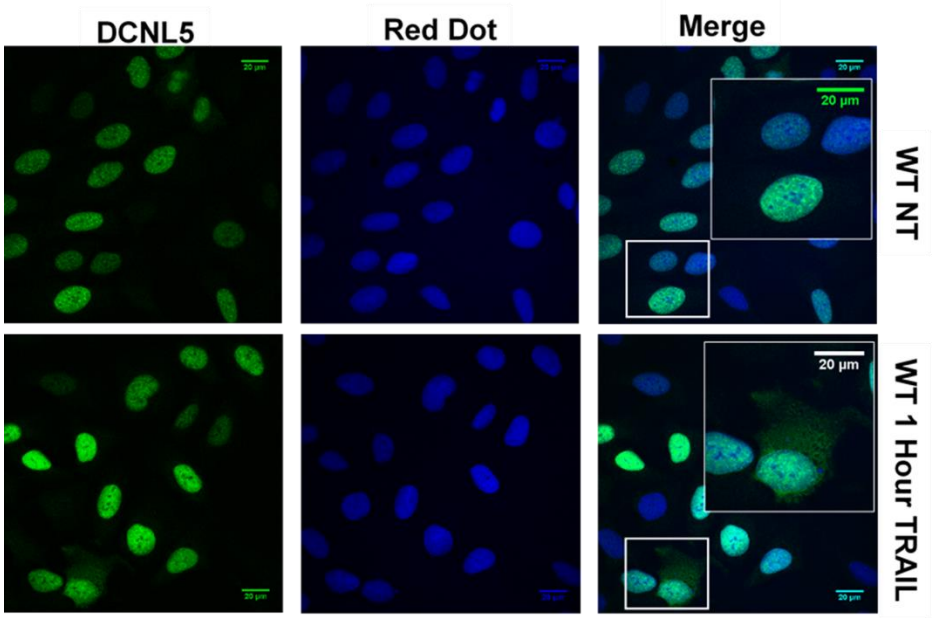



**Figure 4-3 DCNL5 Localises to the Cytoplasm Following TRAIL Stimulation.**
Representative fluorescent images of GFP-tagged DCNL5 show here were obtained following plating of cells on coverslips and fixing them with methanol and incubating them with anti-GFP antibody as well as red dot nuclear stain. TRAIL was used at a concentration of 200ng/ml for treatment times of up to 6 hours, shown here is a 1 hour of treatment. DCNL5 co-localises with the red dot stain in the nucleus. Following 1-hour TRAIL treatment we can see GFP fluorescence outside the nucleus of cells. These images are representative of the majority of cells that were observed.

### 4.2.2 DCNL5 Interacts with Cullin 3 *in vivo*

The established mechanism for caspase 8 activation involves its ubiquitination, followed by its cleavage into intermediate forms, ultimately resulting in formation of the catalytically active p18 hetero-tetramer. It is generally understood that this ubiquitination of caspase 8 is mediated by cullin 3 specifically. Various modifications of caspase 8 occur, including ubiquitination by other proteins, and the modifications can have broad effects on caspase 8 activity. Ubiquitination by cullin 3 is likely one of the only modifications that increases and is essential for caspase 8 activity. The primary binding partners of DCNL proteins are cullins, and given that we have already shown that the role of DCNL5 is likely not only unique amongst the DCNL proteins, but also requires the DAD-patch to confer sensitivity to TRAIL (and TNFα) (since the DAD-mutant cells are as resistant if not more-so than KO cells), we suspected that DCNL5 may be directly required for cullin 3 neddylation and thus its activity as part of the ubiquitin E3-ligase complex.

It has already been shown that all DCNL proteins can interact with all cullin proteins *in vitro* (Monda *et al.*, 2013; Keuss *et al.*, 2016), however little evidence exists for specific roles for individual DCNL proteins in cells. We set out to determine if there is any interaction between DCNL5 and cullin 3 by co-immunoprecipitation. We started by precipitating cullin 3 and probing for interaction with DCNL5 by western blot. As can be seen in Figure 4-4, faint bands at approximately 27kDa and 52kDa can be seen in the WT and WT-rescue cells respectively. These have been highlighted using red asterisks for clarity as the DCNL5 poly-antibody produces a lot of unspecific bands. These bands at 27kDa and 52kDa represent DCNL5 and GFP-tagged DCNL5 in the inducible rescue cell line and are absent in KO cells and DAD-mutant cells, the latter of which are unable to bind to cullin proteins, in this case cullin 3.

There appears to be interaction of the two proteins prior to stimulation with TRAIL, and this interaction increases somewhat with TRAIL treatment although it is hard to say accurately. This represents the first evidence for a specific interaction of DCNL5 with cullin 3 in cells and supports its potential role in mediating the neddylation of the protein in order to enable ubiquitination of caspase 8. The interaction that we have been able to detect here is weak, and

this is likely due to the small amounts of both DCNL5 and cullin 3 that are interacting in response to TRAIL stimulation. The amount of DCNL5 that diffused out of the nucleus was small, and it is known that specific subcellular pools of cullin proteins mediate specific functions. DCNL5 will also interact with other cullin protein to mediate other functions, while other DCNL proteins will interact with cullin 3 in other cellular processes. Further experimental work is required to better elucidate this interaction between DCNL5 and cullin 3 and to determine if this interaction increases with TRAIL treatment.

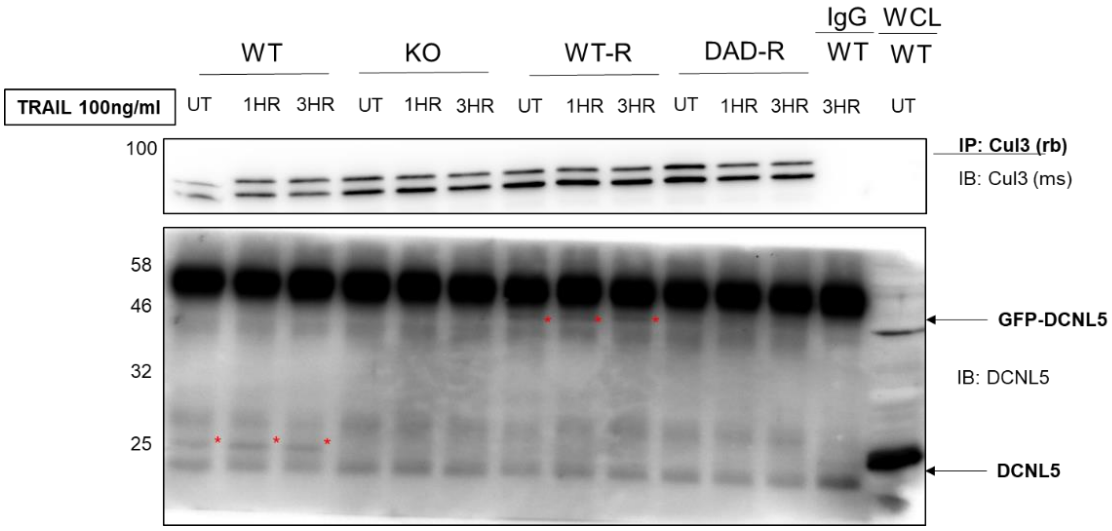



**Figure 4-4 Cullin Interacts with DCNL5 *in vivo*.**

This representative western blot (n = 2) shows the result of immunoprecipitating cullin 3 (using 1µg of antibody) and its interaction with DCNL5 following 100ng/ml TRAIL treatments. The immunoprecipitation was successful and the amount of cullin 3 that was pulled down was consistent across treatment time points and cell lines. There appears to be no major change in amounts of neddylated (upper band) and un-neddylated (lower band) cullin 3. The red asterisks highlight bands at 27 and 52kDa representing DCNL5 and GFP-DCNL5 which are expressed in WT and WT-rescue cells, respectively. DCNL5 KO cells do not express the protein and therefore show no interaction with cullin 3, while the DAD-mutant rescue cells are unable to bind cullin proteins and therefore no interaction is detected here. This represents a good control for the cell line. The western blot has a lot of unspecific bands. *IP = immunoprecipitation, IB - immunoblot*

We also wanted to confirm the reverse of this interaction by pulling on DCNL5 and detecting whether cullin 3 co-immmunoprecipitates with it. We were able to detect cullin 3 as an interactor by western blot as shown in Figure 4-5, however the interaction was very weak. The neddylated (upper band) and un-neddylated (lower band) forms of cullin 3 have been highlighted as the bands are faint and

hard to detect. This weak interaction is due in part to likely low amounts of DCNL5 being immunoprecipitated, as well as the relatively small pools of both DCNL5 and cullin 3 that are interacting. The low amounts of cullin 3 make it difficult to determine whether there is an increase in interaction following TRAIL treatment – although this appears to be the case and is consistent with what we saw in the previous figure. Also shown in the figure are the inputs, and the reduction in pro-caspase 8 indicates successful induction of the pathway and cleavage of the protein. This data along with the data in Figure 4-4 provide evidence of the interaction (albeit a weak one) between DCNL5 and cullin 3 in cells. Further work would be required to investigate if this interaction changes with treatment and if so by how much, including crosslinking, which could be employed to stabilise transient interactions and may thus allow for improved detection of interaction between cullin 3 and DCNL5.

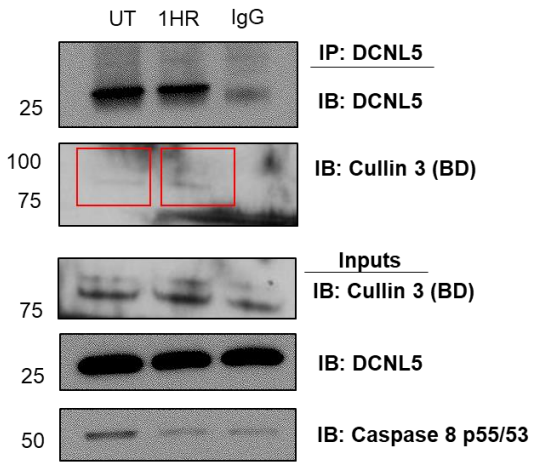



**Figure 4-5 DCNL5 Co-IP Shows Interaction with Cullin 3.**

DCNL5 was immunoprecipitated prior to TRAIL treatment (of 200ng/ml) and following 1 hour of treatment and cullin 3 was shown to interact weakly (red boxes) in both cases, as shown here by a representative western blot.The upper, neddylated band of cullin 3 as well as the lower un-neddylated band can be seen. Cullin 3, DCNL5 and caspase 8 inputs were analysed by western blot and the reduction in full length caspase 8 is consistent with induction of apoptosis. *IP = immunoprecipitation, IB = immunoblot*

### 4.2.3 DCNL5 KO and DAD-Mutant Cells Exhibit Less Caspase 8 Interaction with Cullin 3

In this project we have already shown that DCNL5 KO cells and DCNL5 DAD-mutant cells exhibit a resistance to TRAIL-induced apoptosis and a reduction in caspase 8 cleavage. Cullin 3 is responsible for mediating the ubiquitination of caspase 8 and these two proteins are known to interact directly. We wanted therefore to confirm this interaction in U2OS cells and to see if there were any differences in this interaction in DCNL5 KO or DAD-mutant cell lines. We investigated the interaction of caspase 8 with cullin 3 by co-immunoprecipitation and the results are shown in Figure 4-6 below. Caspase 8 was shown to interact with cullin 3 in all cell lines, most of the interaction was mediated by the full length p55/53 fragment of the protein. Overall, there is little difference in the amount of p55/53 caspase 8 interacting with cullin 3 between the cell lines or treatment times, there is possibly a small drop between 1 and 3 hours of TRAIL stimulation in the WT cells but further optimisation would be required to state this definitively.

1 hour of TRAIL treatment seems to result in no interaction between p43/41 caspase 8 to and cullin 3. Despite this we can observe more pronounced differences in the amount of p43/41 caspase 8 that co-precipitated with cullin 3 after 3 hours of TRAIL treatment (highlighted by red asterisks in Figure 4-6). There appear to be lower levels of this fragment precipitating in KO cells than WT and WT-rescue cells, while DAD-mutant cells have almost no protein at all. This is largely consistent with previous data which shows that caspase 8 cleavage is impaired in DCNL5 KO cells. It is difficult to interpret the significance of this result but it is clear that there is a difference between WT and KO cells which is even more pronounced in the DAD-mutant cells. We can also conclude that the lack of DCNL5 expression has no effect on the interaction of cullin 3 with caspase 8.

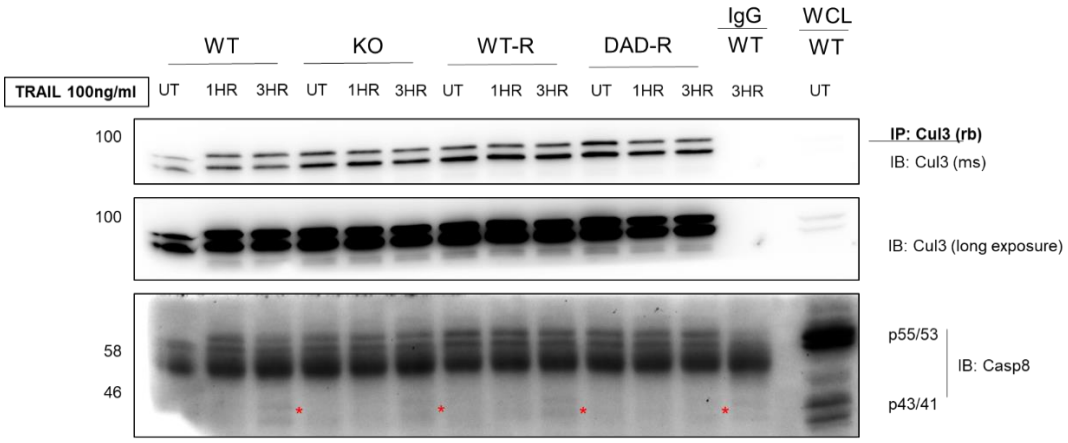



**Figure 4-6 DCNL5 KO and DAD-Mutant Cells Appear to Show a Reduction in p43/41 Caspase Interaction with Cullin 3 Following TRAIL Stimulation.**

This cullin 3 co-immunoprecipitation experiment confirmed interaction of caspase 8 with cullin 3 *in vivo* using U2OS cell lines. The amount of full-length caspase 8 (p55/53) that interacts with cullin 3 is largely unchanged across treatment times and cell lines. There is possibly a reduction between 1- and 3-hour treatment in the WT cells. The cleaved form of caspase 8, p41/41, appears to interact more strongly with cullin 3 following 3 hours of TRAIL treatment in WT and WT-rescue cells, less so in KO cells and there is almost no protein present in the DAD-mutant cells. These results hint at a difference in cleaved caspase 8 interaction with cullin 3 between WT and KO DCNL5 cells but overall confirm that interaction of full-length caspase 8 with cullin 3 is unaffected by DCNL5 expression. *IP = immunoprecipitation, IB = immunoblot*

### 4.2.4 Caspase 8 Does Not Co-Precipitate with DCNL5

It remained a possibility that DCNL5 can bind directly to caspase 8 in some sort of structural role, so we wanted to investigate whether the two proteins interact. We performed another IP using caspase 8 and probed for DCNL5 in the same way as we did for the cullin 3 co-IP reaction. The resulting western blot is shown below in Figure 4-7. The IP was successful as caspase 8 is present in the immunoblot and the amount of protein in each lane is relatively similar. Blotting for DCNL5 revealed non-specific bands (indicated on Figure 4-7 with asterisks) however no DCNL5 was co-precipitated. DCNL5 is present in the input confirming that this was not due to an issue with the antibody. It is possible that the level of DCNL5 present was too low to detect, or simply that DCNL5 does not interact in a complex with caspase 8. This experiment lacks a useful positive control to confirm successful immunoprecipitation (such as cullin 3), and given the small amounts of DCNL5 that are involved in interacting with caspase 8 in this process, it perhaps unsurprising that we were unable to detect interaction of any sort with caspase 8 if this interaction is bridged by cullin 3.

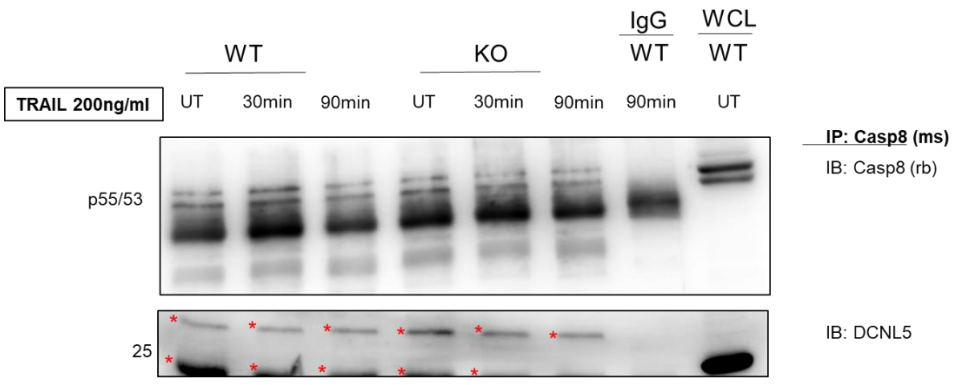



**Figure 4-7 DCNL5 Does Not Interact Directly with Caspase 8.**

This western blot shows the result of a caspase immunoprecipitation experiment. The IP appears to have worked given the presence of caspase 8 in the IP samples. DCNL5 appears not to interacts as there is no protein present in the IP samples – non-specific bands of similar sizes are present and have been highlighted using a red asterisk for clarity. The expected size of DCNL5 is 27kDa and a corresponding band is present in the input (WT cells) for this experiment. *IP = immunoprecipitation, IB = immunoblot*

### 4.2.5 Caspase 8 Co-Precipitates with a Ubiquitinated Protein in WT but not KO Cells

Caspase 8 ubiquitination is essential for its activation and we suspected that in DCNL5 KO cells there may be a lack of this modification occurring. A useful way to observe ubiquitination of proteins is to perform immunoprecipitation reactions under denaturing conditions, which disrupts binding to other proteins but still allows for the interaction of covalent modifications such as the addition of ubiquitin. We used lysis buffer containing 1% SDS to denature protein lysates before diluting the lysates down to 0.1% SDS and incubating overnight with antibody. Due to the loss of tertiary structure under denaturing conditions, we used a caspase 8 antibody that had been shown to bind successfully following denaturing of the protein lysate (ref Lim et al, 2017). Previous work looking at caspase 8 ubiquitination by denaturing immunoprecipitation used TRAIL stimulation at 4°C in order to stabilise aggregate formation and improve detection of modifications (Jin *et al.*, 2009). We did not do this which may account for the lack of signal and difficulty obtaining consistent results for this experiment.

Several attempts at this experiment were made and one set of results is shown below in Figure 4-8 – the results presented are not conclusive however provide some insight into what may be happening in the KO cells. In Figure 4-8A we probed for caspase 8 by immunoblot to confirm the immunoprecipitation was successful. The amount of caspase 8 present in all lanes is very low, which was a consistent theme in optimisation of this experiment, and furthermore the level varied between cell lines and treatment times. WT untreated cells precipitated significantly more caspase 8 than any of the other conditions. Figure 4-8B shows an immunoblot probing for ubiquitin and we were able to detect ubiquitin 'smears' (indicated by the red bracket); it is clear that the levels of ubiquitin bound to caspase 8 varies. WT cells appear to show a drop in ubiquitination following TRAIL stimulation for 30 minutes but factoring in how much less caspase 8 was precipitated in the 30 minute time point compared to untreated, it is likely that there is little change in ubiquitination following treatment, or perhaps even an increase.

The discrepancy in the amount of caspase 8 that was precipitated for this experiment may arise from the masking of the epitope due to the modification by ubiquitination – TRAIL stimulation may indirectly impair the ability of the antibody to bind to caspase 8 The DCNL5 KO cells appear to show a significant drop in caspase 8 ubiquitination following TRAIL stimulation, and given the similar amounts of caspase 8 that were precipitated with and without TRAIL treatment in the KO cells, this result seems accurate. When comparing TRAIL treated WT and KO cells (lanes 2 and 4 in Figure 4-8B) and factoring in the differences in caspase 8 that was precipitated (as shown in Figure 4-8A), there does seem to be less caspase 8 being poly-ubiquitinated in KO cells compared to the WT cells. There is a band just below 100kDa which is present in fairly equal amounts in all lanes which is of unknown origin. It is possible that it represents caspase 8 polyubiquitination (approximately 5 ubiquitin molecules in addition to pro-caspase 8) or it could also represent some sort of caspase 8 dimer. Given that the levels are broadly similar in all lanes it is perhaps non-specific binding by the antibody and could therefore be used as an approximate loading control, in which case the ubiquitin levels we observe are reasonably comparable.

Significant caution needs to be advised when interpreting these results, the aim for these results was to determine if the KO cells are lacking in caspase 8 poly-ubiquitination in response to TRAIL, and while there does appear to be a reduction in caspase 8 modification, further optimisation and experimental validation is required. The overall significance of this is unclear, there are different ubiquitin modifications of caspase 8 that occur, so it remains difficult to analyse these results in isolation. There appears to be a difference in ubiquitination state of caspase 8 following TRAIL stimulation between WT and KO cells, but given that this experiment is often done at 4°C it is possible that we are not detecting all of the modified caspase 8. Overall, these results indicate that KO cells are lacking in ubiquitination of caspase 8 but additional experimental evidence and optimisation is required.

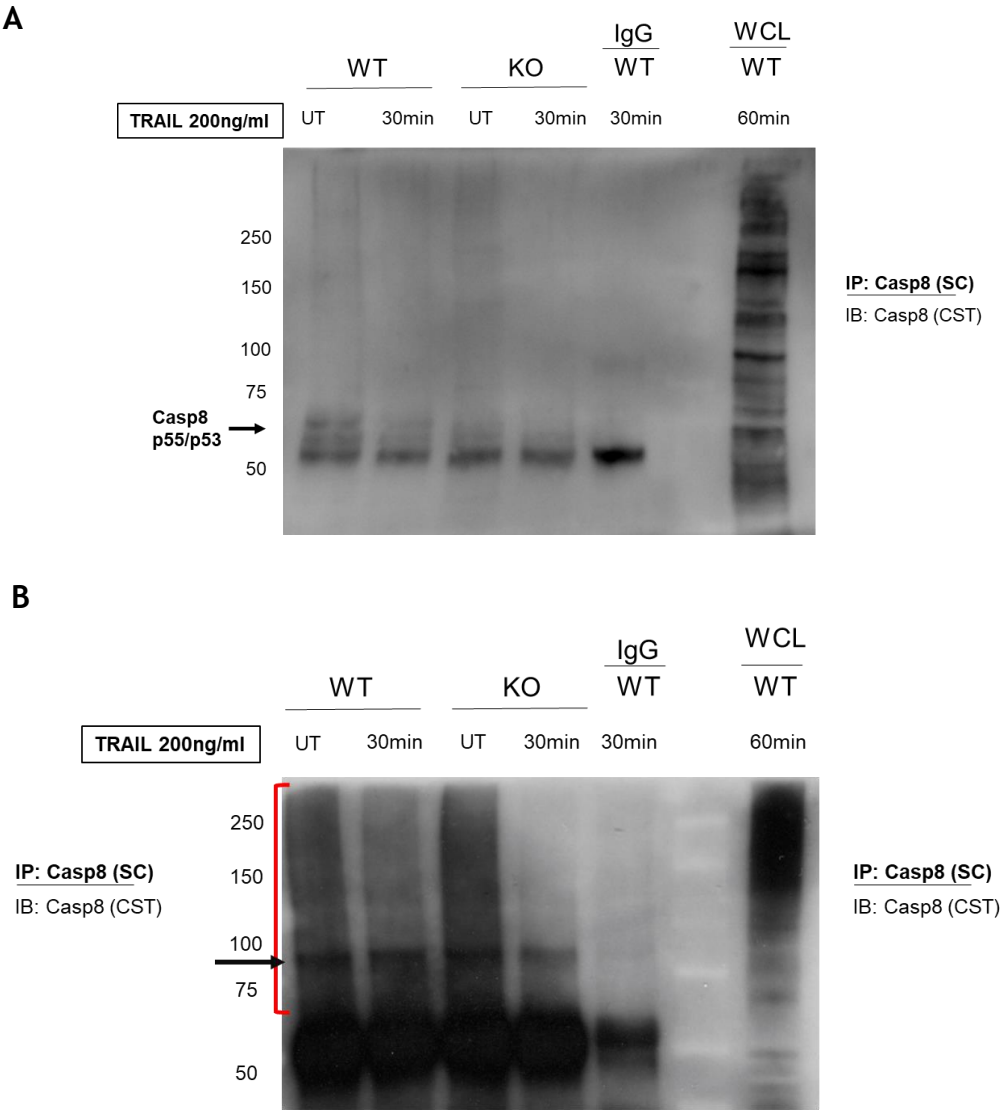



**Figure 4-8 Denaturing Immunoprecipitation Proved Inconclusive.**

(A) This representative caspase 8 immunoblot following denaturing immunoprecipitation shows that low levels of caspase 8 were precipitated in all lanes, with significantly more present in WT untreated cell than other conditions, perhaps due to the reduced stability of the protein upon ubiquitination. (B) Ubiquitination of caspase 8 is shown in this immunoblot and is often referred to as a ubiquitin 'smear' (indicated by the red bracket) and the levels vary significantly. TRAIL treatment causes a drop in ubiquitination in both WT and KO cells, however less caspase 8 was precipitated in WT treated cells suggesting that overall, the level of ubiquitination may in fact be the same or even increased with treatment. The reduction in ubiquitination in the KO cells following TRAIL stimulation appears to be a real result and may indicate a reduced ability of these cells to undergo caspase 8 ubiquitination in response to TRAIL, but the reason for this drop is uncertain. *IP = immunoprecipitation, IB = immunoblot*

Another method that can be used to analyse caspase 8 ubiquitination involves tandem ubiquitin binding entities (TUBEs) which consist of multiple ubiquitin binding associated domains (UBAs) which have an extreme high affinity (in the nanomolar range) for poly-ubiquitin chains. The UBA domains are derived from ubiquilin 1 which is a poly-ubiquitin binding protein that plays a role in protein quality control. Ubiquilin proteins consist of a C-terminal UBA domain and an N-terminal ubiquitin-like (UBL) domain TUBEs can be conjugated to beads of various types, and are available to buy commercially, however the ones we used were made previously in our laboratory and are conjugated to HALO beads. TUBEs allow for the enrichment of poly-ubiquitinated proteins (preferentially over mono-ubiquitinated proteins) from lysates as well as serving protective purposes to inhibit the degradation of these chains. In our experiment, we used TUBEs which were previously purified and then conjugated to magnetic HALO beads by Roland Hjerpe, a former post-doctoral researcher in the group (Hjerpe *et al.*, 2009). We then used these beads in the same way as antibody-conjugated beads for immunoprecipitation reactions.

Figure 4-9A shows a simplified schematic diagram summarising how poly-ubiquitinated proteins, in this case caspase 8, bind to the TUBE-conjugated beads. Individual ubiquitin molecules are added to target substrates to form chains and are linked by lysine residues. Poly-ubiquitin chains have various purposes in cells and often signals proteins to be degraded by the 26S proteasome. In the context of cullin 3-mediated ubiquitination of caspase 8, this modification is a required step for cleavage of the protein. These ubiquitin chains have a high affinity for the UBA domains of the TUBEs so they bind tightly and form complexes which can be eluted from the beads. Given that caspase 8 is known to become ubiquitinated, the hypothesis was that we would precipitate much more caspase 8 in WT DCNL5 cells than the KO cells if the latter lacked modification of caspase 8 as we suspected.

Figure 4-9B confirms the successful application of the HALO-TUBE beads in a ‘pulldown’-type experiment. We can clearly see large amounts of ubiquitin in WT and KO cells before and after TRAIL treatment of 30 and 60 minutes. There is no

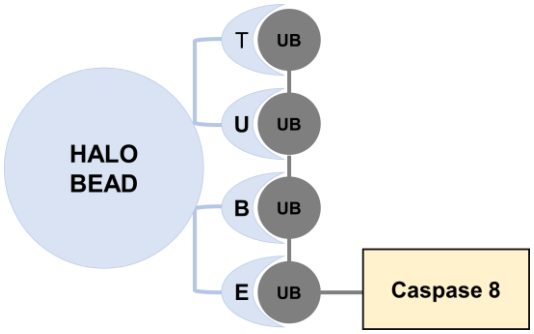
real difference in ubiquitination, but it is important to remember that this is essentially global poly-ubiquitin levels we are detecting here. The amount of ubiquitin is much higher in the pulldowns than in the whole cell lysate which is expected given that we are strongly enriching for this modification. Having shown that the TUBEs had successfully precipitated ubiquitinated proteins, we looked to see if we could detect caspase 8 in the enriched lysates. Probing the same samples with caspase 8 antibody yielded an unexpected result. As can be seen in Figure 4-9C, there is no caspase 8 present in any of the KO cell conditions. WT cells also lack any caspase 8 in the absence of TRAIL stimulation as well as after 30 minutes of stimulation, however after 60 minutes we see the p43/41 caspase 8 fragment co-precipitating as well as a small amount of pro-caspase 8. Pro-caspase 8 has been highlighted with a red box while a red asterisk indicates the p43/41 fragment more clearly. Figure 4-9D shows the inputs from this experiment which confirm that global pro-caspase 8 levels are similar in both cell lines and at all treatment time points.

If we were detecting modified caspase 8, we would expect to see a 'smear' of protein of higher molecular weight representing poly-ubiquitinated protein, likely similar but less strong than the ubiquitin blot shown in Figure 4-9B. Instead we are seeing unmodified caspase 8, and mostly the intermediate cleaved form at that. This indicates that pro-caspase 8 and p43/41 caspase 8 are interacting with a protein that is poly-ubiquitinated in response to TRAIL in WT but not KO cells. The fact that we detect mostly p43/41 caspase 8 is unsurprising since this form is more stable and associates with the DISC while the fully active p18 form is unstable and is not associated with the DISC. The presence of pro-caspase 8 in WT cells indicates that this un-cleaved form of caspase 8 interacts with ubiquitinated protein(s). Since pro-caspase 8 is also present in KO cells as we have shown previously, the lack of caspase 8 cleavage may arise due to a lack of the interaction between un-modified caspase 8 and a ubiquitinated protein which may be a significant finding.

As with the denaturing immunoprecipitation reactions in this section, TRAIL treatment can be performed at 4°C which tends to amplify signal and preserve caspase 8 ubiquitination and prevents degradation which may have enabled us to get a clearer picture of what is happening here. It is possible that we would not

only detect more caspase 8 in this experiment at 4°C, but we may also be able to detect interaction in KO cells in the event that these cells undergo more rapid caspase 8 degradation. We may have also been able to better detect the pro-caspase 8 fragment. It is also possible that un-modified caspase 8 binds to poly-ubiquitinated caspase 8 however we are below the detection threshold for the modified form. From this data, we can conclude that the KO cells are lacking in a ubiquitination event but we cannot say clearly whether this is a lack of caspase 8 ubiquitination or if is the ubiquitination of a caspase 8-interacting protein such as FADD.

A


HALO
BEAD
T
UB
U
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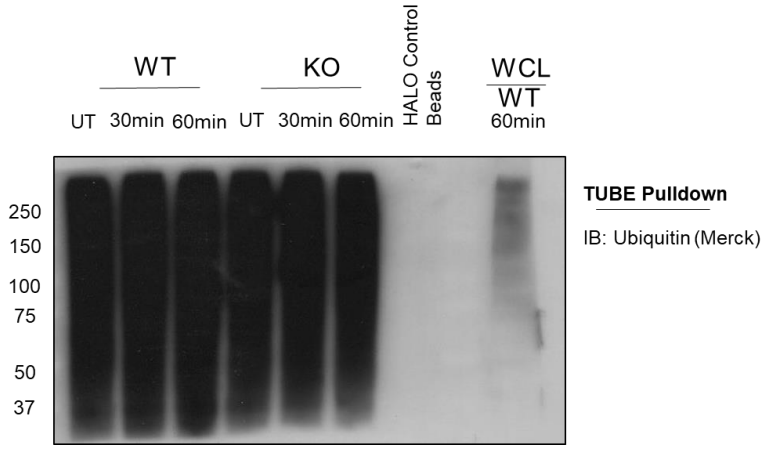
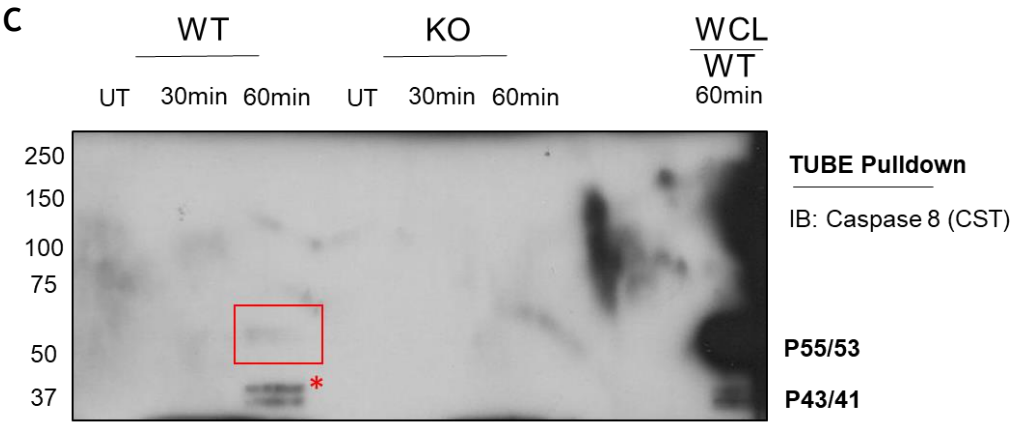
E
UB
Caspase 8

B


WT
KO
HALO Control Beads
WCL
WT
60min
UT
30min
60min
UT
30min
60min
250
150
100
75
50
37
TUBE Pulldown
IB: Ubiquitin (Merck)

C


WT
KO
WCL
WT
60min
UT
30min
60min
UT
30min
60min
250
150
100
75
50
37
*
TUBE Pulldown
IB: Caspase 8 (CST)
P55/53
P43/41

D

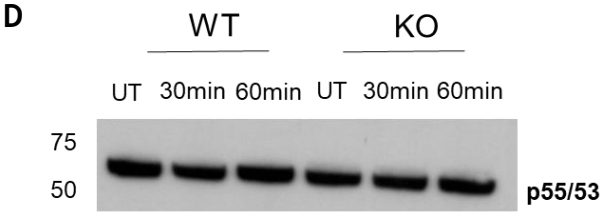

WT
KO
UT
30min
60min
UT
30min
60min
75
50
p55/53

**Figure 4-9 Caspase 8 Co-Precipitates with a Ubiquitinated Protein in WT but not KO DCNL5 Cells.**

(A) This schematic diagram shows the interaction between poly-ubiquitin chains on caspase 8 and the TUBEs which are conjugated to HALO beads. This allows for enrichment of ubiquitinated proteins from lysates and more efficient detection of modified proteins. (B) This western blot shows the result of incubating protein lysates with HALO-TUBE conjugated beads. Ubiquitin 'smears' representing total ubiquitinated proteins in the lysate, are present in both cell lines and in the presence and absence of TRAIL. The levels are broadly similar in all cases, and the ubiquitin signal is much higher in the pulldowns than in the whole cell lysate (WCL). (C) This blot shows the result of probing the same samples with caspase 8 antibody. The only lane that shows any protein, apart from the whole cell lysate, is WT cells after 60 minutes of TRAIL treatment. Interestingly we detect unmodified p43/41 caspase 8 mostly as well as a small amount of pro-caspase 8, which may indicate that this co-precipitates with a protein that is ubiquitinated which was pulled down by the TUBEs. (D) This blot shows pro-caspase 8 inputs from this experiment confirming that pro-caspase 8 levels are similar in all lanes. *Data shown in (B), (C) and (D) are representative of two independent experiments (n = 2). IB = immunoblot*

### 4.2.6 MLN4924 Treatment Does Not Fully Inhibit Caspase 8 Cleavage

The role of cullin 3 in caspase 8 activation has been detailed extensively and it has previously been shown that overexpressing cullin 3 increases caspase 8 ubiquitination while siRNA-mediated knockdown of cullin 3 reduces this modification (ref Jin et al, 2009). We explore modulation of cullin 3 and DCNL5 levels in different cancer cell lines in Chapter 5, however we wanted to determine whether the neddylation state of cullin 3 had an affect on caspase 8 cleavage in U2OS cells. Cullin proteins required neddylation to form active Cullin-RING ligase complexes which mediate ubiquitination. We therefore wished to test if inhibiting the neddylation of cullin 3, which would then render the CRL it forms the core of inactive, and thus inhibit ubiquitination of caspase 8 By using the neddylation inhibitor MLN4924, used previously in this project, we were able to inhibit neddylation of cullin proteins, including cullin 3. We pre-treated DCNL5 WT and KO cells with MLN for one hour followed by stimulation with TRAIL (and included an MLN-free control) and the resulting western blot can be seen in Figure 4-10.

Cullin 3 shows an upper, neddylated band and a lower, un-neddylated band in the absence of MLN treatment. With treatment, only the lower band is present due to the global inhibition of neddylation - this can be seen clearly from the cullin 3 immunoblot. In the WT cells, we observe the presence of p43/41 caspase 8 in the absence of TRAIL stimulation. The level increases slightly with 60-minute TRAIL treatment while formation of catalytically active caspase 8 (p18) can be seen as well. Crucially, pre-treatment with MLN reduces but does not entirely inhibit formation of p18 caspase 8 in the WT cells. This suggests that while cullin neddylation is important for caspase 8 cleavage, it is not absolutely required. This is the first indication that un-neddylated cullin 3 (whose neddylation was inhibited by MLN4924) has a function in caspase 8 activation and is also the first indication of a functional role for cullin proteins that does not require their neddylation. The DNCL5 KO cells show basal p43/41 caspase 8 levels in the absence of TRAIL stimulation, like the WT cells, but significantly less. These levels are unaffected by TRAIL stimulation or by MLN treatment, further confirming that DCNL5 is required for caspase 8 cleavage. This data together suggests that the role of DCNL5 is more important than the role of neddylated cullin 3, however the

question remains whether the role of DCNL5 and the effect of MLN4924 treatment are the same in other cell lines.

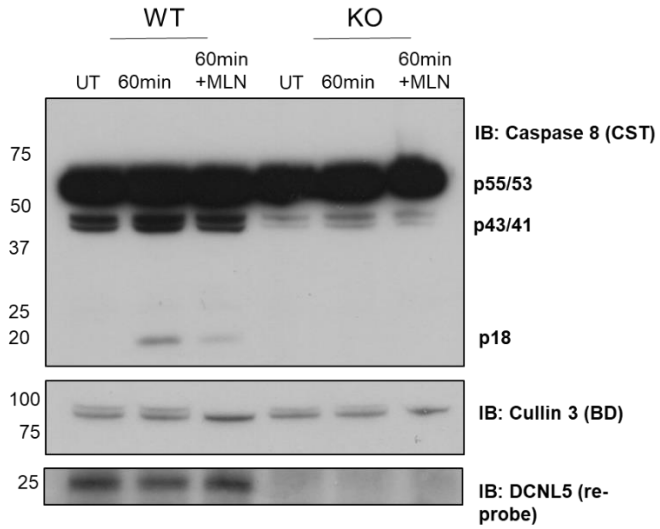



**Figure 4-10 MLN4924 Treatment Reduces But Does Not Fully Inhibit Caspase 8 Cleavage.** This representative western blot shows the effect of MLN4924 on cullin 3 neddylation and on caspase 8 cleavage. Successful inhibition of neddylation can be seen by the presence of only the lower (un-neddylated) molecular weight band of cullin 3 being present following pre-treatment with the drug. 60 minutes of TRAIL treatment causes cleavage of caspase 8 to form the active p18 fragment, and this is reduced but not entirely inhibited by MLN treatment. This suggests that un-neddylated cullin 3 can mediate some of the function required to activate caspase 8. DCNL5 KO cells show basal levels of p41/41 caspase cleavage, as in the WT cells, only less protein is evident in the KO cells. As has been consistent in the data thus far, TRAIL stimulation causes no caspase 8 cleavage in the KO cells. It appears that the role of DCNL5 is more crucial for full caspase activation than neddylated cullin 3. *IB = immunoblot*

## 4.3 Discussion

### 4.3.1 Summary

The key aim of this chapter was to begin to establish a mechanism for the role of DCNL5 in caspase 8 cleavage and activation. We initially showed that DCNL5 is able to translocate from the nucleus to the cytosol upon TRAIL stimulation, providing the first piece of evidence for a role of the protein outside of the nucleus. We were also able to show that DCNL5 and cullin 3, the known cullin protein responsible for caspase 8 activation, were able to interact in cells representing one of the first known specific interactions of DCNL5 in cells. We detected an indication that there may be reduced levels of caspase 8 interacting with cullin 3 by immunoprecipitation in DCNL5 KO and DAD-mutant cells, suggesting that the lack of caspase 8 cleavage in these cells may be due to a reduced interaction between cullin 3 and caspase 8, however the data was not overly clear. From our preliminary data, we were unable to determine whether caspase 8 and DCNL5 interact *in vivo*, further experimental work would be required to probe this interaction further.

We used TUBEs to pull on ubiquitinated caspase 8 in cell lysates treated with and without TRAIL and found that DCNL5 KO cells lack interaction with a ubiquitinated protein that binds to caspase 8, suggesting that ubiquitination of at least one protein within the DISC is lacking in the absence of DCNL5 expression. It is possibly that caspase 8 or another protein became de-ubiquitinated during the purification of the KO cells however this is unlikely since the presence of the TUBEs are known to protect lysates from DUB activity (Hjerpe *et al.*, 2009). Given the importance of cullin 3 in mediating the ubiquitination of caspase 8 at the DISC, we sought to evaluate the role of neddylation in the process. We used the NAE inhibitor MLN4924 to inhibit the neddylation of cullin proteins in WT and KO cells prior to TRAIL stimulation. KO cells showed no caspase cleavage following TRAIL treatment in the absence of presence of MLN. WT cells showed a reduction but not a total loss of caspase 8 cleavage (specifically the p18 fragment) indicating that cullin neddylation is not absolutely required for the ubiquitination and activation of caspase 8. This last finding suggests that the role of cullin 3 in this pathway may somehow occur independently of its ability to form CRL complexes since

neddylation is an essential step in that process. The complex may also be present but inactive in the absence of neddylation, but it is possible that this particular CRL does not require neddylation for its activity. What would the neddylation-independent role of this CRL be? Perhaps it functions as a nexus for recruitment of other proteins which are involved in promoting caspase 8 cleavage and ubiquitination. Perhaps another E3 ligase with common components to this cullin-3 CRL requires anchoring to caspase 8 and thus lack of neddylation of cullin 3 has little impact on caspase 8 cleavage. Questions also remain about the half life of the complex as well as the substrate adaptors involved in this ubiquitination event of caspase 8.

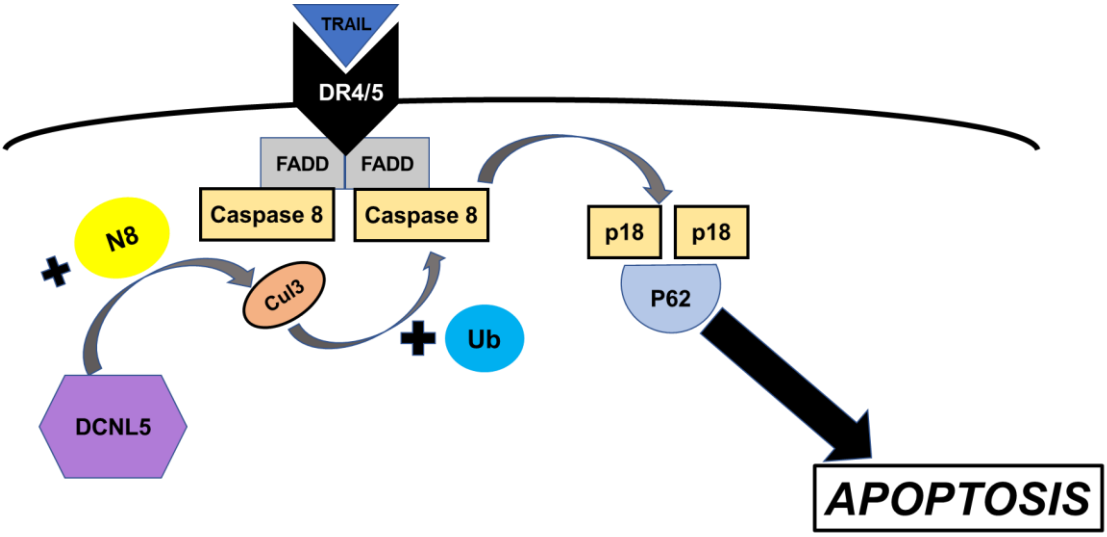



**Figure 4-11 Predicted Role of DCNL5 in Caspase8 Activation.**
Based on the data presented in this chapter, it remains possible that DCNL5 is required for the neddylation of cullin 3 which in turn allows for the ubiquitination of caspase 8 which leads to its cleavage and activation. We have shown that DCNL5 interacts with cullin 3 *in vivo* and we have demonstrated that DCNL5 KO cells lack ubiquitination of a protein which interacts directly with caspase 8. This raises the possibility that DCNL5 is involved in mediating the neddylation of another substrate which regulates caspase 8 activity, however the finding that MLN4924 does not totally ablate caspase 8 cleavage may suggest a cullin-independent mechanism governing caspase 8 cleavage or a role for cullins which is independent of their neddylation.

### 4.3.2 DCNL5 Has Roles Outside the Nucleus

DCNL5 is known to be a predominantly nuclear protein and contains a nuclear localisation sequence in its N-terminus (Keuss *et al.*, 2016). As mentioned in 4.1.1, caspase 8 localisation is known to vary prior to and during apoptotic signalling.

Pro-caspase 8 is thought to be predominantly localised to the mitochondria, and has been shown to be released into the cytosol upon TNFα treatment, however other research has shown that pro-caspase 8 is already localised to the cytosol prior to any apoptotic stimulation (Zhivotovsky *et al.*, 1999). Caspase 8 is well characterised as being cleaved and activated at the DISC in extrinsic apoptosis signalling, which is localised at the plasma membrane (Beaudouin *et al.*, 2013). Active caspase 8 is then released to the cytosol where it can cleave downstream proteins such as caspase 3 and BID.

We were able to show using fluorescence microscopy that DCNL5 diffuses into the cytoplasm following stimulation with TRAIL and that this movement occurred within 1 hour of stimulation. This finding supports our model of DCNL5 playing a direct role in caspase 8 cleavage and activation and provides new evidence for the cytosolic localisation of DCNL5 following initiation of the apoptosis response. Furthermore, this finding raises the question of why DCNL1 is unable to mediate this function of DCNL5. DCNL1 localises to the cytosol and is known to be able to interact with cullin 3 *in vivo,* and inhibitors targeting the N-terminal acetylation site of the NEDD8 E2 UBC12 (UBE2M) specifically reduce cullin 3 neddylation over other cullin proteins in cells (Zhou *et al.*, 2017). We have shown that DCNL1 KO cells are equally sensitive to TRAIL and more sensitive to TNFα-mediated apoptosis compared to WT cells which is at odds with the DCNL5 data. DCNL3, which is located at the plasma membrane (Meyer-Schaller *et al.*, 2009) may not be able to overcome absence of DCNL5 expression however we have not specifically investigated the role of DCNL3 in this response. We hypothesize that given the structural similarities and known overlap in function of DCNL proteins *in vitro* (Keuss *et al.*, 2016), we are likely looking at a highly specific role for DCNL5 in the activation of caspase 8. This may be mediated by its N-terminus which may be required for interaction with the DISC. The fact that MLN4924 treatment shows less of an effect than lack of DCNL5 expression indicates we may be observing a neddylation-independent function for DCNL5 which is mediated by its N-terminus while still requiring its binding to cullins via its DAD-patch.

### 4.3.3 DCNL5 Interacts with Cullin 3 in Cell Extracts

The established mechanism of caspase 8 cleavage requires its ubiquitination as an initial step in its activation and this is mediated by a cullin 3-based E3 ligase in combination with RBX1 (Jin *et al.*, 2009). Cullin proteins require neddylation for their activation and we had postulated that the role of DCNL5 may be in facilitating this neddylation process. In order to test this hypothesis, we needed to demonstrate that DCNL5 and cullin 3 were able to interact *in vivo*. All DCNL proteins have been shown to interact with all cullin proteins *in vitro* (Keuss *et al.*, 2016) and there have been accounts of specific roles for individual DCNL proteins in combination with a specific cullin protein *in vivo* as discussed in section 4.3, however there appears to be little evidence for DCNL5-specific functions, outside of its potential role in the DNA damage repair pathway upon which this project was initially based.

We were able to confirm that DCNL5 interacts with cullin 3 *in vivo* using endogenous protein levels, which is the first evidence of this interaction without overexpressing the proteins. The interactions appeared to be weak, which is perhaps not surprising given the relatively small amounts of DCNL5 we were able to detect translocating out of the nucleus in Figure 4-3 and given the specific nature of the cullin 3 pools required to mediate ubiquitination of caspase 8. In addition to detecting this interaction, we found that there appeared to be a slight increase in interaction between DCNL5 and cullin 3 following TRAIL stimulation, consistent with an increase in caspase 8 processing and thus further highlighting the importance of DCNL5 involvement. This represents a new role for DCNL5 and raises the question of why DCNL3 which localises to the plasma membrane and is known to interact with cullin 3 (Meyer-Schaller *et al.*, 2009) is unable to compensate for the lack of DCNL5 expression in the KO cells to perform the same function. Further experiments are required to verify that DCNL3 does not interact with cullin 3 in response to TRAIL stimulation is required, perhaps by using siRNA-mediated gene silencing and examining the level of caspase 8 cleavage when DCNL3 expression is inhibited. It remains a possibility that part of the N-terminal domain of DCNL5 contains sequences responsible for binding to a specific protein or proteins within the DISC which other DCNL proteins do not have. More recent evidence has suggested that despite the similarity of the conserved C-terminal

PONY domains of the DCNL proteins, not all DCNL protein interact with the NEDD8 E2 family in the same way (Monda *et al.*, 2013) which suggests that there may be unique binding properties of DCNL5 which have yet to be uncovered. DCNL5 crystal structure would provide significant insight into the structural differences between this protein and the other DCNL family members and may elucidate new binding pockets and interaction domains capable of mediating DCNL5's role in apoptosis.

Our approach to investigating the interaction of DCNL5 with cullin 3 was inherently biased but was based on the well established and well studied role of cullin 3 in mediating caspase 8 cleavage and apoptosis induction. Future work needs to be undertaken to screen for other protein-protein interactions which are affected by the absence of DCNL5 expression, perhaps by looking at more general changes in the proteome in DCNL5 KO compared to WT cells in response to TRAIL stimulation using a mass spectrometry-based proteomics screen. It may well be that other cullins interact with DCNL5 during apoptosis and some of our data in the following chapters provides initial evidence of this assertion, and a proteome-wide screen would be an invaluable tool in further investigating this in an unbiased manner.

### 4.3.4 DCNL5 DAD-Patch Interaction is Required for Caspase 8 Cleavage

Previously we showed that DCNL5 KO cells as well as DAD-mutant cells exhibit significantly less caspase 8 cleavage than WT cells and this loss of caspase activity mirrors the reduced sensitivity to apoptosis when measured using a viability assay. The question we wanted to answer was whether caspase 8 is unable to interact with cullin 3 in the absence of DCNL5 and whether there are differences in the amounts of protein interacting. Given the unique role of DCNL5 in this pathway we considered the fact that DCNL5 may play a structural role in anchoring or recruiting proteins to the DISC and in the absence of DCNL5 DISC assembly may be compromised.

We performed an immunoprecipitation reaction using cullin 3 to investigate its interaction with caspase 8. This interaction has been well studied before in other cell lines (Jin *et al.*, 2009) however to our knowledge U2OS cells haven't been used previously. We were able to demonstrate an interaction between cullin 3 and

caspase 8 (as shown in Figure 4-6) and we detected a noticeable difference in the amount of caspase 8 interacting with the different cell lines. DCNL5 KO and DAD-mutant cells showed less p43/41 caspase 8 interacting with cullin 3. This form of caspase 8 is an intermediate cleavage product and suggests that DCNL5 is not required for the interaction of caspase 8 and cullin 3 since the amount of un-cleaved caspase 8 interacting with cullin 3 is largely the same. DCNL5 does however appear to play a role in caspase 8 cleavage and it does this in a cullin-dependent manner, since DAD-mutant cells show even less p43/41 caspase 8 interacting with cullin 3 than the KO cells.

We attempted to verify whether DCNL5 can interact directly with caspase 8, since there is precedent for the role of NEDD8 in caspase activation. Caspase 1 has been shown to require neddylation for efficient self-cleavage to generate its catalytically active subunits following inflammasome activation, however the research involved overexpression and as such the result may not be physiological (Segovia *et al.*, 2015). The researchers did show however that MLN4924 was capable of diminishing caspase 1 processing and reducing IL-1ß maturation which occur as a direct result of inflammasome activation. In the present work, we used an immunoprecipitation reaction, pulling on caspase 8 and subsequently probing for DCNL5 and were unable to detect any interaction. This experiment was only performed once, and the amount of caspase 8 which was precipitated was relatively low, therefore the likelihood of detecting any interaction with DCNL5 was also low. Given the small amount of DCNL5 that is present during the apoptosis response, it would likely require a lot more material to properly detect an interaction and this finding requires further validation. It therefore remains a possibility that there is some direct interaction between DCNL5 and caspase 8.

### 4.3.5 DCNL5 KO Cells Appear to Lack Ubiquitination in Response to TRAIL Stimulation

As highlighted in section 4.1.2, caspase 8 activation requires its dimerization and subsequent cleavage to form catalytically active caspase 8 which is then able to induce the downstream events that are required for successful apoptosis. We have previously sown that DCNL5 KO and DAD-mutant cells lack caspase 8 cleavage in response to TRAIL and TNFα and this lack of caspase 8 activity likely confers

resistance to apoptosis as measured by viability assay. In this results chapter we present evidence for the interaction between cullin 3 and DCNL5 *in vivo* which supports a mechanism whereby DCNL5 is required for the neddylation of cullin 3 in its complex with RBX1 and as yet unknown substrate adaptors to mediate the poly-ubiquitination of caspase 8 at lysine 461 by both K48 and K63 linkages (Jin *et al.*, 2009).

The finding that less cleaved caspase 8 interacts with cullin 3 in KO and DAD-mutant cells indicates that there is a reduction of caspase 8 cleavage occurring in these cell lines. To further investigate this finding we attempted to analyse the extent of caspase 8 ubiquitination by using a denaturing immunoprecipitation reaction, adapted from the one employed by Lim and colleagues who studied the ubiquitination of caspase 8 in cells infected with *Helicobacter* pylori (Lim *et al.*, 2017). The premise of performing an IP reaction under denaturing conditions is to eliminate all non-covalent interactions between the protein of interest and other proteins in lysates that have been treated with apoptosis inducing drugs. In our case, we precipitated using a caspase 8 antibody and then used a general ubiquitin antibody to determine the amount of caspase 8 which was modified in the presence and absence of TRAIL in WT and KO DCNL5 cells. Many repeats of this experiment were performed and included using different ubiquitin antibodies however we found it difficult to successfully precipitate enough protein to consistently visualise caspase 8 ubiquitination. The data from Figure 4-8 did show differences in caspase 8 ubiquitination between cell lines and in the presence of TRAIL however the differences are possibly attributable to the differing amounts of caspase 8 that was precipitated in each lane. Overall, there does appear to be less ubiquitination of caspase 8 in KO cells compared to WT cells, but this result is difficult to interpret given the different ubiquitination modifications that regulate caspase 8 activity.

Polyubiquitination by cullin 3 is the only known positive regulator of caspase 8 activity (Jin *et al.*, 2009) while multiple ubiquitination events have been shown to inhibit caspase 8 activity and are discussed in the following chapter. A key example is the polyubiquitination via K48 linkages by the E3 ligase activity of TRAF2 which initiates a shut-off time which targets caspase 8 for degradation and

thus serves as a key regulator of caspase 8 activity (Gonzalvez *et al.*, 2012). Thus, a reduction in ubiquitination of caspase 8 could also represent a reduction in the negative regulation (via TRAF2) and in fact represent an increase in caspase 8 activity. Great care is required when interpreting caspase 8 ubiquitination data. We did attempt to probe for K63-linked polyubiquitination specifically (data not shown) however the results were less clear than with a general ubiquitin antibody. Optimisation with specific ubiquitin antibodies would allow more accurate determination of the different types of ubiquitination occurring and which modifications are reduced or increased in KO cells compared to WT cells following TRAIL stimulation. Experimentally, these reactions can be performed at 4°C instead of in humidified incubators at 37°C which tends to stabilise modified caspase 8 and improve the signal that can be detected by western blot (Jin *et al.*, 2009) and therefore a repeat of this IP reaction under denaturing conditions at 4°C would likely yield improved signal.

Another way of evaluating caspase 8 ubiquitination status in WT and KO DCNL5 cell lines is to using tandem ubiquitin binding entities (TUBEs) as described in 4.2.5. We used purified TUBEs which had been conjugated to HALO magnetic beads to precipitate poly-ubiquitinated proteins in lysates with and without TRAIL stimulation. Global levels of ubiquitinated proteins were unchanged following TRAIL stimulation since ubiquitination is a common post-translational modification and part of normal cell physiology. When we probed for caspase 8, we had expected to see a ubiquitin 'smear' of high molecular weight following TRAIL stimulation, which is generally what has been observed during immunoprecipitation-type reactions previously. To our surprise, in WT cells, we detected unmodified p43/41 caspase 8 as well as some un-cleaved caspase 8 following TRAIL stimulation of 1 hour, and no caspase 8 in the absence of stimulation. The KO cells were absent of any caspase 8 being detected by western blot.

The finding that unmodified pro-caspase 8 precipitates in WT cells may provide some interesting insight into the potential role of DCNL5 in caspase cleavage. We have previously shown that the KO cells have similar amounts of pro-caspase 8 to WT cells however the TUBE data indicates that there is a lack of ubiquitination of

a protein that interacts with pro-caspase 8 in KO cells. It is not a question of the KO cells lacking pro-caspase 8, but lacking polyubiquitination of a protein or proteins that interact with pro-caspase 8. The lack of such an interaction may explain why the KO cells fail to undergo caspase 8 cleavage. It remains a possibility that pro-caspase 8 stability is lower in KO cells and thus is unable to be detected by the TUBEs – performing the TRAIL treatment at 4°C has been shown to stabilise caspase 8 and increase the signal (Jin *et al.*, 2009). Another consideration is the possibility that the caspase 8 we detect in WT cells is interacting with polyubiquitinated caspase 8. The TUBE result is, to our knowledge, the first-time caspase 8 has been studied in this way and supports the fact that ubiquitination following TRAIL stimulation is lacking in the DCNL5 KO cells. It remains unclear however if caspase 8 itself is unable to be ubiquitinated or whether another protein in the pathway, such as FADD or cFLIP, is unable to be ubiquitinated.

cFLIP is structurally related to caspase 8 but is lacking in proteolytic activity and functions as an endogenous inhibitor of apoptosis. cFLIP is able to interact directly with FADD and caspase 8 at the DISC following TRAIL-mediated apoptosis induction (R Safa, 2013) and is ubiquitinated by the HECT E3 ligase ITCH which has been shown to promote DR5/caspase 8 -mediated apoptosis in tumour cells (Le Clorennec *et al.*, 2019). Interestingly, another HECT E3 ligase called SMURF1 has been shown to require neddylation for its ubiquitin ligase activity and NEDD8 in conjunction with UBC12 allows for the auto-neddylation of SMURF1 on multiple lysine residues (Xie *et al.*, 2014). As has been stated previously, this was all done with overexpression, so these results require verification with endogenous NEDD8 expression levels. It was later shown that NEDD8 has a non-covalent function which is required for the ligase function of SMURF1 (He *et al.*, 2017) highlighting the potentially multifaceted and relatively unknown role of NEDD8 in activating E3 ligases other than CRLs. It is therefore a possibility that NEDD8 may also be involved in the regulation of ITCH activity since it too is a HECT E3 ligase. If there is a role for DCNL5 in facilitating this process, then the lack of DCNL5 expression may account for the reduction in ITCH activity and thus the reduction in cFLIP ubiquitination, resulting in a lack of caspase 8 cleavage and apoptosis. Further experimental work is required to evaluate this hypothesis however it is clear that there is a lack of ubiquitination in DCNL5 KO cells which may responsible for their

resistance to apoptosis, however the key question of what protein or proteins fail to be ubiquitinated in KO cells still remains. Optimisation of the denaturing IP's we employed would allow us to determine which proteins that associate with the DISC are ubiquitinated and by which lysine linkage. This would then allow us to build a better picture of the ubiquitination events that may be lacking in KO cells and thus pinpoint the precise interaction of DCNL5 which is required to promote caspase 8 cleavage in U2OS cells.

### 4.3.6 Neddylation Appears not to be Essential for Caspase 8 Cleavage

The NAE inhibitor MLN4924 has been used clinically for the treatment of some cancers and has shown *in vitro* to be a potent inhibitor of neddylation. Given the well-established role for cullin 3 in the ubiquitination of caspase 8, we suspected that a lack of cullin neddylation would lead to a lack of caspase 8 cleavage and activation. Existing work has shown that neddylated cullin 3 is responsible for mediating the ubiquitination of caspase 8 (Jin *et al.*, 2009) and as has been discussed in the general introduction, cullin proteins require neddylation in order to induce structural re-arrangements through increased flexibility of the C-terminal region of the cullin protein (Duda *et al.*, 2008) which facilitates the addition of ubiquitin to target substrates. Un-neddylated cullin proteins are known to interact with CAND1 which has been postulated to sequester un-modified cullin proteins and function to rapidly exchange substrate receptors for assembly of CRL complexes (Wu *et al.*, 2013). At the time of writing, un-neddylated cullin proteins are not known to have any specific roles beyond existing in specific subcellular pools ready to be neddylated to form active CRL complexes.

MLN4924 pre-treatment was successful in inhibiting cullin 3 neddylation and in DCNL5 KO cells caspase 8 cleavage was absent when cullin 3 neddylation was inhibited and when cullin 3 was neddylated. WT cells showed a reduction but not a total loss of caspase 8 cleavage when cullin 3 was un-neddylated which indicates that in U2OS cells, cullin 3 neddylation and therefore a cullin 3-RBX1-based CRL may not be essential to mediate the cleavage of caspase 8 but it does significantly increase caspase 8 cleavage. This data indicates two things: either negative regulators of caspase 8 ubiquitination are inhibited by MLN as well as the positive

regulation by cullin 3, or there exists a role for cullin 3 independent of its neddylation state and thus catalytic activity as the core of a CRL. Cullin proteins act as scaffolds for CRL assembly and it is therefore possible that they act as scaffolds in caspase 8 activation; perhaps cullin 3 serves as a binding partner for caspase 8 and mediates its recruitment to the DISC along with FADD. What is puzzling is the fact that the role of cullin 3 in H460 cells has been shown to required its neddylation since only neddylated cullin 3 interacts with caspase 8 and the DISC (Jin *et al.*, 2009). However, another interpretation of this is that cullin 3 neddylation may not be required, all that has been shown is that cullin 3 interacting with the DISC is neddylated. Our findings in U2OS cells may indicate that there are cell line-specific nuances in caspase 8 ubiquitination and activation and is explored further in the following chapter where we test the role of DCNL5 and cullin 3 in a range of cancer cell lines.

# 5 Confirmation of the General Role of DCNL5 in the Extrinsic Apoptosis Response in a Variety of Cancer Cell Lines

## 5.1 Introduction

### 5.1.1 TRAIL Signalling Has Diverse Responses in Different Cell Lines

Apoptosis is an incredibly complex and tightly regulated process that has many stages required to initiate the downstream events that mediate cell death. Not only is the process complicated, but the exact signalling pathways and proteins involved can vary from one cell line to the next. Cancer cells have been shown to be more sensitive to TRAIL stimulation than normal cells (Kelley and Ashkenazi, 2004) which prompted a significant increase into research investigating the cytokine's use in cancer treatment. For example, the TRAIL gene has been shown to elicit apoptosis in malignant cells *in vivo* when transfected using adenoviral methods while causing no detectable toxicity in cultured normal fibroblasts or in mouse hepatocytes (Kagawa *et al.*, 2001). Despite the initial promise, numerous cancer cell lines have been shown to be resistant to TRAIL stimulation through a variety of mechanism including loss of caspase 8 expression (Grotzer *et al.*, 2000) or high surface expression of functionally inactive decoy TRAIL receptors in MCF-7 cells (Sanlioglu *et al.*, 2005).

In the present research, we have shown that DCNL5 plays a role in caspase 8 activation, likely at the level of ubiquitination of caspase 8 in conjunction with cullin 3 or possible another cullin protein. We have mostly used U2OS cells for this work, and prior research using these cells has suggested that this cell line is not particularly sensitive to TRAIL and often requires co-treatment with drugs such as cycloheximide (Garnett, Filippova and Duerksen-Hughes, 2007) or the siRNA-mediated knockdown of cFLIP (Zhang *et al.*, 2015). In our hands, we found U2OS cells to be sensitive to TRAIL stimulation alone (with DCNL5 KO cells being resistant), and combination treatment with CHX was found to kill all cells to levels below detectable threshold using our luciferase assay. The finding that cell line responsiveness to TRAIL can vary highlights the complicated nature of TRAIL signalling and sensitivity in tumour cells. In order to determine the general

applicability of our findings we decided to test a variety of cell lines for their response to TRAIL signalling after loss of DCNL5. All the cell lines we used in this chapter undergo caspase 8 cleavage after TRAIL treatment, indicating sensitivity to the drug, except for MCF-7 cells, which as mentioned above, are known to be resistant to the treatment. This resistance has been shown to be linked to the higher expression of decoy TRAIL receptors as well as the increased endocytosis of death receptors 4 and 5 (Zhang and Zhang, 2008), effectively increasing the ratio of decoy-receptors to functional receptors and thus reducing TRAIL effectiveness. MCF-7 cells therefore represent a good control cell line for the studies involving the role of DCNL5 and the caspase 8 ubiquitination and cleavage in cancer cell lines.

### 5.1.2 Caspase 8 Ubiquitination is Regulated by Multiple Ubiquitin E3 Ligases

We have previously discussed the role of caspase 8 ubiquitination, which is mediated by cullin 3, and detailed how this modification is unique in being a positive regulator of caspase 8 activation (Jin *et al.*, 2009). Caspase 8 ubiquitination by other E3 ligases is also known to inhibit its activation and act as negative regulation for apoptosis, which adds a layer of complexity to studying caspase 8 activation. Furthermore, other components of the DISC and downstream signalling complexes are known to be regulated by ubiquitination as well as other post-translational modifications, making interpretation of results challenging.

Caspase 8 ubiquitination by cullin 3 has been shown to be required for cleavage and activation, this process also requires the activity of the scaffold protein p62 (Jin *et al.*, 2009). While not strictly related to TRAIL-induced cell death, the RING E3 ligases TRIM13 has been shown to mediate K63-linked polyubiquitination of caspase 8 (Tomar *et al.*, 2013) which results in its stabilization and activation during ER stress, furthermore TRIM13 expression was shown to sensitise cells to ER stress-induced death through its ability to translocate caspase 8 to autophagosomes (Tomar *et al.*, 2013). Interestingly TRIM13 was shown to act synergistically with p62, similarly to the cullin 3-based E3 ligase for TRAIL signalling (Tomar *et al.*, 2013).

Negative regulation of caspase 8 activity by ubiquitination has also been described. Cullin 7 has been shown to promote tumour cell survival (specifically HeLa cervical cancer cells) through mediating the ubiquitination of caspase 8 at lysine 215 through non-K48-linked polyubiquitin chains (Kong *et al.*, 2019). Cullin 7 was shown to interact with the DED domain of caspase 8 via its C-terminus, and knockdown of cullin 7 protein levels resulted in increased caspase 8 cleavage in response to TRAIL. This modification of caspase 8 was shown to be non-degradative and it was postulated that polyubiquitin chains physically impeded caspase 8 activation at the DISC (Kong *et al.*, 2019).

Caspase 8 polyubiquitination at lysine 215 via K63 linkages has also been shown to inhibit caspase 8 cleavage by the HECT E3 ligase HECTD3 in a non-degradative manner as well (Li *et al.*, 2013). HECTD3 interacts with the DED regions of caspase 8 though its DOC region (destruction of cyclin domain) (Li *et al.*, 2013) which is involved in substrate recognition and is a similar interaction to the interaction between cullin 7 and the DED domain described above. As with cullin 7, siRNA-mediated knockdown of HECTD3 resulted in increased caspase 8 activation and apoptosis in response to activation of the extrinsic branch of the apoptosis pathway (Li *et al.*, 2013).

Another regulator of caspase 8, and one which ties in with the positive regulation by cullin 3, is the polyubiquitination mediated by a protein called TNF receptor associated factor 2 (TRAF2). TRAF family members are known to possess E3 ligase activity and they have been shown to mediate polyubiquitination of a variety of substrates, including activation by ubiquitination of the IκB complex (Deng *et al.*, 2000). TRAF proteins play other roles in in NF-κB signalling (Chen, 2005) ( but TRAF2 specifically is responsible for regulating another pathway: caspase 8 activation during apoptotic signalling (Gonzalvez *et al.*, 2012). TRAF2 has previously been shown to be capable of mediating polyubiquitination (Cardamone *et al.*, 2012) and has also been shown to interact with caspase 8 at the DISC downstream of cullin 3, where TRAF2 mediates the K48-linked polyubiquitination of the p18 catalytic subunit of caspase 8 (Gonzalvez *et al.*, 2012). This modification targets caspase 8 molecules for proteasomal degradation following auto processing and cytoplasmic translocation of p18 and p43 fragments and this

functions as a shutoff timer to attenuate the extrinsic apoptosis response (Gonzalvez *et al.*, 2012). Overall, there are multiple ubiquitination events which regulate caspase 8 activity and thus multiple potential roles for DCNL5 in the extrinsic apoptosis pathway. A question still remains about the importance of NEDD8 in the process since data from the previous results chapter indicates that cullin neddylation may not be essential for the role of DCNL5 in caspase 8 activation.

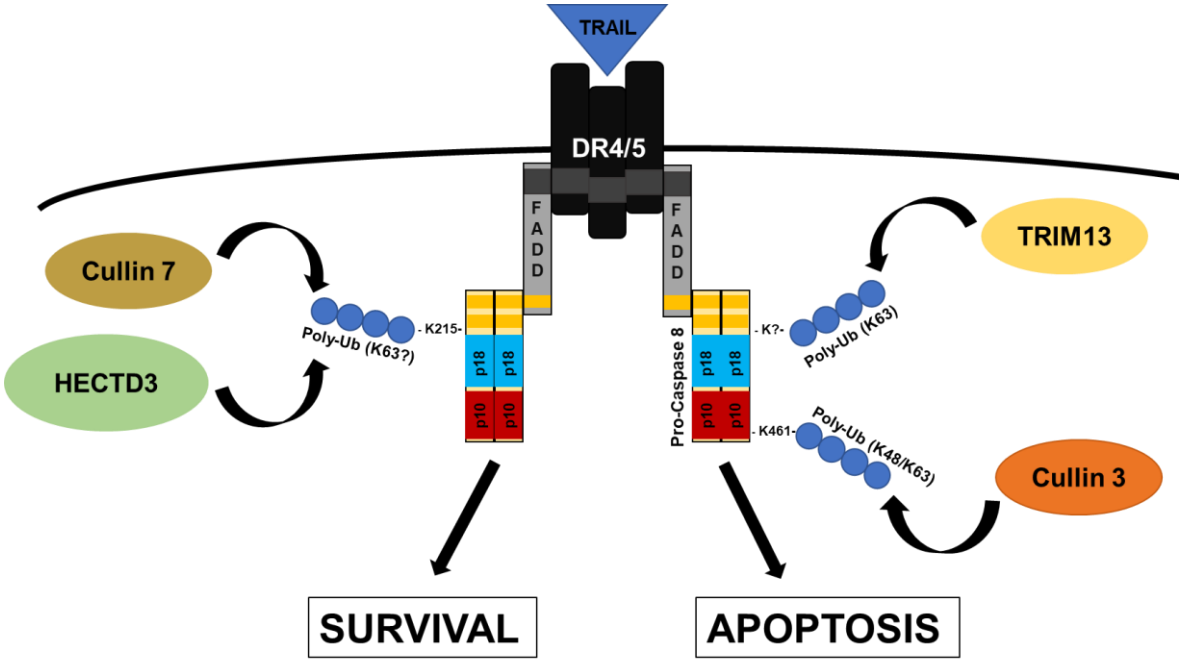



**Figure 5-1 Caspase 8 Regulation by Ubiquitination.**

This diagram outlines some of the major proteins involved in the ubiquitination of caspase 8. Ubiquitination of caspase 8 can have both pro-apoptotic and anti-apoptotic roles, depending on the residue that is modified. Pro-survival modifications occur at lysine 215 which lies between the DED regions of caspase and its p18 domain. Polyubiquitination at this residue by non-K48 linkages promotes cell survival and this polyubiquitination is mediated by a cullin 7-based CRL complex as well as the HECTD3 E3 ligase. Pro-apoptotic signalling can be triggered by polyubiquitination via K63 linkages at an unknown lysine residue within caspase 8 by the E3 ligase TRIM13. This modification is known to occur as a result of ER stress and TRIM13 has been shown to act synergistically with the scaffold protein p62. The better characterised ubiquitination event regarding caspase 8 activity is the polyubiquitination of lysine 461 within the p10 region of caspase 8 via both K48 and K63 linkages. This crucial modification occurs as a result of receptor-mediated apoptosis induction and has been shown to promote the dimerization and cleavage of caspase 8 as well as its subsequent association with p62 in aggregates, no dissimilar to process mediated by TRIM13. This pro-apoptotic ubiquitination event is mediating by cullin 3 in combination with RBX1 and is vital in promoting downstream apoptosis events.

### 5.1.3 The Neddylation Inhibitor MLN4924 Reveals a Role for NEDD8 in Apoptosis

Given the well documented importance of ubiquitination in caspase 8 activation as discussed above, and the well-established role of multiple cullin proteins in mediating this process, it is essential to investigate the importance of NEDD8 in extrinsic apoptosis. NEDD8 addition to cullin proteins is an essential step in the activation of CRL complexes which mediate ubiquitination (Kawakami *et al.*, 2001;

Wu *et al.*, 2002; Sakata *et al.*, 2007). In this chapter, we will present data using the NEDD8 activating enzyme inhibitor MLN4924, and while the mechanism and function of this drug has already been discussed in terms of its activity in inhibiting neddylation, it is important to understand that MLN4924 has been shown to also play roles in the apoptosis pathway. MLN has been shown to synergise with TNFα during treatments of 6-8 hours to induce apoptosis in a caspase 8 dependent manner in rat liver hepatoma cells, however the molecular mechanism underlying the role of the neddylation inhibitor remains unclear in this context (Wolenski *et al.*, 2015). Research has also shown that MLN is able to heighten apoptotic signalling in response to TRAIL stimulation though the degradation of cFLIP in head and neck cancer cells (Zhao *et al.*, 2011).

Other work has been done which shows the MLN treatment in human oesophageal squamous cell carcinoma cells resulted in increased DR5-mediated caspase 8 activation. MLN4924 inhibited the activity of CRLs which lead to the stabilization of ATF4 which in turn lead to transactivation of the transcription factor CHOP which upregulated DR5 expression and increased downstream caspase 8 activation (Chen *et al.*, 2016). In the research by Chen and colleagues they induced apoptosis by stimulating cells for 72 hours with MLN4924 while Wolenski and colleagues used up to 8 hours and Zhao and colleagues used the drug for 24 hours. In our research, we used MLN for 1-hour pre-treatments followed by incubation with TRAIL for 30-90 minutes, representing a much shorter time period over which cells were exposed to the NEDD8 inhibitor. Longer term MLN4924 treatment will affect cells drastically since all ubiquitination events mediated by cullins will be inhibited. Our relatively short treatments with the inhibitor will provide insight specifically on the role of cullins in the apoptosis pathway and have little affect on other cellular processes during the treatment duration. One final complication in using MLN4924 when studying apoptosis is that it has also been shown to induce apoptosis through the intrinsic branch of the pathway (Wang *et al.*, 2015) however this generally does not affect caspase 8 cleavage but will impact downstream caspase cleavage as well as other downstream events.

### 5.1.4 Aims

We wanted to determine whether the role of DCNL5 is conserved across different cancer cell lines, the phenotype observed in U2OS cells is very strong and given the diverse response of cancer cells to TRAIL coupled with the complexity of the TRAIL pathway, it would solidify the role of DCNL5 as a key regulator in the apoptosis response. We also wanted to investigate the importance of neddylation in caspase 8 activation given DCNL5 is a key regulator of neddylation and neddylation is required for CRL activity. Linked to this, we wanted to test the importance of cullin 3 in a variety of cell lines given its established role as the key mediator of caspase 8 ubiquitination. We used a selection of cancer cell lines covering a wide range of tumours for this section of work. H460 cells were selected because these lung carcinoma cells were used extensively in the seminal work by Jin and colleagues who characterised the role of cullin 3 in caspase 8 ubiquitination (Jin *et al.*, 2009). We used MCF-7 cells as a negative control since these cells are not TRAIL sensitive. HeLa cells are a common cell line used to study a wide range of cellular processes and represent an important cell line to study caspase 8 activity, while DU-145 prostate cancer cells and A375 melanoma cells have been shown to be TRAIL sensitive.

## 5.2 Results

### 5.2.1 Successful Generation of CRISPR/Cas9 DCNL5 KO H460 Cells

A common cell line used to study caspase 8 cleavage and activation is the H460 lung carcinoma cell line and these cells have been used extensively in TRAIL-mediated caspase 8 cleavage research (Jin *et al.*, 2009) and thus represented a good additional cell line to investigate the role of DCNL5. We wanted to generate a CRISPR/Cas9 KO cell line to directly compare with the KO U2OS cell line we have used throughout this project. We transfected a guide RNA vector for exon 1 of DCNL5 which was generated previously by mutagenesis PCR of pEsgRNA (Munoz *et al.*, 2014; Keuss *et al.*, 2016) with the target sequence 5'-GCAGCAGTAGCGGAAGACGGAGG-3' (plus strand) into H460 cells stably expressing FLAG-tagged Cas9 which was under tetracycline-inducible promotor control. Expression was induced with tetracycline and we then single cell cloned these cells. After one round of single cell cloning we analysed a selection of clones to evaluate the success of the process and the resulting western blot showing DCNL5 expression can be seen in Figure 5-2A. 15 individual colonies were screened initially and four of these clones appeared to show significant reductions in DCNL5 expression. These clones were then re-analysed in combination with a loading control to more accurately determine whether the reduction in DCNL5 expression was significant. Clones 6, 8 and 15 showed major reductions in DCNL5 expression however they still showed low levels of DCNL5, suggesting that these clones were not complete knockouts and were perhaps mixed populations.

We then opted to re-grow and re-screen these clones hoping to obtain complete KO cells. The result of another round of screening of the initial clone number 6 can be seen in Figure 5-2B. Out of 34 single colonies that grew successfully we found half a dozen potential complete KO clones, which have been underlined in the figure. These clones showed a total lack of DCNL5 expression as determined by western blot, however a higher molecular weight band appeared to be present in most of these. While this band was unexpected, a possible explanation is that the polyclonal DCNL5 antibody is binding to another DCNL protein (likely DCNL4) or another protein entirely in the absence of DCNL5. If this were to be the case,

the size of the band is similar to the size of DCNL4 (34kDa) and this may therefore explain the band we see on the western blot. A simple way of confirming that this band is indeed not a mutant form DCNL5 would be to perform siRNA-mediated silencing of DCNL5 and if the band is still present, it would strongly suggest that it not DCNL5. We wanted to confirm the successful generation of DCNL5 KO cells, so we analysed the sequence of the DCNL5 gene in these clones. Overall, 2 main mutations were present in these clones presumably in each copy of the gene. In all cases, premature stop codons were introduced in exon 1 leading to the lack of formation of the mature and properly folded protein. Clones that showed no reduction in DCNL5 expression were kept and used as WT controls.

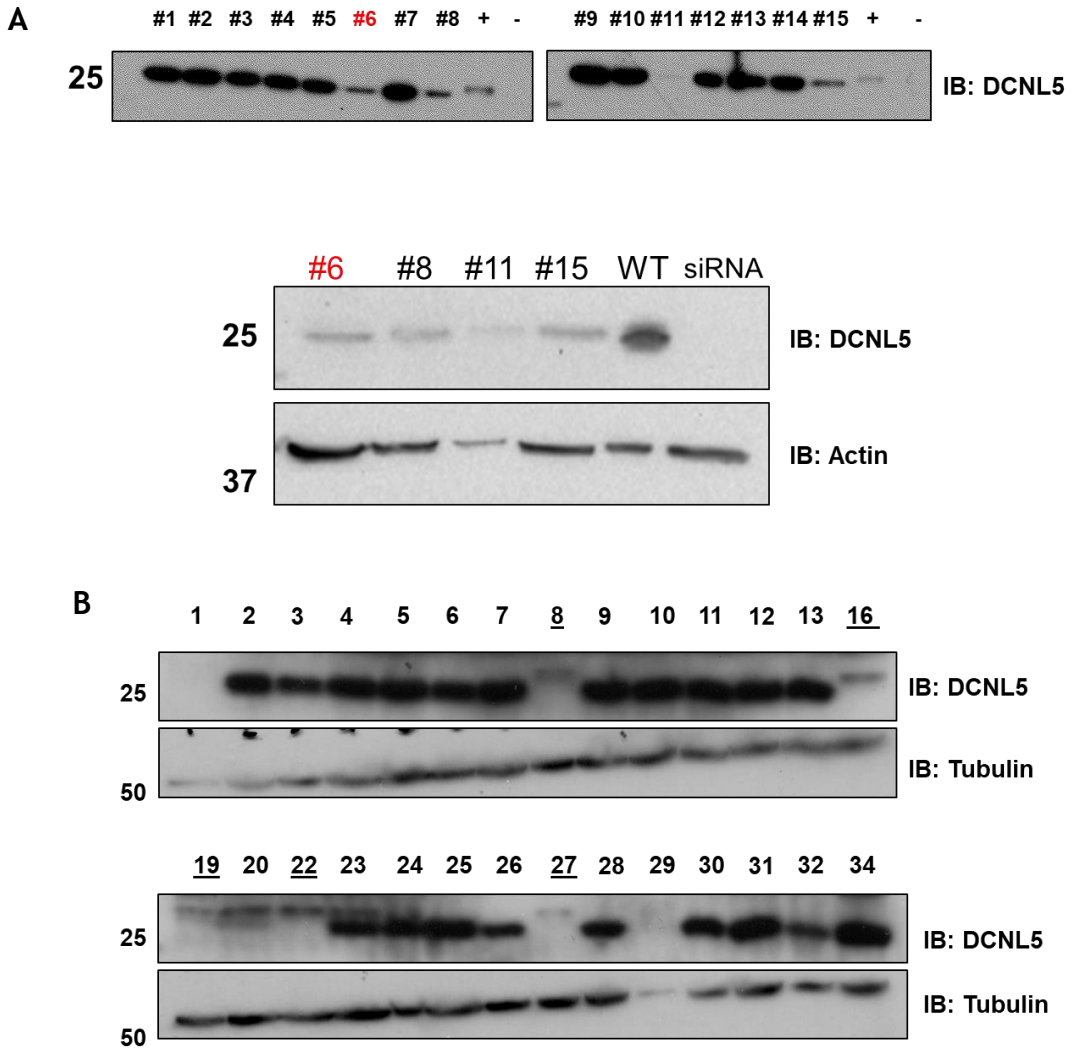



**Figure 5-2 DCNL5 CRISPR Cas9 KO Cells Were Made Following 2 Rounds of Screening.**
(A) Initial screening following single cell plating of cells that had been subject to CRISPR/Cas9-mediated knockout using gRNA for exon 1 of DCNL5 showed partial reduction in DCNL5 expression in 3 clones. These likely represented mixed populations and number 6 (highlighted in red) was used for another round of screening. (B) A second round of single colony plating of clone number 6 resulted in several clones showing complete lack of DCNL5 expression, which have been underlined. These clones were sequenced to confirm successful disruption of the DCNL5 gene.

### 5.2.2 H460 Cells Demonstrate an Increase in Caspase 8 Ubiquitination Following TRAIL Stimulation

In the previous chapter we had attempted to optimise a denaturing immunoprecipitation reaction to show the changes in caspase 8 ubiquitination following TRAIL stimulation. This proved difficult with U2OS cells and we did not perform the TRAIL treatment at 4°C which increases the caspase 8 signal and stabilises ubiquitinated caspase 8 aggregates. We wanted to see if we could detect the modification in H460 cells given that they are one of the cell lines of choice for studying caspase 8. We sought to compare this cancer cell line to a non-cancerous cell line (HEK 293) to highlight the fact that it is only tumour cells and cell lines which respond to TRAIL. The sensitivity of cancer cells to TRAIL (compared to healthy cells) make it a useful drug in chemotherapy and cancer treatments in clinical settings.

We were able to detect what appears to be an increase in caspase 8 ubiquitination after performing a denaturing IP using H460 cells that were stimulated with 200ng/ml of TRAIL for 30 minutes. The western blot in Figure 5-3 shows an increase in caspase 8 modification following stimulation (shown by the ubiquitin smear highlighted by the red bracket), although it is possible there is slightly more caspase 8 that has been precipitated in that lane. By contrast, HEK cells show much less change in caspase 8 ubiquitination which supports the assertion that TRAIL is more selective for tumour cells and cell lines. We anticipate that a repeat of this experiment at 4°C would dramatically increase the caspase 8 signal due to a stabilisation of the modified protein and we predict that U2OS cells would show the same increase in modification following treatment when DCNL5 is present, but not in its absence.

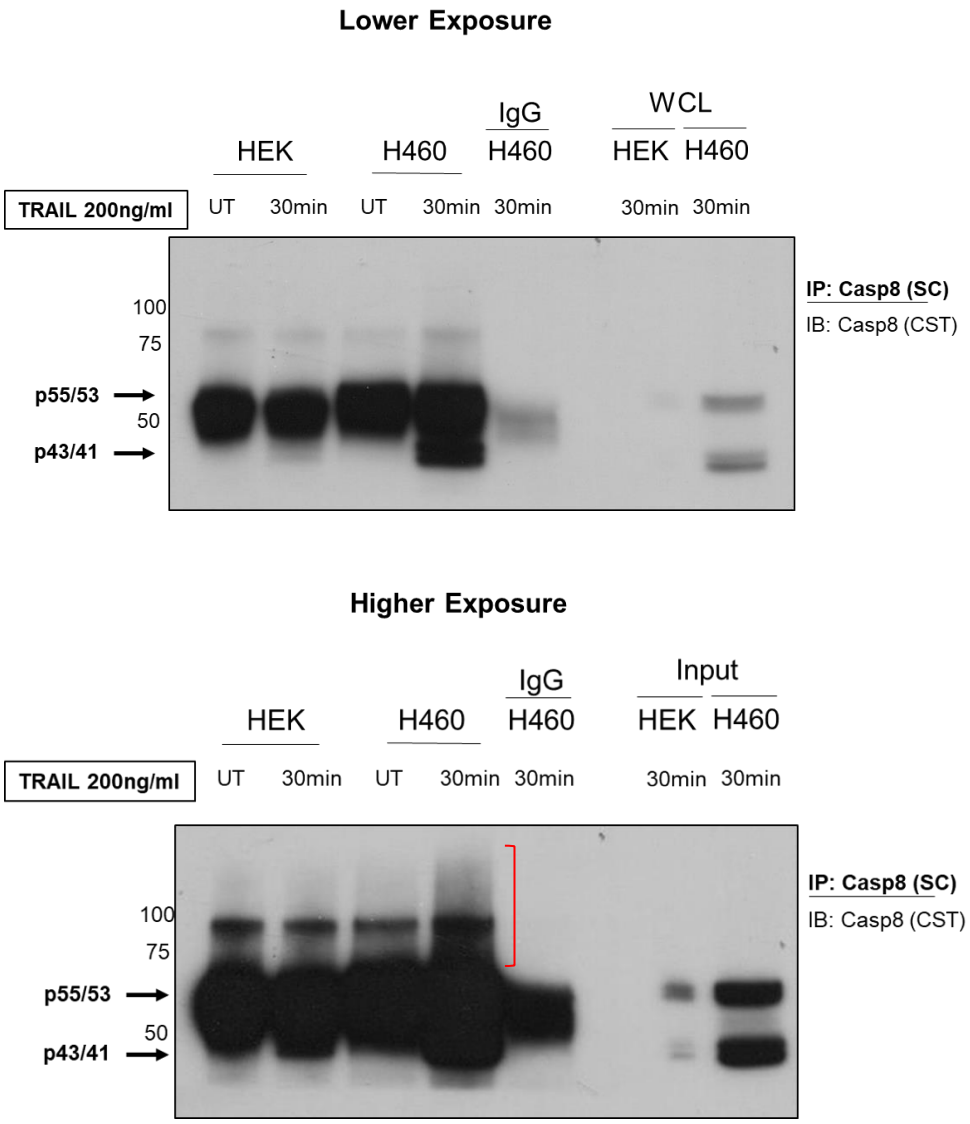



**Figure 5-3 H460 Cells Exhibit an Increase in Caspase 8 Ubiquitination While HEK293 Cells do not.**

This representative western (n = 2) (low and high exposure above and below respectively) shows the result of a denaturing immunoprecipitation reaction performed on H460 and HEK 293 cells with and without 30-minute TRAIL stimulation for 30 minutes. In HEK cells we see no increase in caspase 8 ubiquitination following treatment, however we do see an increase in modification in the H460 cells (highlighted by the red bracket) which supports the fact that TRAIL is selective for tumour cells. There does appear to be slightly more caspase 8 present in the TRAIL-treated lane, however. This also confirms that H460 cells are a useful cell line for studying caspase 8 cleavage and activation.

### 5.2.3 H460 DCNL5 KO Clones and siRNA-Mediated Knockdowns Show Reduced Caspase 8 Cleavage Following TRAIL Treatment

To investigate whether the role of DCNL5 is the same in H460 cells as in U2OS cells, we subjected some of the KO clones we generated (see 5.2.1) to TRAIL treatment and analysed caspase 8 cleavage by western blot. The resulting western blot can be seen in Figure 5-4A. We used two KO clones as well as a WT clone in which the CRISPR-mediated gene inactivation had not been successful. The WT clone shows cleavage of caspase 8 to form both p43/41 and p18 fragments upon TRAIL stimulation. KO clone #22 shows a significant reduction in p18 cleavage following treatment compared to the WT clone, while KO clone #27 shows almost no p18 protein levels, indicating a significant reduction in caspase 8 cleavage in response to the treatment. This data suggests that lack of DCNL5 expression has a clear effect in H460 cells however the variation between the two KO clones requires further investigation – perhaps there is a very small amount of DCNL5 still present which is sufficient to cause some caspase 8 cleavage.

To further validate the role for DCNL5 in H460 cells we used siRNA-mediated gene silencing to inhibit protein expression prior to TRAIL treatment. The result can be seen in the western blot in Figure 5-4B, and it is clear that siRNA-treated H460 cells undergo less caspase 8 cleavage, particularly p18 cleavage, than non-silenced cells. It actually looks as though DCNL5 expression is a bit lower in the treated un-transfected cells than the transfected ones, suggesting that the difference in p18 protein levels may in reality be more significant. This reduction in caspase 8 cleavage is similar to existing data which suggests that only a small reduction in caspase 8 cleavage can represent a significant lack of ubiquitination (Jin *et al.*, 2009). Our siRNA data in combination with the reduced response of KO clones to TRAIL supports the results found in U2OS cells showing DCNL5 is required for cell death and caspase 8 cleavage and provides compelling evidence that the function of DCNL5 is not restricted to one cell line.

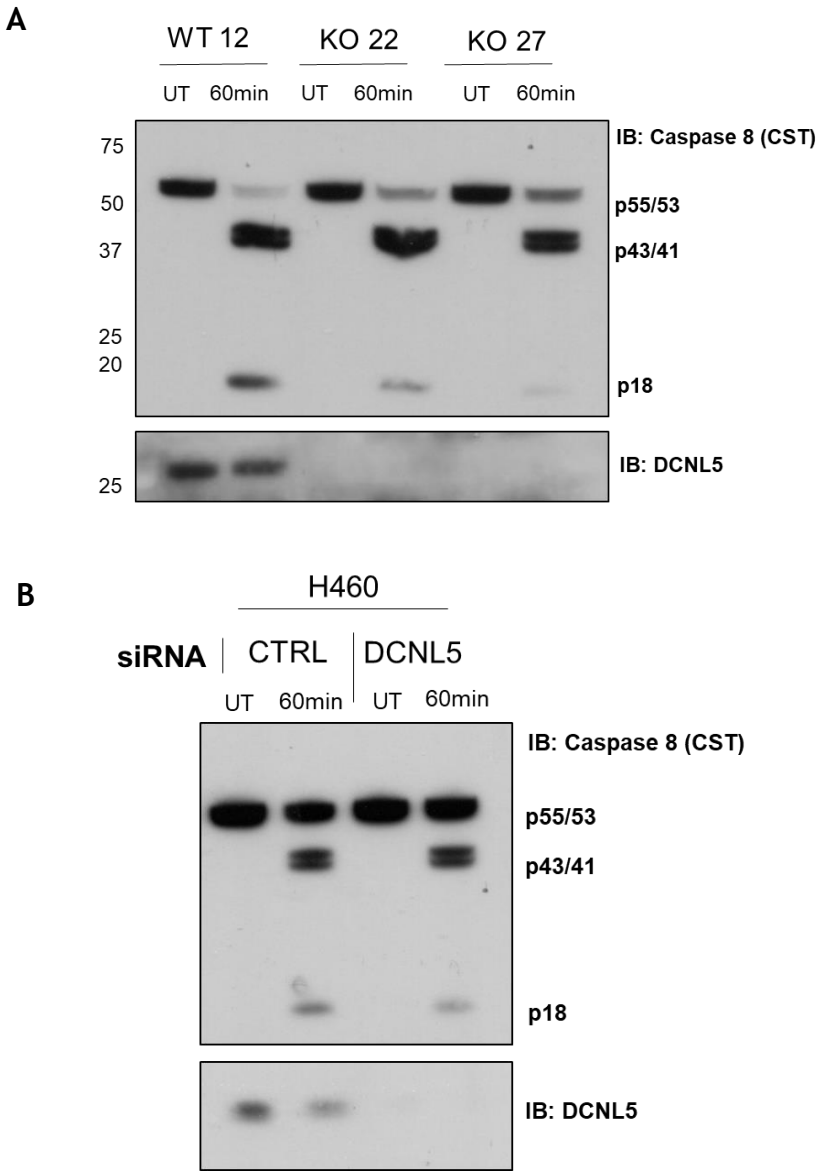



**Figure 5-4 H460 DCNL5 KO Clones Show Reduced Caspase 8 Cleavage and DCNL5 Knockdown has a Similar Effect.**

(A) This representative western blot (n = 2) shows two of the H460 DCNL5 KO clones as well as a WT clone (which has normal DCNL5 expression despite subjecting it to CRISPR/Cas9) and their response to 60 minutes of TRAIL stimulation at a concentration of 200ng/ml. The WT clone shows caspase 8 cleavage to both the p43/41 and p18 forms, while KO clone #22 shows a pronounced reduction in p18 fragment levels after TRAIL treatment, and clone #27 shows an almost total lack of p18 caspase 8 expression. In both KO clones we do still see p43/41 caspase 8 being formed which we saw in U2OS KO cells as well. (B) Representative western blot showing that siRNA-mediated knockdown of DCNL5 in H460 cells produced a similar effect. While not as dramatic as with the KO clones, we do see a marked reduction in p18 caspase 8 levels after TRAIL treatment when DCNL5 expression is inhibited.

### 5.2.4 DCNL5 Knockdown Reduces Caspase 8 Cleavage in Cancer Cells

Having now demonstrated a role for DCNL5 in the caspase 8 activation pathway in U2OS and H460 cancer cell lines, we wanted to investigate this role in more cell lines. We chose A375 cells (skin melanoma), DU-145 cells (prostate carcinoma) and HeLa cells (cervix adenocarcinoma), as well as TRAIL resistant MCF7 (breast adenocarcinoma) cells as negative controls. We used RNAiMAX (ThermoFisher) to transfect each cell line with siRNA targeting DCNL5 for 72 hours (A375 cells were only treated for 48 hours) prior to treating the cells with TRAIL for 60 minutes. U2OS cells were a good positive control for this experiment since we already knew that DCNL5 KO cells exhibit significantly less caspase 8 cleavage than cells expressing DCNL5. siRNA treatment of these cells (shown in Figure 5-5A) caused a significant drop in p43/41 protein levels as well as a drop in p18 levels, however the latter fragment was only faintly visible in un-transfected cells. This independently confirms the role of DCNL5 in U2OS cells, which have been used extensively in this research.

Figure 5-5B shows the effect of DCNL5 knockdown in A375 cells and as we have seen with H460 cells (Figure 5-4B and Figure 5-5C), we can detect a reduction in both cleavage products of caspase 8 following TRAIL stimulation compared to un-transfected cells. The effect of DCNL5 knockdown is strongest in HeLa cells which show a large reduction in p18 caspase 8 compared to un-transfected cells – there is almost no active caspase 8 being formed where DCNL5 expression is inhibited in this cell line. DU-145 cells show no change in caspase cleavage when DCNL5 expression is absent, representing the only cell line we tested that is TRAIL-sensitive to show to no change in caspase cleavage in the absence of DCNL5. 80% of the cell lines we were able to test showed at least some reduction in caspase 8 cleavage in the absence of DCNL5 and previous research has shown that the reduction in caspase 8 cleavage in terms of formation of p43/41 and p18 fragments does not need to be very large in order for the effects to be significant (Jin et al, 2019, supplementary data showing cullin 3 siRNA).

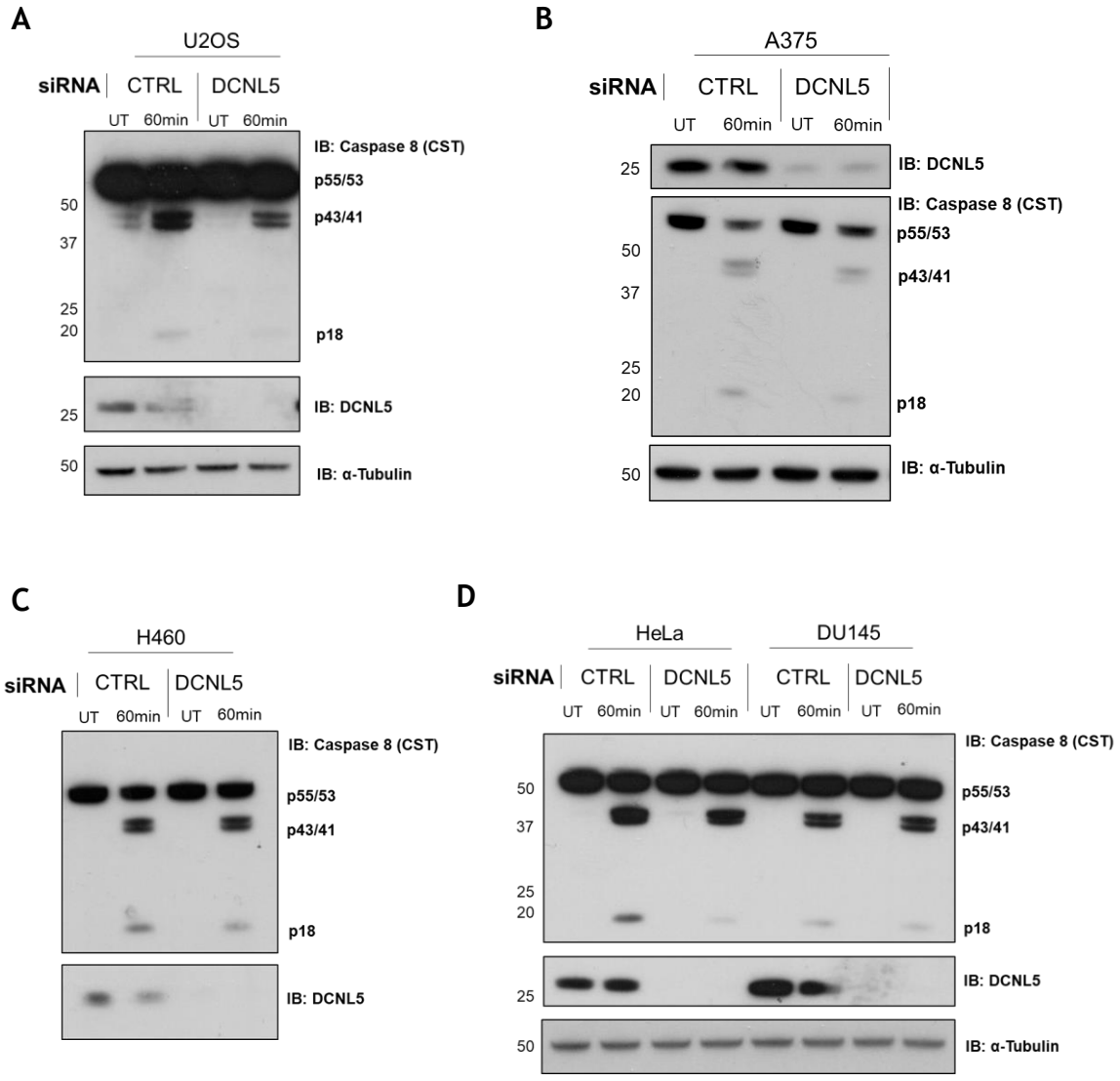



**Figure 5-5 siRNA-Mediated Knockdown in Cancer Cell Lines Reduces Caspase 8 Cleavage.** DCNL5 expression is confirmed by western blot in all panels. (A) This representative western blot shows that inhibition of DCNL5 expression in U2OS cells via siRNA causes a reduction in caspase 8 cleavage, which is consistent with the data from the KO and inducible rescue cell lines used in Chapter 3. (B) Representative western blot showing knockdown of DCNL5 expression in A375 cells also causes a slight reduction in p41/41 and p18 caspase 8 levels following TRAIL stimulation with respect to non-transfected cells. (C) This representative western blot is the same as the previous figure (5-4) and shows reduced caspase 8 cleavage in H460 cells following DCNL5 siRNA. (D) This representative western blot shows the response of both HeLa and DU-145 cells to TRAIL. siRNA-mediated knockdown of DCNL5 in HeLa cells causes a pronounced reduction in p18 caspase 8 compared to un-transfected cells. DU-145 cells show no detectable change in caspase 8 cleavage in the absence of DCNL5 expression.

### 5.2.5 DCNL5 Overexpression May Increase Caspase 8 Cleavage in HeLa Cells

Having established that knockdown of DCNL5 expression in multiple cell lines reduced caspase 8 cleavage in response to TRAIL treatment, we wanted to investigate the effect of overexpressing DCNL5 on this process. We initially used HeLa cells as they showed the strongest response to siRNA suggesting that these cells were susceptible to transient modulation of DCNL5 expression levels. We transfected the cells using Lipofectamine 2000 (ThermoFisher) for 5 hours and allowed the cells to grow for a further 48 hours before treating the cells with TRAIL for 60 minutes. The expression of DCNL5 was greatly increased following the transfection as shown by DCNL5 Western Blot in Figure 5-6. TRAIL stimulation led to caspase 8 cleavage in the presence of TRAIL, and this cleavage was stronger in cells that were overexpressing DCNL5. It is important to note that there does appear to be more pro-caspase 8 present when DCNL5 is overexpressed suggesting that the increase in detectable caspase 8 cleavage may arise due to more precursor caspase 8 being present. Nonetheless, it still appears that there may exist a link between the amount of DCNL5 being expressed in cells and the amount of caspase 8 processing that occurs in response to induction of apoptosis. Absence of DCNL5 results in a reduction of caspase 8 cleavage and increase in DCNL5 expression may result in more processing of the protein, however further validation is required, including using other cell lines.

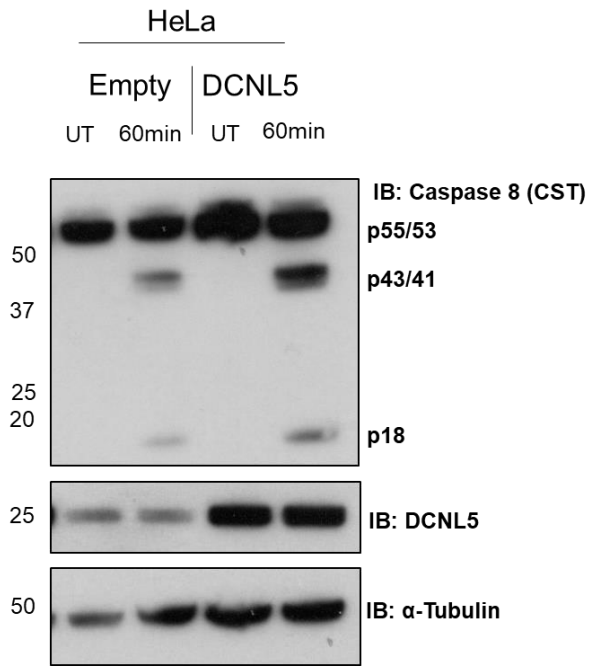



**Figure 5-6 Overexpression of DCNL5 in HeLa Cells Appears to Cause an Increase in Caspase Cleavage.**

Overexpression of DCNL5 in HeLa cells, confirmed here by the increase in protein levels shown by a representative western blot (n = 2), leads to an increase in caspase 8 cleavage following TRAIL stimulation. There is an increase of both p43/41 and p18 caspase 8 levels when DCNL5 expression is increased. There does appear to be globally more pro-caspase 8 with increased DCNL5 expression.

### 5.2.6 MLN4924 Treatment Has no Major Effect on the Response of a Variety of Cancer Cell Lines to TRAIL

The established mechanism for caspase 8 activation involves the ubiquitination of caspase 8 by cullin 3 which is required for cleavage and aggregate formation. To our knowledge, the role of neddylation in this pathway has not been studied, and given that cullin proteins require neddylation for their activity and their roles as the cores of ubiquitin E3 ligases, we wanted to investigate the effect of inhibiting neddylation on the cleavage of caspase 8. We used the neddylation activating enzyme (NAE) inhibitor MLN4924 as we have done previously in this project: 1 hour pre-treatment at a concentration of 3μM. MLN4924 is used in clinical settings as a cancer treatment drug and is actually known to induce apoptosis however in this context of treatments of fairly short duration (2 hours total treatment time) we postulated that short-term treatment would not significantly affect global CRL-governed processes including cell cycle regulation. We have previously shown that MLN4924 reduces but does not entirely inhibit caspase 8 cleavage in U2OS cells and this result is shown again in Figure 5-7B. This result suggested that cullin 3 neddylation is not absolutely required for cleavage of caspase 8 which represents a potentially new role for a cullin protein in its un-neddylated state.

We performed the same experiment using H460, HeLa and DU-145 cells in order to establish whether this result holds up in different cell lines and obtained interesting results. Figure 5-7A shows western blots for each cell line and the success of the MLN4924 treatment can be seen by the presence of only un-neddylated (lower molecular weight) cullin 3 in the lanes where cells were pre-treated with the drug. The H460 cells show a reduction in the levels of the p18 caspase 8 fragment when pre-treated with MLN4924 followed by TRAIL, similar to the U2OS cells shown in panel B. HeLa cells and DU-145 cells however, show an increase in the amount of p18 following pre-treatment with MLN4924 which is not what we expected to see. At the very least it can be said that in these 4 cell lines the neddylation of cullin 3 (or any other cullin is not essential for the processing of caspase 8, with 2 cell lines indicating that this may even enhance the processing.

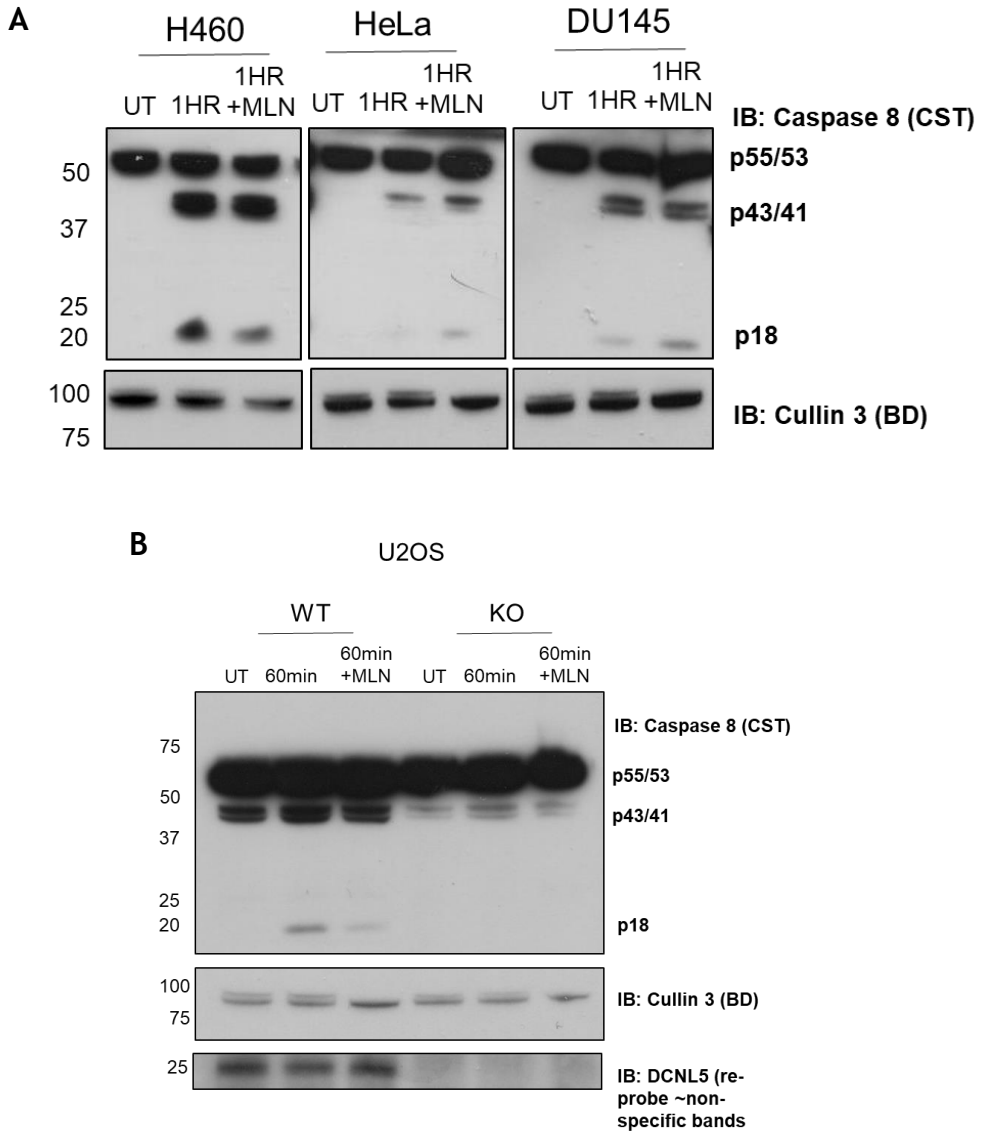



**Figure 5-7 MLN4924 Treatment is Insufficient to Inhibit Caspase 8 Cleavage and May Even Increase Cleavage in Some Cell Lines.**

(A) MLN4924 was used for 1 hour prior to stimulation with TRAIL. Successful inhibition of neddylation is confirmed by the loss of the upper, neddylated band of cullin 3 shown here by representative western blot. In H460 cells MLN treatment reduces p18 caspase 8 levels following TRAIL stimulation. In both HeLa and DU-145 cells MLN treatment actually appears to increase caspase 8 cleavage; a slight increase in p18 protein levels can be observed in both levels with MLN pre-treatment compared to without pre-treatment. (B) This representative western blot showing the effect of MLN treatment on WT and DCNL5 KO U2OS shows that in WT cells MLN treatment causes a reduction in p18 caspase 8, similar to the H460 cells.

### 5.2.7 Cullin 3 siRNA-Mediated Knockdown Has Less of an Effect than DCNL5 Knockdown

In addition to investigating the requirement of cullin 3 to be neddylated to mediate its function in caspase 8 processing, we wanted to establish whether knockdown of the protein had a significant effect on caspase 8 cleavage. Existing data suggests that cullin 3 is absolutely required for the ubiquitination of caspase 8. As with DCNL5, we used siRNA targeting the cullin 3 gene and used RNAiMAX to transfect for 48 to 72 hours prior to TRAIL stimulation. Success of the knockdown was confirmed by western blot for each experiment and in general cullin 3 expression was able to be depleted in most cell lines. H460 cells and A375 cells show strong cullin 3 expression according to our data.

The results of cullin 3 knockdown followed by TRAIL stimulation are shown in Figure 5-8. U2OS cells (Figure 5-8A) show a reduction in p43/41 levels when cullin 3 expression is depleted compared to un-transfected cells following TRAIL treatment. H460 cells show a small reduction in both p43/41 and p18 fragments of caspase 8 following cullin 3 knockdown, as can be seen in the western blot in Figure 5-8B. This result is in line with the data found by Jin and colleagues, the reduction in caspase 8 cleavage does not need to be substantial for it to be indicative of a reduction in ubiquitination (Jin *et al.*, 2009). Figure 5-8C shows that A375 cells also show a reduction in caspase 8 cleavage in the absence of cullin 3 expression (although siRNA was not quite as successful in these cells), it is worth noting that DCNL5 knockdown, shown on the same Western Blot, appears to have a more significant effect than cullin 3 knockdown. As can be seen from the western blots, cullin 3 knockdown was not always completely successful (in the case of H460 cells and A375 cells in particular) therefore the small reduction in caspase 8 cleavage may be due to enough cullin 3 being expressed to mediate processing of the protease. When we performed cullin knockdown using HeLa cells, we unexpectedly found that this actually increased the amount of caspase 8 being cleaved compared to cells expressing cullin 3 normally (see Figure 5-8D).

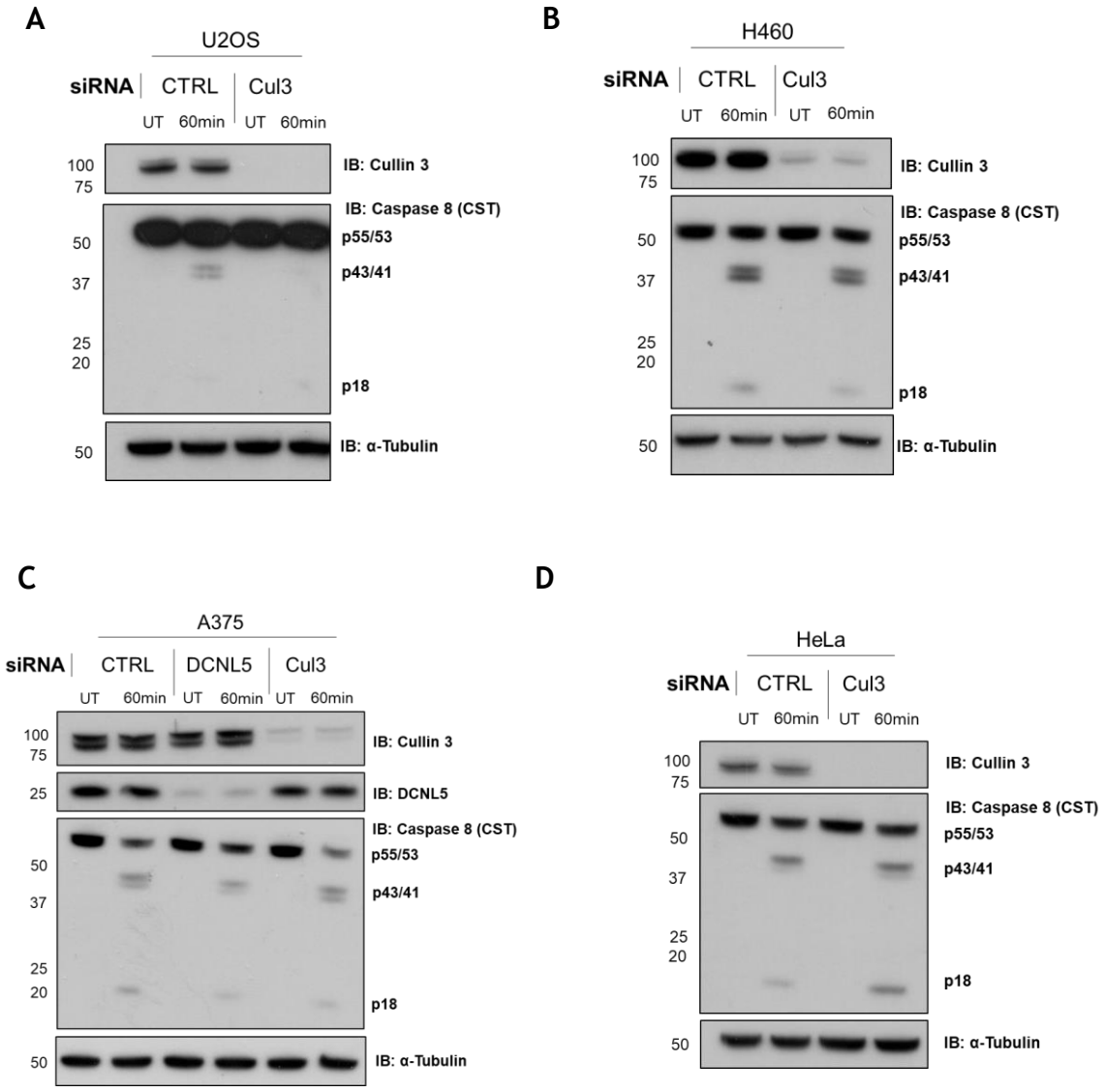



**Figure 5-8 Cullin 3 Knockdown Reduces Caspase 8 Cleavage in U2OS, H460 and A375 Cells, but Increases Caspase 8 Cleavage in HeLa Cells.**

Representative western blot confirming knockdown of cullin 3 expression shown by the reduction in cullin 3 protein levels in (A) U2OS cells and (B) H460 cells, which in turn caused a reduction in p43/41 caspase 8 cleavage. (C) Representative western blot showing that cullin 3 knockdown (as well as DCNL5 knockdown) in A375 cells caused a reduction in caspase 8 cleavage compared to un-transfected cells as a result of TRAIL treatment (D) Representative western blot showing that siRNA-mediated knockdown of cullin 3 in HeLa cells caused in increase in p18 protein levels following TRAIL stimulation compared to un-transfected cells.

In order to ensure that DCNL5 and cullin 3 expression levels have no effect in other pathways that affect caspase 8 cleavage, we used MCF-7 cells which are a breast cancer cell line and are known to be resistant to TRAIL treatment as a negative control. siRNA-mediated knockdown of both proteins, as shown in Figure 5-9, had

no effect on the caspase 8 cleavage following TRAIL treatment. In un-transfected cells we observe no caspase 8 cleavage following induction of apoptosis with TRAIL, which was expected given their insensitivity to the treatment. Knockdown of DCNL5 and cullin 3 expression has no effect. This may seem obvious, but this confirms that the roles for these proteins are specific to mediating caspase 8 cleavage directly and have no off-target effects that may independently contribute to caspase 8 processing. An additional experiment for future work which would better probe the importance of DCNL5 and cullin 3 in this cell line would be to overexpress these proteins and determine if caspase 8 cleavage occurs with excess amounts of these proteins.

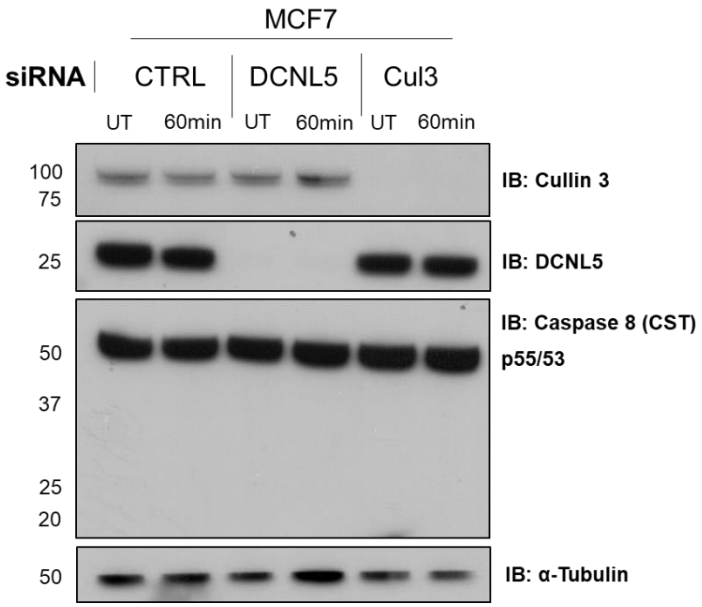



**Figure 5-9 TRAIL Resistant MCF-7 Cells Are Unaffected by Cullin 3 and DCNL5 Knockdown.** Representative western blot showing that caspase 8 cleavage does not occur in MCF-7 cells following TRAIL treatment. Knockdown of DCNl5 or cullin 3 does not change this and these proteins likely act specifically to mediate caspase 8 cleavage directly rather than through another mechanism.

## 5.3 Discussion

### 5.3.1 Summary

We have built upon our finding that DCNL5 is required for caspase cleavage and demonstrated that it likely interacts with cullin 3 to mediate the ubiquitination of the caspase 8. In this chapter we have expanded our analysis to several cancer cell lines, and probed the role of not only DCNL5 in TRAIL-mediated apoptosis, but the role of neddylation in the pathway as well as the role of cullin 3 specifically in caspase 8 ubiquitination. We have shown that the role of DCNL5 is required in multiple cancer cell lines, and that DCNL5 appears to be more important in caspase 8 cleavage than cullin 3. We used MLN4924 to determine the effect of inhibiting cullin neddylation on caspase 8 cleavage. MLN4924 treatment caused slight reductions in the amounts of p18 caspase 8 being formed in U2OS and H460 cells, however it was insufficient to inhibit caspase 8 cleavage completely. Interestingly, DU-145 cells and HeLa cells exhibited a slight increase in p18 caspase 8 levels when pre-treated with the NEDD8 inhibitor compared to without. This result may arise from the role of MLN4924 in enhancing caspase 8 cleavage following TRAIL stimulation, however the treatment time used here was much shorter than in other research. Overall, this data supports the role for DCNL5 in caspase 8 cleavage in numerous cell lines and raises the issue of cullin 3 involvement in these cell lines and the possible function of cullin 3 in the absence of its neddylation as a scaffold protein.

### 5.3.2 DCNL5 is Required for Caspase 8 Cleavage in Multiple Cell Lines

The results in this chapter point to a clear role for DCNL5 in caspase 8 cleavage in not only U2OS cells as used throughout this research, but also in other cancer cell lines. We successfully generated DCNL5 CRISPR/Cas9 KO cells using H460 cells to aid in our investigation of the role of DCNL5. These cells were selected based on their sensitivity to TRAIL and their extensive use in caspase 8 research, including the seminal work by Jin and colleagues in 2009. The KO clones both showed reduced p18 caspase cleavage in response to TRAIL compared to WT cells, and this result was mirrored by siRNA-mediated knockdown of DCNL5 in WT H460 cells

which also exhibited a reduction in caspase 8 cleavage. This provides an indication that the amount of DCNL5 being expressed in cells may relate directly to the amount of caspase 8 which is able to be cleaved. This data supported the findings in U2OS cells and suggested that the role of DCNL5 may be conserved in multiple different cell lines.

In order to determine if our findings are more generally applicable with regards to the role of DCNL5 in cancer signalling, we wanted to test more cell lines for the importance of DCNL5 by using siRNA to ablate the levels of the protein and compare transfected to un-transfected cells in response to TRAIL. We were able to use cell lines from different cancers including A375 cells (skin melanoma), DU-145 cells (prostate cancer), HeLa cells (cervical cancer) and MCF7 cells (breast cancer). MCF7 cells are known to be unresponsive to TRAIL stimulation due to a combination of high ratio of decoy TRAIL receptors to functional TRAIL receptors (Sanlioglu *et al.*, 2005) which may in in part due to the constitutive endocytosis of death receptors 4 and 5 (Zhang and Zhang, 2008). In all the other cell lines we tested except for DU-145 cells, we found that DCNL5 knockdown resulted in a decrease in caspase 8 cleavage. The extent to which caspase 8 cleavage was impaired varied from one cell line to the next, with HeLa cells showing the most significant reduction in cleavage.

The lack of role for DCNL5 in DU-145 prostate cancer cells may arise from the reduced sensitivity of this cell line to TRAIL stimulation. General consensus on these cells seems to be that they are not particularly sensitive to apoptosis induction using recombinant TRAIL, which we have employed in the present report. Research has shown that DU-145 cells (as well as other prostate cancer cell lines) are resistant to 100ng/ml to recombinant TRAIL and require co-stimulation with doxorubicin in order to induce cytotoxicity (Voelkel-Johnson, King and Norris, 2002). It was also shown that adenoviral delivery of full-length TRAIL was able to induce cell death in DU-145 cells as well. Insensitivity of DU-145 cells has also been shown by other researchers (ref Wei et al, 2018) and other work has shown DU-145 cells' resistance to adenoviral delivery of full length TRAIL, which was linked to elevated expression of decoy TRAIL receptor (DcR2).

siRNA-mediated knockdown of DcR2 was able to sensitise the cells to TRAIL (Sanlioglu *et al.*, 2007).

In contrast to other research, DU-145 cells have also been shown to be sensitive to recombinant TRAIL and this was shown to be associated with the expression of a protein called hPEBP4 which is involved in phosphatidylethanolamine binding and is preferentially expressed in breast and ovarian cancer cells. DU-145 cells were shown to express low levels of hPEBP4 which rendered them sensitive to TRAIL while other prostate cancer cells (LNCaP) were found to highly express the protein which conferred the reduced sensitivity to TRAIL (Li *et al.*, 2007). The conflicting data regarding the sensitivity of DU-145 cells to TRAIL makes it difficult to fully interpret our data showing that DCNL5 knockdown made no significant impact on caspase 8 cleavage. The fact that we see cleavage at all suggests that the cells are indeed sensitive to TRAIL on its own and require no additional stimulation or co-treatments, and therefore in this particular cell line DCNL5 may not play a crucial role in caspase 8 cleavage.

As a follow up to the results seen in HeLa cells, which showed the most dramatic reduction in caspase 8 cleavage following siRNA-mediated knockdown of DCNL5 expression, we wanted to determine the effect of overexpressing DCNL5. When compared to un-transfected cells, DCNL5 overexpressing HeLa cells showed a noticeable increase in caspase 8 cleavage when stimulated with. This contrasted neatly with the siRNA data, clearly linking DCNL5 protein levels to caspase 8 cleavage. HeLa cells have been shown to be TRAIL sensitive and have been used extensively for studying TRAIL signalling and caspase 8 activity (Seol *et al.*, 2001; Mohr, Yu and Zwacka, 2015) and thus represent a useful cell line to include in the present research. HeLa cell sensitivity to TRAIL has been shown to depend on a number of factors, including death receptor expression with DR4 expression conveying TRAIL sensitivity in these cells (Mohr, Yu and Zwacka, 2015). Interestingly, some research has shown that HeLa cells contain large amounts of DR5 within the nucleus which has been shown to confer resistance to TRAIL (Kojima *et al.*, 2011), seemingly at odds with the finding that DR4 is the essential mediator of TRAIL signalling in this cell line. The inconsistency in response of HeLa

and other cell lines to TRAIL further highlights the difficulty in studying caspase 8 signalling given the complexity of the pathways.

The consistency in being able to reduce caspase 8 cleavage by inhibiting DCNL5 expression suggests that the role of the protein is critical in many cancer cell lines. DCNL5 may in fact be a general regulator of apoptosis signalling in many cancer cell lines, and based on our results, DCNL5 not playing a role may be an exception rather than the rule. We have previously shown that DCNL5 KO cells are still able to recruit caspase 8 to the DISC in our cullin 3 co-IP experiment which indicates that the role of DCNL5 is likely not in the coordination of caspase 8 binding to the DISC. The deficiency in caspase 8 cleavage has been shown to be DAD-patch dependent by viability assays as well as by western blotting, strongly indicating that cullin proteins may in some way involved in the interaction of DCNL5 in this pathway. Technically, it is possible that DCNL5 interacts with another protein via the DAD patch region in its C-terminal PONY domain.

We have however shown that DCNL5 interacts with cullin 3 *in vivo* for the first time and this lends support to the hypothesis that DCNL5 is interacting with cullin 3 to mediate its neddylation and thus activity. MLN4924 treatment was not as severe in inhibiting caspase 8 cleavage as the lack of DCNL5 expression, which indicates that the function of DCNL5 may occur independently of its neddylation activity. This may suggest that cullin 3 may function as a scaffold protein and given that it is known to interact with caspase 8 *in vivo*, perhaps DCNL5 plays a role in coordinating this interaction. To date, no published data supports a role for DCNL proteins outside of their interactions with cullins, therefore further work is required to establish the binding partner(s) for DCNL5 in this pathway and whether the DAD-patch is an important region for binding to other proteins. We have shown in Chapter 3 that DCNL1 KO cells are sensitive to TRAIL and CHX/TNFα combination treatments but have looked at the role of DCNL3 which is known to neddylate cullin 3 at the plasma membrane (Meyer-Schaller *et al.*, 2009). DCNL1 is the best studied and most ubiquitously expressed DCNL protein and is also known to promote cullin 3 neddylation albeit in a different physiological context (Huang *et al.*, 2017) so it seems likely that some specific characteristic of DCNL5 enables it to localise and interact with the DISC and caspase 8 to promote neddylation of

a cullin protein, possibly a non-cullin protein or simply interact with a protein via its DAD-patch. The likely region of DCNL5 that may mediate this specific interaction in the apoptosis response is the N-terminus which is the least conserved region between the DCNL proteins and is predicted to be unstructured (Keuss *et al.*, 2016). It is possible that despite the similarity between the C-terminal domain of DCNL proteins, only DCNL5 is capable of associating with cullin 3 in this context, and our data suggests that this role may not even involve the transfer of NEDD8 and that DCNL5 may play a key structural role in DISC assembly or caspase 8 activation unable to be performed by other DCNL family members. Our data could therefore indicate a new function for DCNL5 and may be the first evidence for new binding partners for this family of proteins.

### 5.3.3 Neddylation is not Essential for Caspase 8 Cleavage

Cullin proteins are known regulators of caspase 8 ubiquitination in both pro and anti-apoptotic roles. Neddylation is an essential step in the activation of CRL complexes and un-neddylated cullins are thought to be non-functional however still important in forming complexes with CAND1 for efficient substrate exchange and rapid CRL assembly with different substrate adaptors (Wu *et al.*, 2013). We wanted to investigate the effect of inhibiting neddylation prior to and during TRAIL stimulation to determine whether the lack of cullin neddylation would have an impact on caspase 8 ubiquitination. We were specifically wanting to determine whether inhibition of cullin 3 neddylation, which is the only known positive regulator of caspase 8 ubiquitination in extrinsic apoptosis (Jin *et al.*, 2009), would reduce or totally inhibit caspase 8 processing in cells.

Inhibiting neddylation had differing effects on different cell lines. U2OS as well as H460 cells displayed a reduction in caspase 8 cleavage but the formation of catalytically active p18 form was not entirely inhibited. This suggests that caspase 8 processing is able to occur in the absence of the cullin 3-RBX1-based E3 ligase activity (or any active CRL E3 ligase for that matter) which to our knowledge is a novel finding in the context of the role of cullin 3 in this pathway. Another potential explanation for this result is that cullin 3 may play a role which is independent of its activity as the core of a CRL complex, perhaps as a scaffold protein, it would be interesting to determine whether CAND1 interacts with the

DISC since cullin 3 is likely in complex with this protein during MLN4924 treatment. In DU-145 and HeLa cells, MLN4924 treatment actually increased the levels of catalytically active caspase 8 compared to un-treated cells. Interpreting this result is difficult since as described in section 5.1.3, MLN4924 treatment has been shown to promote caspase 8 activation following both TNFα and TRAIL-mediated apoptosis induction.

The research into the role of MLN4924 in extrinsic apoptosis has generally involved exposing cells to longer treatments with the drug, from 6 hours up to 72 hours (Wolenski *et al.*, 2015; Zhao *et al.*, 2011; Chen *et al.*, 2016). Over longer treatment times, MLN will cause accumulation of CRL substrates which are no longer ubiquitinated and degraded including cell cycle regulators which can lead to cell cycle arrest and cell death by other pathways, which may result in cleavage of caspase 8 indirectly. In our research MLN4924 is only used for 2 hours, which is still sufficient to completely inhibit neddylation of all cullins, and this treatment is a significantly shorter time period and less likely to result in off-target effects due to reduction in degradation of CRL substrates. As a result, it is likely that any alterations in caspase 8 cleavage we detect are likely due to the specific inhibition of cullin neddylation. This presents an important issue with regards to the established mechanism involving cullin 3-mediated polyubiquitination of caspase 8: why is caspase 8 cleavage enhanced in the absence of CRL activity? It is possible that the lack of cullin neddylation and thus CRL E3 ligase activity may have an effect on anti-apoptotic modifications of caspase 8 as well as the pro-apoptotic regulation by cullin 3 (see Figure 5-1 for a summary of caspase 8 regulation).

If cullin-based E3 ligases which normally function to inhibit caspase 8 cleavage and activation (e.g. cullin 7) are inactivated by MLN4924, would this account for the increase we see in caspase 8 processing in some cell lines? This may be the case despite the lack of cullin 3 activity in activating caspase 8. It is perhaps a question of the balance of pro and anti-apoptotic ubiquitination modifications that are occurring at the level of caspase 8 which control the degree of caspase 8 cleavage. As mentioned in section 5.1.2, TRAF2 mediates the polyubiquitination of caspase 8 in its p18 catalytic subunit after cullin 3 mediates the polyubiquitination of caspase 8 at its p10 catalytic subunit (Jin *et al.*, 2009;

Gonzalvez *et al.*, 2012). This modification by TRAF2 serves to trigger degradation of catalytically active caspase 8 upon release into the cytosol, thereby setting s shutoff timer for caspase 8 activity. In the context of neddylation, research has shown that another TRAF family member, TRAF6, undergoes neddylation at lysine 124 which is required for it ability to mediate NF-κB activity (Liu *et al.*, 2019). It is therefore possible that NEDD8 also plays a role in TRAF2 activity and MLN4924 treatment may inhibit this negative regulation of caspase 8 and result in less caspase 8 being degraded in the cytosol and therefore more caspase 8 cleavage and activity. It is therefore possible that the negative regulation by some E3 ligases is stronger than the positive regulation by cullin 3.

As far as we are aware, this is the first time MLN4924 has been studied in the context of caspase 8 cleavage via cullin 3-mediated ubiquitination events. Future work in this area could focus on looking at the role of other cullin proteins in caspase 8 activations via siRNA as well as investigating peptides to block different DCNL proteins following the work by Monda and colleagues in determining the structure of DCNL proteins in complex with NEDD8 E2 enzymes (Monda *et al.*, 2013). More recently, inhibitors have been designed which specifically block neddylation of cullin 3 in cells, the first of which was called DI-591. This cell-permeable compound was shown to bind to DCNL1 and DCNL2 with KD values of 21.9nM and 11.2nM respectively (Zhou *et al.*, 2017). Unexpectedly, this compound was shown to specifically reduce the neddylation of cullin 3 only while having minimal effect on the neddylation of other cullin proteins (Zhou *et al.*, 2017). This inhibitor would be useful in the context of hour work, to specifically target cullin 3 neddylation rather than having to inhibit the neddylation of all cullin proteins will MLN4924. This would more accurately tell us the importance of cullin 3 neddylation.

In the work by Jin and colleagues (Jin *et al.*, 2009) they showed that cullin 3 knockdown resulted in a significant reduction in caspase ubiquitination however they did not detect such a dramatic change at the level of caspase 8 cleavage. This suggests that a small reduction in caspase 8 cleavage can represent much more significant reductions in caspase 8 polyubiquitination. In our work we have been able to detect more significant reductions in caspase 8 cleavage than they

showed, suggesting that the impairment in caspase 8 ubiquitination might be even more severe. The fact that DCNL5 knockdown appears to have more of an effect on caspase 8 cleavage than the loss of E3 ligase activity (via neddylation inhibition) may provide insight into the role DCNL5 plays. We have shown that DCNL5 involvement is dependent on DAD-patch interaction, however it may be that cullin neddylation is not an essential step and we may therefore be looking at a neddylation-independent role for cullin 3 as well as DCNL5, as mentioned above.

### 5.3.4 Role of Cullin 3 in Apoptosis is Ambiguous

To further probe the role of cullin 3 in caspase 8 activation in multiple cancer cell lines, we used siRNA to mediate the knockdown of cullin 3 expression prior to stimulation with TRAIL. The importance of neddylation has been questioned by our use of MLN4924 and appears largely to be cell line dependent. We suspected therefore that the role of cullin 3 may follow a similar trend. TRAIL stimulation resulted in less caspase 8 cleavage when cells were treated for cullin 3 siRNA in U2OS, H460 and A375 cell lines. U2OS and H460 cells also showed slight reductions in caspase 8 cleavage following MLN4924 pre-treatment (as discussed in 5.3.3) which mirrors the results seen here. HeLa cells, unexpectedly, showed an increase in caspase 8 cleavage following knockdown of cullin 3 expression. This result also mirrored the MLN4924 data which also showed an increase in caspase 8 processing when neddylation was inhibited. Taken together, this data brings into question the importance of cullin 3 in caspase 8 cleavage. We weren't able to successfully analyse caspase 8 ubiquitination (since we did not perform treatments at 4°C as has been shown to increase signal) so cannot definitively conclude that the lack of caspase cleavage is as a direct result of a lack of ubiquitination, however it has been shown, as mentioned previously, that even a slight reduction in caspase 8 cleavage can be indicative of a dramatic reduction in caspase 8 ubiquitination (Jin *et al.*, 2009). When analysing our cullin 3 knockdown data, it is important to note that incomplete cullin 3 knockdown we saw for some of the cell lines may explain why we see only small reductions in caspase 8 cleavage. Optimisation of this process would clarify the importance of cullin 3 in these cell lines.

Given the well-established role of cullin 3 and its unique function in regulating caspase 8 activity in a positive manner, the finding that HeLa cells appear to

undergo more caspase 8 cleavage in the absence of cullin 3 expression is puzzling. In the case of MLN4924 treatment it can be interpreted that the neddylation inhibitor prevents activation of CRLs and therefore CRL-mediated negative regulation of caspase 8, which may explain the reduction rather than total loss of caspase 8 cleavage, or the increase in caspase 8 cleavage depending upon cell line in question. For cullin 3 knockdown, the increased caspase 8 processing may also occur as a result of a loss of downstream ubiquitination events mediated by cullin 3-based CRLs which may play a feedback role in caspase 8 activation. The other possibility is that in the absence of cullin 3 expression, another cullin protein is able to mediate the ubiquitination of caspase 8 in a pro-apoptotic manner, however to date no other cullins have been known to play such role but this would be an interesting hypothesis to analyse further.

Our data supports previous findings (Jin *et al.*, 2009) that cullin 3 knockdown results in a reduction in caspase 8 cleavage as detectable by western blot in H460 cells. In our hands, U2OS and A375 cells show a similar dependence on cullin 3 for caspase 8 processing. The fact that we only see reductions in cleavage and not a total loss appears to indicate that there are other factors controlling caspase 8 processing beyond cullin 3 which is an interesting finding and one which requires further investigation. The current picture of cullin 3 as being essential and unique in promoting caspase 8 cleavage may not be fully correct. The established mechanism for cullin 3 in mediating caspase 8 may therefore require some revision to account of cell-line specific differences or at the very least, further investigation into other pro-apoptotic regulators. Our use of MLN4924, and thus the investigation into the importance of neddylation, indicates that there may be non-cullin E3 ligases that play a role in activating caspase 8 and our data looking at cullin knockdown highlights the potential role of another cullin protein in regulating caspase 8 ubiquitination in a pro-apoptotic manner. It is also possible that there are other processes governing caspase 8 activity in a positive manner that are ubiquitin dependent but do not involve cullins or the direct ubiquitination of caspase 8. The balance of caspase 8 regulators may dictate the degree of caspase 8 cleavage and activity in cells and this may be regulated by both ubiquitination as well as by neddylation.

# 6 General Discussion

## 6.1 Overview of Findings

The NEDD8 E3 ligase family of proteins known as DCNL proteins, have well established roles in promoting cullin neddylation and thus regulating ubiquitination activity of CRL complexes. There are some limited unique roles for individual DCNL proteins that have been described, however little work has been undertaken into studying the physiological role of DCNL5. In the present work, we have uncovered a novel role for DCNL5 in the apoptosis response. We have shown that in the absence of DCNL5, U2OS cells exhibit a significantly reduced the ability to undergo apoptosis and this stems from a lack of caspase cleavage activity. We demonstrated that DCNL5 KO cells lack caspase 8 cleavage which is the crucial initiator caspase protein involved in mediating extrinsic apoptosis.

We have shown for the first time that DCNL5 is able to interact with cullin 3 in cells at endogenous expression levels, and this is important because cullin 3 is well established as the key positive regulator of caspase 8 activity via polyubiquitination. Our findings support a model whereby DCNL5 may regulate cullin 3 neddylation (or at least interact with cullin 3) to promote its activity as the core of as an as yet uncharacterised CRL complex, perhaps independently of its neddylation, that promotes the polyubiquitination of caspase 8 which is required for its dimerization and cleavage. Using a variety of different cancer cell lines, we have provided compelling evidence that the role of DCNL5 is conserved across different tissue culture cells by manipulating DCNL5 expression using siRNA.

In addition, we have also discovered that while the importance of DCNL5 is conserved across the cancer cell lines we tested, the importance of neddylation is less clear. Using the neddylation inhibitor MLN4924, we showed that while caspase 8 cleavage is reduced by the drug, some cleavage still occurs in the absence of neddylation. This is significant because the established mechanism governing caspase 8 cleavage involves the interaction of neddylated cullin 3 with caspase 8 to promote its polyubiquitination. What was unusual was that in some cell lines, including HeLa cells, MLN4924 actually increased caspase 8 cleavage which seems to be in direct contradiction of the established literature.

Interpretation of our results is difficult because multiple NEDD8-dependent E3 ligases regulate caspase 8 activity in a negative manner, raising the possibility that MLN4924 inhibits this negative regulation as well as the positive regulation by cullin 3. We also queried the role of cullin 3 in caspase 8 cleavage by inhibiting expression of the protein by siRNA. We found that in U2OS and H460 cells, that this resulted in a reduction in caspase 8 processing, but in HeLa cells, we found that the lack of cullin 3 protein levels actually increased caspase 8 cleavage. Overall, our work presents evidence for a key role of DCNL5 in caspase 8 cleavage, and provides initial evidence for the role of cullin 3 as a scaffold-type protein which occurs independently of its neddylation, but also raises the possibility of other cullin proteins playing a role in promoting caspase 8 cleavage in the absence of cullin 3. Our results support the notion that the apoptosis signalling pathway is different in different cell types and thus further research is required to determine a unified model for caspase 8 cleavage and activation.

## 6.2 A New Role for DCNL5

A key finding in this study is the discovery of a new physiological role of DCNL5. Our work is the first time any of the DCNL family members have been investigated for their role in the apoptosis response, and given that ubiquitination is a key regulator of various facets of apoptosis, it is perhaps unsurprising that there may be roles for these proteins in this pathway. We have robustly demonstrated that DCNL5 plays a part in caspase cleavage, having shown that the key initiator caspase, caspase 8, fails to undergo cleavage in the absence of DCNL5. We showed that the role of DCNL5 is contingent on its ability to interact via its DAD-patch, which is responsible for cullin-binding (Kurz *et al.*, 2008) but may theoretically mediate interactions with other proteins. Given that there is significant promiscuity in function of the DCNL proteins in cells (Keuss *et al.*, 2016; Monda *et al.*, 2013) we anticipated that the role we had discovered for DCNL5 was unique and this assertion was supported by the finding that DCNL1 is not essential in this pathway and absence of DCNL1 did not confer resistance to death. We did not interrogate the role of any other of the DCNL proteins, but given that only DCNL1, DCNL3 and DCNL5 have had unique roles described in the literature (Meyer-Schaller *et al.*, 2009; Keuss *et al.*, 2016; Thomas *et al.*, 2018; Wu *et al.*, 2011;

Bommeljé *et al.*, 2014; Broderick *et al.*, 2010), future work could probe the role of DCNL3 in this pathway in addition to DCNL1.

The N-terminus of DCNL5 contains a nuclear localisation sequence which has shown to be essential for its neddylation activity *in vivo* (Bommeljé *et al.*, 2014) however the rest of the N-terminus is uncharacterised. It therefore remains a possibility that DCNL5 is uniquely able to play a role in the apoptosis response due to its N-terminus interacting with one or more proteins. Paradoxically, we show that DCNL5 translocates out of the nucleus into the cytosol following TRAIL stimulation. Does DCNL5 retain its neddylation activity outside the nucleus? And how is DCNL5 shuttled out of the nucleus in response to the stimulus? The *in vitro* data suggests that all DCNL proteins can neddylate all cullins so it is widely accepted that the limitation for DCNL-cullin interaction and neddylation is subcellular localisation in cells, therefore if DCNL5 is able to localise to another subcellular compartment, it should presumably be able to neddylate the available cullin proteins. DCNL1 has been shown to undergo monoubiquitination mediated by the E3 ligase NEDD4-1 and subsequent export out of the nucleus (Wu *et al.*, 2011). It was suggested that the N-terminal UBA domain mediates interaction with ubiquitin or ubiquitin-linked NEDD4-1 to promote the monoubiquitination of DCNL1 at lysine residues within its PONY domain (Wu *et al.*, 2011). It is therefore a possibility that DCNL5 undergoes monoubiquitination in its PONY domain which is the driving mechanism behind its nuclear export in the context of TRAIL signalling. DCNL5 lacks the UBA domain which is required to promote this monoubiquitination, so this hypothesis requires further investigation – mono-ubiquitination of DCNL5 has not been seen experimentally before.

In addition to showing that DCNL5 translocates out of the nucleus in response to TRAIL, we also demonstrated that DCNL5 interacts with cullin 3 in U2OS cells at endogenous expression levels for the first time. This represents the first interaction of DCNL5 with any specific cullin in cells, and provides evidence for the potential role of DCNL5 in promoting the neddylation of cullin 3 which is required for its ability to polyubiquitinate caspase 8, in coordination with RBX1 (Jin *et al.*, 2009). As mentioned previously, DCNL3 has been shown to neddylate cullin 3 (Meyer-Schaller *et al.*, 2009) although there is evidence that it doesn't

actually possess E3 ligase activity and serves to inhibit DCNL1-mediated neddylation of cullins (Huang *et al.*, 2014). This finding is at odds with the convincing evidence that showed that ectopic expression of human DCNL3 (as well as DCNL5) in yeast Dcn1Δ cells restored Cdc53 (yeast cullin homologue) neddylation (Meyer-Schaller *et al.*, 2009), and can therefore be disregarded. DCNL1 has also been shown to neddylate cullin 3 to promote midbody localization and activity of $Cul3^{KLHL21}$ during abscission (Huang *et al.*, 2017). The literature suggests that cullin 3 neddylation is regulated by 2 of the DCNL family members, so while we have shown that DNCL5 interacts with cullin 3, we cannot definitively say that DCNL5 promotes its neddylation in response to TRAL stimulation, however this is one hypothesis for its role in this pathway.

Our data using the cullin non-binding DAD-patch mutant form of DCNL5 has consistently indicated that DCNL5's role in promoting caspase 8 cleavage requires cullin binding, which supports the model in which DCNL5 neddylates cullin 3 to increase caspase 8 polyubiquitination. It is possible that DCNL5 interacts with another protein via its DAD patch; this highly charged (acidic) region which normally interacts with a positively charged region in cullin proteins (Kurz *et al.*, 2008) may also play roles in binding of other proteins via charged interactions and further experiments are required to probe this hypothesis further. Our data using the neddylation inhibitor MLN4924 indicates that neddylation may not be essential in all cancer cell lines which may support a non-cullin interaction mediated by DCNL5 to promote caspase 8 cleavage. If this were to be the case, this would indicate a novel, cullin-independent role for DCNL5 and perhaps all DCNL proteins.

It would have been interesting to use some of the recently described DCNL inhibitors which tend to specifically inhibit cullin 3 neddylation. Multiple of these inhibitors have been synthesized and characterised which specifically inhibit the interaction between DCNL1 (and to a lesser extent DCNL2) and the acetylated N-terminus of UBC12 which tend to preferentially reduce cullin 3 neddylation in cells (Zhou *et al.*, 2017; Scott *et al.*, 2017) as well as cullin 1 neddylation (Kim *et al.*, 2019). It has been shown that despite the conserved interaction between DCNL PONY domains and the acetylated N-termini of NEDD8 E2 enzymes, the affinities of these interactions vary greatly, with DCNL4 and DCNL5 exhibiting less

interaction with either E2 enzyme than DCNL1 and DCNL2 (Monda *et al.*, 2013). This suggests that while the PONY domains are well conserved between then DCNL proteins, some of their *in vivo* functions may differ due to the different affinities towards E2 enzymes and possibly other binding partners. It is difficult to know whether the cullin 3 specificity exhibited by these DCNL inhibitors is common to all cullin 3-based CRLs or simply a subset which are neddylated by DCNL1 and DCNL2 which as mentioned above, tend to be targeted by this class of inhibitors. In terms of DCNL5, it is possible that in conjunction with its distinct N-terminal region, the C-terminal region of the protein may confer specific binding properties which allows it to play a unique role in caspase 8 cleavage.

One of the most intriguing results we obtained, was the finding that DCNL5 KO cells lack a ubiquitination event that may or may not be caspase 8 polyubiquitination. We used TUBEs to bind polyubiquitinated proteins in TRAIL-treated lysates and found that in WT cells but not KO cells, un-modified full length and p43/41 caspase 8 co-precipitated with a polyubiquitinated protein. We cannot say what protein is failing to undergo polyubiquitination in the KO cells however it is highly likely that this event is required for caspase 8 cleavage and involves DCNL5 and this may represent the critical event which DCNL5 regulates. It is possible that another DISC-associating protein fails to become ubiquitinated and this results in less caspase 8 being processed in the absence of DCNL5.

A non-catalytic role for caspase 8 has recently been described in which caspase 8 acts as a scaffold for assembly of a structure which has been termed the FADDosome which consists of caspase 8 FADD and RIP1 and this complex leads to NF-κB-dependent inflammation (Henry and Martin, 2017). This complex was shown to form following TRAIL induction and caspase 8 (crucially including catalytically inactive caspase 8) was shown to be required for this complex formation and specifically recruits RIP1 to promote inflammation (Henry and Martin, 2017). In this role caspase 8 acts as a scaffold for complex assembly, and this complex involves downstream polyubiquitination of RIP1 which may therefore be what is being pulled on in our TUBE experiment, since we detect unmodified caspase 8. The role of this complex in the context of cell death and the role of DCNL5 in this complex is unclear, especially since we have comprehensively shown that caspase

8 fails to undergo cleavage in the absence of DCNL5. If anything, we would perhaps expect that this complex forms more readily in the DCNL5 KO cells since caspase 8 processing is lacking and thus may more readily function as a scaffold for complex assembly.

There exists another complex containing caspase 8 and is involved in apoptosis, called the Ripoptosome (Tenev *et al.*, 2011). The complex contains RIP1, FADD and caspase 8 (similar to the FADDosome) and it has been established to assemble in response to genetic stress-induced depletion of IPAs including XIAP, cIAP1 and cIAP2 (Tenev *et al.*, 2011). The complex is also negatively regulated by cFLIP and has been shown to assemble independently of death receptor mediated signalling (Tenev *et al.*, 2011; Feoktistova *et al.*, 2011). The Ripoptosome can promote both caspase-dependent cell death or caspase-independent cell death (Tenev *et al.*, 2011) but given that it forms as a result of inhibition of IAP expression, it is unclear if we are detecting formation of this complex in our TUBE experiment. IAPs have ubiquitin E3 ligase activity and regulate the apoptosis response (Ni, Li and Zou, 2005) and they have been tentatively described as possessing NEDD8 E3 ligase activity (Broemer *et al.*, 2010). This complex is inactivated by polyubiquitination of RIP1 and possibly caspase 8 by IAPs and it is likely this modification that we would be detecting in the WT cells if this is the complex that we are precipitating with the TUBEs. Much more work is required to elucidate which polyubiquitinated protein we precipitated in WT cells and too understand how DCNL5 coordinates this modification.

## 6.3 A Potential Neddylation Independent Function for Cullin 3

As mentioned throughout this thesis, the established mechanism for cullin 3-mediated ubiquitination of caspase 8 involves RBX1 but this protein was not shown to associate with the DISC (Jin *et al.*, 2009). It is possible that this is due to the transient nature of this interaction or perhaps due to the reduced stability of RBX1 in its interaction with the DISC. This may perhaps indicate that cullin 3 plays a structural role in caspase 8 regulation, independently of its interaction with the RING protein. This would be a totally novel finding, however it is probably unlikely since RBX1 is absolutely required for cullin neddylation and the cullin 3 that

associates with the DISC has been shown to be neddylated (Jin *et al.*, 2009). It is therefore more likely that RBX1 was not detected because the protein is small and may have migrated through the membrane. Our data supports a potential NEDD8-independent role for cullin 3 in promoting caspase 8 cleavage. In HeLa cells and DU-145 cells, MLN4924 treatment appeared to increase caspase 8 cleavage, likely corresponding to an increase in ubiquitination of caspase 8. In these cell lines, cullin 3 may be playing a role independent of its ubiquitination activity as the active core of a CRL E3 ligase complex since it fails to become neddylated. As discussed previously, the inhibition of neddylation may inactivate CRL complexes responsible for negatively regulating caspase 8 cleavage such as cullin 7 (Kong *et al.*, 2019), and in the case of cullin 7 its role as a negative regulator of caspase 8 was described and characterised in HeLa cells. It is therefore possible that the reason we see an increase in caspase 8 cleavage when neddylation is inhibited is because cullin 7-mediated negative regulation of caspase 8 is abolished and this may have more of an effect on caspase 8 cleavage than the lack of cullin 3-mediated positive regulation.

MLN4924 reduced caspase 8 processing in U2OS and H460 cells in our work, however it did not totally abolish caspase 8 cleavage. It has been shown that only a small reduction in caspase 8 cleavage as detected by western blotting can correlate with a significant reduction in caspase 8 ubiquitination (Jin *et al.*, 2009), therefore our data for U2OS and H460 cells may represent significant reductions in caspase 8 polyubiquitination in the absence of neddylation. This largely supports the established role for neddylated cullin 3 in promoting caspase ubiquitination.

In U2OS cells and H460 cells, some caspase 8 is still cleaved in the absence of neddylation which may also be explained by cullin 3 and DCNL5 playing scaffold roles in DISC assembly. It remains possible that this interaction is critical for caspase 8 activation even in the absence of NEDD8. To date, no NEDD8-independent roles for cullin proteins have been described. Un-neddylated cullin proteins are known to exist in complex with CAND1 and DCNL proteins (Keuss *et al.*, 2016) so it would be worth investigating if there is any interaction between CAND1 and caspase 8. This interaction would presumably not be direct and be

bridged by cullin 3. Another issue that has yet to be resolved regarding cullin 3-mediated ubiquitination of caspase 8 is what the substrate adaptor is for this interaction. As mentioned previously, RBX1 was not found to associate with the DISC by both co-IP and mass spectrometry (Jin *et al.*, 2009), which does support a potential scaffold role for cullin 3 in DISC association, however the same researchers comprehensively showed that it is neddylated cullin 3 that interacts with the DISC, and that RBX1 is required for caspase 8 polyubiquitination. This further supports the likelihood that RBX1 was simply not detected at the DISC, raising the question of whether other components were also undetected.

## 6.4 Is Caspase 8 Regulated by Neddylation?

There is some evidence that caspase proteins are regulated by neddylation, both in drosophila and in humans. A systematic *in vivo* RNAi screen identified DEN1, CSN5 and a predicted de-neddylase enzyme called CG1503 as being involved in apoptosis (Broemer *et al.*, 2010). The drosophila effector caspase drICE (human caspase 3 homologue) was then identified as being neddylated *in vivo* under endogenous conditions, however this was expanded upon using His-tagged (and therefore overexpressed) NEDD8 to be mediated by a drosophila inhibitor of apoptosis called DIAP1 and reversed by the deneddylase enzyme DEN1 (Broemer *et al.*, 2010). Human caspase 7 was identified as a target for neddylation and concluded to be mediated by X-linked inhibitor of apoptosis (XIAP) (Broemer *et al.*, 2010) however this was done using NEDD8 overexpression which has been shown to result in the UBE1 (Ub E1 enzyme)-mediated activation and transfer of NEDD8 to targets via ubiquitin machinery (Hjerpe *et al.*, 2012). Other researchers concluded that while XIAP is able to function as a NEDD8 E3 ligase, it does not promote the neddylation of caspase 7 under endogenous conditions (Nagano *et al.*, 2012).

There is also literature indicating that caspase 1 is regulated by neddylation. Caspase 1 plays an inflammatory role and is unrelated in function to caspase 8, however it Is structurally and mechanistically similar and conserved. Caspase 1 was shown to undergo neddylation under endogenous as well as overexpressed conditions, and this neddylation was required to promote its autocatalytic cleavage and processing (Segovia *et al.*, 2015). NEDD8 interacted with the CARD

domain of caspase 1 to promote its autocatalytic processing and cleavage and this was inhibited by MLN4924 (Segovia *et al.*, 2015). While caspase 8 does not possess a CARD domain, it remains a possibility that NEDD8 may directly regulate caspase 8 processing. We were unable to detect interaction between DCNL5 and caspase 8 however we were also unable to optimise that particular co-IP reaction, so cannot definitively conclude that there is no interaction *in vivo*. It is likely that DCNL5 interaction may not be detected by co-IP given that a) a very small amount of DCNL5 and caspase 8 will be involved and b) the interaction may be short-lived and occur at a very specific timepoint post-treatment and would therefore require significant investigation to accurately interrogate this.

Neddylated cullin 3 interacts with caspase 8 (Jin *et al.*, 2009), but it remains to be seen whether any other neddylation machinery associates with caspase 8 or the DISC. As mentioned previously, CAND1 exists in complex with un-neddylated cullins as well as DCNL proteins and since it is neddylated cullin 3 that interacts at the DISC, CAND1 by definition will not be present. The NEDD8 E2 enzymes interact directly with DCNL proteins which themselves bind to cullins (which bind RBX1) so it is possible that the E2 as well as DCNL5 (and the undetectable RBX1) may also be present at the DISC. Our data using MLN4924 supports the role for NEDD8 in TRAIL-mediated caspase 8 cleavage in U2OS and H460 cells in particular, and CHX/TNFα treatment was shown to require neddylation for full processing of caspase 8 to form the catalytically active p18 fragment. The MLN4924 treatment inhibited p18 caspase cleavage to the same extent that the pan-caspase inhibitor QVD-OPh did. The fact that MLN4924 inhibits the full cleavage of caspase 8 in response to TNFα-mediated apoptosis strongly supports a direct role for NEDD8 in regulating caspase 8 processing. DCNL5 would therefore likely play a role in this neddylation event in this scenario, however this is all largely hypothetical and there is little convincing data to support a role for caspases as NEDD8 targets. They key issue with performing these sorts of interactions by overexpressing the components including NEDD8 is that ubiquitin machinery is capable of mediating the addition of NEDD8 to a target substrate that was never a true substrate in the first place (Hjerpe *et al.*, 2012), so great care needs to be taken to ensure that sufficient criteria is met to ensure a target is a bona fide NEDD8 target (Enchev, Schulman and Peter, 2015).

## 6.5 Is a New Model Required for Caspase 8 Cleavage?

Our data indicates that neddylation as well as cullin 3 appear to be less essential for caspase 8 cleavage than the role of DCNL5. Whether DCNL5 functions as a NEDD8 E3 ligase or whether it acts as a scaffold protein or in some other protein-protein interactions remains unclear. The present work questions the requirement for cullin 3 as well as neddylated cullin 3 in caspase 8 activity. siRNA-mediated knockdown of cullin 3 did reduce caspase cleavage in U2OS and H460 cells, consistent with its role in promoting cleavage, however in HeLa cells it had the opposite effect. Is the role of cullin 3 therefore cell line specific? Or are there additional roles for cullin 3 which serve to negatively regulate caspase 8 activity?

It is widely accepted that TRAIL-mediated apoptotic signalling and thus TRAIL sensitivity varies significantly in different cell lines. This is thought to arise in part due to the variation in expression of endogenous inhibitors of apoptosis (ref Huang et al, 2016), the variation in expression of death receptors (Twomey *et al.*, 2015), the diversity in cellular signalling responses (Baskar *et al.*, 2019) and even cell-to-cell variability in TRAIL-induced apoptosis (Spencer *et al.*, 2009). Attempts have been made to establish models of cell-to-cell variability in apoptosis which was shown to explain fractional killing and predicts reversible resistance (Bertaux *et al.*, 2014) and mechanisms underlying TRAIL resistance have been extensively studies for over a decade (Zhang and Fang, 2005; Trivedi and Mishra, 2015). Thus, the study of apoptosis is complicated and the level of variation from once cell line to the next or even from one cell within a population to the next cell, underlies just how complex developing treatments to overcome TRAIL resistance or to target specific interactions can be.

A summary of some of the known regulators of caspase 8 activity as well as potential new roles for DCNL5 are shown in Figure 6-1. A fundamental axiom of extrinsic apoptosis involves the polyubiquitination of caspase 8 by neddylated cullin 3 in an RBX1-dependent process. This ubiquitination event promotes the dimerization and cleavage of caspase 8 and its subsequence association with p62 in cytosolic ubiquitin-rich foci containing caspase 8 aggregates (Jin *et al.*, 2009). The present study provides substantial evidence for the role of the NEDD8 E3 ligase DCNL5 in caspase 8 activation, in a DAD-patch dependent process. We have

queried the role of neddylation through the use of MLN4924 and the finding that different cell lines respond differently to the inhibition of cullin 3 neddylation which is thought to be essential for this process. We take this further by showing that cullin knockdown results in an increase in caspase 8 processing in HeLa cells which raises the possibility that there are other cullins which mediate ubiquitination of caspase 8 in a pro-apoptotic manner.

In the absence of cullin 3, HeLa cells may undergo processing by another cullin/CRL complex which may be unique to these cells or common to a variety of other tissue culture cell lines. To date, cullin 3 is the only known positive regulator of caspase 8 activity in response to apoptosis induction. We consistently found that DCNL5 knockdown resulted in a more significant reduction in caspase 8 cleavage than neddylation inhibition or cullin 3 knockdown, and the role of DCNL5 appeared to be conserved in almost all the cell lines we tested. Further work is required to uncover the mechanism underlying DCNL5 involvement, and to establish whether it acts in coordination with cullin 3 or another cullin, or if DCNL5 can interact with another protein to promote caspase 8 cleavage.

Since DCNL5 has been implicated as an oncogene and is found to be overexpressed in oral and lung squamous cell carcinomas (Bommeljé *et al.*, 2014), it is worth further investigation to determine whether DCNL5 expression correlates with resistance to apoptosis in all cancers and if this is the case, whether mutant DCNL5 is responsible for this. siRNA-mediated knockdown of DCNL5 in cancer cell lines resulted in a decrease in viability of cancer cells with high endogenous DCNL5 expression (Bommeljé *et al.*, 2014) which on the surface seems paradoxical with respect to the data presented in this thesis. We have comprehensively shown that DCNL5 is required for extrinsic apoptosis, but cell viability and disease pathogenesis can be affected by many pathways beyond extrinsic apoptosis and DCNL5 may therefore play roles elsewhere in cellular processes. Our data using the cullin non-binding DAD-patch mutant suggests that expression of this version of DCNL5 conveys significant resistance to TRAIL-mediated apoptosis and extrinsic apoptosis in general, so if similar mutations occur in cancers, this may explain why DCNL5 expression correlates with poor disease survival. The role of DCNL5 in apoptosis represents an interesting field for further investigation. Our data

presents a significant and comprehensive phenotype in tissue culture cells of apoptosis resistance in the absence of DCNL5 expression which may make it a potential therapeutic target in the future. We provide initial evidence for additional regulators of caspase 8 cleavage which may also represent additional targets in cancer therapy and drug development.

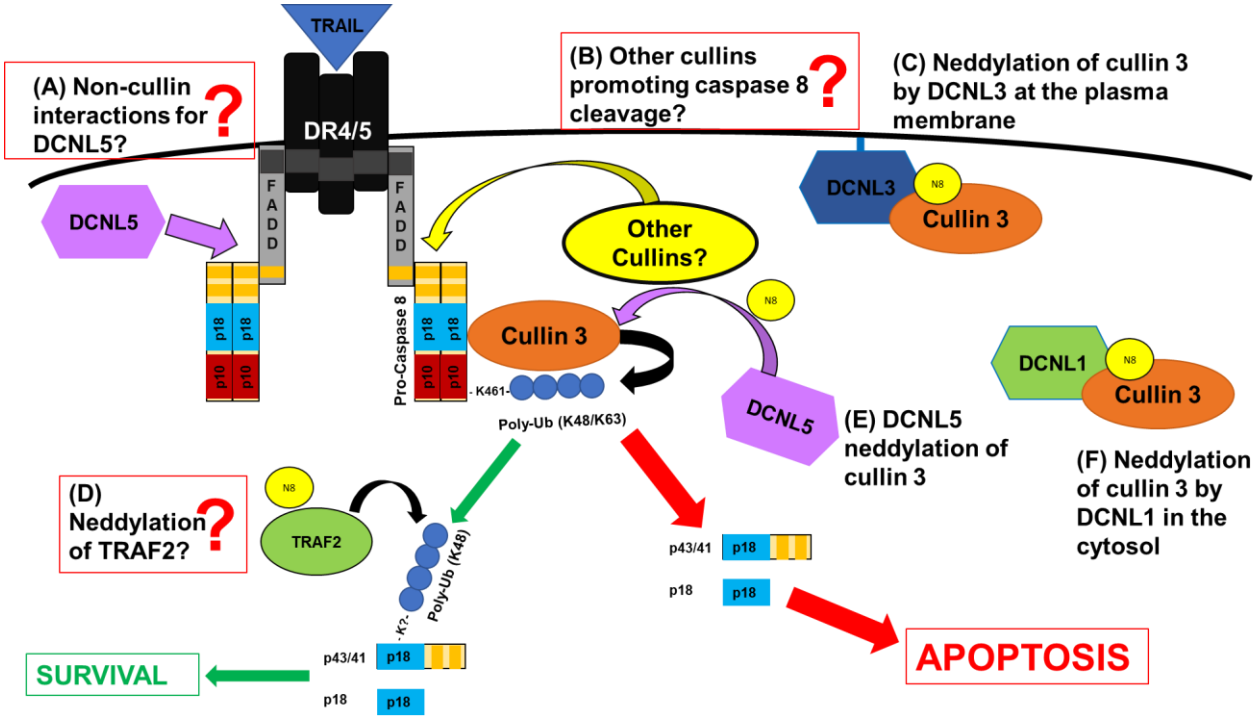



**Figure 6-1 Potential Interactions Involved in Caspase 8 Activation**

This schematic overview summarises potential regulators of caspase 8 activity based on the findings from the present study. (A) It is possible that DCNL5 interacts with FADD, caspase 8 or another protein via its DAD-patch since we have shown that DCNL5 involvement requires these residues and that neddylation may not be essential. (B) Our cullin knockdown data suggests that there may be other cullin proteins that can act as positive regulators of caspase 8 activity. (C) DCNL3 is known to neddylate cullin 3 at the plasma membrane (Meyer-Schaller et al, 2009) but it is currently unknown whether it is DCNL3 which mediate cullin 3 neddylation in the context of caspase 8 activation. (D) There is precedent for the neddylation of TRAF6 (Liu et al, 2019) therefore it is possible that TRAF2 also requires neddylation which may explain why. MLN4924 enhances caspase 8 activity in some cell lines since this may inhibit the degradation of caspase 8 by TRAF2 (Gonzalvez et al, 2012). (E) Our data supports a potential role for DCNL5 in promoting the neddylation of cullin 3, since we have shown that they interact *in vivo* under endogenous conditions and that the role of DCNL5 is dependent on DAD-interaction and therefore cullin binding. Cullin knockdown and MLN4924 supports this hypothesis in U2OS and H460 cells. (F) It is possible that DCNL1 regulates cullin 3 neddylation since this interaction has been shown *in vivo* (ref Huang et al, 2017) and inhibition of DCNL1 has been shown to specifically reduce cullin 3 neddylation (ref Zhou et al, 2017). We found that DCNL1 KO cells were as sensitive if not more so than WT cells to apoptosis, which suggests that while DCNL1 may be responsible for some cullin 3 neddylation, it is not essential in this cellular context. *Red boxes with question marks represent possible interactions not yet demonstrated experimentally and thus potential future avenues of investigation.*

# List of References

Acehan, D., Jiang, X., Morgan, D. G., Heuser, J. E., Wang, X. and Akey, C. W. (2002) 'Three-dimensional structure of the apoptosome: Implications for assembly, procaspase-9 binding, and activation', *Molecular Cell,* 9(2), pp. 423-432.

Ambroggio, X. I., Rees, D. C. and Deshaies, R. J. (2004) 'JAMM: A metalloprotease-like zinc site in the proteasome and signalosome', *PLoS Biology,* 2(1).

Amerik, A. Y. and Hochstrasser, M. (2004) 'Mechanism and function of deubiquitinating enzymes', *Biochimica et Biophysica Acta - Molecular Cell Research,* 1695(1-3), pp. 189-207.

Amir, R. E., Iwai, K. and Ciechanover, A. (2002) 'The NEDD8 pathway is essential for SCfß-TrCP-mediated ubiquitination and processing of the NF-κB precursor p105', *Journal of Biological Chemistry,* 277(26), pp. 23253-23259.

Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L., McMurtrey, A. E., Hebert, A., DeForge, L., Koumenis, I. L., Lewis, D., Harris, L., Bussiere, J., Koeppen, H., Shahrokh, Z. and Schwall, R. H. (1999) 'Safety and antitumor activity of recombinant soluble Apo2 ligand', *Journal of Clinical Investigation,* 104(2), pp. 155-162.

Baek, K., Krist, D. T., Prabu, J. R., Hill, S., Klügel, M., Neumaier, L. M., von Gronau, S., Kleiger, G. and Schulman, B. A. (2020) 'NEDD8 nucleates a multivalent cullin–RING–UBE2D ubiquitin ligation assembly', *Nature,* 578(7795), pp. 461-466.

Bar-nun, S. and Glickman, M. H. (2012) 'Biochimica et Biophysica Acta Proteasomal AAA-ATPases : Structure and function ☆', *BBA - Molecular Cell Research,* 1823(1), pp. 67-82.

Baskar, R., Fienberg, H. G., Khair, Z., Favaro, P., Kimmey, S., Green, D. R., Nolan, G. P., Plevritis, S. and Bendall, S. C. (2019) 'TRAIL-induced variation of cell signaling states provides nonheritable resistance to apoptosis', *Life science alliance,* 2(6), pp. 1-16.

Baumeister, W., Walz, J., Zühl, F. and Seemüller, E. 1998. The proteasome: Paradigm of a self-compartmentalizing protease.

Beaudouin, J., Liesche, C., Aschenbrenner, S., Hörner, M. and Eils, R. (2013) 'Caspase-8 cleaves its substrates from the plasma membrane upon CD95-induced apoptosis', *Cell Death and Differentiation,* 20(4), pp. 599-610.

Bedford, L., Paine, S., Sheppard, P. W., Mayer, R. J. and Roelofs, J. (2010) 'Assembly, structure, and function of the 26S proteasome', *Trends Cell Biol,* 20(7), pp. 391-401.

Bennett, E. J., Rush, J., Gygi, S. P. and Harper, J. W. (2010) 'Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics', *Cell,* 143(6), pp. 951-65.

Bertaux, F., Stoma, S., Drasdo, D. and Batt, G. (2014) 'Modeling Dynamics of Cell-to-Cell Variability in TRAIL-Induced Apoptosis Explains Fractional Killing and Predicts Reversible Resistance', *PLoS Computational Biology,* 10(10).

Besse, A., Besse, L., Kraus, M., Mendez-Lopez, M., Bader, J., Xin, B. T., de Bruin, G., Maurits, E., Overkleeft, H. S. and Driessen, C. (2019) 'Proteasome Inhibition in Multiple Myeloma: Head-to-Head Comparison of Currently Available Proteasome Inhibitors', *Cell Chemical Biology,* 26(3), pp. 340-351.e3.

Birol, M., Enchev, R. I., Padilla, A., Stengel, F., Aebersold, R., Betzi, S., Yang, Y., Hoh, F., Peter, M., Dumas, C. and Echalier, A. (2014) 'Structural and biochemical characterization of the Cop9 signalosome CSN5/CSN6 heterodimer', *PLoS ONE,* 9(8), pp. 1-13.

Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J. and Cerretti, D. P. (1997) 'A metalloproteinase disintegrin that releases tumour-necrosis factor-Ø from cells', *Nature,* 385(6618), pp. 729-733.

Boatright, K. M., Renatus, M., Scott, F. L. and Sperandio, S. (2003) 'A Unified Model for Apical Caspase Activation the cellular effects that distinguish apoptosis from other', *Molecular Cell,* 11, pp. 529-541.

Bommeljé, C. C., Weeda, V. B., Huang, G., Shah, K., Bains, S., Buss, E., Shaha, M., Gönen, M., Ghossein, R., Ramanathan, S. Y. and Singh, B. (2014) 'Oncogenic function of SCCRO5/DCUN1D5 requires its Neddylation E3 activity and nuclear localization', *Clin Cancer Res,* 20(2), pp. 372-81.

Bossy-Wetzel, E., Newmeyer, D. D. and Green, D. R. (1998) 'Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization', *EMBO Journal,* 17(1), pp. 37-49.

Broderick, S. R., Golas, B. J., Pham, D., Towe, C. W., Talbot, S. G., Kaufman, A., Bains, S., Huryn, L. A., Yonekawa, Y., Carlson, D., Hambardzumyan, D., Ramanathan, Y. and Singh, B. (2010) 'SCCRO promotes glioma formation and malignant progression in mice', *Neoplasia,* 12(6), pp. 476-484.

Broemer, M., Tenev, T., Rigbolt, K. T. G., Hempel, S., Blagoev, B., Silke, J., Ditzel, M. and Meier, P. (2010) 'Systematic In Vivo RNAi Analysis Identifies IAPs as NEDD8-E3 Ligases', *Molecular Cell,* 40(5), pp. 810-822.

Brownell, J. E., Sintchak, M. D., Gavin, J. M., Liao, H., Bruzzese, F. J., Bump, N. J., Soucy, T. A., Milhollen, M. A., Yang, X., Burkhardt, A. L., Ma, J., Loke, H. K., Lingaraj, T., Wu, D., Hamman, K. B., Spelman, J. J., Cullis, C. A., Langston, S. P., Vyskocil, S., Sells, T. B., Mallender, W. D., Visiers, I., Li, P., Claiborne, C. F., Rolfe, M., Bolen, J. B. and Dick, L. R. (2010) 'Substrate-Assisted Inhibition of Ubiquitin-like Protein-Activating Enzymes: The NEDD8 E1 Inhibitor MLN4924 Forms a NEDD8-AMP Mimetic In Situ', *Molecular Cell,* 37(1), pp. 102-111.

Brumatti, G., Ma, C., Lalaoui, N., Nguyen, N. Y., Navarro, M., Tanzer, M. C., Richmond, J., Ghisi, M., Salmon, J. M., Silke, N., Pomilio, G., Glaser, S. P., De Valle, E., Gugasyan, R., Gurthridge, M. A., Condon, S. M., Johnstone, R. W., Lock, R., Salvesen, G., Wei, A., Vaux, D. L., Ekert, P. G. and Silke, J. (2016) 'The

caspase-8 inhibitor emricasan combines with the SMAC mimetic birinapant to induce necroptosis and treat acute myeloid leukemia', *Science Translational Medicine,* 8(339).

Burden, D. A. and Osheroff, N. (1998) 'Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme', *Biochimica et Biophysica Acta - Gene Structure and Expression,* 1400(1-3), pp. 139-154.

Cain, K., Bratton, S. B. and Cohen, G. M. (2002) 'The Apaf-1 apoptosome: A large caspase-activating complex', *Biochimie,* 84(2-3), pp. 203-214.

Cardamone, D. M., Krones, A., Tanasa, B., Taylor, H., Ricci, L., Ohgi, K. A., Glass, C. K., Rosenfeld, M. G. and Perissi, V. (2012) 'A Protective Strategy against Hyperinflammatory Responses Requiring the Nontranscriptional Actions of GPS2', *Molecular Cell,* 46(1), pp. 91-104.

Cardozo, T. and Pagano, M. (2004) 'The SCF ubiquitin ligase: Insights into a molecular machine', *Nature Reviews Molecular Cell Biology,* 5(9), pp. 739-751.

Caserta, T. M., Smith, A. N., Gultice, A. D., Reedy, M. A. and Brown, T. L. (2003) 'Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties', *Apoptosis,* 8(4), pp. 345-352.

Chan, F. K. M., Chun, H. J., Zheng, L., Siegel, R. M., Bui, K. L. and Lenardo, M. J. (2000) 'A domain in TNF receptors that mediates ligand-independent receptor assembty and signaling', *Science,* 288(5475), pp. 2351-2354.

Chan, Y., Yoon, J., Wu, J. T., Kim, H. J., Pan, K. T., Yim, J. and Chien, C. T. (2008) 'DEN1 deneddylates non-cullin proteins in vivo', *Journal of Cell Science,* 121(19), pp. 3218-3223.

Chang, D. W., Xing, Z., Capacio, V. L., Peter, M. E. and Yang, X. (2003) 'Interdimer processing mechanism of procaspase-8 activation', *EMBO J,* 22(16), pp. 4132-4142.

Chang, H. Y. and Yang, X. (2000) 'Proteases for Cell Suicide: Functions and Regulation of Caspases', *Microbiology and Molecular Biology Reviews,* 64(4), pp. 821-846.

Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K. and Varshavsky, A. (1989) 'A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein', *Science,* 243(4898), pp. 1576-1583.

Chen, G. and Goeddel, D. V. (2002) 'TNF-R1 signaling: a beautiful pathway', *Science,* 296(5573), pp. 1634-5.

Chen, G., Zhao, X., Tan, Z., Wang, D., Luo, D., Zhang, P., Cao, J., Wang, F., Liu, Q. and Li, L. (2018) 'Investigation of the role of cullin 4A overexpression in human liver cancer', *Molecular Medicine Reports,* 18(3), pp. 2531-2540.

Chen, J., Ghorai, M. K., Kenney, G. and Stubbe, J. (2008) 'Mechanistic studies on bleomycin-mediated DNA damage: multiple binding modes can result in double-stranded DNA cleavage', *Nucleic Acids Res,* 36(11), pp. 3781-90.

Chen, L. C., Manjeshwar, S., Lu, Y., Moore, D., Ljung, B. M., Kuo, W. L., Dairkee, S. H., Wernick, M., Collins, C. and Smith, H. S. (1998) 'The human homologue for the Caenorhabditis elegans cul-4 gene is amplified and overexpressed in primary breast cancers', *Cancer Research,* 58(16), pp. 3677-3683.

Chen, P., Hu, T., Liang, Y., Li, P., Chen, X., Zhang, J., Ma, Y., Hao, Q., Wang, J., Zhang, P., Zhang, Y., Zhao, H., Yang, S., Yu, J., Jeong, L. S., Qi, H., Yang, M., Hoffman, R. M., Dong, Z. and Jia, L. (2016) 'Neddylation inhibition activates the extrinsic apoptosis pathway through ATF4-CHOP-DR5 axis in human esophageal cancer cells', *Clinical Cancer Research,* 22(16), pp. 4145-4157.

Chen, Y., Neve, R. L. and Liu, H. (2012) 'Neddylation dysfunction in Alzheimer's disease', *Journal of Cellular and Molecular Medicine,* 16(11), pp. 2583-2591.

Chen, Z. J. (2005) 'Ubiquitin signalling in the NF-kappaB pathway', *Nat Cell Biol,* 7(8), pp. 758-65.

Ciechanover, A. (2005) 'Proteolysis: from the lysosome to ubiquitin and the proteasome', *Molecular Cell Biology,* 6(January), pp. 9-9.

Ciechanover, A. and Ben-Saadon, R. (2004) 'N-terminal ubiquitination: More protein substrates join in', *Trends in Cell Biology,* 14(3), pp. 103-106.

Claussen, C. A. and Long, E. C. (1999) 'Nucleic acid recognition by metal complexes of bleomycin', *Chemical Reviews,* 99(9), pp. 2797-2816.

Collins, A. R. and Azqueta, A. (2012) 'DNA repair as a biomarker in human biomonitoring studies; further applications of the comet assay', *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis,* 736(1-2), pp. 122-129.

Cope, G. A., Suh, G. S. B., Aravind, L., Schwarz, S. E., Zipursky, S. L., Koonin, E. V. and Deshaies, R. J. (2002) 'Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1', *Science,* 298(5593), pp. 608-611.

Cullinan, S. B., Gordan, J. D., Jin, J., Harper, J. W. and Diehl, J. A. (2004) 'The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase', *Mol Cell Biol,* 24(19), pp. 8477-86.

Degli-Esposti, M. A., Dougall, W. C., Smolak, P. J., Waugh, J. Y., Smith, C. A. and Goodwin, R. G. (1997a) 'The novel receptor TRAIL-R4 induces NF-κB and protects against TRAIL- mediated apoptosis, yet retains an incomplete death domain', *Immunity,* 7(6), pp. 813-820.

Degli-Esposti, M. A., Smolak, P. J., Walczak, H., Waugh, J., Huang, C. P., DuBose, R. F., Goodwin, R. G. and Smith, C. A. (1997b) 'Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family', *Journal of Experimental Medicine,* 186(7), pp. 1165-1170.

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C. and Chen, Z. J. (2000) 'Activation of the Iκb kinase complex by TRAF6

requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain', *Cell,* 103(2), pp. 351-361.

Duda, D. M., Borg, L. A., Scott, D. C., Hunt, H. W., Hammel, M. and Schulman, B. A. (2008) 'Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation', *Cell,* 134(6), pp. 995-1006.

Dunai, Z. A., Imre, G., Barna, G., Korcsmaros, T., Petak, I., Bauer, P. I. and Mihalik, R. (2012) 'Staurosporine induces necroptotic cell death under caspase-compromised conditions in U937 cells', *PLoS ONE,* 7(7), pp. 1-14.

Eisenhaber, B., Chumak, N., Eisenhaber, F. and Hauser, M. T. (2007) 'The ring between ring fingers (RBR) protein family', *Genome Biol,* 8(3), pp. 209.

Emberley, E. D., Mosadeghi, R. and Deshaies, R. J. (2012) 'Deconjugation of Nedd8 from Cul1 is directly regulated by Skp1-F-box and substrate, and the COP9 signalosome inhibits deneddylated SCF by a noncatalytic mechanism', *J Biol Chem,* 287(35), pp. 29679-89.

Enchev, R. I., Schulman, B. A. and Peter, M. (2015) 'Protein neddylation: beyond cullin-RING ligases', *Nat Rev Mol Cell Biol,* 16(1), pp. 30-44.

Enchev, R. I., Scott, D. C., da Fonseca, P. C., Schreiber, A., Monda, J. K., Schulman, B. A., Peter, M. and Morris, E. P. (2012) 'Structural basis for a reciprocal regulation between SCF and CSN', *Cell Rep,* 2(3), pp. 616-27.

Feoktistova, M., Geserick, P., Kellert, B., Dimitrova, D. P., Langlais, C., Hupe, M., Cain, K., MacFarlane, M., Häcker, G. and Leverkus, M. (2011) 'CIAPs Block Ripoptosome Formation, a RIP1/Caspase-8 Containing Intracellular Cell Death Complex Differentially Regulated by cFLIP Isoforms', *Molecular Cell,* 43(3), pp. 449-463.

Fischer, E. S., Scrima, A., Böhm, K., Matsumoto, S., Lingaraju, G. M., Faty, M., Yasuda, T., Cavadini, S., Wakasugi, M., Hanaoka, F., Iwai, S., Gut, H., Sugasawa,

K. and Thomä, N. H. (2011) 'The molecular basis of CRL4DDB2/CSA ubiquitin ligase architecture, targeting, and activation', *Cell,* 147(5), pp. 1024-39.

Fotouhi, O., Kjellin, H., Juhlin, C. C., Pan, Y., Vesterlund, M., Ghaderi, M., Yousef, A., Andersson-Sand, H., Kharaziha, P., Caramuta, S., Kjellman, M., Zedenius, J., Larsson, C. and Orre, L. M. (2019) 'Proteomics identifies neddylation as a potential therapy target in small intestinal neuroendocrine tumors', *Oncogene,* 38(43), pp. 6881-6897.

Fu, S. C., Imai, K., Sawasaki, T. and Tomii, K. (2014) 'ScreenCap3: Improving prediction of caspase-3 cleavage sites using experimentally verified noncleavage sites', *Proteomics,* 14(17-18), pp. 2042-2046.

Fulda, S. and Debatin, K. M. (2006) 'Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy', *Oncogene,* 25(34), pp. 4798-811.

Fulda, S., Meyer, E. and Debatin, K.-m. (2002) 'Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression'.

Garnett, T. O., Filippova, M. and Duerksen-Hughes, P. J. (2007) 'Bid is cleaved upstream of caspase-8 activation during TRAIL-mediated apoptosis in human osteosarcoma cells', *Apoptosis,* 12(7), pp. 1299-1315.

Gazitt, Y., Shaughnessy, P. and Montgomery, W. (1999) 'Apoptosis-induced by TRAIL and TNF-α in human multiple myeloma cells is not blocked by Bcl-2', *Cytokine,* 11(12), pp. 1010-1019.

Gescher, A. (2000) 'Staurosporine analogues - Pharmacological toys or useful antitumour agents?', *Critical Reviews in Oncology/Hematology,* 34(2), pp. 127-135.

Geserick, P., Drewniok, C., Hupe, M., Haas, T. L., Diessenbacher, P., Sprick, M. R., Schön, M. P., Henkler, F., Gollnick, H., Walczak, H. and Leverkus, M. (2008) 'Suppression of cFLIP is sufficient to sensitize human melanoma cells to TRAIL- and CD95L-mediated apoptosis', *Oncogene,* 27(22), pp. 3211-3220.

Gong, L. and Yeh, E. T. (1999a) 'Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway', *J Biol Chem,* 274(17), pp. 12036-42.

Gong, L. and Yeh, E. T. (1999b) 'Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway', *J Biol Chem,* 274(17), pp. 12036-12042.

Gonzalvez, F., Lawrence, D., Yang, B., Yee, S., Pitti, R., Marsters, S., Pham, V. C., Stephan, J. P., Lill, J. and Ashkenazi, A. (2012) 'TRAF2 Sets a Threshold for Extrinsic Apoptosis by Tagging Caspase-8 with a Ubiquitin Shutoff Timer', *Molecular Cell,* 48(6), pp. 888-899.

Grotzer, M. A., Eggert, A., Zuzak, T. J., Janss, A. J., Marwha, S., Wiewrodt, B. R., Ikegaki, N., Brodeur, G. M. and Phillips, P. C. (2000) 'Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced Apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression', *Oncogene,* 19, pp. 4604-4610.

Grunert, M., Gottschalk, K., Kapahnke, J., Gündisch, S., Kieser, A. and Jeremias, I. (2012) 'The adaptor protein FADD and the initiator caspase-8 mediate activation of NF-κB by TRAIL', *Cell Death and Disease,* 3(10), pp. 1-13.

Haas, A. L. and Rose, I. A. (1982) 'The mechanism of ubiquitin activating enzyme. A kinetic and equilibrium analysis', *J Biol Chem,* 257(17), pp. 10329-37.

Haglund, K. and Dikic, I. (2005) 'Ubiquitylation and cell signaling', *EMBO Journal,* 24(19), pp. 3353-3359.

Harper, N., Hughes, M., MacFarlane, M. and Cohen, G. M. (2003) 'Fas-associated death domain protein and caspase-8 are not recruited to the tumor necrosis factor receptor I signaling complex during tumor necrosis factor-induced apoptosis', *Journal of Biological Chemistry,* 278(28), pp. 25534-25541.

Hay, B. A. and Guo, M. (2006) 'Caspase-Dependent Cell Death in Drosophila', *Annual Review of Cell and Developmental Biology,* 22(1), pp. 623-650.

He, S., Cao, Y., Xie, P., Dong, G. and Zhang, L. (2017) 'The Nedd8 Non-covalent Binding Region in the Smurf HECT Domain is Critical to its Ubiquitn Ligase Function', *Scientific Reports*, 7(February), pp. 1-12.

Hecht, S. M. 2000. Bleomycin: New perspectives on the mechanism of action.

Helmstaedt, K., Schwier, E. U., Christmann, M., Nahlik, K., Westermann, M., Harting, R., Grond, S., Busch, S. and Braus, G. H. (2011) 'Recruitment of the inhibitor Cand1 to the cullin substrate adaptor site mediates interaction to the neddylation site', *Molecular Biology of the Cell*, 22(1), pp. 153-164.

Henry, C. M. and Martin, S. J. (2017) 'Caspase-8 Acts in a Non-enzymatic Role as a Scaffold for Assembly of a Pro-inflammatory "FADDosome" Complex upon TRAIL Stimulation', *Molecular Cell*, 65(4), pp. 715-729.e5.

Hershko, A. and Ciechanover, A. (1998) 'The ubiquitin system', *Annu Rev Biochem*, 67, pp. 425-79.

Hjerpe, R., Aillet, F., Lopitz-otsoa, F., Lang, V., England, P., Rodriguez, M. S., Unit, P., Park, B. T., Pasteur, I. and Biophysique, P.-f. D. (2009) 'Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities', *EMBO reports*, 10(11), pp. 1250-1258.

Hjerpe, R., Thomas, Y., Chen, J., Zemla, A., Curran, S., Shpiro, N., Dick, L. R. and Kurz, T. (2012) 'Changes in the ratio of free NEDD8 to ubiquitin triggers NEDDylation by ubiquitin enzymes', *Biochemical Journal*, 441(3), pp. 927-936.

Hoffmann, J. C., Pappa, A., Krammer, P. H. and Lavrik, I. N. (2009) 'A New C-Terminal Cleavage Product of Procaspase-8, p30, Defines an Alternative Pathway of Procaspase-8 Activation', *Molecular and Cellular Biology*, 29(16), pp. 4431-4440.

Hori, T., Osaka, F., Chiba, T., Miyamoto, C., Okabayashi, K., Shimbara, N., Kato, S. and Tanaka, K. (1999) 'Covalent modification of all members of human cullin family proteins by NEDD8', *Oncogene*, 18(48), pp. 6829-34.

Hsu, H., Shu, H. B., Pan, M. G. and Goeddel, D. V. (1996) 'TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways', *Cell,* 84(2), pp. 299-308.

Huang, D. T., Ayrault, O., Hunt, H. W., Taherbhoy, A. M., Duda, D. M., Scott, D. C., Borg, L. A., Neale, G., Murray, P. J., Roussel, M. F. and Schulman, B. A. (2009) 'E2-RING expansion of the NEDD8 cascade confers specificity to cullin modification', *Mol Cell,* 33(4), pp. 483-95.

Huang, D. T., Miller, D. W., Mathew, R., Cassell, R., Holton, J. M., Roussel, M. F. and Schulman, B. A. (2004) 'A unique E1-E2 interaction required for optimal conjugation of the ubiquitin-like protein NEDD8', *Nature Structural and Molecular Biology,* 11(10), pp. 927-935.

Huang, G., Kaufman, A. J., Ramanathan, Y. and Singh, B. (2011) 'SCCRO (DCUN1D1) promotes nuclear translocation and assembly of the neddylation E3 complex', *J Biol Chem,* 286(12), pp. 10297-304.

Huang, G., Kaufman, A. J., Xu, K., Manova, K. and Singh, B. (2017) 'Squamous cell carcinoma–related oncogene (SCCRO) neddylates Cul3 protein to selectively promote midbody localization and activity of Cul3KLHL21 protein complex during abscission', *Journal of Biological Chemistry,* 292(37), pp. 15254-15265.

Huang, G., Stock, C., Bommeljé, C. C., Weeda, V. B., Shah, K., Bains, S., Buss, E., Shaha, M., Rechler, W., Ramanathan, S. Y. and Singh, B. (2014) 'SCCRO3 (DCUN1D3) antagonizes the neddylation and oncogenic activity of SCCRO (DCUN1D1)', *J Biol Chem,* 289(50), pp. 34728-42.

Huang, G., Towe, C. W., Choi, L., Yonekawa, Y., Bommeljé, C. C., Bains, S., Rechler, W., Hao, B., Ramanathan, Y. and Singh, B. (2015) 'The ubiquitin-associated (UBA) domain of SCCRO/DCUN1D1 protein serves as a feedback regulator of biochemical and oncogenic activity', *Journal of Biological Chemistry,* 290(1), pp. 296-309.

Hughes, M. A., Harper, N., Butterworth, M., Cain, K., Cohen, G. M. and MacFarlane, M. (2009) 'Reconstitution of the Death-Inducing Signaling Complex Reveals a Substrate Switch that Determines CD95-Mediated Death or Survival', *Molecular Cell,* 35(3), pp. 265-279.

Hughes, M. A., Powley, I. R., Jukes-Jones, R., Horn, S., Feoktistova, M., Fairall, L., Schwabe, J. W. R., Leverkus, M., Cain, K. and MacFarlane, M. (2016) 'Co-operative and Hierarchical Binding of c-FLIP and Caspase-8: A Unified Model Defines How c-FLIP Isoforms Differentially Control Cell Fate', *Molecular Cell,* 61(6), pp. 834-849.

Hyoung, T. K., Kwang, P. K., Lledias, F., Kisselev, A. F., Scaglione, K. M., Skowyra, D., Gygi, S. P. and Goldberg, A. L. (2007) 'Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages', *Journal of Biological Chemistry,* 282(24), pp. 17375-17386.

Jackson, P. K., Eldridge, A. G., Freed, E., Furstenthal, L., Hsu, J. Y., Kaiser, B. K. and Reimann, J. D. (2000) 'The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases', *Trends Cell Biol,* 10(10), pp. 429-439.

Jin, J., Li, X., Gygi, S. P. and Harper, J. W. (2007) 'Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging', *Nature,* 447(7148), pp. 1135-1138.

Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V. C., Lill, J. R. and Ashkenazi, A. (2009) 'Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling', *Cell,* 137(4), pp. 721-735.

Jo, M., Kim, T. H., Seol, D. W., Esplen, J. E., Dorko, K., Billiar, T. R., Strom, S. C., Strehlow, D., Jodo, S. and Ju, S. T. (2000) 'Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand\rRetroviral membrane display of apoptotic effector molecules', *Nat Med,* 6(5), pp. 564-567.

Julien, O. and Wells, J. A. (2017) 'Caspases and their substrates', *Cell Death and Differentiation,* 24(8), pp. 1380-1389.

Kagawa, S., He, C., Gu, J., Koch, P., Rha, S. J., Roth, J. A., Curley, S. A., Stephens, L. C. and Fang, B. (2001) 'Antitumor activity and bystander effects of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene', *Cancer Research,* 61(8), pp. 3330-3338.

Kallenberger, S. M., Beaudouin, J., Claus, J., Fischer, C., Sorger, P. K., Legewie, S. and Eils, R. (2014) 'Intra- and interdimeric caspase-8 self-cleavage controls strength and timing of CD95-induced apoptosis', *Science Signaling,* 7(316).

Kaminskyy, V. O., Surova, O. V., Piskunova, T., Zborovskaya, I. B., Tchevkina, E. M., Andera, L. and Zhivotovsky, B. (2013) 'Upregulation of c-FLIP-short in response to TRAIL promotes survival of NSCLC cells, which could be suppressed by inhibition of Ca2+/ calmodulin signaling', *Cell Death and Disease,* 4(3), pp. e522-10.

Kamitani, T., Kito, K., Nguyen, H. P. and Yeh, E. T. (1997) 'Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein', *J Biol Chem,* 272(45), pp. 28557-62.

Kanarek, N. and Ben-Neriah, Y. (2012) 'Regulation of NF-κB by ubiquitination and degradation of the IκBs', *Immunological Reviews,* 246(1), pp. 77-94.

Kandala, S., Kim, I. M. and Su, H. (2014) 'Neddylation and deneddylation in cardiac biology', *American Journal of Cardiovascular Disease,* 4(4), pp. 140-158.

Karin, M. (1999) 'How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex', *Oncogene,* 18(49), pp. 6867-74.

Kaustov, L., Lukin, J., Lemak, A., Duan, S., Ho, M., Doherty, R., Penn, L. Z. and Arrowsmith, C. H. (2007) 'The conserved CPH domains of Cul7 and PARC are protein-protein interaction modules that bind the tetramerization domain of p53', *J Biol Chem,* 282(15), pp. 11300-7.

Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F., Omata, M. and Tanaka, K. (2001) 'NEDD8 recruits E2-ubiquitin to SCF E3 ligase', *EMBO Journal,* 20(15), pp. 4003-4012.

Keller, N., Grütter, M. G. and Zerbe, O. (2010) 'Studies of the molecular mechanism of caspase-8 activation by solution NMR', *Cell Death and Differentiation,* 17(4), pp. 710-718.

Keller, N., Mareš, J., Zerbe, O. and Grütter, M. G. (2009) 'Structural and Biochemical Studies on Procaspase-8: New Insights on Initiator Caspase Activation', *Structure,* 17(3), pp. 438-448.

Kelley, S. K. and Ashkenazi, A. (2004) 'Targeting death receptors in cancer with Apo2L/TRAIL', *Current Opinion in Pharmacology,* 4(4), pp. 333-339.

Kelsall, I. R., Kristariyanto, Y. A., Knebel, A., Wood, N. T., Kulathu, Y. and Alpi, A. F. (2019) 'Coupled monoubiquitylation of the co-E3 ligase DCNL1 by Ariadne-RBR E3 ubiquitin ligases promotes cullin-RING ligase complex remodeling', *J Biol Chem,* 294(8), pp. 2651-2664.

Keuss, M. J., Hjerpe, R., Hsia, O., Gourlay, R., Burchmore, R., Trost, M. and Kurz, T. (2019) 'Unanchored tri-NEDD8 inhibits PARP-1 to protect from oxidative stress-induced cell death', *EMBO Journal,* 38(6).

Keuss, M. J., Thomas, Y., Mcarthur, R., Wood, N. T., Knebel, A. and Kurz, T. (2016) 'Characterization of the mammalian family of DCN-type NEDD8 E3 ligases', *J Cell Sci,* 129(7), pp. 1441-54.

Kim, A. Y., Bommeljé, C. C., Lee, B. E., Yonekawa, Y., Choi, L., Morris, L. G., Huang, G., Kaufman, A., Ryan, R. J., Hao, B., Ramanathan, Y. and Singh, B. (2008) 'SCCRO (DCUN1D1) is an essential component of the E3 complex for neddylation', *J Biol Chem,* 283(48), pp. 33211-20.

Kim, H. S., Hammill, J. T., Scott, D. C., Chen, Y., Min, J., Rector, J., Singh, B., Schulman, B. A. and Guy, R. K. (2019) 'Discovery of Novel Pyrazolo-pyridone DCN1

Inhibitors Controlling Cullin Neddylation', *Journal of Medicinal Chemistry,* 62(18), pp. 8429-8442.

Kipreos, E. T., Lander, L. E., Wing, J. P., He, W. W. and Hedgecock, E. M. (1996) 'cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family', *Cell,* 85(6), pp. 829-839.

Kischkel, F. C., Lawrence, D. A., Chuntharapai, A., Schow, P., Kim, K. J. and Ashkenazi, A. (2000) 'Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5', *Immunity,* 12(6), pp. 611-620.

Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K. and Yamamoto, M. (2004) 'Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2', *Mol Cell Biol,* 24(16), pp. 7130-9.

Kojima, Y., Nakayama, M., Nishina, T., Nakano, H., Koyanagi, M., Takeda, K., Okumura, K. and Yagita, H. (2011) 'Importin ß1 protein-mediated nuclear localization of Death Receptor 5 (DR5) limits DR5/Tumor Necrosis Factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced cell death of human tumor cells', *Journal of Biological Chemistry,* 286(50), pp. 43383-43393.

Kong, Y., Wang, Z., Huang, M., Zhou, Z., Li, Y., Miao, H., Wan, X., Huang, J., Mao, X. and Chen, C. (2019) 'CUL7 promotes cancer cell survival through promoting Caspase-8 ubiquitination', *Int J Cancer,* 145(5), pp. 1371-1381.

Krueger, A., Schmitz, I., Baumann, S., Krammer, P. H. and Kirchhoff, S. (2001) 'Cellular FLICE-inhibitory Protein Splice Variants Inhibit Different Steps of Caspase-8 Activation at the CD95 Death-inducing Signaling Complex', *Journal of Biological Chemistry,* 276(23), pp. 20633-20640.

Kurbanov, B. M., Geilen, C. C., Fecker, L. F., Orfanos, C. E. and Eberle, J. (2005) 'Efficient TRAIL-R1/DR4-mediated apoptosis in melanoma cells by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)', *Journal of Investigative Dermatology,* 125(5), pp. 1010-1019.

Kurihara, L. J., Semenova, E., Levorse, J. M. and Tilghman, S. M. (2000) 'Expression and functional analysis of Uch-L3 during mouse development', *Mol Cell Biol,* 20(7), pp. 2498-504.

Kurz, T., Chou, Y. C., Willems, A. R., Meyer-Schaller, N., Hecht, M. L., Tyers, M., Peter, M. and Sicheri, F. (2008) 'Dcn1 functions as a scaffold-type E3 ligase for cullin neddylation', *Mol Cell,* 29(1), pp. 23-35.

Kurz, T., Ozlü, N., Rudolf, F., O'Rourke, S. M., Luke, B., Hofmann, K., Hyman, A. A., Bowerman, B. and Peter, M. (2005a) 'The conserved protein DCN-1/Dcn1p is required for cullin neddylation in C. elegans and S. cerevisiae', *Nature,* 435(7046), pp. 1257-61.

Kurz, T., Özlü, N., Rudolf, F., O'Rourke, S. M., Luke, B., Hofmann, K., Hyman, A. A., Bowerman, B. and Peter, M. (2005b) 'The conserved protein DCN-1/Dcn1p is required for cullin neddylation in C. elegans and S. cerevisiae', *Nature,* 435(7046), pp. 1257-1261.

Kuželová, K., Grebeňová, D. and Brodská, B. (2011) 'Dose-dependent effects of the caspase inhibitor Q-VD-OPh on different apoptosis-related processes', *Journal of Cellular Biochemistry,* 112(11), pp. 3334-3342.

Lan, H., Tang, Z., Jin, H. and Sun, Y. (2016) 'Neddylation inhibitor MLN4924 suppresses growth and migration of human gastric cancer cells', *Sci Rep,* 6, pp. 24218.

Lander, G. C., Estrin, E., Matyskiela, M. E., Bashore, C., Nogales, E. and Martin, A. (2012) 'Complete subunit architecture of the proteasome regulatory particle', *Nature,* 482(7384), pp. 186-91.

Larsen, C. N., Krantz, B. A. and Wilkinson, K. D. (1998) 'Substrate specificity of deubiquitinating enzymes: Ubiquitin C-terminal hydrolases', *Biochemistry,* 37(10), pp. 3358-3368.

Lavrik, I., Krueger, A., Schmitz, I., Baumann, S., Weyd, H., Krammer, P. H. and Kirchhoff, S. (2003) 'The active caspase-8 heterotetramer is formed at the CD95 DISC [2]', *Cell Death and Differentiation,* 10(1), pp. 144-145.

Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G. and Earnshaw, W. C. (1994) 'Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE', *Nature,* 371(6495), pp. 346-347.

Le Clorennec, C., Lazrek, Y., Dubreuil, O., Sampaio, C., Larbouret, C., Lanotte, R., Poul, M. A., Barret, J. M., Prost, J. F., Pèlegrin, A. and Chardès, T. (2019) 'ITCH-dependent proteasomal degradation of c-FLIP induced by the anti-HER3 antibody 9F7-F11 promotes DR5/caspase 8-mediated apoptosis of tumor cells', *Cell Communication and Signaling,* 17(1), pp. 1-16.

Legler, D. F., Micheau, O., Doucey, M. A., Tschopp, J. and Bron, C. (2003) 'Recruitment of TNF receptor 1 to lipid rafts is essential for TNFα-mediated NF-κB activation', *Immunity,* 18(5), pp. 655-664.

Li, H., Wang, X., Li, N., Qiu, J., Zhang, Y. and Cao, X. (2007) 'hPEBP4 resists TRAIL-induced apoptosis of human prostate cancer cells by activating Akt and deactivating ERK1/2 pathways', *Journal of Biological Chemistry,* 282(7), pp. 4943-4950.

Li, H., Zhu, H., Xu, C. J. and Yuan, J. (1998) 'Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis', *Cell,* 94(4), pp. 491-501.

Li, Y., Kong, Y., Zhou, Z., Chen, H., Wang, Z., Hsieh, Y. C., Zhao, D., Zhi, X., Huang, J., Zhang, J., Li, H. and Chen, C. (2013) 'The HECTD3 E3 ubiquitin ligase facilitates cancer cell survival by promoting K63-linked polyubiquitination of caspase-8', *Cell Death Dis,* 4, pp. e935.

Liang, L. J., Si, Y., Tang, S., Huang, D., Wang, Z. A., Tian, C. and Zheng, J. S. (2018) 'Biochemical properties of K11,48-branched ubiquitin chains', *Chinese Chemical Letters,* 29(7), pp. 1155-1159.

Lim, M. C. C., Maubach, G., Sokolova, O., Feige, M. H., Diezko, R., Buchbinder, J., Backert, S., Schlüter, D., Lavrik, I. N. and Naumann, M. (2017) 'Pathogen-induced ubiquitin-editing enzyme A20 bifunctionally shuts off NF-κB and caspase-8-dependent apoptotic cell death', *Cell Death Differ,* 24(9), pp. 1621-1631.

Lin, S., Shang, Z., Li, S., Gao, P., Zhang, Y., Hou, S., Qin, P., Dong, Z., Hu, T. and Chen, P. (2018) 'Neddylation inhibitor MLN4924 induces G2 cell cycle arrest, DNA damage and sensitizes esophageal squamous cell carcinoma cells to cisplatin', *Oncology Letters,* 15(2), pp. 2583-2589.

Lingaraju, G. M., Bunker, R. D., Cavadini, S., Hess, D., Hassiepen, U., Renatus, M., Fischer, E. S. and Thomä, N. H. (2014) 'Crystal structure of the human COP9 signalosome', *Nature,* 512(7513), pp. 161-5.

Liu, J., Furukawa, M., Matsumoto, T. and Xiong, Y. (2002) 'NEDD8 modification of CUL1 dissociates p120CAND1, an inhibitor of CUL1-SKP1 binding and SCF ligases', *Molecular Cell,* 10(6), pp. 1511-1518.

Liu, J. and Nussinov, R. (2011) 'Flexible cullins in cullin-RING E3 ligases allosterically regulate ubiquitination', *Journal of Biological Chemistry,* 286(47), pp. 40934-40942.

Liu, K., Chen, K., Zhang, Q., Zhang, L., Yan, Y., Guo, C., Qi, J., Yang, K., Wang, F., Huang, P., Guo, L., Deng, L. and Li, C. (2019) 'TRAF6 neddylation drives inflammatory arthritis by increasing NF-κB activation', *Laboratory Investigation,* 99(4), pp. 528-538.

Liu, L., Lee, S., Zhang, J., Peters, S. B., Hannah, J., Zhang, Y., Yin, Y., Koff, A., Ma, L. and Zhou, P. (2009) 'CUL4A Abrogation Augments DNA Damage Response and Protection against Skin Carcinogenesis', *Molecular Cell,* 34(4), pp. 451-460.

Liu, X., Reitsma, J. M., Mamrosh, J. L., Zhang, Y., Straube, R. and Deshaies, R. J. (2018) 'Cand1-Mediated Adaptive Exchange Mechanism Enables Variation in F-Box Protein Expression', *Molecular Cell,* 69(5), pp. 773-786.e6.

Liu, X., Yue, P., Khuri, F. R. and Sun, S. Y. (2005) 'Decoy receptor 2 (DcR2) is a p53 target gene and regulates chemosensitivity', *Cancer Research*, 65(20), pp. 9169-9175.

Locksley, R. M., Killeen, N. and Lenardo, M. J. (2001) 'The TNF and TNF receptor superfamilies: Integrating mammalian biology', *Cell*, 104(4), pp. 487-501.

Lv, Y., Li, B., Han, K., Xiao, Y., Yu, X., Ma, Y., Jiao, Z. and Gao, J. (2018) 'The Nedd8-activating enzyme inhibitor MLN4924 suppresses colon cancer cell growth via triggering autophagy', *Korean Journal of Physiology and Pharmacology*, 22(6), pp. 617-625.

Lydeard, J. R., Schulman, B. A. and Harper, J. W. (2013) 'Building and remodelling Cullin-RING E3 ubiquitin ligases', *EMBO Reports*, 14(12), pp. 1050-1061.

Malsy, M., Bitzinger, D., Graf, B. and Bundscherer, A. (2019) 'Staurosporine induces apoptosis in pancreatic carcinoma cells PaTu 8988t and Panc-1 via the intrinsic signaling pathway', *European Journal of Medical Research*, 24(1), pp. 1-8.

Mandruzzato, S., Brasseur, F., Andry, G., Boon, T. and Van Der Bruggen, P. (1997) 'A CASP-8 mutation recognized by cytolytic T lymphocytes on a human head and neck carcinoma', *Journal of Experimental Medicine*, 186(5), pp. 785-793.

Manns, J., Daubrawa, M., Driessen, S., Paasch, F., Hoffmann, N., Löffler, A., Lauber, K., Dieterle, A., Alers, S., Iftner, T., Schulze-Osthoff, K., Stork, B. and Wesselborg, S. (2011) 'Triggering of a novel intrinsic apoptosis pathway by the kinase inhibitor staurosporine: activation of caspase-9 in the absence of Apaf-1', *FASEB J*, 25(9), pp. 3250-61.

Marsters, S. A., Sheridan, J. P., Pitti, R. M., Huang, A., Skubatch, M., Baldwin, D., Yuan, J., Gurney, A., Goddard, A. D., Godowski, P., Ashkenazi, A., Apol, A., Way, D. N. A., Francisco, S. S., Ctttcgatcc, A., Cttctcatgg, A., Acaaagcgtc, G., Cgagcgctcg, C., Tatccaggag, A., Gtcgggaacc, C., Tcctggaccc, A., Aagttcgtcg, C., Cgcggttctg, T., Gggttgactc, C., Ccccggcagg, T. and Ccagcagaca, A. (1997) 'A novel

receptor for Apo2L / TRAIL contains a truncated death domain mRNA transcript showed a unique expression pattern in human tissues and was particularly abundant in fetal liver and adult testis . Upon overexpression , DcR2 did not activate apoptosis', *Current Biology*, pp. 1003-1006.

Mathias, N., Johnson, S. L., Winey, M., Adams, A. E., Goetsch, L., Pringle, J. R., Byers, B. and Goebl, M. G. (1996) 'Cdc53p acts in concert with Cdc4p and Cdc34p to control the G1-to-S-phase transition and identifies a conserved family of proteins', *Molecular and Cellular Biology,* 16(12), pp. 6634-6643.

Medema, J. P., Scaffidi, C., Kischkel, F. C., Shevchenko, A., Mann, M., Krammer, P. H. and Peter, M. E. (1997) 'FLICE is activated by association with the CD95 death-inducing signaling complex (DISC)', *EMBO Journal,* 16(10), pp. 2794-2804.

Meir, M., Galanty, Y., Kashani, L., Blank, M., Khosravi, R., Fernández-Ávila, M. J., Cruz-Garciá, A., Star, A., Shochot, L., Thomas, Y., Garrett, L. J., Chamovitz, D. A., Bodine, D. M., Kurz, T., Huertas, P., Ziv, Y. and Shiloh, Y. (2015) 'The COP9 signalosome is vital for timely repair of DNA double-strand breaks', *Nucleic Acids Research,* 43(9), pp. 4517-4530.

Mendoza, H. M., Shen, L. N., Botting, C., Lewis, A., Chen, J., Ink, B. and Hay, R. T. (2003) 'NEDP1, a highly conserved cysteine protease that deNEDDylates cullins', *Journal of Biological Chemistry,* 278(28), pp. 25637-25643.

Merin, N. M. and Kelly, K. R. (2014) 'Clinical use of proteasome inhibitors in the treatment of multiple myeloma', *Pharmaceuticals,* 8(1), pp. 1-20.

Meyer, H. J. and Rape, M. (2014) 'Enhanced protein degradation by branched ubiquitin chains', *Cell,* 157(4), pp. 910-921.

Meyer-Schaller, N., Chou, Y. C., Sumara, I., Martin, D. D., Kurz, T., Katheder, N., Hofmann, K., Berthiaume, L. G., Sicheri, F. and Peter, M. (2009) 'The human Dcn1-like protein DCNL3 promotes Cul3 neddylation at membranes', *Proc Natl Acad Sci U S A,* 106(30), pp. 12365-70.

Micheau, O. and Tschopp, J. (2003) 'Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes', *Cell,* 114(2), pp. 181-190.

Miyamoto, S., Maki, M., Schmitt, M. J., Hatanaka, M. and Verma, I. M. (1994) 'Tumor necrosis factor α-induced phosphorylation of IκBα is a signal for its degradation but not dissociation from NF-κB', *Proceedings of the National Academy of Sciences of the United States of America,* 91(26), pp. 12740-12744.

Mohr, A., Yu, R. and Zwacka, R. M. (2015) 'TRAIL-receptor preferences in pancreatic cancer cells revisited: Both TRAIL-R1 and TRAIL-R2 have a licence to kill', *BMC Cancer,* 15(1), pp. 1-11.

Monda, J. K., Scott, D. C., Miller, D. J., Lydeard, J., King, D., Harper, W., Bennett, E. J. and Schulman, B. A. (2013) 'Structural conservation of distinctive N-terminal acetylation- dependent interactions across a family of mammalian NEDD8 ligation enzymes', *Structure,* 21(1), pp. 42-53.

Monleón, I., Martínez-Lorenzo, M. J., Monteagudo, L., Lasierra, P., Taulés, M., Iturralde, M., Piñeiro, A., Larrad, L., Alava, M. A., Naval, J. and Anel, A. (2001) 'Differential Secretion of Fas Ligand- or APO2 Ligand/TNF-Related Apoptosis-Inducing Ligand-Carrying Microvesicles During Activation-Induced Death of Human T Cells', *The Journal of Immunology,* 167(12), pp. 6736-6744.

Moreau, P., Richardson, P. G., Cavo, M., Orlowski, R. Z., San Miguel, J. F., Palumbo, A. and Harousseau, J. L. (2012) 'Proteasome inhibitors in multiple myeloma: 10 Years later', *Blood,* 120(5), pp. 947-959.

Morett, E. and Bork, P. (1999) 'A novel transactivation domain in parkin', *Trends in Biochemical Sciences,* 24(6), pp. 229-231.

Mungunsukh, O., Griffin, A. J., Lee, Y. H. and Day, R. M. (2010) 'Bleomycin induces the extrinsic apoptotic pathway in pulmonary endothelial cells', *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 298(5), pp. 696-703.

Munoz, I. M., Szyniarowski, P., Toth, R., Rouse, J. and Lachaud, C. (2014) 'Improved genome editing in human cell lines using the CRISPR method', *PLoS ONE*, 9(10).

Muppidi, J. R. and Siegel, R. M. (2004) 'Ligand-independent redistribution of Fas (CD95) into lipid rafts mediates clonotypic T cell death', *Nature Immunology*, 5(2), pp. 182-189.

Muppidi, J. R., Tschopp, J. and Siegel, R. M. (2004) 'Life and death decisions', *Immunity*, 21, pp. 461-465.

Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S. and Dixit, V. M. (1998) 'An induced proximity model for caspase-8 activation', *Journal of Biological Chemistry*, 273(5), pp. 2926-2930.

Nagai, K., Suzuki, H., Tanaka, N. and Umezawa, H. (1969) 'Decrease of melting temperature and single strand scission of dna by bleomycin in the presence of hydrogen peroxide', *The Journal of Antibiotics*, 22(12), pp. 624-628.

Nagano, T., Hashimoto, T., Nakashima, A., Kikkawa, U. and Kamada, S. (2012) 'X-linked inhibitor of apoptosis protein mediates neddylation by itself but does not function as a NEDD8-E3 ligase for caspase-7', *FEBS Letters*, 586(11), pp. 1612-1616.

Nakatsu, F., Sakuma, M., Matsuo, Y., Arase, H., Yamasaki, S., Nakamura, N., Saito, T. and Ohno, H. (2000) 'A di-leucine signal in the ubiquitin moiety: Possible involvement in ubiqutination-mediated endocytosis', *Journal of Biological Chemistry*, 275(34), pp. 26213-26219.

Ni, T., Li, W. and Zou, F. (2005) 'The ubiquitin ligase ability of IAPs regulates apoptosis', *IUBMB Life*, 57(12), pp. 779-85.

Nikolaev, A. Y., Li, M., Puskas, N., Qin, J. and Gu, W. (2003) 'Parc: A cytoplasmic anchor for p53', *Cell*, 112(1), pp. 29-40.

Obrig, T. G., Culp, W. J., McKeehan, W. L. and Hardesty, B. (1971) 'The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes', *Journal of Biological Chemistry,* 246(1), pp. 174-181.

Ohki, Y., Funatsu, N., Konishi, N. and Chiba, T. (2009) 'Biochemical and Biophysical Research Communications The mechanism of poly-NEDD8 chain formation in vitro', *Biochemical and Biophysical Research Communications,* 381(3), pp. 443-447.

Ohtake, F., Saeki, Y., Ishido, S., Kanno, J. and Tanaka, K. (2016) 'The K48-K63 Branched Ubiquitin Chain Regulates NF-κB Signaling', *Molecular Cell,* 64(2), pp. 251-266.

Ohtake, F., Saeki, Y., Sakamoto, K., Ohtake, K., Nishikawa, H., Tsuchiya, H., Ohta, T., Tanaka, K. and Kanno, J. (2015) 'Ubiquitin acetylation inhibits polyubiquitin chain elongation', *EMBO Rep,* 16(2), pp. 192-201.

Okatsu, K., Sato, Y., Yamano, K., Matsuda, N., Negishi, L., Takahashi, A., Yamagata, A., Goto-Ito, S., Mishima, M., Ito, Y., Oka, T., Tanaka, K. and Fukai, S. (2018) 'Structural insights into ubiquitin phosphorylation by PINK1', *Sci Rep,* 8(1), pp. 10382.

Osaka, F., Kawasaki, H., Aida, N., Saeki, M., Chiba, T., Kawashima, S., Tanaka, K. and Kato, S. (1998) 'A new NEDD8-ligating system for cullin-4A', *Genes Dev,* 12(15), pp. 2263-2268.

Ott, M., Robertson, J. D., Gogvadze, V., Zhivotovsky, B. and Orrenius, S. (2002) 'Cytochrome c release from mitochondria proceeds by a two-step process', *Proceedings of the National Academy of Sciences of the United States of America,* 99(3), pp. 1259-1263.

Ouyang, W., Yang, C., Zhang, S., Liu, Y., Yang, B., Zhang, J., Zhou, F., Zhou, Y. and Xie, C. (2013) 'Absence of death receptor translocation into lipid raftsin

acquired TRAIL-resistant NSCLC cells', *International Journal of Oncology,* 42(2), pp. 699-711.

Pająk, B., Gajkowska, B. and Orzechowski, A. (2005) 'Cycloheximide-mediated sensitization to TNF-α-induced apoptosis in human colorectal cancer cell line COLO 205; role of FLIP and metabolic inhibitors', *Journal of Physiology and Pharmacology,* 56(SUPPL. 3), pp. 101-118.

Pan, G., Ni, J., Wei, Y. F., Yu, G. I., Gentz, R. and Dixit, V. M. (1997a) 'An antagonist decoy receptor and a death domain-containing receptor for TRAIL', *Science,* 277(5327), pp. 815-818.

Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, R., Ni, J. and Dixit, V. M. (1997b) 'The receptor for the cytotoxic ligand TRAIL', *Science,* 276(5309), pp. 111-113.

Pan, Z. Q., Kentsis, A., Dias, D. C., Yamoah, K. and Wu, K. (2004) 'Nedd8 on cullin: Building an expressway to protein destruction', *Oncogene,* 23(11 REV. ISS. 1), pp. 1985-1997.

Pelzer, C., Kassner, I., Matentzoglu, K., Singh, R. K., Wollscheid, H. P., Scheffner, M., Schmidtke, G. and Groettrup, M. (2007) 'UBE1L2, a novel E1 enzyme specific for ubiquitin', *Journal of Biological Chemistry,* 282(32), pp. 23010-23014.

Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D. and Gygi, S. P. (2003) 'A proteomics approach to understanding protein ubiquitination', *Nature Biotechnology,* 21(8), pp. 921-926.

Peter, M. E. and Krammer, P. H. (2003) 'The CD95(APO-1/Fas) DISC and beyond', *Cell Death and Differentiation,* 10(1), pp. 26-35.

Peters, J. M. (1998) 'SCF and APC: The yin and yang of cell cycle regulated proteolysis', *Current Opinion in Cell Biology,* 10(6), pp. 759-768.

Petroski, M. D. and Deshaies, R. J. (2005) 'Function and regulation of cullin-RING ubiquitin ligases', *Nature Reviews Molecular Cell Biology,* 6(1), pp. 9-20.

Pickart, C. M. and Fushman, D. (2004) 'Polyubiquitin chains: Polymeric protein signals', *Current Opinion in Chemical Biology,* 8(6), pp. 610-616.

Pierce, N. W., Lee, J. E., Liu, X., Sweredoski, M. J., Graham, R. L. J., Larimore, E. A., Rome, M., Zheng, N., Clurman, B. E., Hess, S., Shan, S. O. and Deshaies, R. J. (2013) 'Cand1 promotes assembly of new SCF complexes through dynamic exchange of F box proteins', *Cell,* 153(1), pp. 206-215.

Pommier, Y., Leo, E., Zhang, H. and Marchand, C. (2010) 'DNA topoisomerases and their poisoning by anticancer and antibacterial drugs', *Chemistry and Biology,* 17(5), pp. 421-433.

Pop, C., Fitzgerald, P., Green, D. R. and Salvesen, G. S. (2007) 'Role of proteolysis in caspase-8 activation and stabilization', *Biochemistry,* 46(14), pp. 4398-4407.

Povirk, L. F. (1996) 'DNA damage and mutagenesis by radiomimetic DNA-cleaving agents: Bleomycin, neocarzinostatin and other enediynes', *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis,* 355(1-2), pp. 71-89.

Qin, H., Srinivasula, S. M., Wu, G., Fernandes-Alnemri, T., Alnemri, E. S. and Shi, Y. (1999) 'Structural basis of procaspase-9 recruitment by the apoptotic protease-activating factor 1', *Nature,* 399(6736), pp. 549-557.

Qin, Z. H., Wang, Y., Kikly, K. K., Sapp, E., Kegel, K. B., Aronin, N. and DiFiglia, M. (2001) 'Pro-caspase-8 Is Predominantly Localized in Mitochondria and Released into Cytoplasm upon Apoptotic Stimulation', *Journal of Biological Chemistry,* 276(11), pp. 8079-8086.

R Safa, A. (2013) 'Roles of c-FLIP in Apoptosis, Necroptosis, and Autophagy', *Journal of Carcinogenesis & Mutagenesis*.

Raiborg, C., Bache, K. G., Gillooly, D. J., Madshus, I. H., Stang, E. and Stenmark, H. (2002) 'Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes', *Nature Cell Biology,* 4(5), pp. 394-398.

Reverter, D., Wu, K., Erdene, T. G., Pan, Z. Q., Wilkinson, K. D. and Lima, C. D. (2005) 'Structure of a complex between Nedd8 and the Ulp/Senp protease family member Den1', *Journal of Molecular Biology,* 345(1), pp. 141-151.

Riley, B. E., Lougheed, J. C., Callaway, K., Velasquez, M., Brecht, E., Nguyen, L., Shaler, T., Walker, D., Yang, Y., Regnstrom, K., Diep, L., Zhang, Z., Chiou, S., Bova, M., Artis, D. R., Yao, N., Baker, J., Yednock, T. and Johnston, J. A. (2013) 'Structure and function of Parkin E3 ubiquitin ligase reveals aspects of RING and HECT ligases', *Nat Commun,* 4, pp. 1982.

Riley, J. S., Malik, A., Holohan, C. and Longley, D. B. (2015) 'DED or alive: Assembly and regulation of the death effector domain complexes', *Cell Death and Disease,* 6(8), pp. 1-16.

Roos, W. P. and Kaina, B. (2006) 'DNA damage-induced cell death by apoptosis', *Trends Mol Med,* 12(9), pp. 440-50.

Rotin, D. and Kumar, S. (2009) 'Physiological functions of the HECT family of ubiquitin ligases', *Nature Reviews Molecular Cell Biology,* 10(6), pp. 398-409.

Rudolph, M. J., Wuebbens, M. M., Rajagopalan, K. V. and Schindelin, H. (2001) 'Crystal structure of molybdopterin synthase and its evolutionary relationship to ubiquitin activation', *Nature Structural Biology,* 8(1), pp. 42-46.

Sakata, E., Yamaguchi, Y., Miyauchi, Y., Iwai, K., Chiba, T., Saeki, Y., Matsuda, N., Tanaka, K. and Kato, K. (2007) 'Direct interactions between NEDD8 and ubiquitin E2 conjugating enzymes upregulate cullin-based E3 ligase activity', *Nature Structural and Molecular Biology,* 14(2), pp. 167-168.

Saleh, M., Vaillancourt, J. P., Graham, R. K., Huyck, M., Srinivasula, S. M., Alnemri, E. S., Steinberg, M. H., Holan, V., Baldwin, C. T., Hotchkiss, R. S.,

Buchman, T. G., Zehnbauer, B. A., Hayden, M. R., Farrer, L. A., Roy, S. and Nicholson, D. W. (2004) 'Differential modulation of endotoxin responsiveness by human caspase-12 polymorphisms', *Nature,* 429(6987), pp. 75-79.

Sanlioglu, A. D., Dirice, E., Aydin, C., Erin, N., Koksoy, S. and Sanlioglu, S. (2005) 'Surface TRAIL decoy receptor-4 expression is correlated with TRAIL resistance in MCF7 breast cancer cells', *BMC Cancer,* 5, pp. 1-17.

Sanlioglu, A. D., Karacay, B., Koksal, I. T., Griffith, T. S. and Sanlioglu, S. (2007) 'DcR2 (TRAIL-R4) siRNA and adenovirus delivery of TRAIL (Ad5hTRAIL) break down in vitro tumorigenic potential of prostate carcinoma cells', *Cancer Gene Therapy,* 14(12), pp. 976-984.

Sarikas, A., Hartmann, T. and Pan, Z. Q. 2011. The cullin protein family.

Sarkaria, I., O-Charoenrat, P., Talbot, S. G., Reddy, P. G., Ngai, I., Maghami, E., Patel, K. N., Lee, B., Yonekawa, Y., Dudas, M., Kaufman, A., Ryan, R., Ghossein, R., Rao, P. H., Stoffel, A., Ramanathan, Y. and Singh, B. (2006) 'Squamous cell carcinoma related oncogene/DCUN1D1 is highly conserved and activated by amplification in squamous cell carcinomas', *Cancer Research,* 66(19), pp. 9437-9444.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H. and Peter, M. E. (1998) 'Two CD95 (APO-1/Fas) signaling pathways', *EMBO Journal,* 17(6), pp. 1675-1687.

Scheffner, M., Huibregtse, J. M., Vierstra, R. D. and Howley, P. M. (1993) 'The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53', *Cell,* 75(3), pp. 495-505.

Scheffner, M. and Kumar, S. (2014) 'Mammalian HECT ubiquitin-protein ligases: Biological and pathophysiological aspects', *Biochimica et Biophysica Acta - Molecular Cell Research,* 1843(1), pp. 61-74.

Schlesinger, D. H., Goldstein, G. and Niall, H. D. (1975) 'The complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells', *Biochemistry,* 14(10), pp. 2214-8.

Schlierf, A., Altmann, E., Quancard, J., Jefferson, A. B., Assenberg, R., Renatus, M., Jones, M., Hassiepen, U., Schaefer, M., Kiffe, M., Weiss, A., Wiesmann, C., Sedrani, R., Eder, J. and Martoglio, B. (2016) 'Targeted inhibition of the COP9 signalosome for treatment of cancer', *Nat Commun,* 7, pp. 13166.

Schulman, B. A. and Wade Harper, J. (2009) 'Ubiquitin-like protein activation by E1 enzymes: The apex for downstream signalling pathways', *Nature Reviews Molecular Cell Biology,* 10(5), pp. 319-331.

Scott, D. C., Hammill, J. T., Min, J., Rhee, D. Y., Connelly, M., Sviderskiy, V. O., Bhasin, D., Chen, Y., Ong, S. S., Chai, S. C., Goktug, A. N., Huang, G., Monda, J. K., Low, J., Kim, H. S., Paulo, J. A., Cannon, J. R., Shelat, A. A., Chen, T., Kelsall, I. R., Alpi, A. F., Pagala, V., Wang, X., Peng, J., Singh, B., Harper, J. W., Schulman, B. A. and Guy, R. K. (2017) 'Blocking an N-terminal acetylation-dependent protein interaction inhibits an E3 ligase', *Nature Chemical Biology,* 13(8), pp. 850-857.

Scott, D. C., Monda, J. K., Bennet, E. J., Harper, J. W. and Schulman, B. A. (2011) 'N-Terminal Acetylation Acts as an Avidity Enhancer Within an Interconnected Multiprotein Complex', *SCIENCE,* (November), pp. 674-679.

Scott, D. C., Monda, J. K., Grace, C. R. R., Duda, D. M., Kriwacki, R. W. and Kurz, T. (2010) 'Article A Dual E3 Mechanism for Rub1 Ligation to Cdc53', *Molecular Cell,* 39(5), pp. 784-796.

Scott, D. C., Sviderskiy, V. O., Monda, J. K., Lydeard, J. R., Cho, S. E., Harper, J. W. and Schulman, B. A. (2014) 'Structure of a RING E3 trapped in action reveals ligation mechanism for the ubiquitin-like protein NEDD8', *Cell,* 157(7), pp. 1671-1684.

Segovia, J. A., Tsai, S.-Y., Chang, T.-H., Shil, N. K., Weintraub, S. T., Short, J. D. and Bose, S. (2015) 'Nedd8 Regulates Inflammasome-Dependent Caspase-1 Activation', *Molecular and Cellular Biology,* 35(3), pp. 582-597.

Seibenhener, M. L., Babu, J. R., Geetha, T., Wong, H. C., Krishna, N. R. and Wooten, M. W. (2004) 'Sequestosome 1/p62 Is a Polyubiquitin Chain Binding Protein Involved in Ubiquitin Proteasome Degradation', *Molecular and Cellular Biology,* 24(18), pp. 8055-8068.

Seki, N., Hayakawa, Y., Brooks, A. D., Wine, J., Wiltrout, R. H., Yagita, H., Tanner, J. E., Smyth, M. J. and Sayers, T. J. (2003) 'Tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis is an important endogenous mechanism for resistance to liver metastases in murine renal cancer', *Cancer Research,* 63(1), pp. 207-213.

Seol, D. W., Li, J., Seol, M. H., Park, S. Y., Talanian, R. V. and Billiar, T. R. (2001) 'Signaling events triggered by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL): Caspase-8 is required for TRAIL-induced apoptosis', *Cancer Research,* 61(3), pp. 1138-1143.

Sharon, M., Mao, H., Boeri Erba, E., Stephens, E., Zheng, N. and Robinson, C. V. (2009) 'Symmetrical Modularity of the COP9 Signalosome Complex Suggests its Multifunctionality', *Structure,* 17(1), pp. 31-40.

Shen, L. N., Liu, H., Dong, C., Xirodimas, D., Naismith, J. H. and Hay, R. T. (2005) 'Structural basis of NEDD8 ubiquitin discrimination by the deNEDDylating enzyme NEDP1', *EMBO Journal,* 24(7), pp. 1341-1351.

Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, W. I., Goddard, A. D., Godowski, P. and Ashkenazi, A. (1997) 'Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors', *Science,* 277(5327), pp. 818-821.

Shi, Y. (2002) 'Mechanisms of caspase activation and inhibition during apoptosis', *Molecular Cell,* 9(3), pp. 459-470.

Simons, K. and Ikonen, E. (1997) 'Functional rafts in cell membranes', *Nature*, 387(6633), pp. 569-72.

Simons, K. and Toomre, D. (2000) 'LIPID RAFTS AND SIGNAL TRANSDUCTION', *Nature Reviews Molecular Cell Biology*, 1(1), pp. 31-41.

Simons, K. and Van Meer, G. (1988) 'Lipid Sorting in Epithelial Cells', *Biochemistry*, 27(17), pp. 6197-6202.

Smith, B. L., Bauer, G. B. and Povirk, L. F. (1994) 'DNA damage induced by bleomycin, neocarzinostatin, and melphalan in a precisely positioned nucleosome. Asymmetry in protection at the periphery of nucleosome-bound DNA', *Journal of Biological Chemistry*, 269(48), pp. 30587-30594.

Song, J. H., Tse, M. C. L., Bellail, A., Phuphanich, S., Khuri, F., Kneteman, N. M. and Hao, C. (2007) 'Lipid rafts and nonrafts mediate tumor necrosis factor-related apoptosis-inducing ligand-induced apoptotic and nonapoptotic signals in non-small cell lung carcinoma cells', *Cancer Research*, 67(14), pp. 6946-6955.

Soucy, T. A., Dick, L. R., Smith, P. G., Milhollen, M. A. and Brownell, J. E. (2010) 'The NEDD8 Conjugation Pathway and Its Relevance in Cancer Biology and Therapy', *Genes Cancer*, 1(7), pp. 708-16.

Soucy, T. A., Smith, P. G., Milhollen, M. A., Berger, A. J., Gavin, J. M., Adhikari, S., Brownell, J. E., Burke, K. E., Cardin, D. P., Critchley, S., Cullis, C. A., Doucette, A., Garnsey, J. J., Gaulin, J. L., Gershman, R. E., Lublinsky, A. R., McDonald, A., Mizutani, H., Narayanan, U., Olhava, E. J., Peluso, S., Rezaei, M., Sintchak, M. D., Talreja, T., Thomas, M. P., Traore, T., Vyskocil, S., Weatherhead, G. S., Yu, J., Zhang, J., Dick, L. R., Claiborne, C. F., Rolfe, M., Bolen, J. B. and Langston, S. P. (2009) 'An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer', *Nature*, 458(7239), pp. 732-6.

Soung, Y. H., Lee, J. W., Kim, S. Y., Sung, Y. J., Park, W. S., Nam, S. W., Kim, S. H., Lee, J. Y., Yoo, N. J. and Lee, S. H. (2005) 'Caspase-8 gene is frequently

inactivated by the frameshift somatic mutation 1225_1226delTG in hepatocellular carcinomas', *Oncogene,* 24(1), pp. 141-147.

Spencer, S. L., Gaudet, S., Albeck, J. G., Burke, J. M. and Sorger, P. K. (2009) 'Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis', *Nature,* 459(7245), pp. 428-32.

Sprick, M. R., Weigand, M. A., Rieser, E., Rauch, C. T., Juo, P., Blenis, J., Krammer, P. H. and Walczak, H. (2000) 'FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2', *Immunity,* 12(6), pp. 599-609.

Stennicke, H. R., Jürgensmeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Bredesen, D., Green, D. R., Reed, J. C., Froelich, C. J. and Salvesen, G. S. (1998) 'Pro-caspase-3 is a major physiologic target of caspase-8', *Journal of Biological Chemistry,* 273(42), pp. 27084-27090.

Stepczynska, A., Lauber, K., Engels, I. H., Janssen, O., Kabelitz, D., Wesselborg, S. and Schulze-Osthoff, K. (2001) 'Staurosporine and conventional anticancer drugs induce overlapping, yet distinct pathways of apoptosis and caspase activation', *Oncogene,* 20(10), pp. 1193-1202.

Stewart, M. D., Ritterhoff, T., Klevit, R. E. and Brzovic, P. S. (2016) 'E2 enzymes : more than just middle men', *Nature Publishing Group,* 26(4), pp. 423-440.

Sträter, J., Hinz, U., Walczak, H., Mechtersheimer, G., Koretz, K., Herfarth, C., Möller, P. and Lehnert, T. (2002) 'Expression of TRAIL and TRAIL receptors in colon carcinoma: TRAIL-R1 is an independent prognostic parameter', *Clinical Cancer Research,* 8(12), pp. 3734-3740.

Stubbe, J. and Kozarich, J. W. (1987) 'Mechanisms of Bleomycin-Induced DNA Degradation', *Chemical Reviews,* 87(5), pp. 1107-1136.

Swaney, D. L., Rodríguez-Mias, R. A. and Villén, J. (2015) 'Phosphorylation of ubiquitin at Ser65 affects its polymerization, targets, and proteome-wide turnover', *EMBO Rep,* 16(9), pp. 1131-44.

Swords, R. T., Erba, H. P., Deangelo, D. J., Bixby, D. L., Altman, J. K., Maris, M., Hua, Z., Blakemore, S. J., Faessel, H., Sedarati, F., Dezube, B. J., Giles, F. J. and Medeiros, B. C. (2015) 'Pevonedistat (MLN4924), a First-in-Class NEDD8-activating enzyme inhibitor, in patients with acute myeloid leukaemia and myelodysplastic syndromes: A phase 1 study', *British Journal of Haematology,* 169(4), pp. 534-543.

Tait, S. W. G. and Green, D. R. (2008) 'Caspase independent cell death: leaving the set without the final cut', *Oncogene,* 27(50), pp. 6452-6461.

Tang, Z., Li, B., Bharadwaj, R., Zhu, H., Özkan, E., Hakala, K., Deisenhofer, J. and Yu, H. (2001) 'APC2 cullin protein and APC11 RING protein comprise the minimal ubiquitin ligase module of the anaphase-promoting complex', *Molecular Biology of the Cell,* 12(12), pp. 3839-3851.

Tenev, T., Bianchi, K., Darding, M., Broemer, M., Langlais, C., Wallberg, F., Zachariou, A., Lopez, J., MacFarlane, M., Cain, K. and Meier, P. (2011) 'The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs', *Mol Cell,* 43(3), pp. 432-48.

Terlizzi, M., Di Crescenzo, V. G., Perillo, G., Galderisi, A., Pinto, A. and Sorrentino, R. (2015) 'Pharmacological inhibition of caspase-8 limits lung tumour outgrowth', *British Journal of Pharmacology,* 172(15), pp. 3917-3928.

Thibaudeau, T. A. and Smith, D. M. (2019) 'A practical review of proteasome pharmacology', *Pharmacological Reviews,* 71(2), pp. 170-197.

Thomas, Y., Scott, D. C., Kristariyanto, Y. A., Rinehart, J., Clark, K., Cohen, P. and Kurz, T. (2018) 'The NEDD8 E3 ligase DCNL5 is phosphorylated by IKK alpha during Toll-like receptor activation', *PLoS ONE,* 13(6), pp. 1-19.

Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. J. F., Egger, L. A., Gaffney, E. P., Limjuco, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T.-T., Lee, T. D., Shively, J. E., MacCross, M., Mumford, R. A., Schmidt, J. A. and Tocci, M. J. (1992) 'A novel heterodimeric cysteine protease is required for interleukin-1ßprocessing in monocytes', *Nature,* 355(6372), pp. 242-244.

Thrower, J. S. (2000) 'Recognition of the polyubiquitin proteolytic signal', *The EMBO Journal,* 19(1), pp. 94-102.

Tomar, D., Prajapati, P., Sripada, L., Singh, K., Singh, R. and Singh, A. K. (2013) 'TRIM13 regulates caspase-8 ubiquitination, translocation to autophagosomes and activation during ER stress induced cell death', *Biochim Biophys Acta,* 1833(12), pp. 3134-3144.

Trivedi, R. and Mishra, D. P. (2015) 'Trailing TRAIL resistance: Novel targets for TRAIL sensitization in cancer cells', *Frontiers in Oncology,* 5(APR).

Twenytman, P. R. (1983) 'Bleomycin - Mode of Action With Particular Reference to the Cell Cycle', *Pharmacology & Therapeutics,* 23(3), pp. 417-441.

Twomey, J. D., Kim, S. R., Zhao, L., Bozza, W. P. and Zhang, B. (2015) 'Spatial dynamics of TRAIL death receptors in cancer cells', *Drug Resistance Updates,* 19, pp. 13-21.

Van Der Reijden, B. A., Erpelinck-Verschueren, C. A. J., Bob, L. and Jansen, J. H. (1999) 'TRIADs: A new class of proteins with a novel cysteine-rich signature', *Protein Science,* 8(7), pp. 1557-1561.

van der Veen, A. G. and Ploegh, H. L. (2012) 'Ubiquitin-Like Proteins', *Annual Review of Biochemistry,* 81(1), pp. 323-357.

van Loo, G., Saelens, X., Matthijssens, F., Schotte, P., Beyaert, R., Declercq, W. and Vandenabeele, P. (2002) 'Caspases are not localized in mitochondria during life or death', *Cell Death and Differentiation,* 9(11), pp. 1207-1211.

Varfolomeev, E., Maecker, H., Sharp, D., Lawrence, D., Renz, M., Vucic, D. and Ashkenazi, A. (2005) 'Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand', *Journal of Biological Chemistry,* 280(49), pp. 40599-40608.

Varfolomeev, E. E. and Ashkenazi, A. (2004) 'Tumor Necrosis Factor: An Apoptosis JuNKie?', *Cell,* 116(4), pp. 491-497.

Vernole, P., Tedeschi, B., Caporossi, D., Maccarrone, M., Melino, G. and Annicchiarico-Petruzzelli, M. (1998) 'Induction of apoptosis by bleomycin in resting and cycling human lymphocytes', *Mutagenesis,* 13(3), pp. 209-215.

Voelkel-Johnson, C., King, D. L. and Norris, J. S. (2002) 'Resistance of prostate cancer cells to soluble TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) can be overcome by doxorubicin or adenoviral delivery of full-length TRAIL', *Cancer Gene Therapy,* 9(2), pp. 164-172.

Wada, H., Kito, K., Caskey, L. S., Yeh, E. T. H. and Kamitani, T. (1998) 'Cleavage of the C-terminus of NEDD8 by UCH-L3', *Biochemical and Biophysical Research Communications,* 251(3), pp. 688-692.

Wajant, H., Pfizenmaier, K. and Scheurich, P. (2003) 'Tumor necrosis factor signaling', *Cell Death and Differentiation,* 10(1), pp. 45-65.

Walczak, H., Degli-Esposti, M. A., Johnson, R. S., Smolak, P. J., Waugh, J. Y., Boiani, N., Timour, M. S., Gerhart, M. J., Schooley, K. A., Smith, C. A., Goodwin, R. G. and Rauch, C. T. (1997) 'TRAIL-R2: A novel apoptosis-mediating receptor for TRAIL', *EMBO Journal,* 16(17), pp. 5386-5397.

Walczak, H. and Krammer, P. H. (2000) 'The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems', *Experimental Cell Research,* 256(1), pp. 58-66.

Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R. G., Rauch, C. T., Schuh, J. A. C. L. and Lynch, D. H. (1999) 'Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo', *Nature Medicine*, 5(2), pp. 157-163.

Walden, H., Podgorski, M. S. and Schulman, B. A. (2003) 'Insights into the ubiquitin transfer cascade from the structure of the activating enzyme for NEDD8', *Nature*, 422(6929), pp. 330-4.

Walden, H. and Rittinger, K. (2018) 'RBR ligase-mediated ubiquitin transfer: A tale with many twists and turns', *Nature Structural and Molecular Biology*, 25(6), pp. 440-445.

Wang, L., Du, F. and Wang, X. (2008) 'TNF-alpha induces two distinct caspase-8 activation pathways', *Cell*, 133(4), pp. 693-703.

Wang, W., Zhang, M., Sun, W., Yang, S., Su, Y., Zhang, H., Liu, C., Li, X., Lin, L., Kim, S., Okunieff, P., Zhang, Z. and Zhang, L. (2013) 'Reduction of Decoy Receptor 3 Enhances TRAIL-Mediated Apoptosis in Pancreatic Cancer', *PLoS ONE*, 8(10).

Wang, Y., Luo, Z., Pan, Y., Wang, W., Zhou, X., Jeong, L. S., Chu, Y., Liu, J. and Jia, L. (2015) 'Targeting protein neddylation with an NEDD8-activating enzyme inhibitor MLN4924 induced apoptosis or senescence in human lymphoma cells', *Cancer Biology and Therapy*, 16(3), pp. 420-429.

Wenzel, D. M., Lissounov, A., Brzovic, P. S. and Klevit, R. E. (2011) 'UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids', *Nature*, 474(7349), pp. 105-8.

Whitby, F. G., Xia, G., Pickart, C. M. and Hill, C. P. (1998) 'Crystal structure of the human ubiquitin-like protein NEDD8 and interactions with ubiquitin pathway enzymes', *J Biol Chem*, 273(52), pp. 34983-91.

Wolenski, F. S., Fisher, C. D., Sano, T., Wyllie, S. D., Cicia, L. A., Gallacher, M. J., Baker, R. A., Kirby, P. J. and Senn, J. J. (2015) 'The NAE inhibitor pevonedistat (MLN4924) synergizes with TNF-α to activate apoptosis', *Cell Death Discovery*, 1(1), pp. 1-9.

Wolf, D. A., Zhou, C. and Wee, S. (2003) 'The COP9 signalosome: an assembly and maintenance platform for cullin ubiquitin ligases?', *Nat Cell Biol*, 5(12), pp. 1029-1033.

Wu, K., Chen, A., Tan, P. and Pan, Z. Q. (2002) 'The Nedd8-conjugated ROC1-CUL1 core ubiquitin ligase utilizes Need8 charged surface residues for efficient polyubiquitin chain assembly catalyzed by Cdc34', *Journal of Biological Chemistry*, 277(1), pp. 516-527.

Wu, K., Yamoah, K., Dolios, G., Gan-Erdene, T., Tan, P., Chen, A., Lee, C. G., Wei, N., Wilkinson, K. D., Wang, R. and Pan, Z. Q. (2003) 'DEN1 is a dual function protease capable of processing the c terminus of Nedd8 and deconjugating hyper-neddylated CUL1', *Journal of Biological Chemistry*, 278(31), pp. 28882-28891.

Wu, K., Yan, H., Fang, L., Wang, X., Pfleger, C., Jiang, X., Huang, L. and Pan, Z. Q. (2011) 'Mono-ubiquitination drives nuclear export of the human DCN1-like protein hDCNL', *Journal of Biological Chemistry*, 286(39), pp. 34060-34070.

Wu, S., Zhu, W., Nhan, T., Toth, J. I., Petroski, M. D. and Wolf, D. A. (2013) 'CAND1 controls in vivo dynamics of the cullin 1-RING ubiquitin ligase repertoire', *Nature Communications*, 4, pp. 1-9.

Xie, P., Zhang, M., He, S., Lu, K., Chen, Y., Xing, G., Lu, Y., Liu, P., Li, Y., Wang, S., Chai, N., Wu, J., Deng, H., Wang, H. R., Cao, Y., Zhao, F., Cui, Y., Wang, J., He, F. and Zhang, L. (2014) 'The covalent modifier Nedd8 is critical for the activation of Smurf1 ubiquitin ligase in tumorigenesis', *Nature Communications*, 5(May).

Yadav, S. S., Prasad, C. B., Prasad, S. B., Pandey, L. K., Singh, S., Pradhan, S. and Narayan, G. (2015) 'Anti-tumor activity of staurosporine in the tumor

microenvironment of cervical cancer: An in vitro study', *Life Sciences,* 133, pp. 21-28.

Yu, H., Peters, J. M., King, R. W., Page, A. M., Hieter, P. and Kirschner, M. W. (1998) 'Identification of a cullin homology region in a subunit of the anaphase-promoting complex', *Science,* 279(5354), pp. 1219-1222.

Zachariae, W., Shevchenko, A., Andrews, P. D., Ciosk, R., Galova, M., Stark, M. J. R., Mann, M. and Nasmyth, K. (1998) 'Mass spectrometric analysis of the anaphase-promoting complex from yeast: Identification of a subunit related to cullins', *Science,* 279(5354), pp. 1216-1219.

Zhang, L. and Fang, B. (2005) 'Mechanisms of resistance to TRAIL-induced apoptosis in cancer', *Cancer Gene Ther,* 12(3), pp. 228-37.

Zhang, Y. and Zhang, B. (2008) 'TRAIL resistance of breast cancer cells is associated with constitutive endocytosis of death receptors 4 and 5', *Molecular Cancer Research,* 6(12), pp. 1861-1871.

Zhang, Y. P., Kong, Q. H., Huang, Y., Wang, G. L. and Chang, K. J. (2015) 'Inhibition of c-FLIP by RNAi enhances sensitivity of the human osteogenic sarcoma cell line U2OS to TRAIL-induced apoptosis', *Asian Pacific Journal of Cancer Prevention,* 16(6), pp. 2251-2256.

Zhang, Y. X., Yu, S. B., Ou-Yang, J. P., Xia, D., Wang, M. and Li, J. R. (2005) 'Effect of protein kinase C alpha, caspase-3, and survivin on apoptosis of oral cancer cells induced by staurosporine', *Acta Pharmacologica Sinica,* 26(11), pp. 1365-1372.

Zhao, L., Yue, P., Lonial, S., Khuri, F. R. and Sun, S. Y. (2011) 'The NEDD8-activating enzyme inhibitor, MLN4924, cooperates with TRAIL to augment apoptosis through facilitating c-FLIP degradation in head and neck cancer cells', *Molecular Cancer Therapeutics,* 10(12), pp. 2415-2425.

Zhao, Y., Shen, Y., Yang, S., Wang, J., Hu, Q., Wang, Y. and He, Q. (2010) 'Ubiquitin ligase components Cullin4 and DDB1 are essential for DNA methylation in Neurospora crassa', *Journal of Biological Chemistry,* 285(7), pp. 4355-4365.

Zhao, Y. and Sun, Y. (2013) 'Cullin-RING Ligases as attractive anti-cancer targets', *Curr Pharm Des,* 19(18), pp. 3215-25.

Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, D. M., Elledge, S. J., Pagano, M., Conaway, R. C., Conaway, J. W., Harper, J. W. and Pavletich, N. P. (2002) 'Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex', *Nature,* 416(6882), pp. 703-709.

Zhivotovsky, B., Samali, A., Gahm, A. and Orrenius, S. (1999) 'Caspases: Their intracellular localization and translocation during apoptosis', *Cell Death and Differentiation,* 6(7), pp. 644-651.

Zhou, H., Lu, J., Liu, L., Bernard, D., Yang, C. Y., Fernandez-Salas, E., Chinnaswamy, K., Layton, S., Stuckey, J., Yu, Q., Zhou, W., Pan, Z., Sun, Y. and Wang, S. (2017) 'A potent small-molecule inhibitor of the DCN1-UBC12 interaction that selectively blocks cullin 3 neddylation', *Nature Communications,* 8(1), pp. 1-12.

Zong, H., Yin, B., Chen, J., Ma, B., Cai, D. and He, X. (2009) 'Over-expression of c-FLIP confers the resistance to TRAIL-induced apoptosis on gallbladder carcinoma', *Tohoku Journal of Experimental Medicine,* 217(3), pp. 203-208.