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O'É Gaillimh
NUI Galway

**The Molecular Mechanisms of 5-Fluorouracil-Induced
Apoptosis in Human Colorectal Cancer Cells and the Use of
5-FU as a Sensitising Agent to TRAIL**

By

Laura Marie Deedigan B.Sc.

This thesis is submitted to the National University of Ireland, Galway, as partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Supervisors: Dr. Ralf Zwacka & Dr. Andrea Mohr

“THE LAST ENEMY THAT SHALL BE DESTROYED IS DEATH”

- J.K. ROWLING



*This thesis is dedicated in loving memory of my family
members who have lost their fight to cancer.*



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ABSTRACT

Colorectal cancer is the third most common cause of death worldwide and despite many advances in recent years resistance to apoptosis induced by chemotherapeutic drugs, such as 5-fluorouracil (5-FU), remains a serious issue. A better understanding of how chemotherapeutic drugs induce apoptosis in colon cancer cells will better inform us on how to develop novel therapeutic strategies to overcome this resistance. We investigated cell death induced by 5-FU in the colon cancer cell line HCT116, and found that not only cell death, but also cytochrome-c release was dependent on an upstream caspase activity. Upon further investigation, we discovered a large (1-2 MDa) apoptosis-inducing complex forming following 5-FU-mediated RNA stress. This complex could be purified and identified by sucrose gradient density fractionation and was found to contain the core components caspase-8, FADD and RIP1 with interaction from Bid. In the absence of caspase-8 and FADD, complex formation and apoptosis was abolished. This complex forms without involvement of death receptors such as CD95 or the TRAIL receptors DR4 and DR5 despite a clear upregulation of DR5 protein levels following 5-FU. Furthermore, knocking down DR5 had no effect on initial caspase-8 cleavage and did not prevent complex formation. This complex forms upstream of the mitochondria, evidenced by overexpression of Bcl-2 or knocking down Bax or Bid. In addition we could demonstrate the inducible interaction between the complex members caspase-8 and FADD as well as FADD with RIP1 by co-immunoprecipitation, and the inducible interaction of FADD molecules following 5-FU stimulation.

We also explored the contribution of the 5-FU-induced DR5 upregulation to Tumour necrosis-factor related apoptosis-inducing ligand (TRAIL)-induced apoptosis in HCT116 cells. TRAIL selectively induces rapid apoptosis in most tumour cell types, and represents a promising anti-cancer agent. Subtoxic doses of TRAIL-induced apoptosis could be enhanced by co-treatment with low doses of 5-FU in a caspase-dependent manner. Sensitisation to TRAIL involved enhanced DR5 expression and activation of the caspase cascade. Our results demonstrate that caspase-8 expression is necessary for this 5-FU-mediated TRAIL sensitivity. Finally, we demonstrated that low-dose 5-FU pre-treatment sensitises HCT116 cells to Mesenchymal stem cell-mediated delivery of sTRAIL *in vitro*.

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LIST OF ABBREVIATIONS

Ad	Adenovirus
APS	Ammonium persulphate
Apaf-1	Apoptotic protease-activating factor-1
ATP	Adenosine triphosphate
ATM	Ataxia telangiectasia mutated
ATR	ATM- and RAD3-related
Bad	Bcl-2-antagonist of cell death
Bak	Bcl-2-antagonist killer 1
Bax	Bcl-2-associated X protein
BD	BD Pharmingen
BCA	Bichinchoninic acid assay
Bcl-2	B-cell lymphomas
Bcl-X_L	Bcl-2-like 1 long
BH	Bcl-2 homology
Bid	BH3 interacting domain against death agonist
Bim	Bcl-2 interacting mediator of cell death
BIR	Baculovirus IAP repeat
BSA	Bovine serum albumin
C-3	Caspase-3
C-8	Caspase-8
C-9	Caspase-9
CaCl₂	Calcium chloride
CAD	Caspase-activated DNase
CARD	Caspase recruitment domain

Caspase	CysteinyI aspartate specific proteinases
CD95	Cluster of differentiation 95
c-FLIP_L	Cellular-FLICE-like inhibitory protein long
c-FLIP_S	Cellular-FLICE-like inhibitory protein short
c-IAP	Cellular-IAP
CPT-11	Camptothecin-11
CO₂	Carbon dioxide
CRC	Colorectal cancer
CS	Cell Signaling Technology
C-terminus	Carboxy-terminus
CuZnSOD	Copper Zinc Superoxide dismutase
DcR	Decoy receptor
DD	Death-domain
DED	Death effector domain
DEF	Death effector filaments
dH₂O	Distilled water
DIABLO	Direct inhibitor of apoptosis protein binding protein with low pI
DISC	Death-inducing signalling complex
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DN	Dominant Negative
DNA	Deoxyribonucleic acid
DNA-PK	DNA-activated protein kinase
dNTP	Deoxynucleotide triphosphate
DR	Death receptor

DsRed	Red fluorescent Protein
dTMP	Thymidine monophosphate
dTTP	Thymidine triphosphate
dUMP	Deoxyuridine monophosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra-acetic acid
EGFP	Enhanced green fluorescent protein
FACS	Fluorescence-activated cell sorting
FADD	Fas associated death domain
FasL	Fas Ligand
FBS	Foetal bovine serum
FdUMP	5-fluorodeoxyuridine monophosphate
FdUTP	5-fluorodeoxyuridine triphosphate
Fig	Figure
FLICE	Fas-associated death-domain-like IL-1 β -converting enzyme
FUTP	5-fluorouridine triphosphate
g	gram
H₂O	Water (Dihydrogen monoxide)
HBS	HEPES-buffered saline
HCl	Hydrogen chloride
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
h	Hour
ICE	Interleukin-1 β -converting enzyme
ICAD	Inhibitor of caspase-activated Dnase
IgG	Immunoglobulin G

IFN-γ	Interferon gamma
IP	Immunoprecipitation
IPLA₂	Phospholipase A2
PGE₂	Prostaglandin E2
JNK	c-Jun N-terminal kinase
kDa	Kilo-Dalton
KCl	Potassium chloride
L	Litre
LB	Luria-Bertani medium
μg	Microgram
μl	Microliter
μM	Micromolar
MDa	Mega-dalton
MDM-2	Murine double minute-2
MEF	Mouse embryonic fibroblasts
mg	Milligram
MgCl₂	Magnesium chloride
min	Minute
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MW	Molecular weight
NaCl	Sodium chloride
NaF	Sodium fluoride

NP-40	Nonyl phenoxypolyethoxylethanol
NSAID	Non-steroid anti-inflammatory drugs
N-terminus	Amino-terminus
OMM	Outer mitochondrial membrane
O/N	Overnight
p21	Protein 21
p53	Protein 53
PAK-2	p21-activated kinase
PBS	Phosphate buffered saline
pFADD	Phosphorylated FADD
pH	The Potential of Hydrogen
PI	Propidium iodide
PUMA	p53 upregulated modulator of apoptosis
RIP1	Receptor-interacting protein-1
RNA	Ribonucleic acid
RNAi	RNA interference
ROCK-1	Rho-associated, coiled coil containing protein kinase-1
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	Standard error
shRNA	Short hairpin RNA
Smac	Second mitochondrial-derived activator of caspase
t-Bid	Truncated-Bid

TEMED	N, N, N', N' –tetramethylethylenediamine
TNF	Tumour necrosis factor
TNFR-1	Tumour necrosis factor receptor -1
TRADD	Tumour necrosis factor receptor-associated death domain
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
rhTRAIL	Recombinant TRAIL
sTRAIL	Soluble TRAIL
Tris	Tris (hydroxymethyl) aminomethane
TS	Thymidylate synthase
UV	Ultraviolet
V	Volume
v/v	Volume/volume
w	Weight
w/w	Weight/weight
wt	Wild type
XIAP	X-chromosome-linked IAP
w/v	Weight/volume
Z-VAD-FMK	Benzyloxycarbonil-Val-Ala-DL-Asp-fluoromethylketone
%	Percent
°C	Degrees centigrade
-/-	Null
α	Alpha
β	Beta
5-AZA	5-aza-2' deoxycytidine
5-FU	5-Fluorouracil

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DECLARATION

I declare that all the work in this thesis was performed personally unless stated otherwise. No part of this work has been submitted for consideration as part of any other degree or award.

CHAPTER 1

INTRODUCTION

1.1 COLORECTAL CANCER & CURRENT TREATMENT REGIMENS

Colorectal Cancer

Colorectal cancer (CRC) is a significant health issue in the Western world, where Ireland has the highest mortality rate for colorectal cancer in Western Europe and according to GLOBOCAN (2002) had the fourth highest mortality rate amongst men worldwide (Parkin et al. 2005). In Ireland it is the second most commonly diagnosed cancer among both men and women and every year there are approximately 1,900 new cases of colorectal cancer diagnosed. In addition colorectal cancer is the second most common fatal cancer among men and women in Ireland where around 930 people die from colorectal cancer each year. It is estimated that over the last 15 years the number of cases of colorectal cancer has risen by approximately 20% in both sexes and according to statistics by 2020 the number of new cases of colorectal cancer diagnosed each year in Ireland is projected to increase by 79% in men and 56% in women. This projected growth is to a large extent caused by an increasing and ageing population (O' Brien, 2008).

In general cancers are said to develop from alterations at the genetic and epigenetic level, which then further progress to dysregulation in the many complex molecular pathways that control normal cell proliferation and growth (Markowitz & Bertagnolli 2009). In the case of colorectal cancer, it is most often said to arise sporadically, with a smaller number of cases arising as a result of inherited mutations or resulting from inflammatory bowel disease (Berg & Søreide 2011). This disease typically originates from a benign polyp through an adenoma with dysplasia into a carcinoma with metastatic potential (Berg & Søreide 2011) and can be subdivided into three molecular based groups termed MSI (microsatellite instability) (Søreide et al. 2006), CIN (chromosomal instability) (Lengauer et al. 1997) or CIMP (CpG island methylator phenotype) (Baylin et al. 1986; Toyota et al. 1999).

The current treatment regimen for colorectal cancer patients differs dependent on disease stage, which is based on tumour size and degree of penetration (see **Table**

1.1). When diagnosed at an early, localised disease stage, where the tumour is small and has not penetrated the mucosal layer (Stage I), patients undergo curative surgical resection. Stage II patients, in which there is localised spread into the muscle wall with no lymph node involvement, are treated surgically with or without 5-Fluorouracil (5-FU)-based adjuvant chemotherapy.

Table 1.1 Staging of Colorectal Cancer (adapted from Obel, 2006.)

Stage	T Stage	N Stage	M Stage	5 Year Survival (%)
I	T1 or T2	N0	M0	93.2
IIa	T3	N0	M0	84.7
IIb	T4	N0	M0	72.2
IIIa	T1 or T2	N1	M0	83.4
IIIb	T3 or T4	N1	M0	64.1
IIIc	Any T	N2	M0	44.3
IV	Any T	Any N	M1	8.1

T1 = Limited to mucosa and submucosa, **T2** = Extension into but not through muscularis propria, **T3** = Invasion of perirectal fat, **T4** = Invasion of adjacent structures, **N0** = No involvement of lymph nodes, **N1** = Fewer than four regional nodes involved, **N2** = More than four regional nodes involved, **M0** = No metastasis, **M1** = Distant metastasis.

The 5-year survival of Stage II patients undergoing surgical resection alone is approximately 75%, indicating that only/maximal 25% of Stage II patients may potentially benefit from adjuvant chemotherapy (Hector & Prehn 2009). In the most advanced stages of the disease, characterised by nearby lymph node involvement (Stage III) or in the rare case of metastasis to remote organs (Stage IV), the current treatment paradigm is surgical resection followed by 5-FU adjuvant-based chemotherapy (Hector & Prehn 2009).

Treatment Strategies: Past and Present

First line treatment for patients diagnosed with colorectal cancer is usually surgery followed by chemotherapy to kill any residual disease (Rosen et al. 2000). Surgery is curative in about 60% of patients but many patients progress to develop metastatic lesions to distant sites, which require further chemotherapy. Traditionally, the most common chemotherapeutic treatment for advanced colorectal cancer has been 5-FU since its development in 1957 by Heidelberger (Curreri et al. 1958). 5-FU is a molecule that is converted to an active metabolite and inhibits DNA and RNA synthesis and repair by forming a stable ternary complex with thymidylate synthase (TS) (de Gramont et al. 1997). Unfortunately as a monotherapy 5-FU has disappointing response rates of between 10-15% (Goldberg & Gill 2004; André et al. 2004; Peters & van Groeningen 1991). In light of these less than satisfactory improvements with 5-FU monotherapy, there have been considerable strides to implement changes in the treatment regimens to include novel chemotherapeutic agents and the treatment of colorectal cancer has changed significantly since the initial FDA approval of 5-FU in 1962. More recently, combination therapies have been used to greater success, but generally these are all still based on 5-FU as the core drug (Goldberg & Gill 2004; André et al. 2004).

5-FU is most commonly used in combination with Leucovorin (i.e. folinic acid), which appears to stabilise the binding of the drug to TS, and can often be combined with Irinotecan (a topoisomerase inhibitor that blocks DNA repair and possibly down-regulated expression of TS) often termed FOLFIRI (FOL from folinic acid, F from 5-FU and IRI from Irinotecan). This combination demonstrated statistically significant clinical benefits, including improved tumour control and prolonged survival in a number of clinical studies (Saltz, 2000; Douillard et al., 2000). When combined with Oxaliplatin it is often termed FOLFOX (OX from Oxaliplatin) (Goldberg & Gill 2004). Oxaliplatin and Irinotecan exert their cytotoxic effects via induction of the DNA damage response, leading to cell cycle arrest and/or induction of apoptosis.

The main concern with the current FOLFIRI and FOLFOX combinations is the associated toxicities which include diarrhoea, nausea and neurotoxicity (Falcone et al. 2007; Ramanathan et al. 2003). By treating patients with a FOLFOXIRI combination, improved response rates have been achieved although this is at the expense of

increased, but generally manageable, toxicities when compared to FOLFIRI treatment (Anon 1995; Falcone et al. 2007; Montagnani et al. 2010). The drawback of such side effects is that they ultimately lead to limitations in patient treatment therefore an important goal of novel therapeutic strategies is to improve the efficacy of treatment while reducing such side effects. Although the introduction of combination therapies of oxaliplatin and irinotecan with 5-FU have evolved the response rates of 5-FU monotherapy from 10-15% (Giacchetti et al., 2000) to 40-50% (Douillard et al., 2000) new strategies for therapy and resistance reversal are required (Zhang et al., 2008). Indeed, resistance (both intrinsic and acquired), to chemotherapeutic agents remains one of the major reasons for death of colon cancer patients after surgery. This requires that we understand the determinants of 5-FU sensitivity and resistance in colon cancer cells. Therefore there is a great importance in identifying the key mechanisms of 5-FU-induced cell death to help develop new patient-tailored treatment strategies for colorectal cancer patients. This would enable screening of patients to determine whether they are likely to benefit from 5-FU therapy or whether a different combination therapy might be more successful.

1.2 KNOWN MECHANISMS OF ACTION OF 5-FU

5-FU has been the mainstay of colorectal cancer treatment for over 40 years, whereby it improves overall and disease-free survival of patients with resected stage III colorectal cancer (*IMPACT*, 1995). However, response rates for 5-FU in advanced colorectal cancer are modest (Johnston & Kaye 2001). Although combining 5-FU with newer chemotherapeutic agents Oxaliplatin and Irinotecan has improved response rates to between 40-50% (Giacchetti et al. 2000; Douillard et al. 2000), new therapeutic strategies are necessary. Understanding the molecular mechanism of action of 5-FU is needed if drug resistance is to be overcome by improvements to current treatment regimens.

5-FU is a heterocyclic aromatic organic compound with a structure similar to that of the pyrimidine molecules of DNA (thymine) and RNA (uracil); it is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen (see **Fig. 1.1**) (Rutman et al. 1954). Due to its structure, 5-FU interferes with nucleoside metabolism and can be incorporated into RNA and DNA, leading to cytotoxicity and cell death

(Thomas & Zalcborg 1998; Noordhuis 2004).

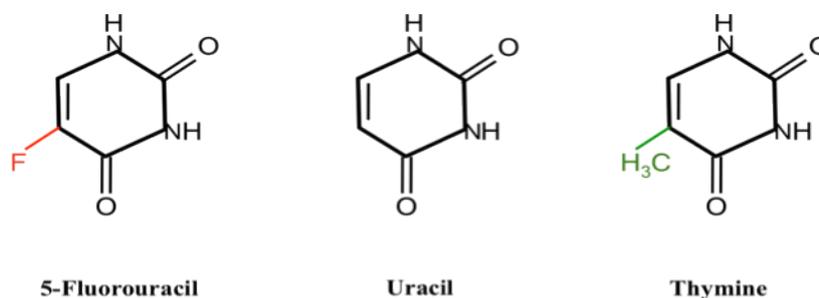


Fig. 1.1 Chemical Structure of 5-FU, Uracil and Thymine (adapted from Laar et al. 1998).

Mechanism of Action

Briefly, in mammalian cells 5-FU is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). The active metabolites of 5-FU disrupt RNA synthesis (FUTP), inhibit the action of thymidylate synthase (TS) – a nucleotide synthetic enzyme (FdUMP) – and can also be directly misincorporated into DNA (FdUTP) (See **Fig. 1.2**) (Wyatt & Willson, 2009).

Historically, the principal mechanism of action of fluoropyrimidines has been considered to be the inhibition of TS, and TS is thought to be the main target of 5-FU. More recently however, studies have shown that DNA and RNA damage play a more pertinent role in the mechanism of action of 5-FU. Pritchard *et al.* reported that TS was not the major locus of action of 5-FU by demonstrating that Thymidine failed to relieve 5-FU-induced cytotoxicity and folinic acid failed to increase it. Furthermore they were able to relieve apoptosis induced by a quinazoline-based antifolate, Tomudex (a pure TS inhibitor) with Thymidine but not the apoptosis induced by 5-FU. This is in line with experiments from many laboratories, that the toxicity of 5-FU to some cell types is not due to inhibition of TS but to its incorporation into RNA (Pritchard et al. 1997) and a pharmacological analysis that compiled gene expression profiles for human cancer cell lines treated with different drugs showed that 5-FU clusters with inhibitors of RNA synthesis (Scherf et al. 2000), which suggests that RNA-mediated mechanisms, such as reduced synthesis of ribosomes and inhibition of the nuclear RNA surveillance pathway, contribute significantly to the therapeutic properties of 5-FU (Silverstein et al. 2011).

The extent to which each of the pathways mentioned above contributes to the cytotoxicity of 5FU probably varies among cancer types, individual genetic backgrounds and administration schemes (Silverstein et al. 2011). The two mechanisms of most interest in the present study are the cell death induced by the DNA and RNA stress caused by 5-FU within the cell.

DNA and RNA Misincorporation

As previously mentioned, 5-FU is a pyrimidine analogue that can be misincorporated into both RNA and DNA in place of uracil or thymine. The interference with the normal biosynthesis or function of nucleic acids is therefore another possible mechanism of action for 5-FU (Zhang et al. 2008) and has come to be accepted as the main mechanism through which 5-FU exerts its cytotoxic effects.

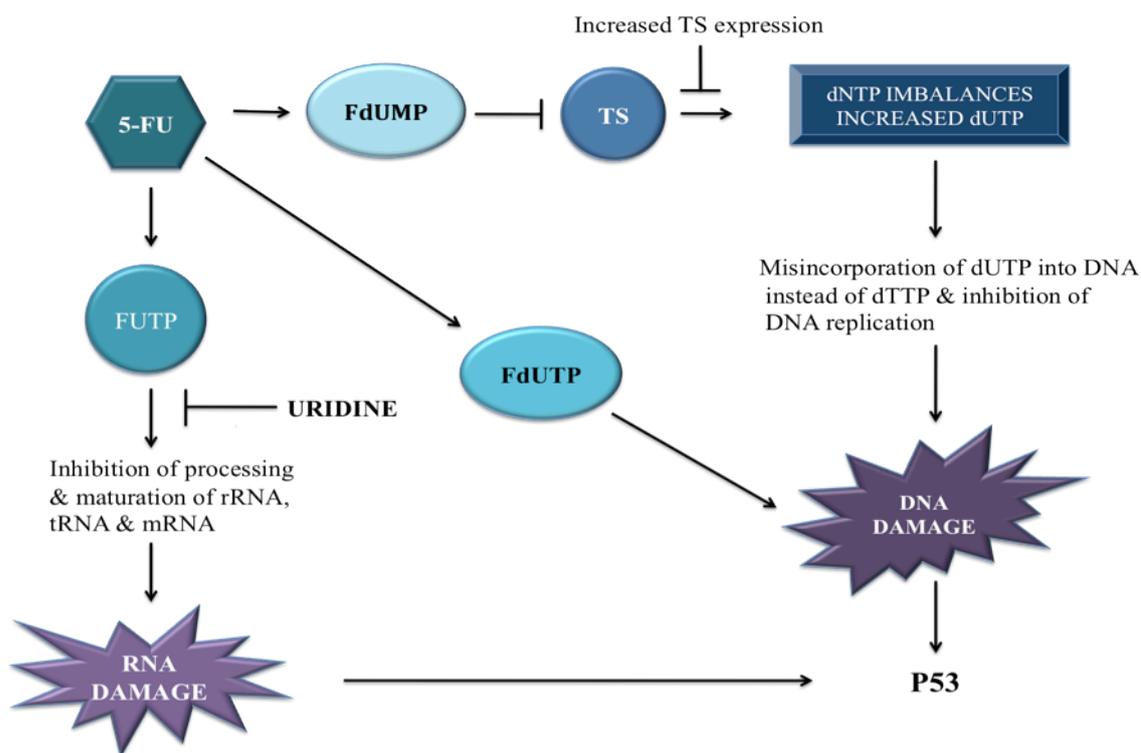


Fig. 1.2 Mechanism of Action of 5-FU resulting in RNA or DNA damage. 5-FU can activate p53 by more than one mechanism: incorporation of fluorouridine triphosphate (FUTP) into RNA, incorporation of fluorodeoxyuridine triphosphate (FdUTP) into DNA and inhibition of thymidylate synthase (TS) by fluorodeoxyuridine monophosphate (FdUMP) with resultant DNA damage. TS-directed cytotoxicity is abrogated by increased TS expression, whereas increasing the intracellular levels of Uridine can abrogate RNA-directed cytotoxicity (adapted from Longley et al. 2003).

It is thought that deoxynucleotide pool imbalances severely disrupt DNA synthesis and repair, resulting in lethal DNA damage (Houghton et al. 1995; Yoshioka et al. 1987). The misincorporation of the 5-FU metabolite fluorodeoxyuridine triphosphate (FdUTP) prevents repair of uracil- and 5-FU-containing DNA mediated by the base excision repair enzyme uracil-DNA-glycosylase (UDG) (Lindahl 1974). This eventually leads to DNA strand breaks. The capacity of a cancer cell to either repair DNA or instigate cell death can determine resistance to chemotherapeutic drugs that induce DNA damage such as 5-FU. Signalling pathways have evolved to arrest the cell cycle following DNA damage to allow more time for DNA to repair and only when repair is incomplete (when DNA damage is too extensive) will cells undergo cell death.

The 5-FU metabolite FUTP is also extensively incorporated into RNA, disrupting RNA processing and evidence suggests that RNA-based effects play a significant role in its cytotoxicity. In a study by Linke *et al.* it was shown that 5-FU was incorporated *in vitro* into RNA but not DNA from HCT116 colon cancer cells, which was inhibited by incubation with uridine and relieved toxicity whereas thymidine had no effect of 5-FU toxicity (Linke et al. 1996). Pritchard *et al.* also demonstrated that p53-dependent apoptosis induced *in vivo* was very significantly reduced by administration of uridine but not by thymidine (Pritchard et al. 1997). Therefore the incorporation of 5-FU into newly synthesised RNA appears to be the primary determinant of its cytotoxicity (Glazer & Lloyd 1982). More specifically, 5-FU misincorporation has been found to inhibit processing of pre-rRNA into mature rRNA (Kanamaru et al. 1986; Ghoshal & Jacob 1994) and disrupt post-transcriptional modification of transfer RNAs (tRNAs), which also play an essential role in mRNA translation (Santi & Hardy 1987; Randerath et al. 1983). Results also suggest that rRNA and tRNA maturation are important targets for 5-FU (Gustavsson & Ronne 2008; Giaever et al. 2004; Lum et al. 2004). There is also evidence to suggest that 5-FU inhibits premRNA splicing (Zhao & Yu 2007). The action is not only inhibiting the processing of pre-rRNA into mature rRNA, but also disrupting post-transcriptional modifications of tRNAs and the assembly activity of snRNA/protein complexes, thereby inhibiting splicing of pre-mRNA (Samuelsson 1991). As a result, significant correlations between 5-FU misincorporation into RNA and loss of clonogenic potential have been demonstrated in human colon and breast cancer cell lines (Kufe & Major 1981; Glazer & Lloyd

1982).

RNA stress is not just responsible for the main cytotoxicity of 5-FU but is also reported to be the mechanism of action of other anti-cancer chemotherapy agents and is an important component of their action. In the case of Cisplatin, it forms adducts on both DNA and RNA, and inhibits translation in cancer cells by crosslinking of mRNA to ribosomal RNA or ribosomal RNA to itself (Jamieson & Lippard 1999). Adriamycin (doxorubicin), is an anthracycline drug, which intercalates between base pairs of double-helical nucleic acids, and has been shown to bind to RNA substrates and inhibit RNA helicase, an activity essential for RNA synthesis, processing, transport and turnover (Zhu et al. 1999).

It was also demonstrated in HCT116 cells that 5-FU treatment although clearly primarily perturbing RNA metabolism, later induced alkali-sensitive DNA strand breaks (Li et al. 2009). Although these DNA strand breaks may be secondary to perturbations of RNA, they could be the ultimate signal for the induction of a p53-induced cell death. Whether perturbations in RNA structure and/or metabolism *per se* or DNA damage, which is in some way secondary to the effects on RNA, is sensed by p53 requires further investigation. It is plausible that RNA damage occurs in the first instance, which results in DNA damage at a later stage as suggested by the study by Li et al. (2009) and therefore there may be more than one pathway occurring within the cell that are responsible for the overall toxicity induced by 5-FU. Understanding of the first event that signals the consequential cascade of events would be an important discovery to aid in the development of specific drugs to initiate this effect. As both DNA and RNA damage can initiate cell death via activation of the protein p53, this protein is also an important factor to be considered.

p53 activation, mutation and its effects on colorectal cancer response

The activation of the RNA and/or DNA damage response in colorectal cancer cells can both result in the activation of a common mediator of decision of cell fate, p53. The p53 protein was first identified in 1979 (DeLeo et al. 1979), and following on from its discovery, the generation of the first p53 null mice gave evidence of the highly important role of p53 as a potent tumour suppressor (Donehower et al. 1992). P53 has been termed the guardian of the genome (Lane 1992) due to the vastly

diverse and pivotal functions within the cell regarding maintenance of cellular and genetic integrity. The longstanding view of p53 as simply a tumour suppressor is becoming outdated and more recently it has become more apparent that p53 is also a key regulator of metabolic pathways that have a pivotal role in cancer. P53 is capable of contributing to the regulation of such metabolic processes as: glycolysis; glutaminolysis; oxidative phosphorylation; nucleotide biosynthesis and mitochondrial integrity among others (See et al., Maddocks & Vousden, 2011 for full review). It is thought that loss of p53 in cancers may initially serve to drive metabolism in support of tumorigenesis. The gene encoding p53, TP53, is the most frequently mutated gene in human cancers (Hollstein et al. 1991; Kastan & Bartek 2004), with about 50% of all tumours estimated to carry a mutation (Levine 1997).

Under normal, unstressed conditions p53 is kept at low levels, primarily through a mechanism in which the negative regulator Mdm2 targets p53 to degradation. Mdm2 is one of p53's target genes and thus any increase of p53 normally leads to an increase in Mdm2 levels, which then pushes p53 back down to a low steady state level. Following a number of intra and extracellular stimuli such as the previously discussed RNA or DNA damage caused by stimuli such as ionising radiation, UV radiation or treatment with cytotoxic drugs (including 5-FU (Longley et al. 2002)) and chemotherapeutic agents, wild-type p53 and p53-target genes are activated (Longley et al. 2002) which functions to regulate a diverse range of biological functions (Vousden & Lu 2002; Vogelstein et al. 2000; Levine 1997). For example, DNA damage results in activation of upstream kinases such as ATM (Ataxia-telangiectasia mutated), ATR (ATM and Rad-3 related), and DNA-PK (DNA-dependent protein kinase), which can directly or indirectly activate p53 (Ljungman 2000). This can then result in phosphorylation of p53 and Mdm2, disrupting the p53-Mdm2 interaction, leading to stabilization of p53 and an increase in p53 transcriptional activity.

P53 activation of its target genes is achieved by binding of p53 to specific sites in the promoter regions of p53 responsive genes which strongly enhances their transcription (Kern et al. 1991). When irrevocable damage is induced, p53 can initiate apoptosis or programmed cell death, in order to eliminate these unwanted damaged cells. The apoptosis-related genes regulated by p53 include Bax (Bcl-2-associated X protein) (Miyashita et al. 1994; Thornborrow et al. 2002), DR5/KILLER (death receptor 5) (Takimoto & el-Deiry 2000), Fas/CD95 (Cell Death Signalling Receptor) (Schuler &

Green 2001; Yu et al. 1999), Puma (p53-upregulated modulator of apoptosis) (Nakano & Vousden 2001; Yu et al. 2001), NOXA (Oda et al. 2000), PIDD (p53-induced protein with death domain) (Lin et al. 2000) and others.

In vitro studies have reported that loss of p53 function reduces chemosensitivity to 5-FU (Longley et al. 2002; Bunz et al. 1999). Furthermore a number of clinical studies have found that increased p53 expression (a surrogate marker for p53 mutation) correlated with resistance to 5-FU-based chemotherapy (Elsaleh et al. 2001; Liang et al. 2002; Ahnen et al. 1998), although one study failed to find a correlation (Paradiso et al. 2000). It has also been demonstrated that p53 mutations are a hallmark in colorectal cancer progression and that p53 protein stabilisation and mutations are associated with poor clinical prognosis (Visca et al. 1999; Graziano & Cascinu 2003; Buglioni et al. 1999).

Due to the relationship between p53 activation upon DNA/RNA stress and its role in apoptosis induction of the cell, a more in depth understanding of not only the apoptotic pathways activated upon RNA/DNA stress induced by current treatment regimens, but more specifically the apical event that mediates the 5-FU response is necessary for our understanding of 5-FU-induced cytotoxicity.

1.3 APOPTOSIS AND ITS ROLE IN COLORECTAL CANCER

Apoptosis

The end goal of all anti-cancer therapies, including 5-FU, is to induce apoptosis in the tumour cell. Apoptosis, or programmed cell death, is a morphologically and biochemically distinct form of cell death, characterised by a number of structural changes including cell shrinkage, chromatin condensation and extensive nuclear and cellular fragmentation and membrane blebbing (Kerr et al. 1972; Hersey & Zhang 2003; Fulda & Debatin 2006b) (see **Fig. 1.3**).

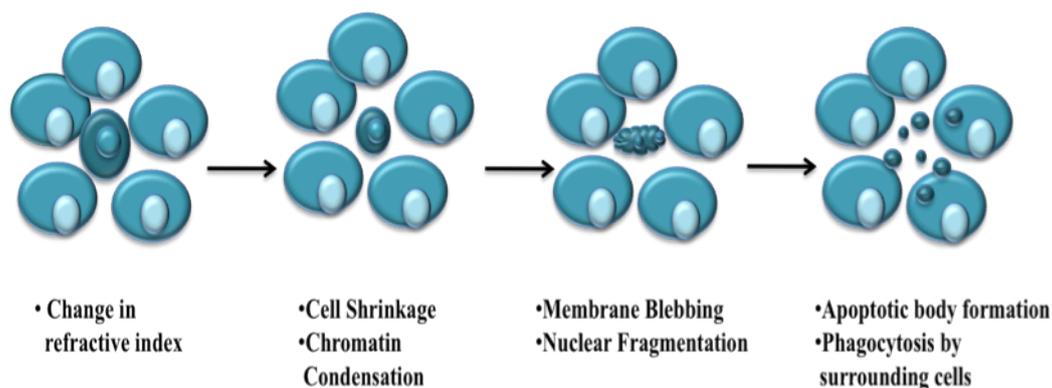


Fig. 1.3 Morphological Features of Apoptosis

Apoptosis plays an important role in many physiologic and pathologic processes. Amongst others, an important function of apoptosis lies in the elimination of damaged cells. For example, cells with genetic damage caused by exposure to carcinogens may be deleted by undergoing apoptosis, thereby preventing their replication and the accumulation of clones of abnormal cells. Defects in apoptotic machinery vary among individual cancer cells, and the efficacy of chemotherapy in killing cancer cells depends on the successful induction of apoptotic pathways (Zhang et al. 2011b; Melet et al. 2008; Rodriguez-Nieto & Zhivotovsky 2006). There is increasing evidence to support the hypothesis that failure of apoptosis may be an important factor in the evolution of colorectal cancer and its poor response to chemotherapy and radiation. Through studies of spontaneous apoptosis, large differences in resistance or susceptibility to apoptosis in colon cancer cells have been observed (Li et al. 2008). In addition, colorectal cancer cells resistance to apoptosis has also been shown to correlate with their invasive/metastatic capabilities (Li et al. 2008). A large number of studies have assessed the proportion of cells undergoing apoptosis, both *in vitro* and in human colorectal tissue sections and several *in vitro* studies have shown changes in colorectal carcinogenesis affecting apoptosis (Bedi et al. 1995; Merritt et al. 1995; Bienz & Clevers 2000). From these studies it appears that a gradual resistance to induction of apoptosis emerges during cancer development.

As dysregulation of apoptosis is a major cause for drug resistance (Melet et al. 2008; Tan & White 2008) it is important to dissect the molecular pathways of apoptosis induced by such chemotherapeutic agents as 5-FU to understand how cells can develop resistance to their cytotoxic effects.

There are two classically described pathways of apoptosis, the mitochondrial

(intrinsic) and death receptor (extrinsic) pathways of apoptosis. These pathways can act both independently and are also capable of crosstalk with each other and act in concert with one another to enhance the overall apoptotic signal. Cells are classified as either type I or type II cells based mainly on whether the intrinsic apoptotic pathway contributes to the amplification of the caspase cascade (Hao & Mak 2010). HCT116 colon cancer cells (used in the present study) have previously been shown to behave as type II cells (Ozören & El-Deiry 2002), meaning that they require amplification of the apoptotic signal generated through the extrinsic pathway by the contribution of the mitochondrial pathway.

The Extrinsic Pathway

In the extrinsic pathway of apoptosis, the events are initiated via ligation of trans-membrane death receptors of the tumour necrosis factor receptor (TNFR) type superfamily, including TNF- α , Fas (CD95/Apo-1) and Tumour Necrosis Related Apoptosis Inducing Ligand (TRAIL), which are characterised by the presence of multiple cysteine-rich repeats in the extracellular domain and the protein-protein interaction module known as the death domain (DD) in the cytoplasmic tails. Upon interacting with their respective ligands the death receptors undergo multimerisation to form the intracellular death-inducing signaling complexes (DISCs) that may include multiple adaptor molecules. In the case of Fas (CD95/Apo-1) and TRAIL receptors, the adaptor molecule FADD, through its C-terminal death effector domains (DED), is recruited to its cytosolic tail by a multi-step mechanism. FADD then recruits caspase-8 zymogens via homophilic interactions with their N-terminal DEDs. When bound to the DISC, several procaspase-8 molecules are in close proximity to each other and therefore are assumed to activate each other by proteolysis (Walczak & Krammer, 2000). In colorectal cancer the death receptor Fas (CD95) and its ligand FasL are thought to be involved in disease progression. This may be in part due to the FasL overexpression of colon cancer cells, which allows cells to avoid death by the immune response (O'Connell et al. 2001). Immunohistochemical staining of FasL is frequently elevated in tumours and FasL positivity is associated with late stage disease and poorer survival. However another study has shown conflicting results with greater FasL expression associated with better outcome and early stage disease

(Sheehan et al. 2003). The Fas pathway has also been implicated in the mechanism of action of 5-FU (Tillman et al. 1999) and therefore its potential as a chemotherapy response marker has also warranted examination. However, in a study of metastatic Stage IV patients, the expression of neither Fas nor FasL correlated with response to 5-FU (Bezulier et al. 2003). A second important ligand of the extrinsic pathways is TRAIL.

Tumour Related Apoptosis Inducing Ligand (TRAIL)

For the Tumour Necrosis Factor Receptor Super Family (TNFRSF) death receptor TRAIL, much interest has been generated in recent years in the cancer research field as TRAIL has been shown to induce apoptosis in a wide variety of tumour cells *in vitro* as well as *in vivo*. The physiological role of TRAIL *in vivo* has been investigated using mouse models. TRAIL^{-/-} mice are viable and display no apparent haematological or reproductive defects. It was also thought that the expression patterns of TRAIL might be indicative of its natural function and TRAIL mRNA and protein expression is found in a variety of cells and tissues. Furthermore studies in mice and humans show that TRAIL is not expressed at the surface of freshly isolated T-cell, B-cells, monocytes, dendritic cells, natural killer (NK) cells or NKT-cells. After stimulation these cells can express TRAIL at their surface suggesting an important role of TRAIL in innate immune response. Furthermore TRAIL contributes to host immunosurveillance against primary tumour development (Duiker et al., 2006). Besides studies performed in mice, several studies report increased serum levels *in vivo* of soluble TRAIL (sTRAIL) in patients with neoplastic, autoimmune and infectious diseases. These results confirm a physiological role of TRAIL in various immune reactions in the human situation (Duiker et al., 2006).

In addition to its physiological role, TRAIL appears to be a promising new anti-cancer agent which, unlike TNF- α and FasL, seems to induce tumour-selective apoptosis, while sparing normal cells (Walczak et al. 1999; French & Tschopp 1999). An additional benefit of TRAIL is that recombinant human (rh) TRAIL was demonstrated to initiate apoptosis independently of the p53 status of a tumour (Ravi et al. 2004). Approximately 80% of human cancer cell lines, including colorectal tumours, were sensitive at least to some extent to TRAIL, whereas the majority of normal cells were

relatively resistant (Ashkenazi et al. 1999; Kelley et al. 2001; Lawrence et al. 2001; Mitsiades et al. 2001; Nagane et al. 2000; Walczak et al. 1999; Pitti et al. 1996; Wiley et al. 1995). In terms of the physiological role of TRAIL, one study in a murine model demonstrated that TRAIL was partly responsible for surveillance of tumour metastasis by natural killer cells, suggesting a potential role as a tumour suppressor (Takeda et al. 2001). Five TRAIL receptors have been identified to date (see Fig. 1.4): two cell death-inducing receptors, DR4/TRAIL-R1 (Pan, Ni, et al. 1997a) and DR5/TRAIL-R2 (Screaton et al. 1997; Pan, O'Rourke, et al. 1997b; Sheridan et al. 1997; Walczak et al. 1997), two non-cell-death-inducing receptors, DcR1/TRAIL-R3 (Pan, Ni, et al. 1997a; Sheridan et al. 1997; Degli-Esposti et al. 1997) and DcR2/TRAIL-R4 (Marsters et al. 1997) both of which are cell surface molecules and osteoprotegerin (OPG) (Simonet et al. 1997), a secreted soluble protein, which is also capable of binding to TRAIL, albeit with a lower affinity (Emery et al. 1998).

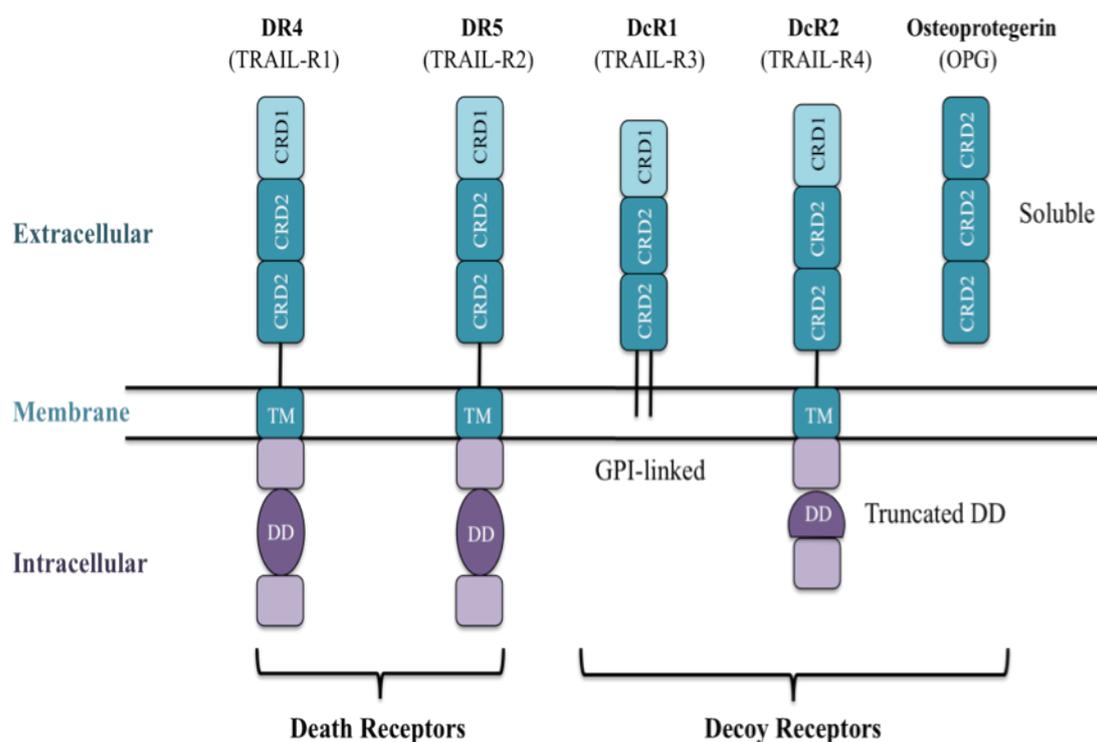


Fig. 1.4 Structural illustration of the TRAIL death and decoy receptors. DR4 and DR5 are death receptors whereas DcR1, DcR2 and OPG are decoy receptors. DcR3 is GPI (glycosyl phosphatidylinositol) -linked, devoid of a death domain (DD), whereas DcR2 harbours a non-functional truncated DD. OPG is a soluble protein capable of binding to TRAIL. CRD = Cysteine Rich Domain. TM = transmembrane (adapted from Huang & Sheikh, 2007).

TRAIL mRNA and DR4 and DR5 transcripts are expressed in several tissues, including normal colon epithelium and colon adenocarcinomas (Ashkenazi & Dixit 1998) and studies examining the protein levels of the TRAIL receptors DR4 and DR5 all show higher expression of both receptors in colonic tumours compared to normal tissue (Koorstra et al. 2003; Sträter et al. 2002; van Geelen et al. 2006). The manner by which TRAIL induces apoptosis in tumour cells but leaves healthy cells unharmed has led to some speculation and several theories exist, including the decoy hypothesis. This hypothesis assumes that sensitivity of cells towards TRAIL is mediated by the lack or the presence of specific receptors (Gura, 1997). For this theory to be successful, normal cells would be protected by the expression of the non-signalling receptors DcR1 and DcR2. This theory was supported by a study by Büneker et al. who found a good correlation between TRAIL-sensitivity and the expression of DR5 alone or an even better correlation if the ratio of DR5 and the decoy receptor expression were taken into account (Büneker et al. 2009). Mérimo et al. also demonstrated the importance of decoy receptor expression to TRAIL-sensitivity and demonstrated that the decoy receptors can inhibit TRAIL-induced apoptosis through distinct mechanisms. DcR1 was found to be a competitor for TRAIL binding preventing DR5-associated DISC assembly and DcR2 impaired DISC processing and initiator caspase activation (Mérimo et al. 2006). However, a contrasting analysis by examination of a large panel of cultured tumour cells showed no correlation between the expression of DcR1 and DcR2 mRNA and the resistance or sensitivity to TRAIL treatment (Griffiths et al. 1999). In addition, it has been shown that the expression and localisation of TRAIL receptors varies between different cells and that resistance to TRAIL is mediated by different mechanisms such as the differential subcellular localisation of decoy receptors and intracellular inhibitors of apoptosis (Zhang et al. 2000). These studies suggest the mechanisms of TRAIL resistance are complex and can vary between different cell types.

Despite the lack of a comprehensive understanding regarding the mechanism of TRAIL-mediated apoptosis in normal and tumour cells, the cell killing ability of TRAIL has encouraged much research in developing it as a potential anti-cancer drug therapy (de Vries et al. 2000). There have been some drawbacks in the utilisation of TRAIL when hepatotoxicity or brain toxicity associated with the use of TRAIL have been detected following experiments where polyhistidine- and FLAG-tagged

recombinant versions of human TRAIL were found to induce apoptosis *in vitro* in isolated hepatocytes and brain tissue slices, respectively (Jo et al. 2000; Nitsch et al. 2000). However, in contrast to this observation, polyhistidine-tagged TRAIL and native rhTRAIL were nontoxic to cultured hepatocytes (Lawrence et al. 2001). In addition, two relevant non-human primate models have indicated that systemic administration of native rhTRAIL is unlikely to cause major toxicity to the liver or other organs (Lawrence et al. 2001; Ashkenazi & Dixit 1999).

In colon cancer cells rhTRAIL induces apoptosis *in vitro* and in xenograft models (Naka et al. 2002; van Geelen et al. 2003), whereas normal human colon epithelium is resistant to TRAIL-induced apoptosis (Sträter et al. 2002). Also the TRAIL receptor, DR5, was shown to be important for sensitivity to 5-FU (Wang & El-Deiry 2004) and various chemotherapeutic agents have been shown to cooperate with rhTRAIL and facilitate an additive p53-independent apoptosis of colon cancer cells (Shimoyama et al. 2002; Ravi et al. 2004). Therefore a synergistic therapy approach combining 5-FU with TRAIL may prove to be a successful path forward to overcome resistance. This concept will be explored in Chapter 3.2 of this thesis. The TRAIL-mediated pathway of apoptosis recruits the important adaptor protein, FADD, which will now be discussed in more detail.

Fas associated protein with death domain (FADD)

FADD (Mort1) was discovered in 1995 (Chinnaiyan et al. 1995; Boldin et al. 1995) as a novel adapter molecule capable of interacting with death receptors expressed at the cell membrane. The role of FADD in Fas, TNF-R1 and DR4 and DR5 mediated apoptosis has been clearly established (Yeh et al. 1998; Chinnaiyan et al. 1995; Varfolomeev et al. 1996; Kuang et al. 2000), and the essential role of FADD in apoptosis initiated by all the death domain - containing TNF receptor family members is underscored by the dramatic phenotype of FADD-deficient mice. These mice die by day 9.5 of gestation due to abnormal cardiac development (Yeh et al. 1998; Zhang et al. 1998). In addition to its pivotal role in death receptor-dependent apoptosis, there have also been reports that FADD is responsible for a death receptor-independent

form of cell death through formation of cytoplasmic death effector filaments (DEF) by oligomerisation of DED-containing proteins (Siegel et al. 1998). However the existence of DEF *in vivo* has not been established and there is increasing evidence to support the concept that DEF could be artefactual structures resulting from protein over expression (Tourneur et al. 2005).

Due to the central role FADD plays in the induction of death-receptor induced apoptosis, it is no surprise that its dysregulation is implicated in tumourigenesis. It has previously been demonstrated that overexpression of FADD induced apoptosis in 85% of malignant glioma cells regardless of Fas/Apo1 expression levels (Kondo et al. 1998). Furthermore, in a recent report, *in vivo* overexpression of FADD increased the efficacy of 5-FU-induced inhibition of tumour growth in nude mice (Yin et al. 2010) which was in line with the findings of Micheau et al. (1999). Therefore the presence of FADD appears to be an important factor in tumour cell sensitivity. The likelihood of FADD mutation or absence was subsequently studied in a number of reports. There was reported FADD mutations in respect to colorectal cancer where Soung et al. (2004) analysed the entire coding region and all splice sites of the human FADD gene to detect somatic mutations in 98 colon cancers. Overall they detected a somatic missense mutation of the FADD gene in one colon carcinoma case, which was the first report of FADD gene mutation in gastrointestinal cancers and suggested that the FADD gene is rarely mutated in human colon cancers. In addition, in a mouse model of thyroid adenoma/adenocarcinoma, the disappearance of FADD protein expression during the course of tumour development was demonstrated (Tourneur et al. 2003). This observed absence of FADD protein expression in cancer cells has also been shown to be a phenomenon in human malignancies, where absent or low FADD protein expression was observed in AML cells and was found to be prognostic of response to chemotherapy (Tourneur et al. 2004). The importance of the FADD molecule lies in its ability to bridge the death receptors to caspase-8 through its DD to form the DISC, which results in the activation of the downstream effector caspases.

The Caspase Family

Caspases belong to the interleukin-1 β -converting enzyme family and are cysteine proteases, capable of cleaving substrates after aspartic residues. They are expressed in a cell as inactive zymogens, in which form they are termed procaspases. Caspases share common structures within three domains: an N-terminal prodomain, a large subunit containing the cysteine within a conserved QACXG motif and a C-terminal small subunit. An aspartate cleavage site separates the prodomain from the large subunit and an interdomain linker containing one or two aspartate cleavage sites separates large and small subunits from one another (Wolf & Green, 1999). Activation of caspases involves autoactivation of their proforms to produce a heterodimer containing a big and a small subunit and two of such heterodimers in turn form an enzymatic active heterotetramer. Caspases can be subdivided based on their homology in amino acid sequences, into initiator and executioner caspases. Initiator caspases harbour protein-protein interaction domains at their amino terminus, which are either death effector domains (DED) or caspase recruitment domains (CARD). These serve for their recruitment to specific activator platforms, where they become activated by induced proximity (Salvesen & Dixit 1999; Muzio et al. 1998).

Fourteen caspases have been identified to date, with eleven caspases in humans (Earnshaw et al., 1999; Fuentes-Prior et al., 2004; MacKenzie & Clark, 2012). These include four initiator caspases, Caspase-8 and Caspase-10, Caspase-9 and Caspase-2. The long prodomain contains the DED in procaspase-8 and -10, or the CARD in procaspase-2 and -9 (see **Fig. 1.5**).

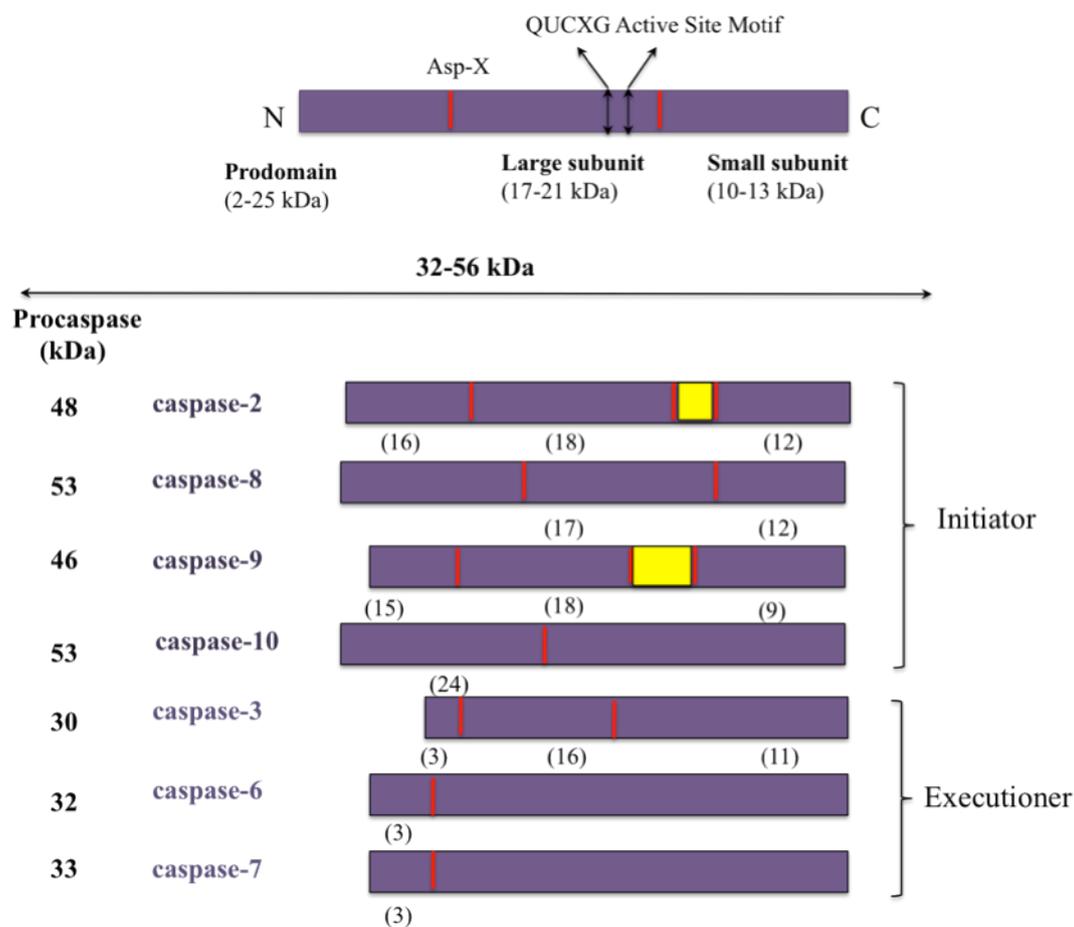


Fig. 1.5 Structure of the initiator and executioner caspases of apoptosis (adapted from Rupinder et al. 2007).

Upon apoptotic stimuli their proforms are recruited to specific activator platforms. Procaspase-8 (and the related Procaspase-10) is recruited to the DISC where two linear subunits of caspase-8 may interact with each other followed by procaspase-8 autoactivation to caspase-8 (Medema et al. 1997; Sprick et al. 2002). Caspase-9 is activated in a large protein complex termed the apoptosome. The apoptosome forms when cellular stress signals trigger the release of cytochrome-c from the mitochondria. Cytochrome-c induces a conformational change in the adapter protein Apaf-1 causing it to oligomerise and recruit procaspase-9 (Zou et al. 1999). Caspase-2 is activated upon genotoxic stress in a large protein complex termed the PIDDosome (Tinel & Tschopp 2004). Caspase-1, -4, -5, and -12 are structurally similar to initiator caspases and play a role in inflammation, but not in apoptosis (Tinel & Tschopp 2004). Whereas caspase-14 shares the structure with executioner caspases and appears to be involved in skin differentiation (Denecker et al. 2008).

The subgroup of executioner caspases consists of caspase-3, -6 and -7 (Taylor et al. 2008). Executioner caspases are substrates of initiator caspases that cleave and thereby activate them (Shi, 2002). Once the executioner caspases are activated, they cleave and process multiple cellular substrates. Some notable substrates include the DNase inhibitor ICAD, where cleavage of ICAD by caspase-3 liberates the active CAD nuclease that mediates apoptotic DNA fragmentation, or cleavage of ROCK-1 and PAK2, implicated in membrane blebbing (Fischer et al. 2003). Caspases also destroy several proteins involved in maintenance of cytoskeletal architecture such as intermediate filaments cytokeratin-18 and vimentin (Fischer et al. 2003).

Caspases and Their Role in Tumourigenesis

There is some evidence of alterations in the expression of caspase family members in colorectal cancer, at both the genetic and protein levels. Palmerini *et al.* examined the expression of caspase-7 and -9 in matched tumour and normal tissue and showed a decrease in their protein expression in the tumour tissue (Palmerini et al. 2001). Soung *et al.* identified some mutations in both the caspase-3 (Soung et al. 2004) and -7 (Soung et al. 2003; Yoo et al. 2007) genes in a number of tumour types and a small percentage of colon cancers. One recent study showed elevated procaspase-3 protein in colorectal cancer tumour tissue compared to adjacent normal tissue (Putt et al. 2006). The gene encoding for caspase-9 does not appear to be either mutated or silenced in cancers (Yoo et al. 2002; Soung et al. 2006; Abel et al. 2002), however decreased expression levels (compared to normal mucosa) have been reported in 46% of colon cancer (Palmerini et al. 2001). For caspase-2 there is not much information available, but its expression has been reported to be lost frequently in gastric cancer in contrast to normal musoca (Yoo et al. 2004).

There is also evidence to demonstrate that caspases 3 and 7 can also promote tissue regeneration. Loss of their expression markedly reduced the rate of tissue repair. They were shown to cleave and activate iPLA2 to trigger the production of the growth signal prostaglandin E2 (PGE2), showing that not only can these caspases contribute to the death process, but also participate in the production of paracrine signals (Li et al., 2010). Because this study suggested a role for cell death in promoting tissue regeneration, the mechanism was termed the “Phoenix Rising” pathway of tissue

regeneration (Li et al., 2010).

Caspase-8

Caspase-8 is one of the initiator caspases, and plays a central role in the execution phase of apoptosis (Degterev et al. 2003). Caspase-8 contains 480 amino acids and is a 55 kDa protein with two N-terminal DEDs, which function as platforms for protein-protein interaction (Barnhart et al. 2003). A number of different isoforms of caspase-8 have been reported. Isoforms caspase-8a and caspase-8b are the pro-apoptotic variants that are most commonly expressed in mammalian cells (Scaffidi et al. 1997), whereas caspase-8 long (caspase-8L) is a splice variant, which leads to the production of a truncated protein, which contains only the two N-terminal DED domains, but lacks the C-terminal protease domain (Horiuchi et al. 2000; Himeji et al. 2002). Caspase-8L can be recruited to the DISC via its DED domains, but remain proteolytically inert due to absence of its protease domain (Miller et al. 2006; Mohr et al. 2005). Consequently, caspase-8L interferes in a dominant-negative manner with the transduction of the death signal from activated death receptors.

Mutations of caspase-8 have been detected at relatively low frequency in colorectal and gastric cancer (Soung et al. 2005). Mutational analysis of the caspase-8 gene revealed the presence of an inactivating mutation in a small subset of patients with invasive colorectal carcinoma (Kim et al. 2003). These mutations were not present in colonic adenomas indicating that these mutations may contribute to the pathogenesis of the disease. In its mutated form caspase-8 interferes with the recruitment of wild-type caspase-8 to activated death receptors in a dominant negative fashion (Kim et al. 2003; Mandruzzato et al. 1997). Additionally, homo- or heterozygous genomic deletions of caspase-8 as well as allelic imbalance on chromosome 2q associated with alterations of the caspase-8 gene have also been described (Teitz et al. 2000; Takita et al. 2001). Epigenetic silencing of caspase-8 expression by hypermethylation of regulatory sequences of the caspase-8 gene has been detected in multiple cancers (Teitz et al. 2000; Fulda et al. 2001; Hopkins-Donaldson et al. 2003; Pingoud-Meier et al. 2003; Harada et al. 2002; Hopkins-Donaldson et al. 2000b; Iolascon et al. 2003; Takita et al. 2000; Grotzer et al. 2000; Shivapurkar et al. 2002b; Shivapurkar et al. 2002a). An additional mechanism of caspase-8 inactivation is caused by inhibitory

phosphorylation on tyrosine 308 of caspase-8 (Cursi et al. 2006).

Flice Like Inhibitory Protein (FLIP)

FLIP (Flice-like inhibitory protein) is an important modulator that has been found to render many types of cells resistant to cell death receptor-mediated apoptosis. In 1997, Irmeler *et al.* reported characterisation of this inhibitor of apoptosis, which was subsequently designated FLIP (Irmeler et al. 1997). At this time FLIP was also characterised by a number of other groups and therefore was known under various other aliases: Casper, CASH, FLAME-1, MRIT, CLARP, USURPIN (Goltsev et al. 1997; Han et al. 1997; Hu et al. 1997; Inohara et al. 1997; Shu et al. 1997; Srinivasula et al. 1997; Rasper et al. 1998). Irmeler reported two forms of cellular FLIP (c-FLIP), a short form, FLIP_S, containing two death effector domains and a long form, FLIP_L. c-FLIP_L contains the two death effector domains and a caspase-like domain with sequence homology to caspase-8, however it lacks key features required for substrate catalysis. FLIP_S and FLIP_L were found to interact with the adaptor protein FADD and caspase-8 and potentially inhibit apoptosis by death receptors (Irmeler et al. 1997; Tschopp et al. 1998).

FLIP deficient mice and cells were subsequently generated following these findings. The FLIP^{-/-} embryos did not survive past day 10.5 of embryogenesis and exhibited impaired heart development (Yeh et al. 2000), a phenotype similar to previously observed FADD^{-/-} and caspase-8^{-/-} embryos (Varfolomeev et al. 1998; Yeh et al. 1998). FLIP^{-/-} embryonic fibroblasts (MEF) were highly sensitive to FasL or TNF- α -induced apoptosis and showed rapid induction of caspase activation. These findings were in contrast to FADD and caspase-8^{-/-} MEF, which were highly resistant to death receptor-induced apoptosis. It is interesting to note that the phenotypes of all knock-out cells were similar despite their different roles.

According to Micheau, the inhibition of pro-apoptotic activity of caspase-8 is more likely to occur through FLIP_L and not other caspase-8 inhibitors, since enhanced expression of FLIP but not c-IAP1 potentially blocks TNF-mediated cell death (Micheau et al. 2001). To combat the interference of FLIP with the proapoptotic activity of caspase-8, agents already exist which are known to lower FLIP expression including DNA-damaging agents (Cisplatin and Doxorubicin) and RNA synthesis inhibitors (actinomycin D) (Kataoka et al. 1998).

FLIP_L has been shown to be more highly expressed in adenocarcinomas of the colon compared to premalignant polyps and normal colonic tissue at both the mRNA and protein levels, indicating that alterations in FLIP_L levels may contribute to the malignant phenotype (Ryu et al. 2001) and overexpression of FLIP_L is thought to be prognostic of a clinically more aggressive phenotype (Ullenhag et al. 2007). A recent study correlating FLIP expression to colorectal cancer patient survival indicates that strong immunohistochemical staining of FLIP_L but not FLIP_S correlates with poor prognosis (Ullenhag et al. 2007). Longley et al. (2006) studied the effect of FLIP on apoptotic response to chemotherapies used in colorectal cancer, namely 5-FU, Oxaliplatin and Irinotecan, and found that simultaneous knockdown of both FLIP isoforms synergistically enhanced chemotherapy-induced apoptosis in the CRC lines. Furthermore overexpression of FLIP_L but not FLIP_S potentially inhibited apoptosis induced by chemotherapy (5-FU, Oxaliplatin or Irinotecan) in HCT116 cells suggesting FLIP_L was the more important splice form in mediating chemoresistance (Longley et al. 2006). After discussing the pathway upstream of the mitochondria, we will now discuss the intrinsic pathway, linked to the extrinsic pathway by the proapoptotic protein Bid.

The Intrinsic Pathway

The second pathway of apoptosis activation is either activated in response to signals originating from inside the cell (intrinsic) (ionising radiation, some chemotherapeutic drugs, or mitochondrial damage) or can be activated via the death receptor-mediated pathway. Following recruitment by FADD, activated caspase-8 can subsequently cleave and activate Bid. The activated c-terminal fragment resulting from this event is termed truncated Bid (tBid) (see **Fig 1.6**). Bid is a member of the Bcl-2 family of proteins (discussed in more detail below) that regulate permeabilisation of the outer mitochondrial membrane (OMM), a critical event during apoptosis. The main function of Bid appears to be to link the death receptor pathway and OMM permeabilisation (Li et al., 1998; Luo et al., 1998). Although full length Bid is capable of exerting some apoptotic function, the full activity of Bid lies in its proteolytic

cleavage.

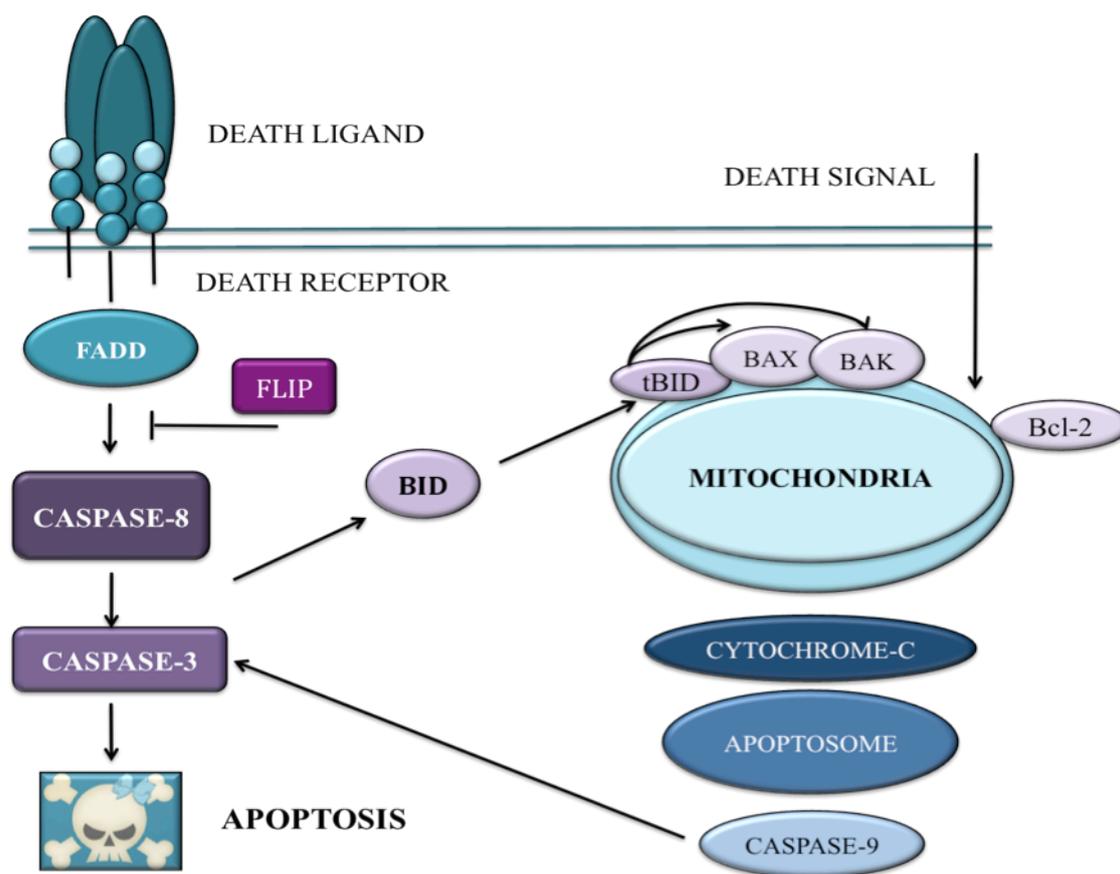


Fig. 1.6 Pathways of apoptosis

tBid can then migrate to the mitochondria and insert itself into the OMM (Gross et al., 1999) where it subsequently drives the translocation and insertion of Bax into the OMM (Eskes et al., 2000), eventually leading to OMM permeabilisation that is dependent on Bax or Bak (Wei et al., 2001).

There has been little work done on examining the role of Bid as a biomarker in colorectal cancer. One immunohistochemical study of Stage II patients indicated that in comparison of tumour and matched adjacent normal tissue; Bid was elevated in 57% of 60 patients but that did not correlate with disease survival (Krajewska et al. 2002). Further analysis by this same group and extension of the patient cohort (n=100) found no difference in Bid expression between tumour and matched normal tissue (Krajewska et al. 2005). Another recent immunohistochemical study by Sinicrope et al. showed that in addition to elevated expression of Bid protein in tumours compared to normal tissue there was also a correlation between high Bid expression and longer survival in Stage II and III patients receiving 5-FU based

chemotherapy (Sinicrope et al. 2008).

Following the appropriate death trigger either through death receptor-mediated tBid or intrinsic signals, the OMM may become selectively permeable, which results in release of apoptogenic factors such as cytochrome-c, AIF, smac/DIABLO, Omi/HtrA2 or endonuclease G from the mitochondrial intermembrane space (Candé et al., 2002; Saelens et al., 2004). The release of cytochrome-c into the cytosol triggers caspase-3 activation through formation of the ‘apoptosome’ (Zou et al. 1999). The apoptosome is a multimeric protein complex containing Apaf-1 (apoptotic protease activating factor 1), cytochrome c and the cofactor dATP/ATP (Liu et al. 1996; Li et al. 1997; Zou et al. 1999; Zou et al. 1997). The central component of this complex is Apaf-1 which recruits caspase-9 via its N-terminus CARD (Qin et al. 1999).

The Bcl-2 Family

The overexpression of members of the Bcl-2 family of apoptosis can inhibit the events downstream of the mitochondria. At least 15 Bcl-2 family member proteins have been identified in mammalian cells, including proteins that promote apoptosis and those that prevent it. The pro-survival Bcl-2 like subfamily members include Bcl-2 (Hockenbery et al. 1990; Vaux et al. 1988), Bcl-x_L (Boise et al. 1993), Mcl-1 (Kozopas et al. 1993; Zhou et al. 1997) and Bcl-w (Gibson et al. 1996); all of which have been examined in a variety of human cancers including colon cancer. Bcl-2 was first identified as an oncogene in B-cell lymphoma with constitutive overexpression resulting from the t(14:18) chromosomal translocation (Hua et al. 1988) and was subsequently identified as a key inhibitor of apoptosis (Söreide et al. 2006; Hockenbery et al. 1990; Vaux et al. 1988). Bcl-2 is one of the most frequently examined apoptotic proteins for potential clinical targeting in cancer therapy. Aberrant expression of this protein has been shown in a number of solid tumours (Chan et al. 2000; Liu et al. 1998; Walker et al. 1995). In normal colonic mucosa, Bcl-2 has a distinct protein expression pattern, expressed solely in the base portion of colonic crypts where there is very low levels of physiological apoptosis (Watson

2004), an expression pattern which is lost in the progression of colorectal cancer (Sinicrope et al. 1995; Visca et al. 1999). In addition, overexpression of Bcl-2 has also been associated with resistance to cytotoxic drugs such as 5-FU, CPT-11, and Cisplatin in various cancer model systems (An et al. 2007a; Violette et al. 2002; Yang et al. 2004; DiPaola & Aisner 1999). In contrast to Bcl-2-like proteins, the Bax family are proapoptotic.

The Bax Family

Bax and Bak are essential in mitochondrial mediated apoptosis, as their insertion into the mitochondrial membrane triggers the release of cytochrome-c into the cytosol, leading to caspase activation and committing the cell to apoptosis (Wolter et al. 1997; Gross et al. 1998; Griffiths et al. 1999; Wei et al. 2001). Bax and Bak have very similar roles, therefore knocking out one is said to not alter the cells ability to undergo apoptosis, while knocking out both Bax and Bak leads to prevention of apoptosis (Wei et al. 2001; Kandasamy et al. 2003; Lindsten et al. 2000). Of the Bax family of proteins, Bax is the most extensively studied in colorectal cancer. Two studies have shown that Bax positively correlated with better survival outcomes than Bax negative tumours in advanced metastatic colorectal cancer (Ogura et al. 1999; Sturm et al. 1999; Nehls et al. 2007; Nehls et al. 2005). In genetic studies there are indications that in approximately half of colorectal tumours there are frameshift mutations in the bax gene (Miquel et al. 2005; Rampino et al. 1997; Schwartz et al. 1999; Trojan et al. 2004; Abdel-Rahman et al. 1999). Downstream of the mitochondria, there are certain proteins capable of blocking the apoptotic signal executed by caspases, these are known as Inhibitors of Apoptosis or the IAP family.

Inhibitors of Apoptosis (IAPs)

IAPs were initially identified by Crook et al. in 1993 (Crook et al. 1993) in the genome of baculovirus as suppressors of apoptosis in host cells. Endogenous IAPs are found in a variety of organisms and contain a conserved baculovirus IAP repeat (BIR) domain, (Takahashi et al. 1998). IAP family members (c-IAP1, c-IAP2, survivin, NIAP, XIAP) can bind to Caspase-9, -7 and -3 and prevent the activation of the caspase cascade (Deveraux et al. 1997; Roy et al. 1997; Deveraux et al. 1998; Tamm et al. 1998; Maier et al. 2002).

Among these IAP members, XIAP appears to be the most potent direct caspase inhibitor. The importance of XIAP is further highlighted by studies that indicate that XIAP is probably the only *bona fide* caspase inhibitor (Eckelman et al. 2006). The expression of the IAP family members, XIAP, survivin and livin, have all been shown in colorectal cancer cells (Yang et al. 2003a; Yagihashi et al. 2003; Endo et al. 2004) and in line with its prominent anti-apoptotic role, XIAP in particular has been shown to be over expressed in tumour cells (Fong et al. 2000; Tamm et al. 2000; Ferreira et al. 2001; Parton et al. 2002; Shiraki et al. 2003; Yang et al. 2003a; Nachmias et al. 2004). Takeuchi *et al.* determined that XIAP expression levels were higher in colorectal cancer tumours than in colorectal normal mucosa and colorectal adenoma and furthermore that XIAP expression levels correlated with colorectal cancer tumours pathology stage. They hypothesised that XIAP is likely to be involved in both colorectal cancer development and tumour progression including metastasis (Takeuchi et al. 2005). Furthermore it was demonstrated that generating stable XIAP knockdown clones in the colorectal cancer cell line HCT116 rendered them more sensitive to TRAIL and irradiation *in vitro* and drug-induced tumour suppression *in vivo* (Connolly et al. 2009; Dai et al. 2008). Furthermore experimental down-regulation of XIAP by anti-sense approaches and RNAi has been able to sensitise cancer cells to Cisplatin and a variety of other apoptosis-inducing agents such as TRAIL (Sasaki et al. 2000; Hu et al. 2003; Chawla-Sarkar et al. 2004; Zhang et al. 2005; Vogler et al. 2007; Braeuer et al. 2006; Mohr et al., 2010). This effect of XIAP down-regulation has been reported to be associated with changes in gene expression that favour increased tendency towards apoptosis, decreased cell proliferation and

angiogenesis potential (Qiao et al. 2009). Therefore, in order to take advantage of these findings, a number of IAP antagonist compounds have been designed to inhibit XIAP to kill tumour cells (Nikolovska-Coleska et al. 2008; Sun et al. 2007; Gao et al. 2007). Most of these compounds have been designed on the basis of the action of the natural inhibitor of XIAP, Smac/DIABLO (Du et al. 2000). Smac can release the inhibition of caspases by XIAP. It is a dimeric protein and normally resides in the mitochondria. Upon triggering of apoptosis it is released from mitochondria into the cytosol, where its binding to the BIR2 and BIR3 domains of XIAP creates a steric hindrance that is essential for preventing binding of XIAP linker regions to effector caspases, thereby neutralising the XIAP inhibition of caspase activation (Gao et al. 2007; Du et al. 2000; Deveraux et al. 1998).

It is clear from the studies discussed thus far, that the complex array of proteins that contribute to the pathways of apoptosis can have profound effects on the sensitivity of a tumour cell to drug-induced apoptosis. Understanding of the exact molecular mechanism with which a particular cytotoxic drug exerts its effects, can enable us to better improve future therapeutic strategies.

1.4 BACKGROUND TO RESEARCH QUESTION

Our group studied 5-FU-induced apoptosis in HCT116 cells and the p53-deficient derivative line HCT p53^{-/-}, and found that not only cell death, but also cytochrome-c release to be dependent on caspase activity in both cell lines. These results pointed to a central caspase activity upstream of the mitochondria. A combination of kinetic caspase activation studies and RNAi approaches revealed that contrary to the classical view of drug-induced apoptosis, caspase-8, not caspase-9, is the initial caspase activated.

1.5 SPECIFIC AIMS OF THE PH.D THESIS

Investigation of the Initial Events in 5-FU-induced Apoptosis

The first aim of the Ph.D thesis was to continue to investigate the initial activation of apoptosis in HCT116 cells induced by 5-FU. This involved i) identification of putative members of a novel 5-FU-induced protein complex, ii) attempt to prove complex member interaction and iii) demonstrate the effect of knocking down putative complex members on apoptosis and caspase-8 and -3 activation.

Development of Alternative Treatment Strategies for Colorectal Cancers

Since the available established colorectal cancer therapies are dealing with several limitations, alternative strategies have to be developed. A promising alternative is represented by the tumour-specific therapy, TRAIL. The second aim of this thesis was therefore the development/improvement of a tumour therapy concept that combines i) a lower dose of 5-FU to overcome associated toxicity and side effects and ii) cancer cell targeting through TRAIL to avoid harming healthy cells with iii) tumour site specific delivery using Mesenchymal Stem Cells (MSCs) as a transgene delivery vehicle to produce TRAIL locally, overcoming the issue of bioavailability at the tumour site. This aims to result in a less toxic, more cancer cell-specific therapeutic treatment of colorectal cancer cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS, REAGENTS & EQUIPMENT

2.1.1 PRIMARY ANTIBODIES

Table 2.1 List of all primary antibodies

ANTIGEN	MW (kDa)	ANTIBODY	ISOTYPE	DILUTION
Bax	20	Cell Signaling Technology, Beverly, MA, USA	Rabbit IgG	1/1000
Bcl-2	26	Cell Signaling Technology, Beverly, MA, USA	Mouse IgG	1/1000
BCL-X_L	30	Cell Signaling Technology, Beverly, MA, USA	Rabbit IgG	1/1000
Bid	23	R&D Systems, Minneapolis, MN, USA	Mouse IgG	1/1000
Bid	23	BD Pharmingen, Franklin Lakes, NJ, USA	Rabbit IgG	1/1000
Caspase-2	45/49	BD Pharmingen, Franklin Lakes, NJ, USA	Mouse IgG	1/1000
Caspase-3	Pro 35 Active 19/17	Clone 3CSP03 Imgenex, San Diego, CA, USA	Mouse IgG	1/1000
Caspase-8	Pro 55/50 Active 40/36 23	BD Pharmingen, Franklin Lakes, NJ, USA	Mouse IgG	1/1000
Caspase-8	Pro 57 Active 43/18	Cell Signaling Technology, Beverly, MA, USA	Mouse IgG	1/1000
Caspase-9	Pro 47 Active 32/17	Novus Biologicals, Littleton, CO, USA	Mouse IgG	1/1000

2.1.1 PRIMARY ANTIBODIES CONTINUED

Table 2.1 List of all primary antibodies continued

ANTIGEN	MW (kDa)	ANTIBODY	ISOTYPE	DILUTION
Caspase-10	Pro 58 Active 43/30	MBL Woburn, MA, USA	Mouse IgG	1/1000
Flip	FLIP _L 55 FLIP _S 30	(NF6) Alexis Biochemicals, San Diego, CA, USA	Mouse IgG	1/200
CD95	36	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Rabbit IgG	1/1000
CuZnSOD	19	The BindingSite, Birmingham, England	Sheep IgG	1/2000
DR4	56	(H-130) Santa Cruz Biotechnology, Santa Cruz, CA, USA	Rabbit IgG	1/500
DR5	40 (mature) 48 (precursor)	Acris Antibodies, Herford, Germany	Rabbit IgG	1/1000
DR5	40 (mature) 48 (precursor)	Cell Signaling Technology, Beverly, MA, USA	Rabbit IgG	1/500
FADD	28	Clone 1F7, Upstate (Millipore), Billerica, MA, USA	Mouse IgG	1/1000
FADD	28	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Rabbit IgG	1/1000
FLAG	-	M2 Stratagene, La Jolla, CA, USA	Mouse IgG	1/1000
HA	-	Origene Technologies, Rockville, MD, USA	Mouse IgG	1/1000

2.1.1 PRIMARY ANTIBODIES CONTINUED

Table 2.1 List of all primary antibodies continued

ANTIGEN	MW (kDa)	ANTIBODY	ISOTYPE	DILUTION
Myc	-	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Mouse IgG	1/500
p53	53	BD Pharmingen, Franklin Lakes, NJ, USA	Mouse IgG	1/1000
p21	21	BD Pharmingen, Franklin Lakes, NJ, USA	Mouse IgG	1/1000
Phospho FADD	28	Upstate (Millipore), Billerica, MA, USA	Rabbit IgG	1/1000
RIP1	78	BD Pharmingen, Franklin Lakes, NJ, USA	Mouse IgG	1/1000
TRAIL	33.7	Peprotech, Rocky Hill, NJ, USA	Rabbit IgG	1/1000
V5	-	Invitrogen, Carlsbad, CA, USA	Mouse IgG	1/1000
XIAP	53	BD Pharmingen, Franklin Lakes, NJ, USA	Mouse IgG	1/1000

2.1.2 SECONDARY ANTIBODIES

Table 2.2 List of all secondary antibodies

ISOTYPE	ANTIBODY	SPECIES	DILUTION
Anti-Sheep IgG	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Rabbit	1/1000
Anti-Rabbit IgG	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Goat	1/1000
Anti-Mouse IgG	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Goat	1/000
Anti-Rat IgG	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Goat	1/1000
Anti-Goat IgG	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Donkey	1/1000
Anti-Mouse IgG	Ebioscience TrueBlot® ULTRA, San Diego, CA, USA	Mouse	1/1000
Anti-Rabbit IgG	Ebioscience TrueBlot®, San Diego, CA, USA	Rabbit	1/1000

2.1.3 BUFFERS & SOLUTIONS

Table 2.3 List of buffers and solutions required

NAME	RECIPE
Anode I Buffer	300 mM Tris, 20% Methanol
Anode II Buffer	25 mM Tris, 20% Methanol
Antibody Dilution Buffer	3% Bovine Serum Albumin (BSA) in TBS-Tween
Blocking Buffer	5% (w/v) Semi-Skimmed Dry Milk in TBS-Tween
CaCl₂ Solution	250 mM in H ₂ O; sterilised
Cathode Buffer	25 mM Tris; 40 mM 6-Aminohexanoic acid; 20% methanol
HBS 2X	140 mM NaCl; 1.5 mM Na ₂ HPO ₄ ; 50 mM HEPES [pH 7.2]; sterilised and stored at -20°C
Nicoletti Buffer	0.1 % (w/v) Sodium Citrate; 0.1 % (v/v) Triton X-100; 50 µg/ml Propidium Iodide.
NP-40 Lysis Buffer	50 mM Tris [pH 7.4] ; 10% Glycerol ; 0.5% NP40 ; 150 mM NaCl ; 1 mM MgCl ₂ ; 1 mM CaCl ₂ ; 1 mM KCl ; Complete Protease Inhibitors (Roche, Basel, Switzerland) 1/50ml.
Phosphate-Buffered Saline (PBS)	5 X Tablets/1000ml diH ₂ O
PBS- Dulbecco 1X	Gibco/Invitrogen, Carlsbad, CA, USA
Reducing Sample Buffer (RSB)	8 mM Tris/HCl [pH 6.8]; 10 % Glycerol (v/v); 2 % SDS (w/v); 4 % β-Mercaptoethanol (v/v); 0.2 % Bromophenol blue (w/v); 0.1% Sodium Azide in diH ₂ O.
RIPA Lysis Buffer	50mM Tris-HCl [pH 7.4]; 150mM NaCl; 2mM EDTA; 1% NP-40; 0.1% SDS ; Complete Protease Inhibitors (Roche, Basel, Switzerland) 1/50ml.
SDS-PAGE 10X Running Buffer	Tris/HCl 25mM; Glycine 192mM; 1% SDS (w/v) in 1L diH ₂ O
Sucrose Buffer 10%	10g Sucrose (w/w) in 90ml dH ₂ O
Sucrose Buffer 30%	30g Sucrose (w/w) in 70ml dH ₂ O
Sucrose Buffer 35%	35g Sucrose (w/w) in 75ml dH ₂ O

2.1.3 BUFFERS & SOLUTIONS CONTINUED

Table 2.3 List of buffers and solutions required continued

NAME	RECIPE
Sucrose Buffer 40%	40g Sucrose (w/w) in 60ml dH ₂ O
Sucrose Buffer 50%	50g Sucrose (w/w) in 50ml dH ₂ O
Tris-Buffered saline (TBS)	Tris-HCl 25 mM [pH 8.0] ; NaCl 137 mM
TBS-Tween	Tris-HCl 25 mM [pH 8.0]; NaCl 137 mM; dH ₂ O, 1% Tween 20
Tris-HCl 1.5M pH 8.8	90.75g Tris, 400ml dH ₂ O, adjust to pH 8.8 with HCl, adjust to 500ml with dH ₂ O
Tris-HCl 0.5M pH 6.8	30.3g Tris, 400ml dH ₂ O, adjust to pH 6.8 with HCl, adjust to 500ml with dH ₂ O
Triton X-100 Lysis Buffer	50mM Tris-HCl [pH 7.4]; 150mM NaCl; 2mM EDTA; 1% Triton X-100 (v/v); 10% Glycerol; 0.1% SDS ; Complete Protease Inhibitors (Roche, Basel, Switzerland) 1/50ml.

2.1.4 REAGENTS

Table 2.4 List of reagents

REAGENT	SUPPLIER
Acrylamide-Bis-Acrylamide	30% ratio 29:1 Sigma-Aldrich, St.Louis, MO, USA
Ammoniumpersulfate (APS)	10% (w/v) Sigma-Aldrich, St.Louis, MO, USA
6-Aminohexanoic acid	Sigma-Aldrich, St.Louis, MO, USA
Ampicillin Sodium Salt	Sigma-Aldrich, St.Louis, MO, USA
β-Mercaptoethanol	Sigma-Aldrich, St.Louis, MO, USA
BCA Protein Assay Reagent (bicinchoninic acid)	Pierce/Thermoscientific, Waltham, MA, USA
BlastocidinTM	Invivogen, San Diego, CA, USA
Bromophenol Blue	Sigma-Aldrich, St.Louis, MO, USA

2.1.4 REAGENTS CONTINUED

Table 2.4 List of reagents continued

REAGENT	SUPPLIER
BSA (Bovine Serum Albumin)	Carl Roth, Karlsruhe Germany
Calcium Chloride	Sigma-Aldrich, St.Louis, MO, USA
Cesium Chloride	Roche, Basel, Switzerland
Caspase Inhibitor Z-VAD-FMK	Santa Cruz, Santa Cruz, CA, USA
Complete Protease Inhibitor Cocktail Tablets	Roche, Basel, Switzerland
DMSO	Sigma-Aldrich, St.Louis, MO, USA
Enhanced chemiluminescent (ECL) Western Blotting Substrate	Pierce/Thermoscientific, Waltham, MA, USA
Ethanol	Sigma-Aldrich, St.Louis, MO, USA
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich, St.Louis, MO, USA
Fetal Calf Serum (FCS)	Gibco/Invitrogen, Carlsbad, CA, USA
5-Flourouracil (5-FU)	Sigma-Aldrich, St.Louis, MO, USA
FuGENE®6 Transfection Reagent	Roche, Basel, Switzerland
FuGENE ®HD Transfection Reagent	Roche, Basel, Switzerland
Geneticin (G418)	Sigma-Aldrich, St.Louis, MO, USA
Glycerol	Sigma-Aldrich, St.Louis, MO, USA
Glycine	Sigma-Aldrich, St.Louis, MO, USA
HEPES	Sigma-Aldrich, St.Louis, MO, USA
Hydrochloric Acid	Sigma-Aldrich, St.Louis, MO, USA
Kanamycin	Sigma-Aldrich, St.Louis, MO, USA
LB Agar	Sigma-Aldrich, St.Louis, MO, USA
LB Broth Powder	Sigma-Aldrich, St.Louis, MO, USA
MagnaBind Goat Anti-Mouse IgG Beads	Thermoscientific/Pierce Waltham, MA, USA
MagnaBind Goat Anti-Rabbit IgG Beads	Thermoscientific/Pierce Waltham, MA, USA

2.1.4 REAGENTS CONTINUED

Table 2.4 List of reagents continued

REAGENT	SUPPLIER
Magnesium Chloride	Sigma-Aldrich, St.Louis, MO, USA
Marvel Dried Skimmed Milk Powder	Chivers, Dublin, Ireland
Methanol	Sigma-Aldrich, St.Louis, MO, USA
Nonidet P-40 (NP-40)	Sigma-Aldrich, St.Louis, MO, USA
Penicillin/Streptomycin Solution (100X)	Gibco/Invitrogen, Carlsbad, CA, USA
Phosphate-Buffered Saline Tablets	Sigma-Aldrich, St.Louis, MO, USA
PhosStop Phosphatase Inhibitor Cocktail Tablets	Roche, Basel, Switzerland
Ponceau-S Solution	Sigma-Aldrich, St.Louis, MO, USA
Potassium Chloride	Sigma-Aldrich, St.Louis, MO, USA
Pre-Stained Protein Marker, Broad Range (7-175Kda)	New England Biolabs, UK
Propanol	Sigma-Aldrich, St.Louis, MO, USA
Recombinant Human TRAIL	R&D Systems, Minneapolis, MN, USA;
Sodium Azide	Sigma-Aldrich, St.Louis, MO, USA
Sodium Citrate	Sigma-Aldrich, St.Louis, MO, USA
Sodium Dodecyl Sulphate Solution (20% in H₂O)	Sigma-Aldrich, St.Louis, MO, USA
Sodium Fluoride	Sigma-Aldrich, St.Louis, MO, USA
Sodium Hydroxide	Sigma-Aldrich, St.Louis, MO, USA
Sucrose	Sigma-Aldrich, St.Louis, MO, USA
1,2-Bis-(dimethylamino)-Ethane (TEMED)	Sigma-Aldrich, St.Louis, MO, USA
Trichloroacetic Acid (TCA)	Sigma-Aldrich, St.Louis, MO, USA
Trizma® Base	Sigma-Aldrich, St.Louis, MO, USA
Triton® X-100	Sigma-Aldrich, St.Louis, MO, USA
Trypsin EDTA Solution	Sigma-Aldrich, St.Louis, MO, USA
Tween®20	Sigma-Aldrich, St.Louis, MO, USA

2.1.5 CELL LINES

Table 2.5 List of Cell Lines and culture conditions

CELL LINE	CELL ORIGIN	SUPPLIER	CULTURE MEDIUM
HCT116	Epithelial human colon carcinoma	ATCC, Manassas, VA	McCoy's, 10% FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin
HCT116 p53-/-	Epithelial human colon carcinoma	ATCC, Manassas, VA	McCoy's, 10% FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin
HEK293 FT	Human Embryonic Kidney 293 cells	ATCC, Manassas, VA	D-MEM- high glucose, 10% FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin
Murine MSC	Murine Mesenchymal Stem Cells	Generated by S.M. Albarenque (Mohr et al., 2010)	D-MEM- low glucose, 15% FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin

2.1.6 CELL CULTURE MATERIALS AND REAGENTS

Table 2.6 Cell Culture Products

REAGENT/MATERIAL	SUPPLIER
D-MEM- High Glucose (Dulbecco's Minimal Essential Medium)	Invitrogen (Carlsbad, CA)
D-MEM-Low Glucose	Invitrogen (Carlsbad, CA)
McCoy's	Cambrex; New Jersey, USA
Foetal Calf Serum	Gibco/Invitrogen, UK
Penicillin/Streptomycin 10,000U/ml	Sigma Aldrich, St.Louis, MO
Trypsin/EDTA 1X	Sigma Aldrich, St.Louis, MO
Dulbecco's Phosphate Buffered Saline 1X	Invitrogen (Carlsbad, CA)

2.1.6 CELL CULTURE MATERIALS AND REAGENTS CONTINUED

Table 2.6 Cell Culture Products continued

REAGENT	SUPPLIER
25cm² cell culture flasks	Sarstedt, Nümbrecht, Germany
75cm² cell culture flasks	Sarstedt, Nümbrecht, Germany
24-well cell culture plates	Sarstedt, Nümbrecht, Germany
6-well cell culture plates	Sarstedt, Nümbrecht, Germany
100mm cell culture dishes	Sarstedt, Nümbrecht, Germany
150mm cell culture dishes	Sarstedt, Nümbrecht, Germany
2ml cryovials	Nalgene, Nunc™, Penfield, NY, USA

2.2 METHODS

2.2.1 MOLECULAR BIOLOGY METHODS

2.2.1.1 TRANSFORMATION OF COMPETENT CELLS

Plasmid DNA was transformed into competent *E. Coli*. An aliquot of 25-100 μ l in a 1.5 ml microcentrifuge tube of competent bacteria previously stored at -80°C was thawed on ice and added to 1 μ g of plasmid mixture. Cells were incubated for 30 min on ice, and then heat-shocked for 90 sec at 42°C . After flash cooling on ice for 1 min, 500 μ l of LB both was added and the cultures were incubated shaking at 37°C for 45 min to allow cell growth. Cells were plated onto agar medium containing appropriate antibiotics. Plates were incubated at 37°C overnight (O/N) and checked for colonies.

2.2.1.2 PREPARATION OF PLASMID DNA FROM BACTERIA

Small and large-scale plasmid purifications were performed using plasmid mini- and maxiprep kits from Sigma-Aldrich or Qiagen according to the manufactures recommendations. Briefly, a single colony was scraped using a sterile pipette tip and immersed in a sterile 15 ml falcon tube containing LB media (3 ml) supplemented with either Ampicillin or Kanamycin as appropriate. Falcon tubes were incubated shaking at 37°C over day (at least 6 h). When solution reached a reasonable level of cloudiness/turbidity due to bacterial growth, it was used either for mini-prep or for maxi-prep. For maxiprep, the 3 ml culture was added to a sterile conical flask (500 ml) containing 150-200 ml of LB media supplemented with either Ampicillin or Kanamycin and incubated shaking at 37°C O/N and then plasmid DNA was prepared as per manufacturers instructions.

2.2.1.3 ESTIMATION OF DNA QUALITY AND PURITY

DNA quantification was measured using a Thermo Scientific Nanodrop ND-1000 Spectrophotometer at 260 nm and 280 nm, where a ratio of ~ 1.8 is generally accepted as pure.

2.2.1.4 RNAi KNOCK-DOWN AND OVEREXPRESSION CONSTRUCTS

All RNAi knock-down constructs were generated in the lab previously. The following small hairpin (sh) RNA motif was used to target: caspase-8 (5'-GGGTCATGCTCTATCAGAT-3'), caspase-9 (5' – GCTTCGTTTCTGCGAACTAAC -3'), DR5 (5'-GCTAGAAGGTAATGCAGACTCTGCCATGTC -3'), DR4 (5'-GCTGTTCTTTGACAAGTTGC-3'), Bid (5' –GAAGACATCATCCGGAATA-3'), XIAP (5'-GTGGTAGTCCTGTTTCAGC-3'), FADD (5' – GTGCAGCATTTAACGTCATAT-3'). Briefly, sense and antisense oligos containing the sh-sequence and 5' overhang representing a restricted BbsI site and EcoRI site on the 3' side were hybridized to generate double-stranded DNA fragments. These fragments were then cloned into a modified pU6.ENTR plasmid (Invitrogen). The resulting pU6.ENTR plasmid was used to generate the pBlockIt.shc8, pBlockIt.shDR5, pBlockIt.shDR4, pBlockIt.shFADD, pBlockIt.shBid and pBlockIt.XIAP plasmid using the pBlockIt vector (Invitrogen) and LR Clonase II. This was used to generate stable caspase-8, DR4 and DR5 knockdown clones of HCT116 cells.

2.2.1.5 CLONING OF sTRAIL CONSTRUCT

The cloning of the sTRAIL construct was performed in the lab previously (Mohr et al. 2010). Briefly the soluble portion of human TRAIL (aa 114-281) was first subcloned into the NheI/NotI sites of the pcDNA3 vector (Invitrogen) resulting in pcDNA3.sTRAIL. Then the signal peptide sequence fused with the Furin cleavage sites sequence (Furin CS) and isoleucine-zipper sequence (ILZ) were cloned into the BamHI/NheI sites of the pcDNA3.sTRAIL vector.

2.2.1.6 ADENOVIRUS RE-AMPLIFICATION

(Note: all DNA plasmids & Adenoviral vectors were previously generated in the lab)

Adenoviral vectors were generated in the lab previously by Dr. Chirlei Klein. Briefly, recombinant E1/E3-eleted adenoviral vectors expressing sTRAIL (Ad.sTRAIL vector

biolabs), Bcl-2 (Ad.Bcl-2, vector biolabs), FLIP (Ad.FLIP, vector biolabs), DN-caspase-9 (Ad.DN-C9 (C287A mutant), vector bioloabs), DsRed (Ad.DsRed), and enhanced fluorescent protein (EGFP; Ad.EGFP), from the cytomegalie virus (CMV) promoter/enhancer element as well as adenoviral vectors encoding small hairpin constructs targeted against the XIAP (Ad.shXIAP), DR4 (Ad.shDR4), DR5 (Ad.shDR5), caspase-8 (Ad.shc-8), caspase-9 (Ad.shc-9) and EGFP (Ad.shEGFP) gene products, respectively, under the control of the human U6 promoter, were generated using the ViraPower adenoviral expression system (Invitrogen) as described earlier (Behrend et al. 2005).

For the present study, Ad.sTRAIL was re-amplified. 1 µl of purified virus was used to re-infect larger quantities of cells and to produce adenovirus on a larger scale. The last amplification was executed with twenty 15 cm dishes and the once the cells went in CPE they were harvested and the crude extract, after freeze-thawing, were resuspended into 10 ml of TBS buffer (see section 2.2.1). 5 g of CsCl was added to the crude lysates. The solution was then transferred to a 14 x 89 mm *Beckman* tube and centrifuged for approximately 20 h at 32,000 rpm at 4°C in a Sorval 100 SE centrifuge, using a Sorval TH-641 rotor. In the CsCl gradient, the band containing the virus could be collected with a syringe and then purified with PD10 columns (G&E). To the final 2 mls of purified adenovirus solution glycerol was added to a final concentration of 10% to enable the virus to be stored safely at -80°C.

The concentration of adenovirus was determined by Dr. Richard Jäger and expressed in virus particles/µl using RT-PCR of viral particles/µl using a method adapted from Thomas et al. (2007).

2.2.1.7 ADENOVIRUS TRANSDUCTION

Cells were plated at a density of 10^5 cells per well in a 6-well plate and left to adhere overnight. Cells were transduced with virus at 1×10^9 virus particles/cell and the plates were spun at 800 x g for 90 min at 37°C. The virus was left on the cells for 6 h and then washed off.

2.2.2 BIOCHEMICAL METHODS

2.2.2.1 CELL CULTURE

Cell lines

Cell lines were maintained in 5% CO₂ at 37°C in a humidified Hera Cell Incubator (Heraeus) in the following culture conditions with the addition of 1% penicillin/streptomycin to all media.

Passage of Cell Lines

Cells were passaged by first rinsing with warmed sterile 1X PBS. Following this brief wash, trypsin/EDTA solution was added (per T75 flask: 2 ml for all cell lines) and cells were incubated under the hood for up to 5 min until they detached. The trypsin/EDTA solution was neutralised by the addition of 10 ml of culture medium. The cell/trypsin/medium mix was pipetted several times to generate a single cell suspension, before generating a 1 in 7 dilution with pre-warmed culture medium and transferring this mix to a new T75 flask. This method was scaled up or down depending on the flask or dish required for further experiments.

Freezing and thawing of mammalian cell lines

Using a modification to the previous protocol, cells to be frozen in the -80°C freezer were dissociated using trypsin and pipetted up and down to obtain a single cell suspension and centrifuged at 1500 rpm for 5 min. The pellet was then resuspended in a freezing mixture composed of FCS and 10% (v/v) DMSO. 1 ml aliquots were transferred to 2 ml Nunc cryovials. Vials were placed on ice for 10 mins, inverted 2-3 times and stored at -80°C. Frozen cells were quickly thawed by warming to 37°C, then transferred to a T75 flask containing 12 mls pre-warmed culture medium. After the cells had attached overnight, the medium was exchanged for fresh prewarmed culture medium to remove any residual DMSO.

2.2.2.2 WESTERN BLOTTING

Preparation of cells extracts

Cells were harvested by washing in ice-cold phosphate-buffered saline (1X PBS), followed by trypsinisation. The cells were washed once in ice-cold PBS and cells were pelleted at 1500 rpm for 5 min. Cell pellets were extracted for 30-60 minutes in the appropriate ice-cold cell lysis buffer (containing Complete Mini Protease Inhibitors (Roche, Basel, Switzerland)) depending on the downstream application. All the following steps were carried out at 4°C or on ice. Cells were then centrifuged at 14,000 rpm for 15 min at 4°C. The clear supernatant was used for protein determination and/or aliquoted and stored at -20°C.

Preparation of membrane and cytosolic fractions

Following the cell lysis, the nuclear debris was pelleted at 3,000 rpm for 10 min. The supernatant was then transferred to a fresh microcentrifuge tube and centrifuged at 13,000 rpm for 20 min to pellet the membrane fraction. The supernatant contained the cytosolic fraction and was transferred to a fresh microcentrifuge tube. The membrane pellet was resuspended in an equal volume of protein lysis buffer as the supernatant containing the cytosolic fraction.

Protein Quantification.

According to the manufacturer's instructions, the BCATM protein assay kit (Pierce) was used to determine the concentration of proteins in cell extracts, using dilutions of a BSA standard (2 mg/ml) in a 96-well plate.

Sodium dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot Analysis.

SDS-PAGE was performed following a protocol adapted from (Laemmli 1970) using 4% stacking and 12% separating gels (1.5 mm thick). The SDS-PAGE apparatus

(Mini-Protean, Bio-Rad) was assembled according to the manufacturer's instructions. Separating gel was prepared (Table 2.7), poured to a level of approx. 3 cm from the top, and approximately 0.5 ml of isopropanol was carefully added with a Pasteur pipette to overlay the gel. After polymerisation (20-30 min), this isopropanol layered was poured off, and washed twice with diH₂O, before Whatmann paper was used to absorb any residual water. The stacking gel solution was then added (Table 2.8), a 15 well comb inserted, and the gel left to polymerise (10-20 min). The gel was loaded into the tank, which was subsequently filled with 1X running buffer and the comb was carefully removed.

Fifty µg (unless otherwise stated) of protein from each sample was mixed with an appropriate volume of 4X RSB (see section 2.1.3, Table 2.3) to give a final concentration of 1X RSB (Total volume ≤ 40 µl for loading). The samples were boiled at 95°C for 5 min and then loaded on the stacking gel along with a prestained protein marker (broad range 6-175 kDa, Biolabs) to determine the molecular mass of proteins. Gels were run at 80 V until the dye front has moved through the stacking gel. Voltage was then increased to 120 V and the gel run until the dye reached the bottom of the gel.

Table 2.7 Separating Gel Recipe

12% Gel	
diH₂O	3.4 ml
1.5M Tris-HCl pH 8.8	2.5 ml
10% SDS	100 µl
30% Acrylamide	4 ml
10% APS	50 µl
TEMED	5 µl

Table 2.8 Stacking Gel Recipe

4% Gel	
diH₂O	1.8 ml
0.5M Tris-HCl pH 6.8	750 µl
10% SDS	30 µl
30% Acrylamide	390 µl
10% APS	15 µl
TEMED	3 µl

Protein transfer onto PVDF (*GE Healthcare*) membrane: Prior to protein transfer, the PVDF membrane was soaked in methanol (pre-activation) followed by Anode II buffer. The blotting sandwich was assembled by firstly placing two pieces of Anode I-soaked whatmann paper on the bottom. In the middle, one piece of Anode II-soaked whatmann was placed on top and the PVDF membrane was placed on top of this. The Gel was carefully overlaid on top of the PVDF membrane, ensuring there were no bubbles present. Finally these were covered with three pieces of Cathode-soaked

whatmann paper. Transfer was carried out at 50 mA constant current for 60 min. The transfer efficiency and equal loading of protein was routinely checked by Ponceau-S staining, after transfer of proteins. The membrane was blocked in blocking buffer (5% non-fat dry milk solution in TBS, supplemented with 0.1% Tween20®) for 1 h at room temperature. For phospho-proteins, the milk was replaced with BSA. This solution was used for all other antibody incubations and washing steps. Protein detection was performed by diluting anti-human Ab 1:1000 (unless stated otherwise) in antibody dilution buffer (TBS 0.1% Tween, 3% BSA) and the primary antibody was then incubated overnight at 4°C, after which the membrane was washed four times to remove excess of primary antibody. The secondary (anti-rabbit/Mouse/Sheep/Goat/Rat) IgG antibody conjugated to horse-radish-peroxidase (S.C.) was added at a dilution of 1:1000. This was incubated for 1 h at RT. Following incubations with the secondary antibody, the membrane was washed four times in blocking buffer, four times in TBST and finally twice in TBS/PBS depending on the phosphorylation state of the protein of interest.

Antibody Detection by Enhanced Chemiluminescence

Protein detection was performed using enhanced chemiluminescence (ECL) Western Blot Chemiluminescence Reagent (GE Healthcare) and a digital imaging system (Alpha Innotech Fluorchem, San Leandro, CA, USA). HRP-conjugated secondary antibodies were used for ECL. Following the final washing steps the membranes were visualised with ECL western blot chemiluminescence reagent (Pierce) as per manufacturer's instructions (Solution A and B, mixed 1:1). The membranes were exposed automatically with a digital imaging system (Alpha Innotech Fluorchem, San Leandro, CA, USA).

2.2.3 CELL BIOLOGY METHODS

2.2.3.1 DRUG TREATMENTS

5-FU (5-400 μ M) treatment was carried out 24 h after cells were seeded, 24 h after adenovirus transduction expressing a transgene or 48 h after adenovirus transduction expressing a short hairpin, or 4-6 h post-transfection with calcium phosphate or FuGENE®. For co-immunoprecipitation experiments, 5-FU was added 4 - 6 h post-transfection for 15 h. TRAIL (0.5 -10 ng/ml) treatment was carried out 24 h after cells were seeded, or 24 h after 5-FU pretreatment.

2.2.3.2 5-FU AND RHTRAIL DOSE-RESPONSE CURVE

To determine a dose-dependent response to rhTRAIL, HCT116 cells were seeded at 1×10^4 cells per well into a 24 well plate. After 24 h of plating, media was removed and cells were treated for 24 h with rhTRAIL at various concentrations (0.125-5 ng/ml). One well without rhTRAIL served as an untreated control. After 24 h, cells were harvested and apoptosis measured. The same procedure was carried out for 5-FU where the concentrations used was 5 - 400 μ M. After 48 h, cells were harvested and assayed for apoptosis. The concentrations of rhTRAIL and 5-FU used in the experiments were 1 ng and 10 μ M respectively.

2.2.3.3 SUPERNATANT TRANSFER EXPERIMENTS

HCT116 cells were seeded onto 6-well plates at a density of 1×10^5 cells per well and left to adhere for 24 h. MSCs were transduced with Ad.sTRAIL or Ad.DsRed for 24 h. MSC.sTRAIL or MSC.DsRed supernatant was diluted 1:5 and 1:10 with MSC culture medium and added to the untreated and 5-FU pretreated HCT116 cells which had been seeded 24 h prior. The untreated HCT116 cells and the MSC.DsRed supernatant served as controls. After 24 h cells were harvested and assayed for apoptosis.

2.2.3.4 COCULTURE EXPERIMENTS

In order to analyse apoptosis induction in MSC.sTRAIL HCT116 cells by concomitant exposure to MSC.sTRAIL and subapoptotic genotoxic damage in vitro, HCT116 cells were seeded into 6-well plates at a density of 1×10^5 cells per well, and left to adhere for 24 h. The cells were then treated with 5-FU (10 μ M) for 24 h or left untreated as a control. The HCT116 cells were then harvested and mixed with MSC.sTRAIL cells or MSC.DsRed cells (1×10^4) and plated in 6-well plates at this 1:10 ratio of MSCs to HCT116 cells. After 24 h cocultivation, cells were harvested and apoptosis was measured. Cocultures of MSC.DsRed cells served as controls.

2.2.3.5 MEASUREMENT OF CELL DEATH

Nicoletti Assay

For determination of cell death, cells were treated with the appropriate concentration of either 5-FU or TRAIL for the indicated amount of time. The leakage of fragmented DNA from apoptotic nuclei was measured using a method adapted from Nicoletti *et al.* (1991). Briefly, the cells were prepared by harvesting with the supernatant and cells were pelleted at 1500 rpm for 5 min at 4°C. The supernatant was discarded and the cells were subsequently lysed in a hypotonic buffer (termed Nicoletti buffer in Section 2.1.3, Table 2.3) and were subsequently analysed by flow cytometry following incubation for 2-16 h at 4°C in the dark. The volume of Nicoletti buffer used was approximately 1 ml for 5×10^5 cells. Nuclei containing hypodiploid DNA were considered apoptotic. All flow cytometry analyses were performed on a BD FACSCanto A flow cytometer analyser (6 colour) (Becton Dickinson, Heidelberg, Germany) by using BD FACSDiva™ Software. To discriminate between true apoptotic nuclei and nuclear debris, we used negative and positive cell controls of untreated and treated cells, and also added Z-VAD-FMK to treated cells to demonstrate the specificity.

2.2.3.6 TRANSFECTION OF EUKARYOTIC CELLS

Transfection is a method to deliver nucleic acids into a mammalian cell (Azzam & Domb 2004). In the present study, three variations of transfection were employed throughout the experiments. These included calcium phosphate based transfection and FuGENE® based methods. For each of the transfection methods, cells were seeded 24 h prior to transfection and the medium was changed 24 h post transfection. To determine the efficiency of the transfection, pEGFP.N1 plasmid was transfected in addition to the transgene of interest and the percentage of green fluorescent cells was estimated 24 or 48 h post transfection via microscopy.

Ca₃(PO₄)₂ transfection

Cells were seeded to about 50-70% confluency 24 h prior to transfection. Prior to adding transfection, medium was changed to 2% reduced serum medium. For each 6-well plate/dish, 0.5 ml 2X HBS was aliquoted into a sterile 1.5 ml microcentrifuge tube and in a separate tube 3-12 µg of DNA (dependent on the plasmid and size of dish) were mixed with 2.5M CaCl₂ and enough distilled water to bring the total volume to 0.5 ml (see Table 2.9 below for different area dishes). The CaCl₂/DNA mix was then added to the HBS (accurate pH is essential) drop-wise under vortex. After 20 min of incubation, the DNA Ca₃(PO₄)₂ coprecipitate was added slowly dropwise directly to the surface of the media containing the cells. Dishes were then placed into the incubator for 4 h. The media was then removed and cells were washed twice with PBS, then fresh complete medium was added and the incubation was resumed for 24-48 h prior to further treatment or assaying.

Table 2.9 Amounts of transfection components required for different surface areas

FORMAT	CULTURE MEDIA (2% FCS)	TRANSFECTION SOLUTION
6-well plate	1.5 ml	0.5 ml
10 cm plate	6.0 ml	1.0 ml
15 cm plate	15 ml	6.0 ml

Transfection with FuGENE®6 or FuGENE®HD

For HEK 293FT cells, Calcium phosphate transfection was sufficient, however, for HCT116 cells we required Fugene6 to reach optimal transfection efficiencies (as visualised with EGFP). FuGENE®6/FuGENE® HD transfection was carried out according to the manufacturer's instructions (ROCHE) on 50-80% confluent cells.

Typically 3 µg of DNA was added to 100 µl of 0% serum medium in a 1.5 ml microcentrifuge tube. This was flicked repeatedly to ensure the mixture was homogenous. The FuGene was added at a ratio of 1:3 DNA to FuGENE to the DNA/medium mixture and was flicked gently. The mixture was left to sit for 20 min at RT before adding dropwise to each well.

2.2.3.7 GENERATION OF STABLE CLONES

3 days post FuGENE® (6/HD) transfection of HCT116 cells with the plasmid expressing a short hairpin or overexpression plasmid, the cells were split into two 15 cm dishes and kept under selection with Blastocidin (10 mg/ml) or G418 respectively. The antibiotic selected cells that had the expected plasmid containing the Blastocidin/G418 resistance and every cell that contains the plasmid grew into a single colony. After approximately 3 weeks, isolated clones were visible and picked with cloning discs or were isolated by suction using a 100 µl pipette tip and transferred to 24-well plates until confluent. The clones were scaled-up and tested by Western blots for appropriate protein silencing/overexpression.

2.2.3.8 CO-IMMUNOPRECIPITATION EXPERIMENTS

HEK293FT cells were plated overnight in 6-well plates and 24 h later the medium was changed to 2% FCS containing medium. The cells were then transfected with the indicated expression vectors and ~ 4-6 h later the 2% FCS medium was removed and cells were washed twice with PBS. Fresh 10% FCS medium was added, and 400 µM 5-FU was added to appropriate cells for 15 h. Untreated transfected cells served as controls.

Cells were collected, washed in PBS, and lysed in the appropriate

immunoprecipitation buffer, containing protease inhibitor cocktail tablets (see table below for buffer conditions). Lysates were incubated on a rotating wheel, for 4-6 h at 4°C in the presence of MagnaBind Goat Anti-Mouse IgG or Goat Anti-Rabbit IgG beads and appropriate antibody. Following this incubation, using the MagnaBind magnet the lysates were separated from the magnetic beads and the supernatant was discarded. The beads were subsequently carefully resuspended in appropriate immunoprecipitation wash buffer and incubated on the magnet for 5 min on ice before wash buffer was removed. This step was repeated 4 more times. The MagnaBind beads were finally resuspended in 20 µl RSB and boiled for 5 min before analysis by Western blotting for appropriate proteins.

Table 2.10 Immunoprecipitation Conditions

IP	ANTIBODY	ANTIBODY CONC.	LYSIS/WASH BUFFER	PROTEIN CONC (µg)	IP VOL (µl)	INCUBATION TIME (h)
C-8	α-c8 (BD)	1µg/mg	RIPA	600-1200	~ 400	4-6
FADD	α-FADD (S.C.)	1µg/mg	NP-40	600-1200	~ 400	4-6
DR5	α-DR5 (Acris)	1µg/mg	X-100	600-1200	~ 400	4-6
V5	α-V5	1µg/mg	RIPA	600-1200	~ 400	4-6
Myc	α-Myc	1µg/mg	RIPA	600-1200	~ 400	4-6
Flag	α-Flag	1µg/mg	RIPA	600-1200	~ 400	4-6

2.2.3.9 SUCROSE DENSITY GRADIENT FRACTIONATION

The following sucrose gradient protocol was adapted from Feig et al. (2007). Cells that had been treated with 5-FU for 15 h are harvested and postnuclear lysates were generated. Briefly cells were washed once with ice-cold PBS at the end of drug stimulation kinetics and resuspended in 300 µl 1% Triton X-100 buffer. Cell lysis was carried out for 10 min on ice and lysates were cleared of debris by centrifugation at 14,000 rpm for 10 min at 4°C.

The step gradient from 10 - 50% (w/w) sucrose was prepared manually by first pipetting 646 µl of the highest density sucrose buffer, 50%, at the bottom of the 3.5

ml *Beckman* ultracentrifuge tube. The other buffers (40%, 35%, 30% and 10%) were layered carefully on top of the 50% sucrose buffer. 270 μ l of protein lysate was carefully overlaid on top of the sucrose gradient, which was then subjected to ultracentrifugation at 37,500rpm for approximately 16 h.

14 Equal fractions of 250 μ l were then collected from the top of the gradient carefully by pipette and aliquoted into 1.5 ml microcentrifuge tubes. 30 μ l of sample was mixed with 7.5 μ l of RSB and boiled for 5 min. The 14 samples were then subjected to SDS-PAGE and Western blotting for appropriate proteins.

According to Feig *et al.* a larger heavier protein will sediment into a denser gradient area and thus appear in a higher fraction number than a small protein. The second dimension results from resolution of the individual fractions on SDS-PAGE, providing information about the apparent molecular mass of a protein or protein complex subunit (Feig & Peter 2008).

Sucrose Calibration and Estimation of Sedimentation Coefficient, S

In order to calibrate our sucrose gradient we employed the standard size markers Aldolase and Thyroglobulin. 5 mg of each of the lyophilised standard proteins was dissolved in 270 μ l of 1% Triton protein lysis buffer (see **Table 2.3**). These were then loaded onto separate sucrose gradients (10-50%) for estimation of the sedimentation coefficient of the complex. The sedimentation coefficient or *S* value is affected by the molecular mass, density, shape and hydration of the proteins within the complex (Marks, 2001). We detected the peak elution fraction for Aldolase, a molecular weight of approx. 160 KDa, to be in Fraction 5, and Thyroglobulin, a molecular weight of approx. 670 KDa in fraction 7, using a BCA protein determination of sucrose fractions.

This allows us to generally estimate our complex to be larger than 670 KDa, given that the standard molecular weight protein Thyroglobulin (~670 KDa) (see **Table 3.1**) elutes in Fraction 7 (~35% sucrose), and we visualise our complex members in Fraction 14 (~50%) of the gradient.

Table 3.1 Sedimentation Coefficient, Partial Specific Volume, and Corresponding Molecular Weight of Typical Protein Standards

Protein	$S_{20,w}$ (S)	Molecular Weight	v (ml/g)
Bovine heart cytochrome c	1.7	12,300	0.728
Lysozyme	1.9	14,400	0.726
Sperm whale myoglobin	2.0	17,200	0.741
Chicken ovalbumin	3.6	42,881	0.748
Bovine serum albumin	4.6	66,000	0.734
Human transferrin	4.9	77,049	0.765
Human serum IgG	7.1	155,000	0.735
Rabbit aldolase	7.3	158,000	0.742
Bovine catalase	11.3	247,000	0.730
Rat β -glucuronidase	12.5	288,000	0.731
Horse heart ferritin	16.5	460,000	0.738
E. coli β -galactosidase	15.9	540,000	0.730
Thyroglobulin	19.3	667,000	0.720
Human serum α -macroglobulin	19.6	720,000	0.731

Adapted from Marks, (2001)

Determination of Sedimentation Coefficients by Extrapolation from migration of standard proteins

Using the protocol outlined in Marks (2001), the sedimentation coefficient was estimated as follows. The sedimentation coefficient, S , of an unknown sample can be calculated directly from the parameters of the fractionation and the migration of the sample. However, S values are said to vary depending on the temperature and the medium through which the sample is sedimenting, and therefore must be converted to a sedimentation coefficient under the standard state of water at 20°C ($S_{20,w}$) for their to be any meaningful relationship to the molecular weight. If the properties of the standard proteins and the sample are similar, then relative sedimentation coefficients can be easily estimated directly by comparing the migration of the standard proteins to that of the sample. A more precise analysis is based on other measurable or calculable parameters.

1. A ratio, R , was calculated based on the distance traveled by the standard and the experimental sample from the meniscus. According to the equation from Martin and Ames (1961),

$$R = \frac{\text{distance travelled from meniscus by sample}}{\text{distance travelled from meniscus by standard}} \quad (1)$$

Assuming that fractions of equal size were taken, we substitute the distance travelled by the quantity [peak fraction # -1].

The value of [peak fraction # -1] is taken because elution with fraction 1 is equivalent to no migration (this should be approx. The volume loaded onto the gradient = 270 μ l).

$$\text{Using Thyroglobulin : } R = \frac{[3500 - 270]}{[1750 - 270]} = \frac{3230}{1480} = 2.18$$

Where total volume of gradient is 3500 μ l, with 14 fraction of 250 μ l each.

2. Using the value of R and the known $S_{20,w}$ value of the standard, we can compute the $S_{20,w}$ value of the sample.

Since macromolecules move linearly through the gradient over time, the ratio R also represents the sedimentation values, such that

$$R = \frac{S_{20,w} \text{ of sample}}{S_{20,w} \text{ of standard}} \quad (2)$$

Therefore, $S_{20,w}$ of sample = $R \times S_{20,w}$ of standard.

These calculations assume that the S value observed is proportional to that under standard conditions of water at 20°C. If the sedimentation buffer has physiological levels of salt and is near neutral pH, this assumption is a reasonable one.

$$\text{Using Thyroglobulin: } S_{20,w} \text{ of sample} = 2.18 \times 19.3 = 42.074S$$

Determination of Sedimentation coefficients by conversion of $S_{T,m}$ to $S_{20,K}$ values

A more accurate assessment of sedimentation coefficient can be made if one eliminates assumptions regarding any similarity between sample and standard regarding shape and the value of the partial specific volume. This method uses first a calculation of a sedimentation coefficient $S_{T,m}$ for the conditions of the experiment.

Determination of the value of $S_{T,m}$

As described by Martin and Ames (1961), the sedimentation coefficient S at a given temperature, T in a solvent m is defined as $S_{T,m} = (dr/dt)/\omega^2 r$, in which dr/dt is the distance traveled over time or velocity in mm/sec (constant in sucrose gradients), r is the distance from the axis of rotation in mm, and ω is the angular velocity of the rotor in radians/second. These parameters can be determined from the measurements of the experiment.

1. We calculate the value of dr/dt , as described in Clarke (1975), by measuring the distance travelled by the molecule in the gradient during the centrifugation: $dr/dt = (R - r_0)/t$, in which r_0 is the position of the sample when applied at the top of the gradient, R is the position of the sample at the end of the run, and t is the time of centrifugation in seconds.

The value of $(R - r_0)$ is the distance that the band has migrated in the tube. To calculate this, we measure the length of the tube and multiply by the relative position of the sample after centrifugation.

Sample eluted in Fraction 14, all the way to the end of the tube $(R - r_0) = 51\text{-mm} \times 1 = \mathbf{51\text{mm}}$

$$dr/dt = (R - r_0)/t \quad (3)$$

$$dr/dt_{\text{sample}} = (51)/57600 = \mathbf{0.00088542} \text{ (Note: 16hours = 57600 sec)}$$

2. We calculate the angular velocity, expressed in radians/second, as $\omega = 2\pi \text{ rpm}/60$, in which rpm is the speed of rotation of the rotor during the experiment.

Using the **TH 660** rotor at **37,500 rpm**, $\omega = (2\pi)(37500)/60 = \mathbf{3925 \text{ radians/sec.}}$

3. We calculate the average distance, r_{avg} , taken as $r_{\text{avg}} = r_{\text{min}} + (R - r_0)/2$, in which r_{min} is the distance from the center of the rotor to the top of the tube (provided by the manufacturer for the rotor to be **6.46cm**).

The distance travelled by the macromolecule will have constantly changed during the course of the experiment. However, one can calculate the average S value by using the average value of r during the course of the experiment, r_{avg} , which is defined as the position halfway between the starting point (r_0) and ending point (R) of travel for the sample.

$$r_{avg} = r_{min} + (R - r_0)/2 \quad (4)$$

$$\text{Sample } r_{avg} = 64.6\text{mm} + (51\text{mm}/2) = \mathbf{90.1\text{mm}}$$

Where r_{min} was taken from the manufacturers specifications for the Sorvall TH 660 rotor
([http://www.tslabor.hu/depo/Thermo Scientific Rotor Guide.pdf](http://www.tslabor.hu/depo/Thermo_Scientific_Rotor_Guide.pdf))

4. Using these values, we calculate $S_{T,m}$ as

$$S_{T,m} = \frac{dr}{dt} = \frac{(R-r_0)/t}{\omega^2 r_{avg} (2\pi \text{rpm}/60)^2 [r_{min} + (R-r_0/2)]} \quad (5)$$

$$\begin{aligned} S_{T,m} \text{ Sample} &= \frac{51/57600}{(3925^2) \times 90.1} = \\ &= 6.4 \times 10^{-13} \text{ Sec or } \mathbf{6.4S} \end{aligned}$$

Determination of the Value of Partial Specific Volume (v)

The partial specific volume is defined as the increase in volume of an infinite volume of solvent by dissolving 1 g of solute, and it is expressed in liter/g or ml/mg.

First most soluble globular proteins or protein complexes have partial specific volumes that fit within the range of 0.72 to 0.75 ml/mg. If a protein or protein complex can be expected to fit this description, then we start by assuming a similar value for its v and enter into the equation below for $S_{20,w}$ values.

Determination of the Value of $S_{20,w}$

Once the $S_{T,m}$ has been calculated and the value of v has been estimated, the value of $S_{T,m}$ must be corrected to the value of $S_{20,w}$ at the “standard state” of 20°C in water. This correction as described by Martin and Ames (1961), relies not only on the partial specific volume (v) of the protein but also on the viscosity (η) and density (ρ) of the medium used in the experiment. The correction is applied as

$$S_{20,w} = S_{T,m} \frac{\eta_{T,m}}{\eta_{20,w}} \frac{(1/v - \rho_{20,w})}{(1/v - \rho_{T,m})} \quad (6)$$

in which $\eta_{T,m}$ and $\rho_{T,m}$ are the viscosity and density, respectively, of the medium m at the temperature T of the experiment, and $\eta_{20,w}$ and $\rho_{20,w}$ are the viscosity and density of water at 20°C. For a sample at the position r_{avg} , halfway between the starting point and endpoint of migration, one can determine the values of $\eta_{T,m}/\eta_{20,w}$ and $\rho_{T,m}$ from published tables on the basis of experimentally determined densities of sucrose at each eluted fraction.

1. We determine the fraction corresponding to the average distance travelled by the sample, r_{avg} , which is the fraction halfway between the origin (fraction 1) and the endpoint of migration for the sample, keeping in mind that fraction 1 should be equivalent to no migration.

The sample migrated to end of the 10-50% sucrose and the gradient was eluted into 14 tubes of equal volume, therefore the sample would have eluted with a peak fraction at 14, and the r_{avg} would have been obtained in fractions $[(14-1)/2 + 1]$ or fraction 7.5 – this is halfway between fraction 7 and 8.

2. Using the sucrose concentrations determined from published tables, and the value of r_{avg} determined above, we calculate the concentration of sucrose at r_{avg} .

Sample fractions 7 and 8 would have sucrose concentrations of approx. 30% and 35% therefore at r_{avg} the sucrose concentration is estimated at approx. 32.5%.

3. From published tables, we determine the values of $\eta_{T,m}/\eta_{20,w}$ and $\rho_{T,m}$ corresponding to the sucrose concentration at r_{avg} .

Sample $\eta_{T,m}/\eta_{20,w}$ at r_{avg} would be ~6.405 and $\rho_{T,m}$ at r_{avg} would be ~1.146.

4. We use the values of $\eta_{T,m}/\eta_{20,w}$, $\rho_{T,m}$ and $S_{T,m}$ at r_{avg} and the value of v to calculate $S_{20,w}$ according the equation above.

In this example, assuming a “typical” v of 0.74 liter/g, the value of $S_{20,w}$ is computed as

$$\text{Sample } S_{20,w} = (6.4S)(6.405) \frac{(1/0.74 - 1.00)}{(1/0.74 - 1.1439)} = \mathbf{69.4S}$$

Therefore we *estimate* the Sedimentation Coefficient or S value to be approximately **69.4S**. Although this value is based on numerous assumptions made in the calculation that may not be accurate. From the gel filtration analysis performed in the lab previously, coupled with extrapolation from the molecular weight protein standard graph the estimated molecular weight of the complex is between 1 and 2.4 MDa.

2.2.3.10 STATISTICAL ANALYSES

If not otherwise stated, three independent experiments were performed in triplicate. Experimental values are expressed as mean value \pm standard error (S.E.). For significance analyses ANOVA and Student’s t-tests, were used where applicable and $p < 0.05$ (*) was considered significant and $p < 0.001$ (**) as highly significant.

CHAPTER 3

RESULTS & DISCUSSION

PART I

Cytotoxic Drug-Induced Apoptosis: Complex Identification and Molecular Pathway Elucidation in Human Colon Cancer HCT116 Cells

3.1.1 BACKGROUND TO STUDY RATIONALE

A major hurdle with current colorectal cancer treatment modalities is the acquired or intrinsic resistance observed in tumour cells. An understanding of how chemotherapeutic drugs, such as 5-FU, activate cell death pathways is important in order to be able to overcome cytotoxic drug resistance as evasion of cell death is one of the prominent hallmarks of cancer (Yang et al. 2003a; Yagihashi et al. 2003; Endo et al. 2004) and many tumours are resistant due to failure in activating their intrinsic apoptotic machinery.

The difficulty in delineating the response of tumour cells to chemotherapy lies in the complexity of the cell signalling pathways that can play a role in execution of the cell death program. The signals initiating these pathways of cell death can involve a multiplicity of signalling platforms such as the DISC (Lavrik et al. 2005), TNF complex II (Micheau & Tschopp 2003), PIDDosome (Tinel & Tschopp 2004), and the Apoptosome (Cain et al. 1999) among others. All such platforms are capable of initiating cell death through distinct cellular pathways. In spite of the identification of the many modes of cell death and the signalling pathways involved, the regulation of the apoptotic signal transduced by cytotoxic drugs is far from being fully elucidated

(Vandenabeele et al. 2010).

Initial studies revealed that cytotoxic drugs can activate CD95 signalling pathways via upregulation of the receptor and/or ligand and this mechanism may be an essential factor in the early phase of drug-induced cell death, depending on the cell type used. Some cells, however, appear not to require CD95 receptor/ligand interaction for drugs to be effective. Although it has been demonstrated that chemotherapeutic drugs can induce CD95L-independent, FADD-mediated activation of the CD95 pathway, by inducing clustering of CD95 receptors. There is also evidence to suggest that there are additional cell death pathways as well as those involving CD95 signalling. Whether the additional apoptotic pathways may involve other major death receptor pathways such as TRAIL has not been fully investigated. Drug-induced apoptosis may also involve mitochondrial-regulated apoptosis and in line with this, cytochrome-c release has been seen in cancer cells exposed to Cisplatin and 5-FU. The release of cytochrome-c can be regulated by Bcl-2 family members (particularly bax), which can directly interact with mitochondrial membranes. As the ability to evade cell death presents tumour cells with noteworthy advantage, it stands to reason that all of the described modes of cell death can potentially be altered in cancer cells. Overexpression of antiapoptotic molecules such as Bcl-2, Bcl-x_L, IAPs or survivin is commonly linked to poor prognosis of patients. Similarly the loss of proapoptotic factors for example death receptors, adaptor molecules or caspases has been demonstrated to confer a resistant phenotype to tumour cells.

From these studies the data demonstrates that chemotherapeutic drugs can activate different apoptotic pathways and at present it is not clear to what extent different pathways contribute to the molecular mechanism of action of different chemotherapeutic drugs. An understanding of this would help in choosing effective combination of drugs to overcome resistance.

This provided us with the rationale for the present investigation into the molecular pathways activated in response to 5-FU treatment. We could then potentially tailor patient treatment according to the molecular phenotype of tumour and patient, with the aim to result in increased tumour response rates. Patients will therefore be spared toxic side effects of a treatment from which they are unlikely to benefit. Higher response rates and decreased toxicity would also reduce cost of patient care whereas expensive treatments such as Oxaliplatin and Irinotecan could be used in a more

targeted manner.

Our specific aim in Chapter 3.1 was to identify the pathway(s) involved in mediating the 5-FU response and how they are initiated. In Chapter 3.2 we continue on to address the issue of tumour-specific cell death and the synergistic approach of combining two chemotherapeutic agents to exert a strong anti-tumour effect, namely 5-FU and TRAIL. The mechanism of action behind the combined therapy will also be investigated at the molecular level.

The questions asked and hypotheses drawn from the observations we make in the following study should provide a rational step forward towards improving current cancer chemotherapy based on scientific understanding and rational targeting of the tumour cell.

3.1.2 RESULTS

3.1.2.1 *Cell death induced by 5-FU in HCT116 cells is caspase-dependent*

The HCT116 colon cancer cell line is a widely used tumour cell line that is sensitive to a number of apoptosis-inducing agents (Yamaguchi et al. 2003; Galligan et al. 2005). Here we used the chemotherapeutic drug 5-FU to trigger apoptosis and to examine potential mechanisms for caspase activation in this cell line. In the following experiments we used various approaches to dissect the sequence of events. The initial goal of this study was to confirm whether the cell death induced by 5-FU in HCT116 cells was indeed caspase-dependent as this has been reported previously (Olsson et al. 2009). To this end we employed an irreversible pan-caspase inhibitor Z-VAD-FMK. HCT116 cells were either treated with 5-FU (200 μ M, 48 h), 5-FU (200 μ M, 48 h) plus Z-VAD-FMK (5 μ g/ml 48 h) or untreated which served as a control. The cells were subsequently harvested using a method adapted from Nicoletti et al. and apoptosis was visualised by flow cytometry (Nicoletti et al. 1991). We found that apoptosis was inhibited by the addition of Z-VAD-FMK, with a reduction from 24.7% to 0.3% (**Fig. 3.1.1 A**). This result indicated that the process is indeed caspase-dependent.

3.1.2.2 Caspase-9 does not play an important role in 5-FU-induced apoptosis in HCT116 cells.

We subsequently investigated the proteolytic processing of initiator caspases by treating HCT116 cells with 5-FU (200 μ M) for 24, 48 and 72 h and observed caspase-2, -8 and -9 to be processed, increasing with time of 5-FU exposure, the highest processing seen at 72 h (**Fig. 3.1.1 B**). Despite the cleavage of caspase-2, it has been ruled out as the initiator caspase of 5-FU-induced apoptosis within our group using an adenoviral shcaspase-2 construct. In the absence of caspase-2, apoptosis and caspase-8 and -3 cleavage were not inhibited (*Mohr et al., unpublished data*).

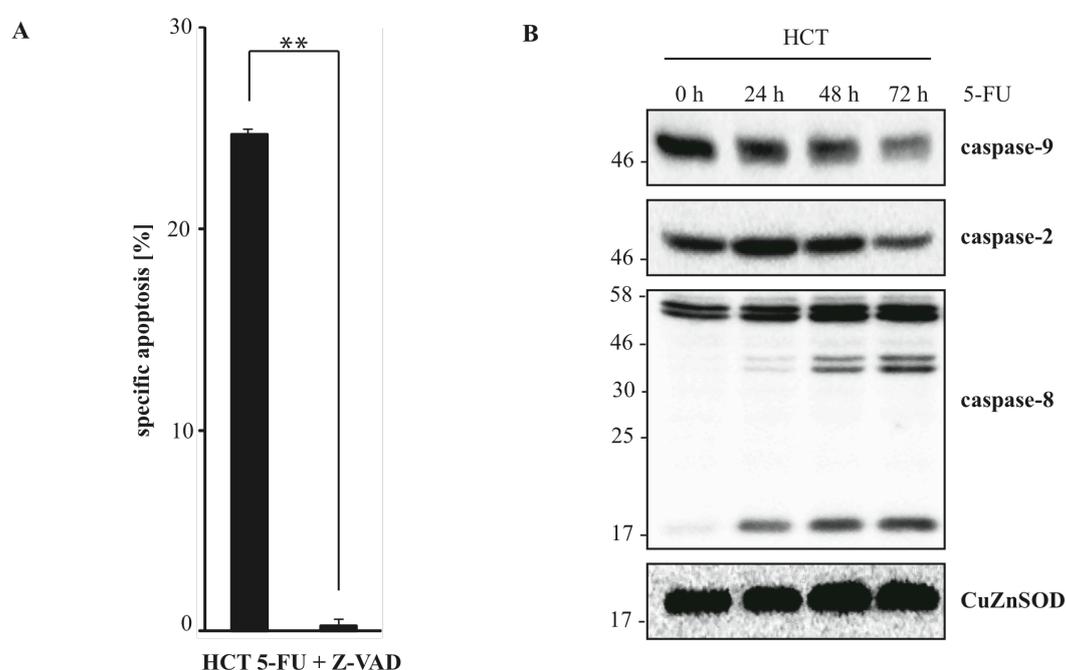


Fig. 3.1.1 5-FU-induced apoptosis in HCT116 cells is caspase-dependent. (A) HCT116 cells were stimulated with 5-FU (200 μ M) for 48 h or prestimulated with Z-VAD-FMK caspase inhibitor (5 μ g/ml), followed by 5-FU (200 μ M) for 48 h. Cell death was characterised by a Nicoletti Assay as described in Chapter 2. (B) HCT116 cells were stimulated with 5-FU (200 μ M) for 24, 48 and 72 h, or untreated as control. Cellular lysates were analysed by Western blotting for caspase-9, -2 and -8 (CS). CuZnSOD served as a loading control and molecular weight markers are indicated. ** = $P < 0.001$. Representative of $n = 4$ experiments.

Early reports on the molecular mechanism underlying the sequential activation of caspases, had led to a model in which caspase-9 is activated upon chemotherapy, and caspase-8 is activated by death receptor signalling (Sun et al. 1999; Budihardjo et al. 1999; Zhuang & Cohen 1998). Nevertheless, some reports have described the

activation of procaspase-8 after chemotherapy (Olsson et al. 2009; Ferreira et al. 2000; Slee et al. 1999; Boesen-de Cock et al. 1999; Perkins et al. 2000; Ekert et al. 1999; Huang et al. 1997). Other reports claim this activation to be downstream and a secondary event that follows caspase-9 activation (Sun et al. 1999; Slee et al. 1999; Boesen-de Cock et al. 1999; Perkins et al. 2000) In recent years, the apical role of caspase-9 in cytotoxic drug-induced apoptosis has been questioned (Marsden et al. 2002; Olsson et al. 2009). As caspase-9 has been reported to induce processing of caspase-2 and -8 in some experimental systems (Samraj et al. 2007) and because of the classical role of caspase-9 in drug-induced apoptosis, we investigated this possibility in HCT116 cells using the following strategies. First we utilised an adenoviral vector which knocked-down caspase-9 to barely detectable levels as determined by Western blotting (**Fig. 3.1.2 A**). These cells were then treated with 5-FU (200 μ M, 48 h) and subsequently were harvested and apoptosis was visualised by flow cytometry. We observed minor difference in apoptosis levels induced by 5-FU in HCT.Adshctrl versus HCT.Adshc-9 (**Fig. 3.1.2 B**). As a positive control, we treated HCT116 cells with the drug Etoposide, and in contrast to the 5-FU, we found apoptosis induced by Etoposide to be completely inhibited in the absence of caspase-9 (*Mohr et al. unpublished data*). In addition, we also generated protein lysates from these cells and found that caspase-8 (**Fig. 3.1.2 D**) and -3 cleavage (**Fig. 3.1.2 E**) was not affected. The caspase-8 Western blot was reprobbed with caspase-9 to demonstrate the near undetectable levels of caspase-9 (**Fig. 3.1.2 C**).

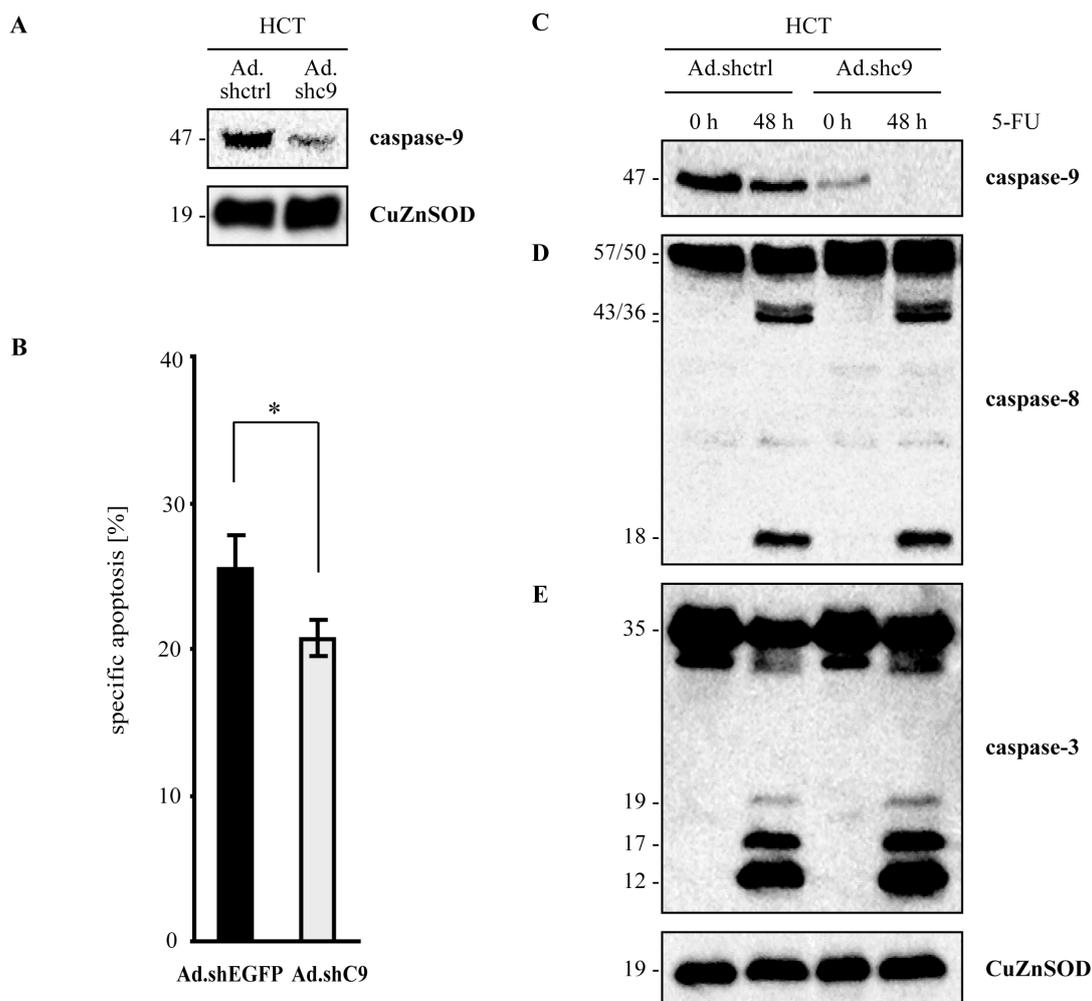


Fig. 3.1.2 Knocking down caspase-9 using adenovirus does not protect HCT116 cells from 5-FU-induced apoptosis (A) HCT116 cells were transduced with adenoviral vector containing shcaspase-9 or shctrl. 48 h post-transduction cells were analysed by Western blotting to demonstrate caspase-9 is knocked down. HCT116 cells were transduced with adenoviral vector containing shcaspase-9 or shcontrol. 48 h post-transduction cells were stimulated with 5-FU (200 μ M). (B) Cell death was characterised by a Nicoletti Assay as described in Chapter 2 Cellular lysates were analysed by Western blotting for (C) caspase-9, (D) caspase-8 (CS) and (E) caspase-3. CuZnSOD served as a loading control and molecular weight markers are indicated. * = $P < 0.05$. Representative of $n = 2$ experiments. Note: apoptosis data was generated by Dr. Andrea Mohr.

Similarly we employed an adenoviral vector that over-expressed a version of caspase-9 that lacks caspase activity due to an introduced point mutation, which can interfere with the formation of the caspase-9/Apaf-1 complex formation. This was termed adenovirus dominant negative caspase-9 (AdDNc-9). As a control we used an adenovirus overexpressing a red fluorescent protein from *Discosoma* (Ad.DsRed) (Fig. 3.1.3 A). We observed similar results as with the Adshc-9, where apoptosis rates were only lowered by approximately 5% (Fig. 3.1.3 B) and caspase-8 (Fig. 3.1.3 D) and -3 cleavage (Fig. 3.1.3 E) were not affected. The caspase-3 Western blot was reprobbed with caspase-9 to demonstrate the level of AdDNc-9 overexpression (Fig.

3.1.3 C).

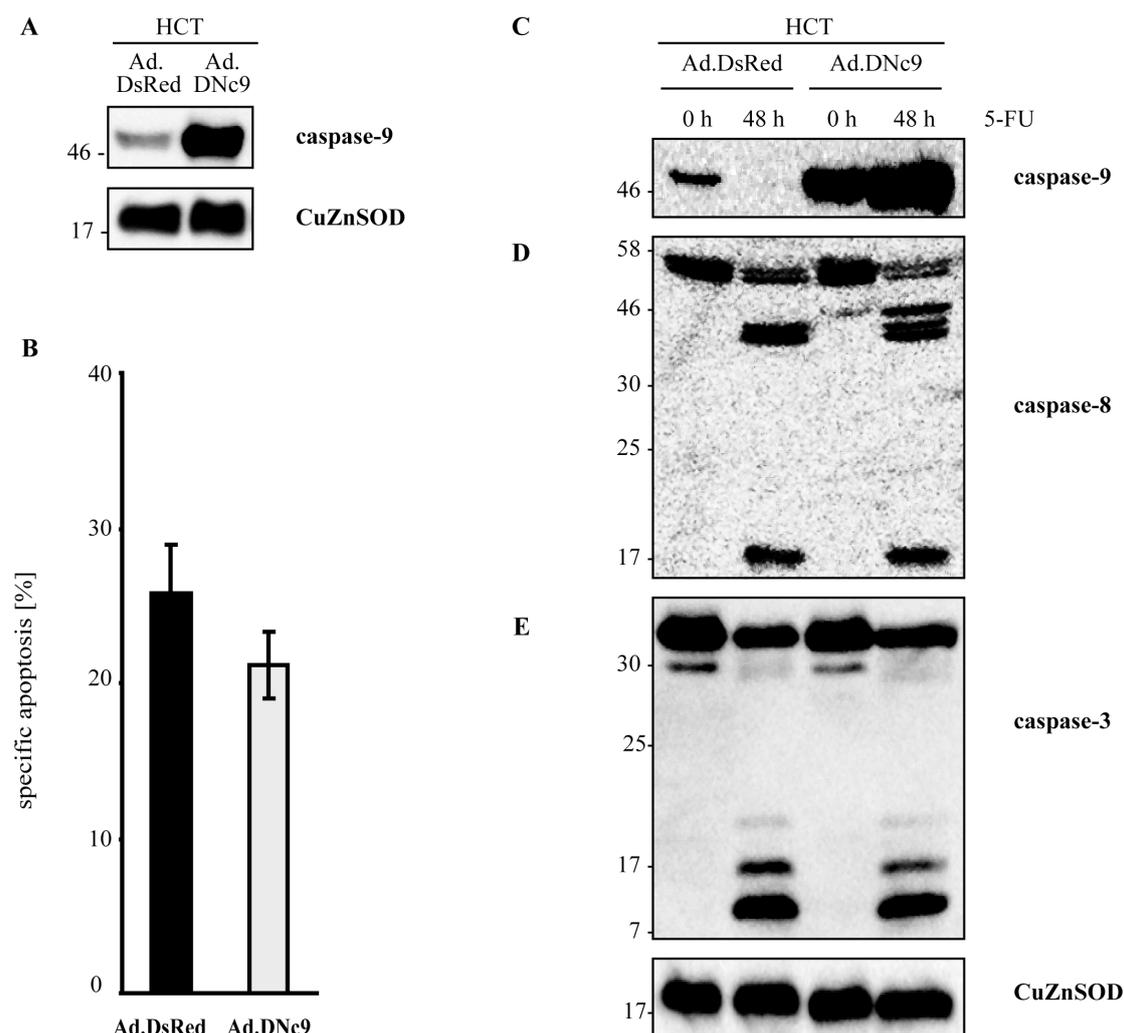


Fig. 3.1.3 Overexpression of Ad.DNc-9 does not interfere with apoptosis induced by 5-FU. (A) HCT116 cells were transduced with adenoviral vector containing DNc-9 or DsRed. 48 h post-transduction cells were analysed by Western blotting to demonstrate DNc-9 is overexpressed. HCT116 cells were transduced with adenoviral vector containing DNc-9 or DsRed. 48 h post-transduction cells were stimulated with 5-FU (200 μ M) for 48 h. (B) Cell death was characterised by a Nicoletti Assay as described in Chapter 2. Cellular lysates were analysed by Western blotting for caspase-9, (C) caspase-8 (CS) and (D) caspase-3. CuZnSOD served as a loading control and molecular weight markers are indicated. Representative of n = 2 experiments. **Note:** [apoptosis data was generated by Dr. Andrea Mohr.](#)

Finally we generated stable knockdown clones of caspase-9 in HCT116 cells, termed HCT.shc-9 (Fig.3.1.4 A) and subjected this clone to the same testing and found, once again, apoptosis rates were only slightly affected (Fig. 3.1.4 B), caspase-8 (Fig. 3.1.4 C) and -3 cleavage were not affected (Fig. 3.1.4 D). These findings demonstrated that caspase-9 was not required for 5-FU-induced apoptosis in HCT116 cells.

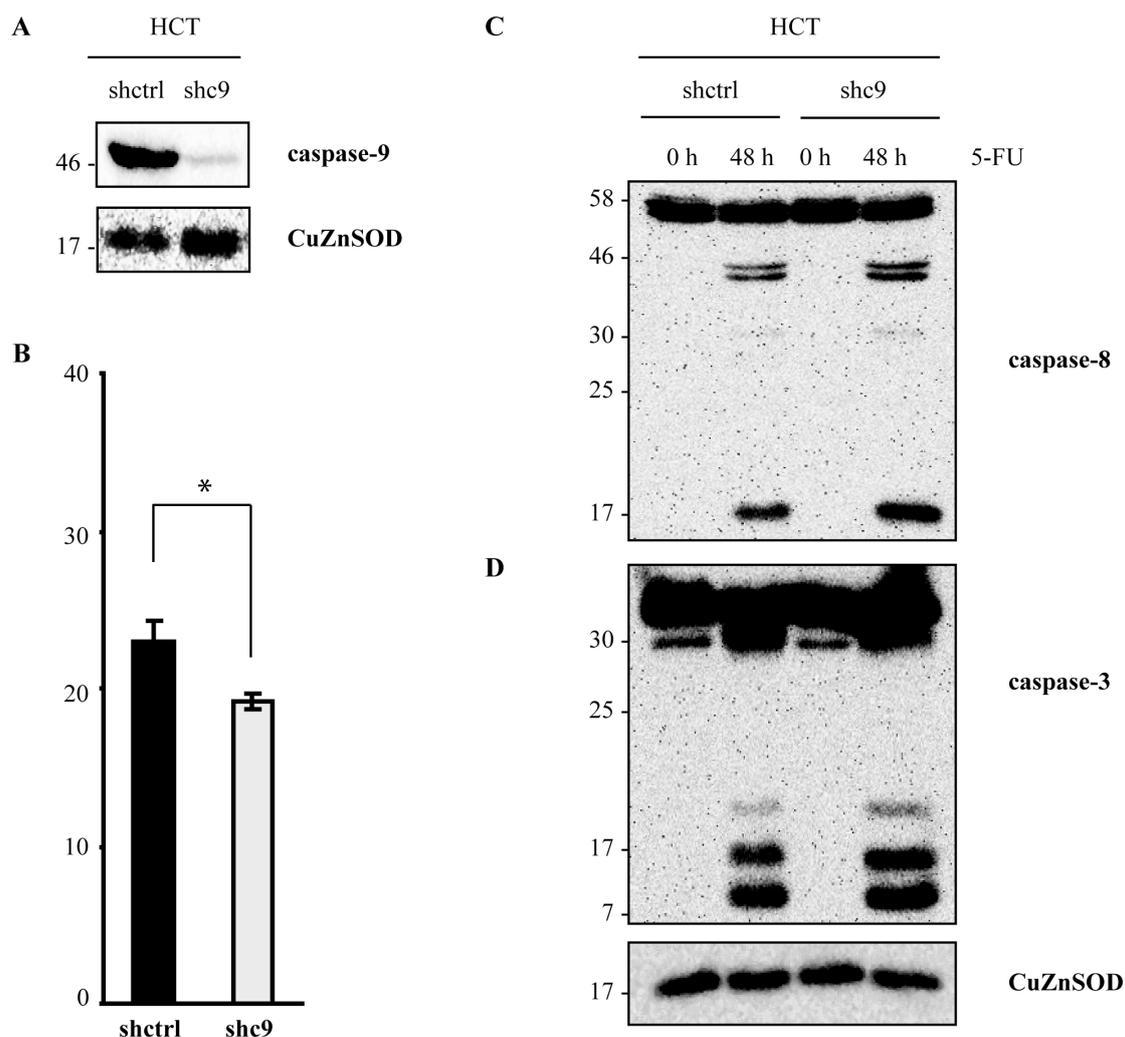


Fig. 3.1.4 Generation of stable shcaspase-9 clones in HCT116 demonstrates that in the absence of caspase-9, 5-FU maintains its effects. **(A)** Stable shcaspase-9 (shc-9) clones were generated in HCT116 cells, termed HCT.shc-9. A shctrl clone served as a control. Cell lysates were analysed by Western blotting to demonstrate that c-9 was knocked down. **(B)** HCT.shc-9 cells were stimulated with 5-FU (200 μ M) for 48 h. Cellular lysates were analysed by Western blotting for c-9, **(C)** c-8 (CS) and **(D)** c-3. CuZnSOD served as a loading control and molecular weight markers are indicated. * = $P < 0.05$. Representative of $n = 4$ experiments. Note: apoptosis data was generated by Dr. Andrea Mohr.

3.1.2.3 Caspase-8 is indispensable for 5-FU-induced apoptosis and is the apical caspase in this cascade.

As caspase-8 cleavage was not affected by the silencing/inhibition of caspase-9 in HCT116 cells, we hypothesised that caspase-8 activation was not a downstream secondary event following caspase-9 activation as has been suggested by other studies (Sun et al. 1999; Slee et al. 1999; Boesen-de Cock et al. 1999; Perkins et al. 2000). Caspase-8 is traditionally associated with the death receptor pathway of apoptosis however recent publications have highlighted the pivotal role caspase-8 plays in

cytotoxic drug-induced apoptosis (Tenev et al., 2011; Feoktostiva et al., 2011). To determine whether caspase-8 activation played an apical role in 5FU-induced apoptosis, we continued to study caspase-8 processing in HCT116 cell lysates. To investigate the effects of silencing caspase-8, we employed an adenoviral vector to silence caspase-8, termed HCT.Adshc-8 (**Fig. 3.1.5 A**). We found apoptosis was inhibited (**Fig. 3.1.5 B**). Caspase-3 cleavage was almost completely abolished compared to the Ad.shctrl sample (**Fig. 3.1.5 C**).

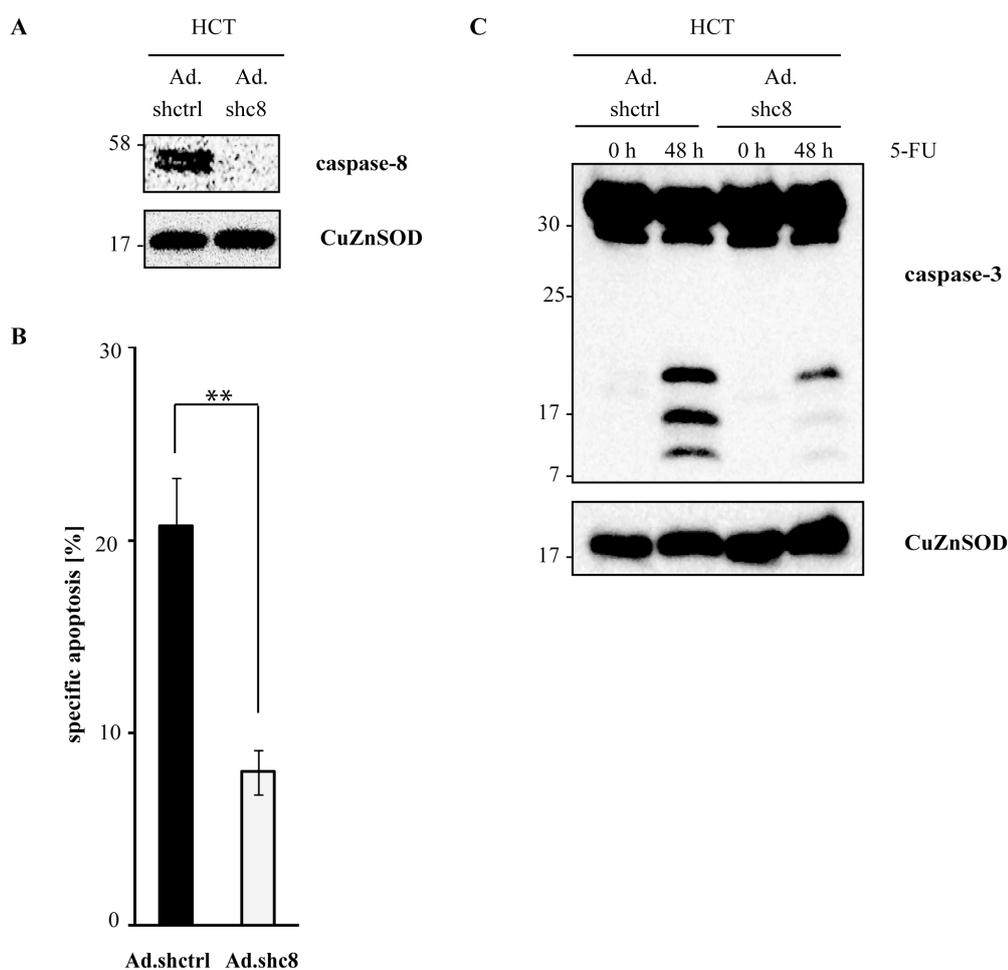


Fig. 3.1.5 Caspase-8 is indispensable for 5-FU-induced apoptosis. (A) HCT116 cells were transduced with adenoviral vector containing shc-8 or shcontrol. 48 h post-transduction cells were analysed by caspase-8 (BD) Western blot to demonstrate caspase-8 is knocked down. (B) Cell death was characterised by a Nicoletti Assay as described in Chapter 2. (C) HCT116 cells were transduced with adenoviral vector containing shc-8 or shcontrol. 48 h post-transduction cells stimulated with 5-FU (200 μ M) for 48 h, or untreated as control. Cellular lysates were analysed by Western blotting for caspase-3. CuZnSOD served as a loading control and molecular weight markers are indicated. ** = $P < 0.001$. Representative of $n = 2$ experiments.

We then generated stable caspase-8 knock down clones in HCT116 cells, termed HCT.shc-8, which were shown to harbour almost no detectable levels of caspase-8

(Fig. 3.1.6 A). Accordingly knock down of caspase-8 suppressed 5-FU-mediated cell death (Fig. 3.1.6 B) and cleavage of the effector caspase-3 (Fig. 3.1.2 C).

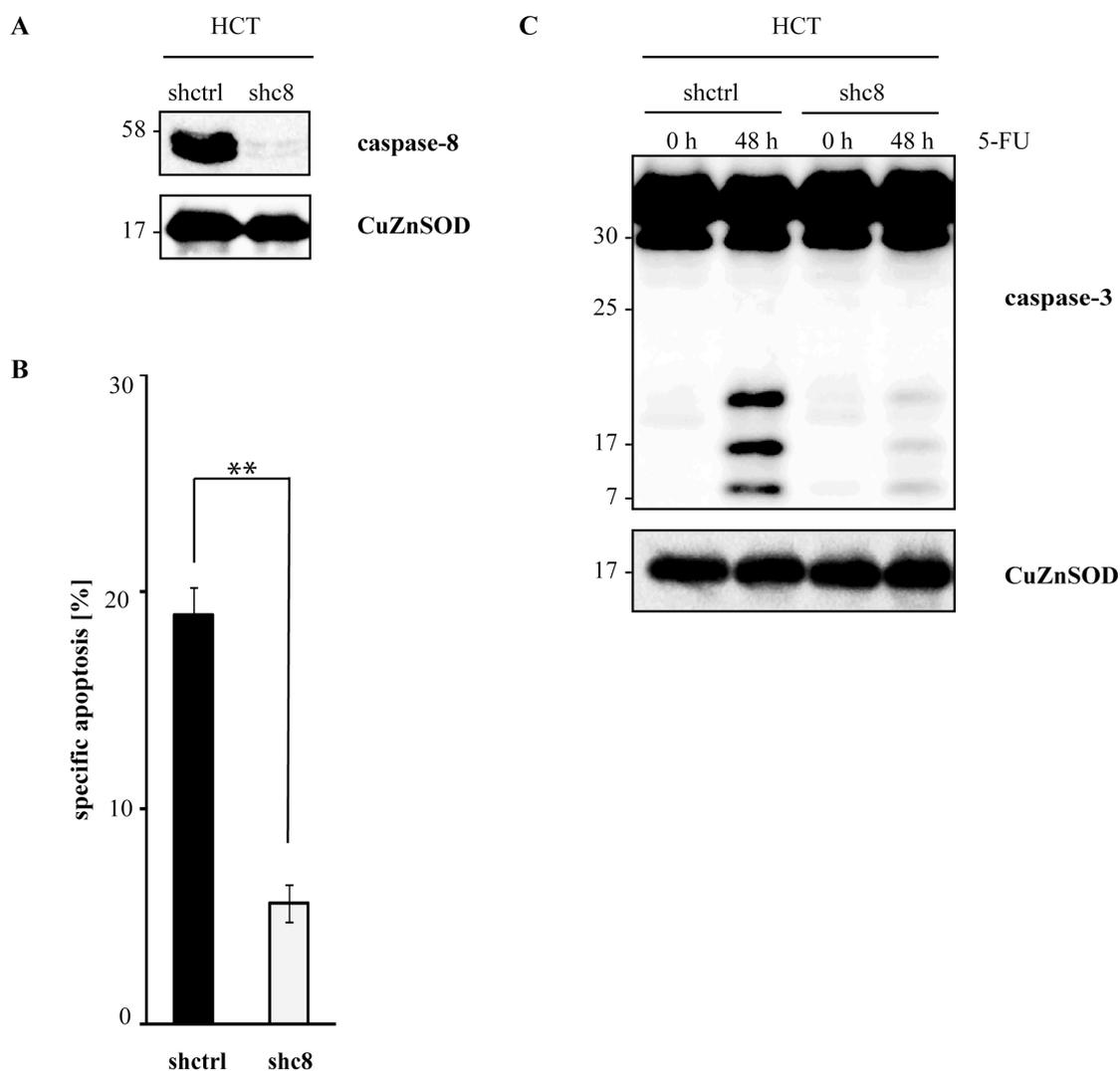


Fig. 3.1.6 Stable shcaspase-8 clones further demonstrate caspase-8 is the apical caspase (A) Stable shcaspase-8 knockdown clones were generated in HCT116 cells termed HCT.shc-8. These cells were analysed by caspase-8 (BD) Western blot to demonstrate caspase-8 is knocked down. (B) Cell death was characterised by a Nicoletti Assay as described in Chapter 2 (C) HCT.shc-8 cells were stimulated with 5-FU (200 μ M) for 48 h. Cell lysates were analysed by Western blotting for caspase-3. CuZnSOD served as a loading control and molecular weight markers are indicated. ** = $P < 0.001$. Representative of $n = 4$ experiments.

These results indicate that caspase-8 processing is an important and necessary event in this apoptotic pathway and silencing caspase-8 but not caspase-9 renders the HCT116 cells resistant to 5-FU.

3.1.2.4 Caspase-8 forms part of a high molecular weight protein complex upon 5-FU stimulation

Previous experimental work within our lab has identified a large protein complex forming following 5-FU in HCT116 cells utilising the technique of gel filtration chromatography. The complex was estimated to be at least 1 MDa in size, and was found to contain caspase-8 and FADD in the same fraction when the collected fractions were tested via Western blot (*Mohr et al., unpublished data*). Another goal of the present study was to confirm the presence of a large protein complex forming in HCT116 cells following 5-FU treatment, containing caspase-8 and FADD and to attempt to identify additional putative complex members. To this end we performed analyses using a sucrose gradient. We chose a continuous gradient of 10-50% (w/w) sucrose, adapted from Feig *et al.* which allows separation of proteins and native protein complexes according to their stoke's radius with medium to very high molecular weights (Feig *et al.* 2007; Feig & Peter, 2008). Briefly, supernatants of HCT116 cells were separated using sucrose density gradient centrifugation and 14 equal fractions were manually collected by pipetting, starting from the top of the gradient (low density) to the bottom (high density) and subsequently ran on Western blots for appropriate proteins.

In un-stimulated cells, caspase-8 was identified in the fractions that best correlated with the molecular weight of the monomeric proteins in the low-density fractions 1-5 (**Fig. 3.1.7 A**). This changed dramatically when cells were stimulated with 5-FU (400 μ M, 15 h). In addition to the monomeric proteins, higher molecular weight structures were detected in terms of their sedimentation in the gradients in the high-density region of fractions 13 and 14 (**Fig. 3.1.7 B**). Caspase-8 sedimented to the high-density fraction of the gradient, suggesting that following 5-FU treatment caspase-8 forms part of a higher molecular weight complex. This gradient was calibrated using the molecular weight protein standards Aldolase (160 KDa/7.3S) and Thyrogobulin (670 KDa/19.3S) (see Chapter 2). Using extrapolation from the peak elution fractions of the molecular weight standard proteins, we estimated the molecular weight of this complex to be approx. 2.4 MDa, with an estimated Sedimentation coefficient of 71.8S (see Chapter 2 for calculation). This value is within a reasonable range as, for example, the bacterial Ribosome is reported to be 2.5 MDa with a sedimentation

coefficient of 70S (Hamburg et al., 2009). We hypothesised that other potential complex members will co-sediment to this fraction along with caspase-8 following 5-FU treatment. As one form of validation for the sucrose gradient we tested whether the complex movement was caspase-dependent in line with our apoptosis studies. The cells were treated with Z-VAD-FMK (5 µg/ml) at the same time as 5-FU (400 µM) or as an additional control, Z-VAD-FMK (5 µg/ml) was added 15 min prior to harvesting (15 h post-5-FU). In the former, the complex movement was inhibited (**Fig. 3.1.7 C**) however with the 15 min pre-treatment with Z-VAD-FMK the complex was still able to form and migrate within the gradient (**Fig. 3.1.7 D**). Z-VAD-FMK has been demonstrated to inhibit apoptosis by irreversibly binding to the catalytic site and blocking the activation of caspases into their active form (Slee et al. 1996).

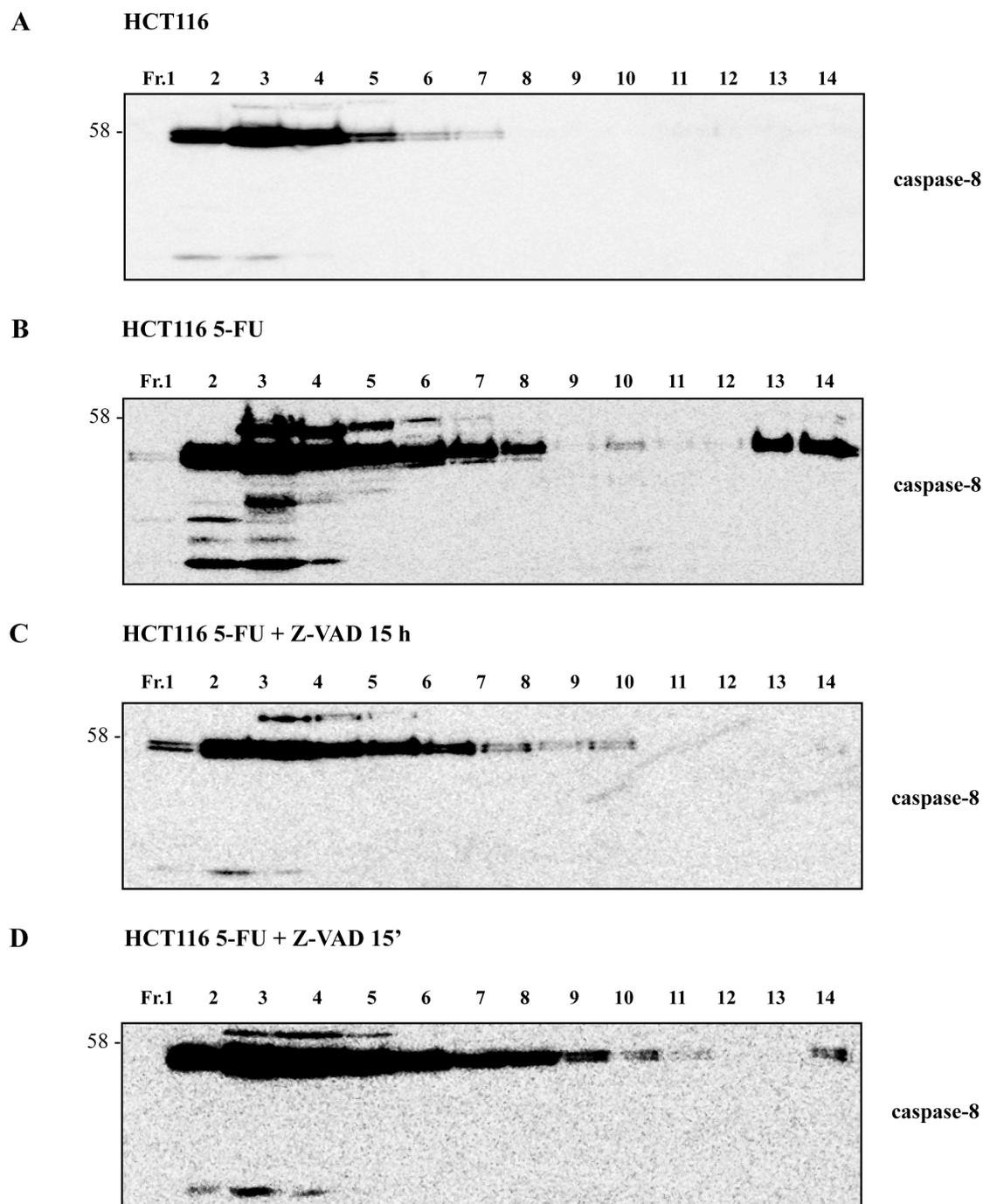


Fig. 3.1.7 Caspase-8 forms part of a high molecular weight complex following 5-FU (A) HCT116 cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for caspase-8 (BD). (B) HCT116 cells were stimulated with 5-FU (400 μ M) for 15 h and cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for caspase-8 (BD). (C) HCT116 cells were either stimulated with 5-FU (400 μ M) in the presence of Z-VAD-FMK (5 μ g/ml) for 15 h or (D) stimulated with 5-FU (400 μ M) for 15 h and Z-VAD-FMK (5 μ g/ml) was added 15 min prior to harvesting as control. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for caspase-8 (BD). Representative of $n = > 4$ experiments (A, B), and $n = 1$ experiments (C, D).

We also wanted to generally determine whether our complex was residing in the cytosol of the cell or whether it was localised at the membrane, potentially as part of a

membrane protein complex. Therefore prior to addition of our 5-FU (400 μ M, 15 h) pre-treated protein lysate to a sucrose gradient, homogenates were cleared by centrifugation at 3,000 rpm for 10 min at 4°C to remove intact cells and nuclei. The heavy membrane fraction was spun down at 13,000 rpm for 20 min at 4°C and cytosolic supernatant was further cleared by repeated centrifugation at 13,000 rpm for 10 min at 4°C. The cytosolic and membrane fractions were each loaded onto two separate sucrose gradients.

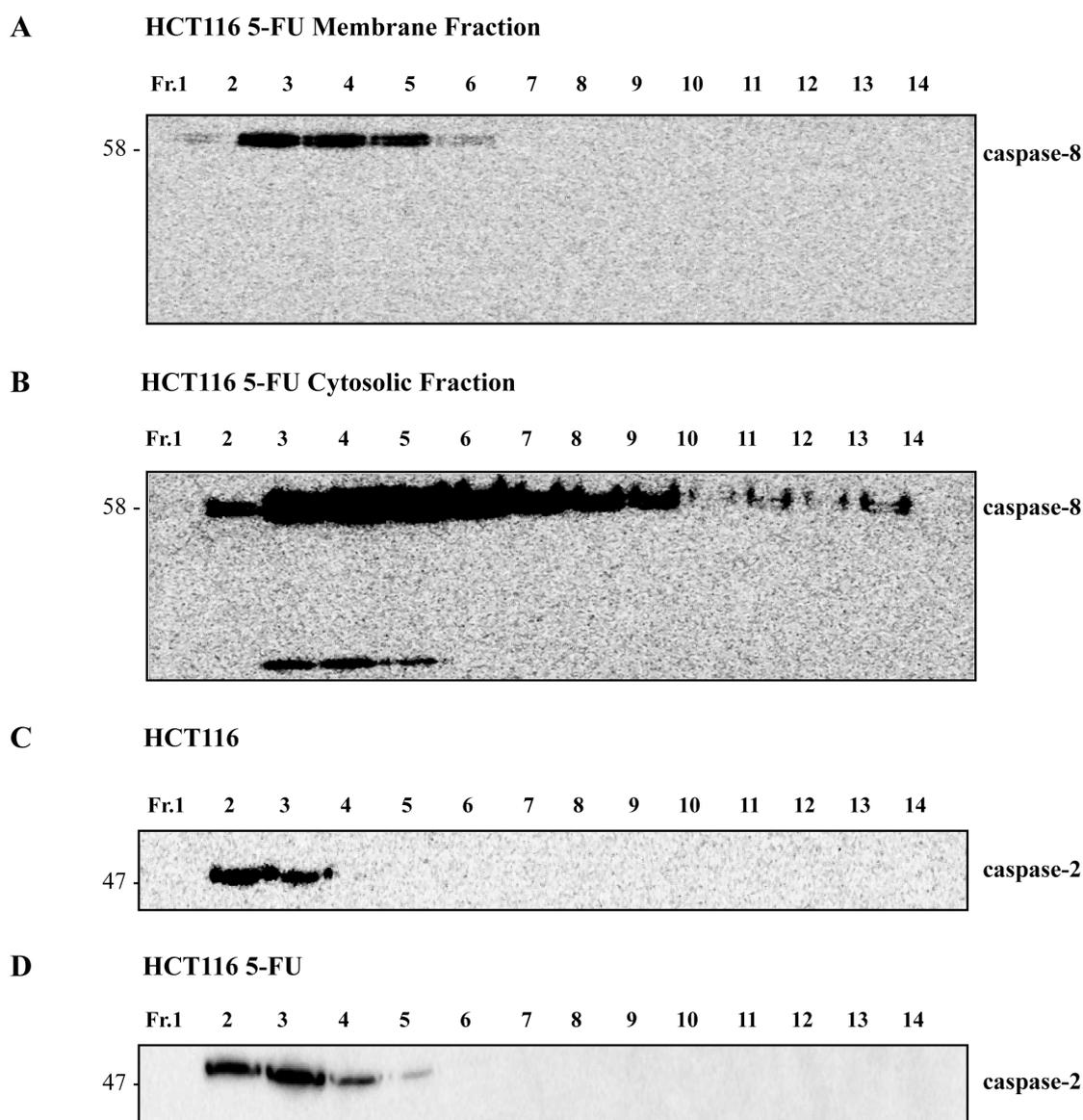


Fig. 3.1.8 The caspase-8 complex resides in the cytosolic, but not the membrane fraction of the cell HCT116 cells were stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were separated into their (A) membrane and (B) cytosolic fractions which were then used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for caspase-8 (BD). (C) HCT116 cells were either unstimulated as control or (D) stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for caspase-2. Molecular weight markers are indicated.

The resulting 14 fractions from both gradients were then Western blotted for caspase-8. In the membrane fraction, there appeared to be no caspase-8 movement to the higher density region of the gradient (**Fig. 3.1.8 A**), however in contrast to this, in the cytosolic fraction, caspase-8 sedimented to the higher density region (**Fig. 3.1.8 B**). This result gives us a general indication of the localisation of the complex within the cell, pointing to a complex that is independent of membrane bound receptors. However, further experimentation is required to validate this observation.

To both further validate the caspase-8 movement and underline that caspase-2 has no involvement with the complex formation, we Western blotted our 5-FU treated HCT116 cell sucrose gradient for caspase-2. There was no change in caspase-2 movement from the low to the high-density region of the gradient prior to (**Fig. 3.1.8 C**) or following 5-FU treatment (**Fig. 3.1.8 D**).

3.1.2.5 Caspase-8 immunoprecipitation establishment

Prior to further identification of any putative caspase-8 complex partners, we wanted to establish a caspase-8 immunoprecipitation intended to for enrich caspase-8 itself and potentially any co-immunoprecipitated proteins, to aid us in visualisation in our future studies. We first titrated the required protein amount and determined that from 500 to 1000 μg of protein was sufficient (**Fig. 3.1.9 A**). We next established the immunoprecipitation time course from 15 min to 4 h, and observed that the immunoprecipitation should be incubated for a minimum of 2 h to bind sufficiently detectable levels of caspase-8 (**Fig. 3.1.9 B**). Finally we validated the specificity of our immunoprecipitation using our HCT.shc8 stable clone and demonstrated there was almost no caspase-8 immunoprecipitated in the HCT.shc8 sample (**Fig. 3.1.9 C**).

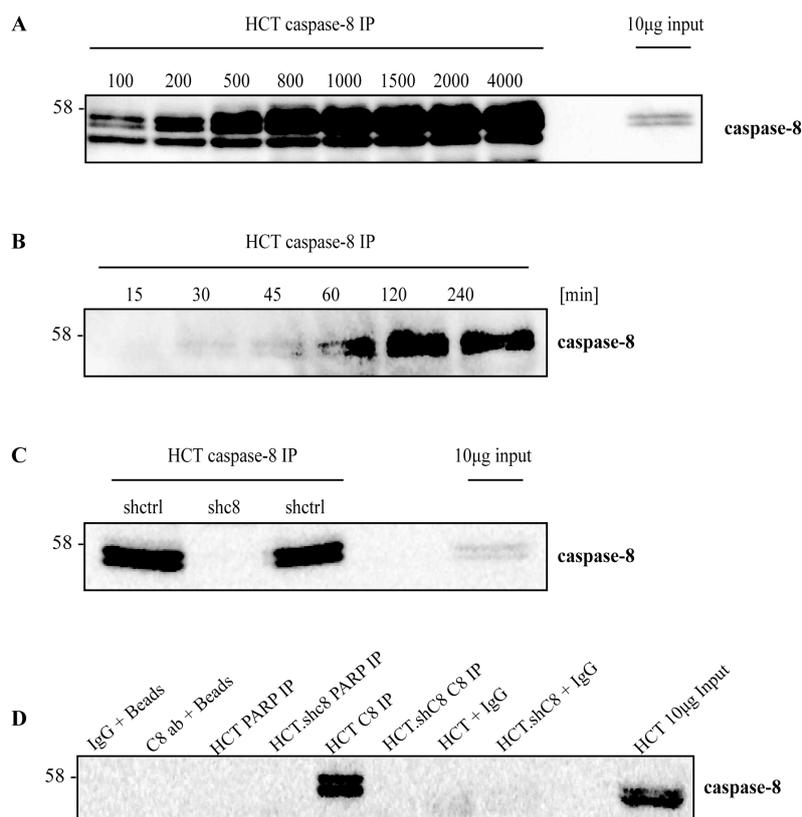


Fig. 3.1.9 Caspase-8 immunoprecipitation establishment (A) HCT116 cells were subjected to caspase-8 (BD) immunoprecipitation with increasing concentrations of protein from 100 µg - 4000 µg. 10 µg of cellular lysate served as an input control. (B) HCT116 cells were subjected to caspase-8 (BD) immunoprecipitation with increasing periods of incubation from 15-240 min. (C) HCT116 cells were subjected to caspase-8 (BD) immunoprecipitation. HCT.shc-8 cells were used to demonstrate immunoprecipitation specificity. 10 µg of cellular lysate served as an input control. Molecular weight markers are indicated. Representative of n = 2 experiments.

Once we had established that caspase-8 was a crucial member of the protein complex, demonstrated it's movement to the higher density region of the sucrose gradient, and in addition established a caspase-8 immunoprecipitation conditions we continued to dissect the pathway and investigate the other potential complex members.

3.1.2.6 FADD is an important mediator of this pathway and its absence leads to abrogation of caspase-8 cleavage and resistance to 5-FU

Fas-associated death domain (FADD) is a common factor that functions as a critical adaptor protein to recruit caspase-8 and -10 through its DED. FADD associates directly with Fas, DR4 and DR5 receptors and allows caspase-8/-10 molecules to be in close proximity to each other to allow their activation. As mentioned in section 3.1.2.3, previous studies within the group identified a high molecular weight protein

complex forming in HCT116 cells following 5-FU treatment using gel filtration chromatography analysis. This complex was found to contain caspase-8 and FADD within the same fraction (*Mohr et al., unpublished data*). With this in mind, we next wanted to dissect the potential role of FADD in 5-FU-induced apoptosis. We generated HCT.shFADD stable knock down clones (**Fig. 3.1.10 A**), and when these cells were treated with 5-FU (200 μ M, 48 h) we observed that apoptosis resistance was conferred upon the cells (**Fig. 3.1.10 B**) and in line with this finding, both caspase-8 (**Fig. 3.1.10 C**) and -3 cleavage were abolished (**Fig. 3.1.10 D**). These results revealed that FADD is an important mediator of 5-FU-induced apoptosis in HCT116 cells. The silencing of FADD was repeated using an Ad.shFADD vector in HCT116 cells and additionally in a second colon cancer cell line, HT-29 (*Mohr et al., unpublished data*). The findings were similar to those presented in this study.

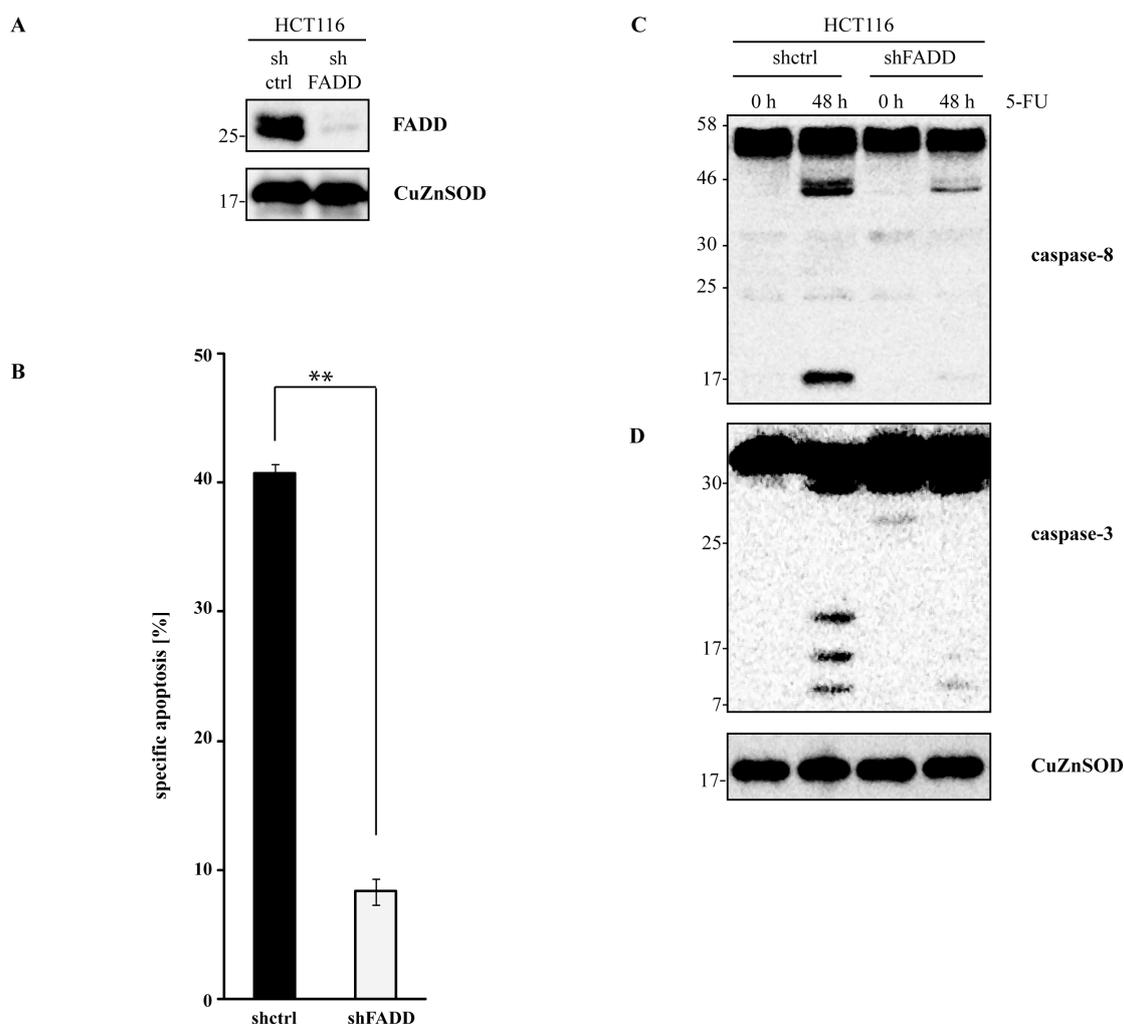


Fig. 3.1.10 FADD is an important mediator of this pathway and its absence leads to abrogation of caspase-8 cleavage and 5-FU resistance. (A) Stable shFADD clones were generated in HCT116 cells termed HCT.shFADD. HCT.shctrl served as

control cells. HCT.shFADD cells were analysed by Western blotting to demonstrate FADD is knocked down. **(B)** HCT.shFADD cells were stimulated with 5-FU (200 μ M) for 48 h. Cell death was characterised by a Nicoletti Assay as described in Chapter 2. **(C)** HCT.shFADD cells were stimulated with 5-FU (200 μ M) for 48 h. Cellular lysates were analysed by Western blotting for caspase-8 (CS) and -3. CuZnSOD served as a loading control and molecular weight markers are indicated. ** = $P < 0.001$. Representative of $n = 3$ experiments.

3.1.2.7 FADD and caspase-8 migrate to high-density region of sucrose gradient

We next examined whether FADD co-sedimented with caspase-8 to the higher density region of the sucrose gradient following 5-FU. We treated HCT116 cells with 5-FU (400 μ M, 15 h) and untreated as control and ran the lysates on two separate sucrose gradients. We Western blotted the collected fractions for FADD and found that following 5-FU, but not in the untreated control (**Fig. 3.1.11 A**), FADD co-sedimented with caspase-8 to Fraction 14 (**Fig. 3.1.11 B**).

We employed our HCT.shFADD stable clone to test whether the caspase-8 complex could form in the absence of FADD and found that caspase-8 sedimentation to the high density region of the sucrose gradient following 5-FU treatment was prevented when FADD is not present (**Fig. 3.1.11 C**). We also employed our HCT.shc8 clone to the same end and observed that in the absence of caspase-8, FADD is prevented from sedimenting to Fraction 14 (**Fig. 3.1.11 D**). We therefore concluded that FADD and caspase-8 form part of the same high density protein complex following 5-FU and that both proteins are essential for the formation of this complex and the apoptosis induced by 5-FU in HCT116 cells.

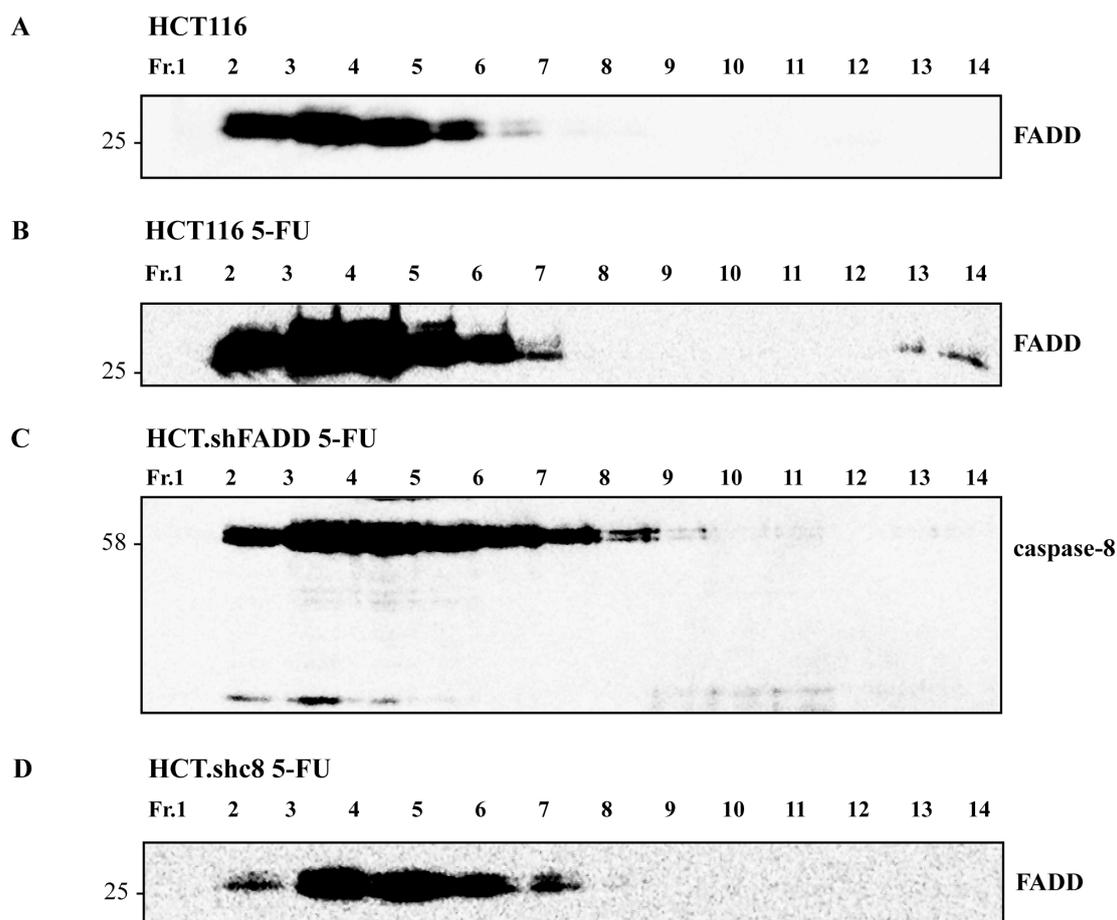


Fig. 3.1.11 FADD co-sediments with caspase-8 as part of the high molecular weight complex following 5-FU (A) HCT116 cells were either unstimulated as control or (B) stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for FADD. (C) HCT.shFADD cells were stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for caspase-8 (BD). (D) HCT.shc-8 cells were stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for FADD. Molecular weight markers are indicated. Representative of $n = 3$ experiments.

3.1.2.8 A physical interaction between FADD and caspase-8 following 5-FU can be demonstrated by co-immunoprecipitation

We next utilised HEK293 cells as a model to investigate our complex. We first tested if HEK293 cells had relevant amounts of the important apoptotic factors via Western blot to ensure a similar response to 5-FU. HEK293 cells expressed comparable levels of the well-known important apoptotic members such as caspase-8, FADD, Bid and RIP1 to HCT116 cells (Fig. 3.1.12 A). We then treated HEK293 cells with 5-FU (200 μ M, 48 and 72 h) and compared the level of apoptosis with HCT116 cells to

determine if they responded in a similar manner. HEK293 cells had a delayed apoptosis but reached comparable HCT116 levels 24 h later (**Fig. 3.1.12 B**).

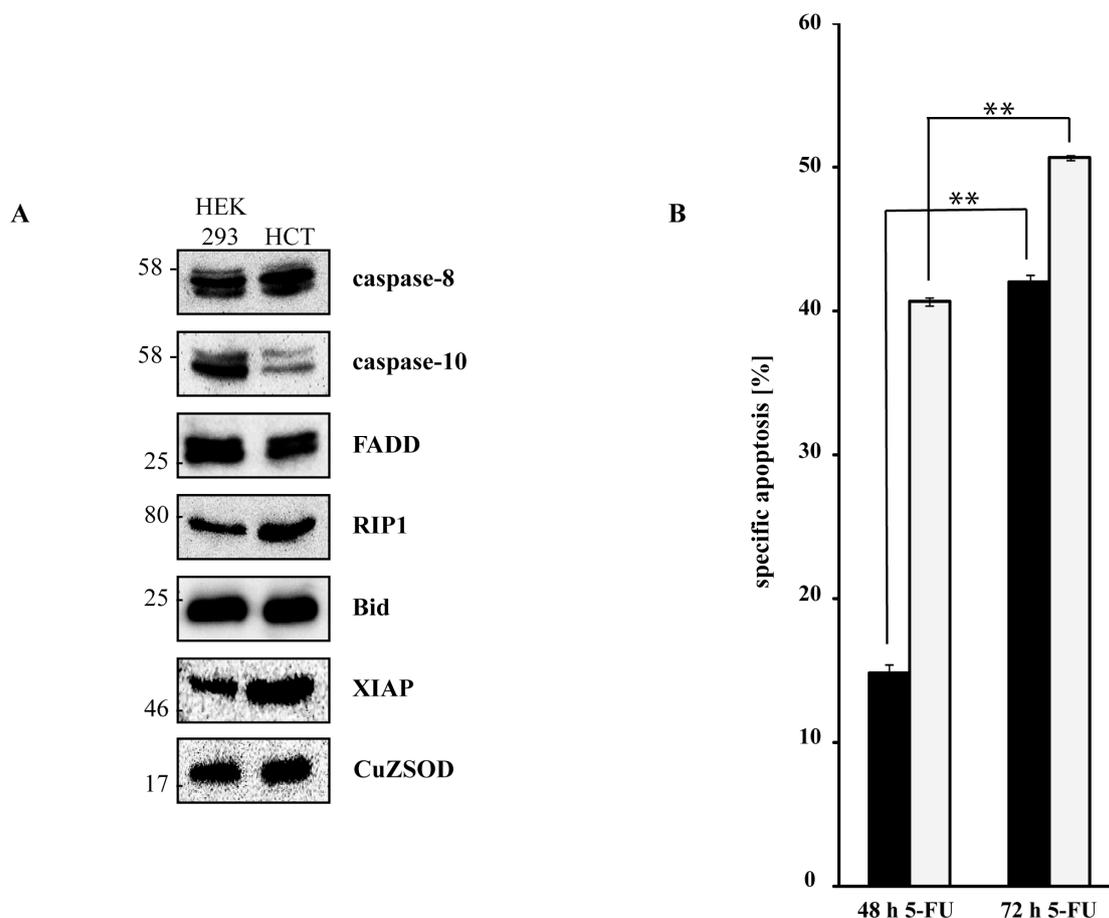


Fig. 3.1.12 HEK293 cells express similar levels of apoptotic proteins as HCT116 cells (A) HEK293 cells and HCT116 cells were harvested and the cellular lysates were analysed by Western blot for caspase-8 (BD), caspase-10, FADD, RIP1, Bid and XIAP. CuZnSOD served as a loading control and molecular weight markers are indicated. **(B)** HEK293 cells and HCT116 cells were stimulated with 5-FU (200 μ M) for 48 and 72 h. Cell death was characterised by a Nicoletti Assay as described in Chapter 2. The Black bars represent 48 h treated cells and the grey bars represent 72 h 5-FU treated cells, with HEK293 cells on the left-hand side and HCT116 cells on the right-hand side. ** = $P < 0.001$. Representative of $n = 2$ experiments.

We transiently over expressed full-length FADD in HEK293 cells (3 μ g/well) and 6 h post-transfection we treated with 5-FU (400 μ M, 15 h) or left untreated as control. We then subjected the lysates to a caspase-8 immunoprecipitation and Western blotted for FADD. In the 5-FU treated sample, FADD was co-immunoprecipitated with caspase-8, but not in the untreated control (**Fig. 3.1.13 A**). The blot was re-probed with caspase-8 as a control for the total protein input (**Fig. 3.1.13 B**). Therefore these results show the physical interaction between FADD and caspase-8 induced by 5-FU.

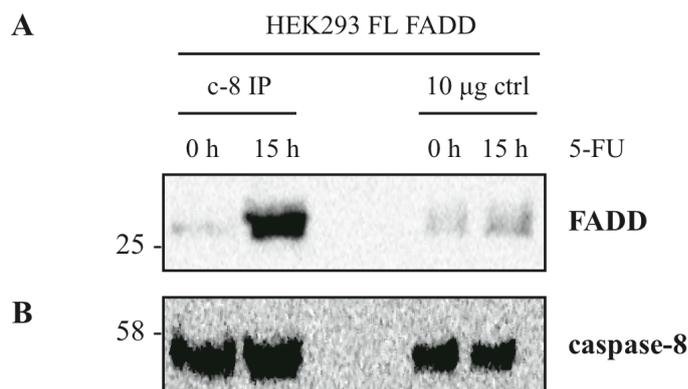


Fig. 3.1.13 A physical interaction between caspase-8 and FADD following 5-FU can be demonstrated by immunoprecipitation (A) HEK293 cells were transfected with plasmid containing Full-length FADD. 6 h post-transfection cells were stimulated with 5-FU (400 µM) for 15 h or unstimulated as control. Cellular lysates were subjected to caspase-8 immunoprecipitation, followed by Western blotting for FADD. 10µg of cell lysate was loaded as input control **(B)** FADD Western blot was reprobed with caspase-8 (BD) as a control. Representative of n = 3 experiments.

3.1.2.9 5-FU treatment induces FADD-FADD interaction and this can contain both phosphorylated and non-phosphorylated FADD

To further dissect the role of FADD in this 5-FU-induced complex formation we next wanted to investigate the initial event that occurs prior to complex formation. We hypothesised that the dimerisation of at least two FADD molecules could occur in the first instance, subsequently recruiting at least two caspase-8 monomers in close proximity to one another, which in turn would lead to the recruitment of remaining complex members. It is already documented that caspase-8 molecules dimerise and proteolytically cleave one another (Chang et al. 2003). However a recent report put forth the concept of a DED chain assembly, whereby a caspase-8 molecule can be bound to a FADD molecule, and the caspase-8 can recruit up to 9-fold more caspase-8 molecules than FADD molecules (Dickens et al. 2012). To examine the possibility of FADD/FADD interaction we required two tagged versions of FADD, therefore we generated FADD-V5, FADD-Myc/Flag and FADD-HA constructs. In **Fig. 3.1.14** the expression and levels of cross-reactivity of the different tagged FADDs were analysed. The FADD-V5 construct was the only construct to be detected by the V5 antibody (**Fig. 3.1.14 A**). The FADD-Myc/Flag construct was the strongest signal detected by the α -Myc antibody however the dominant lower band of the FADD-V5

construct was detected to some extent and the FADD-HA to a lesser extent (**Fig. 3.1.14 B**). The Flag antibody was superior to the α -Myc antibody at solely detecting the FADD-Myc/Flag construct (**Fig. 3.1.14 C**). Finally the α -HA antibody was the least desirable as it detected the FADD-V5 construct almost to the same extent as the FADD-HA and the FADD-Myc/Flag to a lesser degree (**3.1.14 D**). Therefore we did not continue with the FADD-HA construct due to the unacceptable levels of cross-reactivity of the HA antibody.

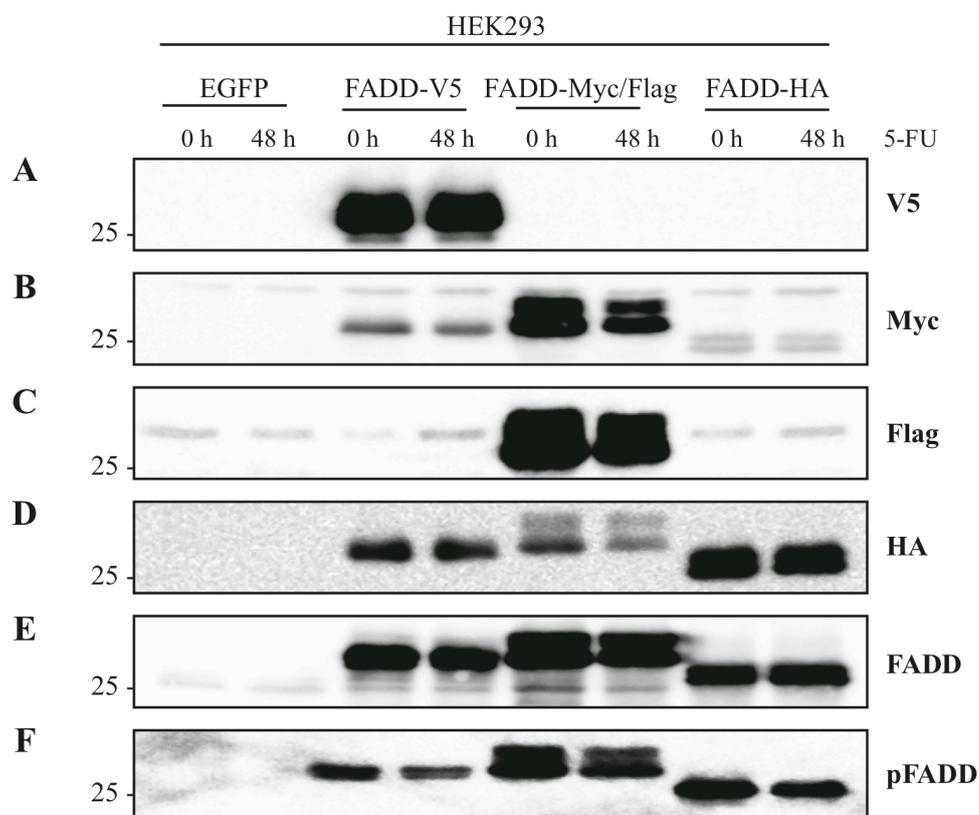


Fig. 3.1.14 The cross-reactivity of the various protein tag antibodies. HEK293 cells were transfected with a plasmid containing either Flag/myc-tagged FADD, V5-tagged FADD, HA-tagged FADD or EGFP as control. 4-6 h post-transfection these cells were stimulated with 5-FU (200 μ M) for 48 h. The cellular lysates were analysed by Western blotting for **(A)** V5, **(B)** Myc, **(C)** Flag, **(D)** HA, **(E)** FADD and **(F)** pFADD. Molecular weight markers are indicated. Representative of n = 1 experiments.

We transfected HEK293 cells with tagged versions of FADD, (FADD-V5 and FADD-Myc/FLAG) which we intended to continue with in the FADD-FADD interaction study. Firstly, to ensure the equivalent complex was forming in the 293 cells as in the HCT116 cells we treated the transfected cells 6 h post-transfection with 5-FU (400

μM , 15 h) and ran on a sucrose gradient. Following harvesting of the sucrose gradient fractions, they were subsequently Western blotted for caspase-8. Following 5-FU treatment, endogenous caspase-8 sedimented to fraction 14 in the HEK293 cell sucrose gradient (**Fig. 3.1.15 A**). We subsequently Western blotted for V5 (**Fig. 3.1.15 B**) and Myc (**Fig. 3.1.15 C**) respectively and found both FADD-V5 and FADD-Myc/Flag co-sedimented to Fraction 14 with caspase-8. We were then satisfied that HEK293 cells were a comparable model of our complex formation for our FADD-FADD interaction study.

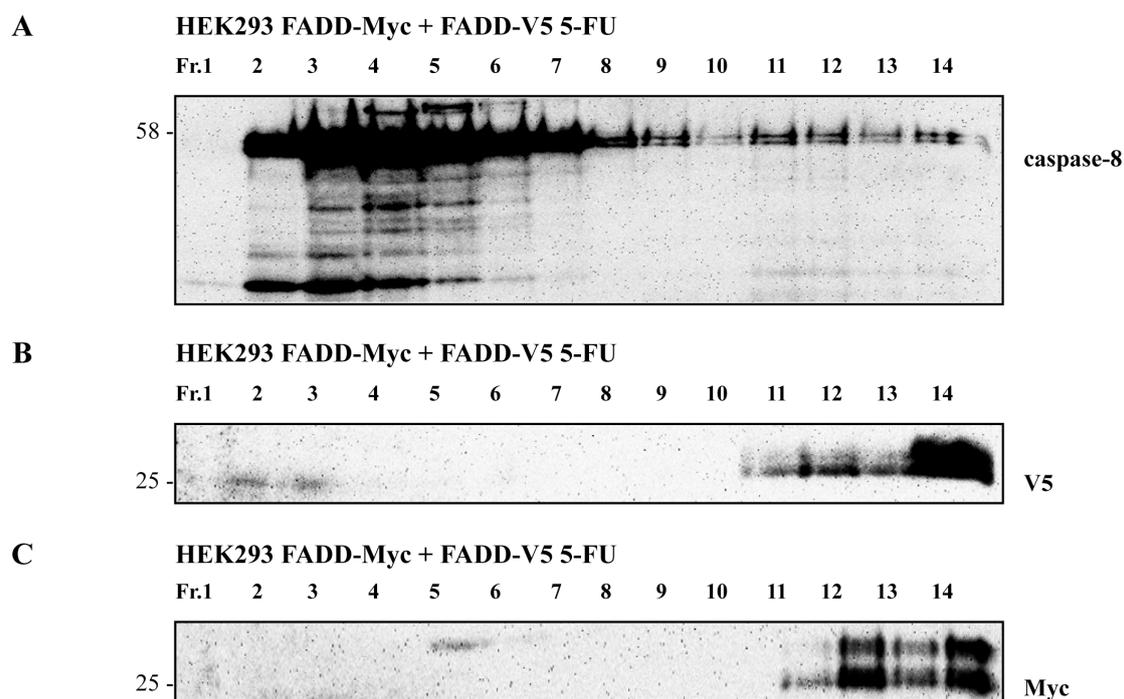


Fig. 3.1.15 A similar complex forms in HEK293 cells following 5-FU. HEK293 cells were co-transfected with a plasmid containing V5-tagged FADD and Flag/Myc-tagged FADD and 4-6 h post-transfection were stimulated with 5-FU (400 μM) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for (A) caspase-8 (BD), (B) V5 and (C) Myc. Molecular weight markers are indicated. Representative of $n = 2$ experiments.

Prior to immunoprecipitating our FADD constructs for the interaction study, we set up an additional control experiment to ensure the low levels of cross-reactivity observed with the α -Myc antibody for the FADD-V5 construct would not produce false positive results with our immunoprecipitations. To this end we transfected HEK293 cells with either EGFP, FADD-Myc/Flag or FADD-V5 and subjected each sample to a V5 immunoprecipitation. The V5 immunoprecipitations were then

Western blotted for V5. V5 was only detected in the FADD-V5 sample (**Fig. 3.1.16 A**). This blot was then re-probed for Myc. FADD-Myc was not co-immunoprecipitated with the α -V5 antibody when no FADD-V5 is present. FADD-Myc was only detected in the 10 μ g input control (**Fig. 3.1.16 B**). Therefore we were satisfied that any results observed in the dimerisation study would be genuine.

We co-over expressed both FADD-V5 (1.5 μ g/well) and FADD-Myc (1.5 μ g/well) in 293 cells and either treated with 5-FU (400 μ M, 15 h) or left untreated as control. The protein lysates were subjected to immunoprecipitation with α -Myc antibody (1 μ g/1000 μ g protein) and Western blotted for V5. Following 5-FU treatment, FADD-V5 was co-immunoprecipitated with FADD-Myc (**Fig. 3.1.16 C**). In the untreated control sample, FADD-V5 was not visible. Therefore the co-immunoprecipitation of both FADD constructs was induced by 5-FU treatment. The lower portion of this blot was re-probed with Myc to demonstrate equal FADD-Myc immunoprecipitated in both samples and the top portion was re-probed with caspase-8, where we observed caspase-8 to be co-immunoprecipitated with the FADD dimer. This experiment was repeated using a FLAG IP with similar results. FADD-V5 was co-immunoprecipitated with FADD-Myc/Flag following 5-FU (**Fig. 3.1.16 D**). This blot was re-probed with α -FLAG antibody to demonstrate equal FADD-Myc/Flag was pulled. Furthermore this experiment was repeated the opposite way around using a V5 immunoprecipitation and Myc Western blot and the results were confirmed (**Fig. 3.1.16 E**). As a control the blot was re-probed for Myc to show equal FADD-Myc/Flag was pulled.

These results indicate that following 5-FU treatment, FADD molecules interact and this process could be either due to FADD molecules actively converging or because they are bound to (a number of) caspase-8 molecules as part of the complex. Future work will include performing the FADD-FADD interaction study in a caspase-8 null background to investigate whether this FADD interaction occurs prior to caspase-8 recruitment or as a result of it.

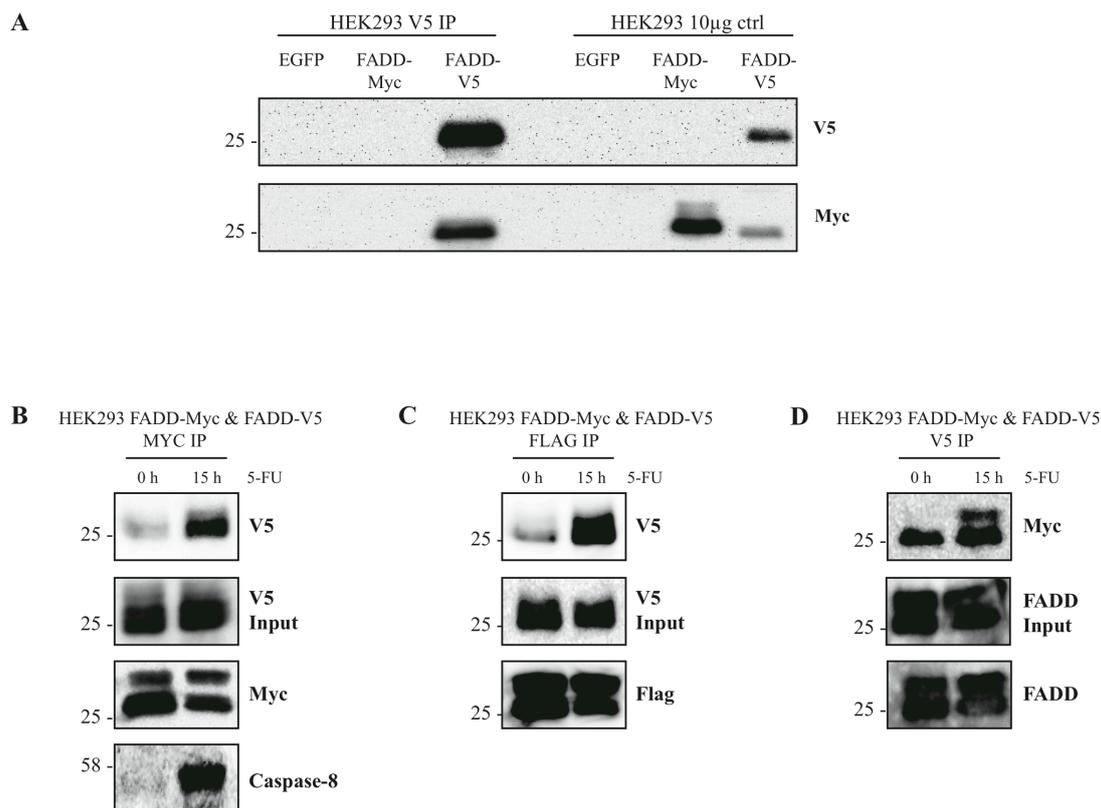


Fig. 3.1.16 An inducible FADD-FADD interaction forms following 5-FU (A) HEK293 cells were transfected with a plasmid containing Flag/Myc-tagged FADD, V5-tagged FADD or EGFP as control. 24 h post-transfection the cellular lysates were harvested and subjected to a V5 immunoprecipitation, followed by Western blotting for V5 and subsequently reprobred for Myc. 10 µg of cellular lysate was loaded as an input control. HEK293 cells were co-transfected with a plasmid containing Flag/Myc-tagged FADD and V5-tagged FADD. 4-6 h post-transfection the cells were stimulated with 5-FU (400 µM) for 15 h. The cells were then subjected to either a (B) Myc IP, (C) FLAG IP or (D) V5 IP. These immunoprecipitations were analysed by Western blotting for (B) V5 (reprobed with Myc and caspase-8), (C) V5 (reprobed with Flag) or (D) Myc (reprobed with FADD) respectively. 10 µg of protein was loaded as an input control. Molecular weight markers are indicated. Representative of n = 1 experiments (A) and n = 3 experiments (B, C, D).

We then questioned what event was the molecular driver behind the putative FADD-FADD interaction. One potential explanation could be a post-translational modification of FADD. A common modification examined in the literature is the phosphorylation of FADD, described to affect the functions of FADD in various studies (Tishler et al. 1992; Geard et al. 1993; Gagandeep et al. 1999; Shimada et al. 2004). FADD is phosphorylated at a single location in human cells, Serine149 (Shimada et al. 2004). To investigate the role, if any, that phosphorylation of FADD played in 5-FU-induced apoptosis in HCT116 cells, we first Western blotted for phosphorylated FADD (pFADD) in 5-FU treated (200 µM, 48 h) and untreated

HEK293 cells using a pFADD-specific antibody and found that there was no obvious increase/decrease in the protein levels of pFADD following 5-FU (**Fig. 3.1.14 F**). This blot was reprobed with FADD to control for total FADD protein (**Fig. 3.1.4 14 E**). We then attempted to visualise pFADD in HCT 5-FU-treated sucrose gradient but this proved difficult due to a poor signal. This experiment was repeated by precipitating the sucrose fractions using methanol/chloroform or TCA however this did not improve the signal strength (data not shown). Therefore we transfected HEK293 cells with FADD-V5 to aid in visualising pFADD in the sucrose gradient. We treated the cells with 5-FU (400 μ M, 15 h) and ran on a sucrose gradient, which was then Western blotted for pFADD. pFADD co-sedimented to the high-density region of the gradient following 5-FU (**Fig. 3.1.17 A**).

We then repeated our FADD-FADD interaction experiment using a V5 immunoprecipitation and Western blotted for pFADD and detected an increase in pFADD upon 5-FU induction (**Fig. 3.1.17 B**). This was repeated using a Myc immunoprecipitation with similar results (data not shown). These results would suggest that there is more pFADD immunoprecipitated because there is more FADD pulled in the 5-FU treated sample in general. Therefore while pFADD is present as part of the FADD interaction this does not mean that it is comprised exclusively of pFADD, or indeed that it is even required.

In order to study in greater detail the role of phosphorylated FADD, we generated a phospho-dead mutant of FADD-V5, termed S194A-V5, where the Serine at position 194, was replaced with an Alanine. Using the FADD-Myc plasmid and the phospho-dead FADD S194A-V5 mutant we repeated our co-immunoprecipitation interaction experiment and found that while the upper V5 band representing the pFADD-V5 was no longer present, the lower V5 band representing the unphosphorylated FADD-V5 still resulted in induction following 5-FU treatment (**Fig. 3.1.17 C**). This blot was reprobed with Flag to demonstrate equal FADD-Myc/Flag was immunoprecipitated. Therefore, we can conclude that the pFADD-dead S194A-V5 mutant did not prevent the formation of a FADD-FADD interaction. In order to fully examine the importance of pFADD in the FADD-FADD interaction, we will need to over express both a FADD-V5 and a FADD-Myc phospho-dead mutant and perform our interaction experiment. In addition both mutants will need to be expressed in our HCT.shFADD stable clone and both apoptosis and caspase-8 and -3 cleavage induced by 5-FU will

be compared to regular FADD over expression to attempt to elucidate the necessity of pFADD in this system.

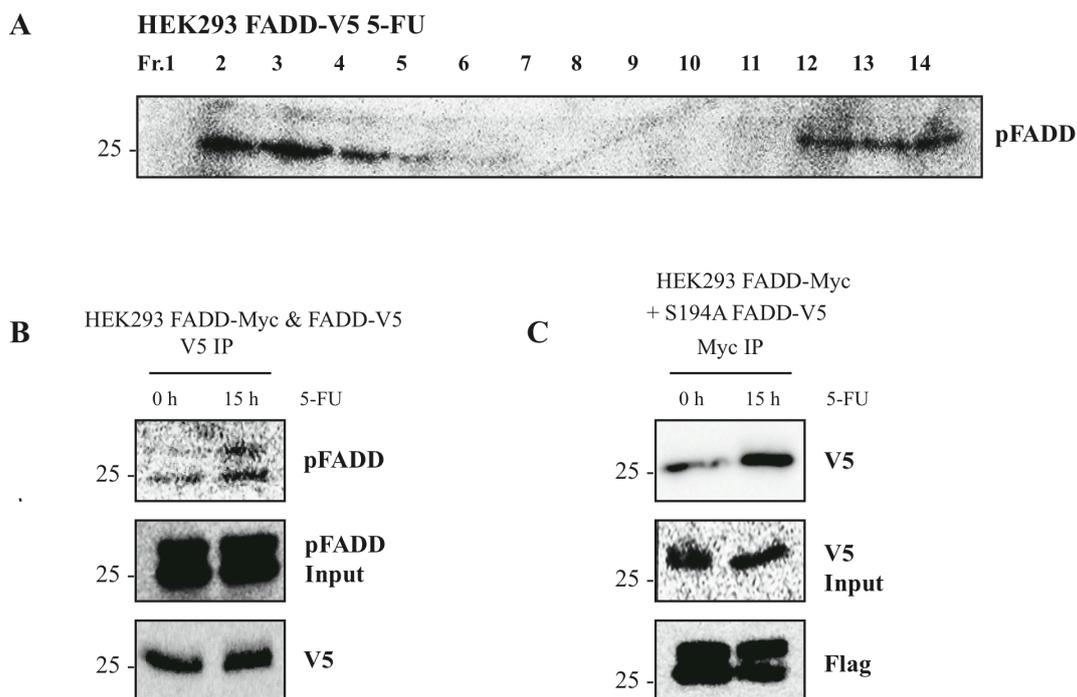


Fig. 3.1.17 Phosphorylated FADD is present as part of the protein complex and within the FADD-FADD interaction (A) HEK293 cells were transfected with a plasmid containing V5-tagged FADD and 4-6 h post-transfection were stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for pFADD. **(B)** HEK293 cells were co-transfected with a plasmid containing Flag/Myc-tagged FADD and V5-tagged FADD. 4-6 h post-transfection the cells were stimulated with 5-FU (400 μ M) for 15 h. The cells were then subjected to a V5 IP. This immunoprecipitation was analysed by Western blotting for pFADD. 10 μ g of cellular lysates were used as input control. **(C)** HEK293 cells were transfected with a plasmid containing S194A V5-tagged FADD. 4-6 h post-transfection the cells were stimulated with 5-FU (400 μ M) for 15 h. The cells were then subjected to a Myc immunoprecipitation. This immunoprecipitation was analysed by Western blotting for V5 (reprobed with Flag). 10 μ g of cellular lysates were used as input control. Molecular weight markers are indicated. Representative of $n = 2$ experiments.

3.1.2.10 Death receptors such as DR4 and DR5 do not play a role in the initial cleavage of caspase-8 but DR5 is important in overall 5-FU-mediated apoptosis.

We next questioned the involvement of death receptors known to commonly interact with FADD in the 5-FU-induced caspase-8 cleavage and complex formation in HCT116 cells. Ordinarily, the cascade led by caspase-8 is involved in death receptor-mediated apoptosis, such as the one triggered by FasL, TNF and TRAIL. On activation, these receptors bind to FADD, which in turn recruits procaspase-8, which

leads to its cleavage into its active form. Due to the clear importance of caspase-8 and FADD in the present pathway, we questioned the role of those death receptors known to commonly interact with both of these proteins. Interestingly, treatment with chemotherapeutic drugs is capable of up regulating protein levels (Nimmanapalli et al. 2001) and mRNA of the TRAIL receptors DR4 and DR5 in p53-dependent or independent ways (Sheikh et al. 1998). We observed this increase in protein expression of DR4 (minimally) (**Fig. 3.1.18 A**) and DR5 (to a large extent) (**Fig. 3.1.18 B**) in the HCT116 cells following 5-FU.

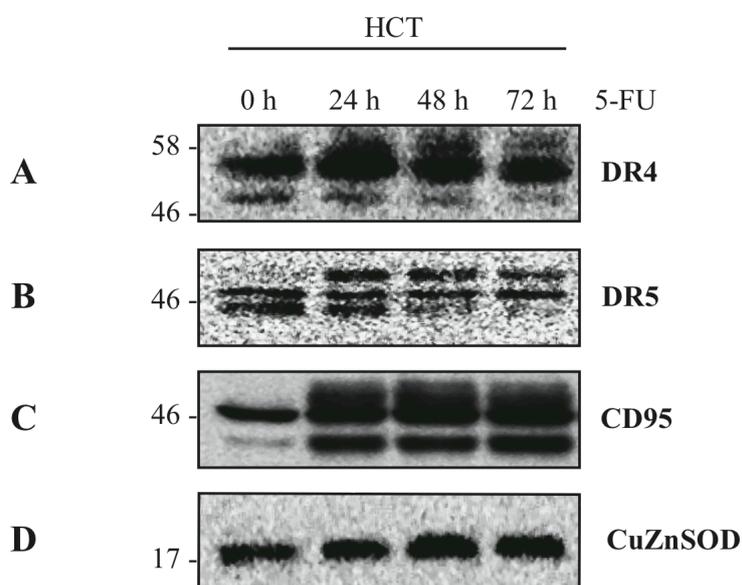


Fig. 3.1.18 5-FU stimulation upregulates the expression of DR5 and CD95 in HCT116 cells. HCT116 cells were stimulated with 5-FU (200 μ M) for 24, 48 and 72 h. The cellular lysates were analysed by Western blot for (A) DR4, (B) DR5 and (C) CD95. CuZnSOD served as a loading control and molecular weight markers are indicated. Representative of n = 3 experiments.

To elucidate any involvement of DR4 and/or DR5 in the mechanism of action of 5-FU, we generated HCT116 stable knock down clones termed HCT.shDR4 and HCT.shDR5 respectively. While the TRAIL receptor DR4 can also bind to TRAIL and induce apoptosis, several studies have shown that DR4 has different physiological roles to DR5 and that DR5 transduces the TRAIL signal more efficiently. In **Fig. 3.1.19 A** the DR4 protein levels of HCT.shDR4 in comparison to HCT.ctrl cells were demonstrated via Western blot. The DR4 levels could not be up-regulated in HCT.shDR4 after 5-FU stimulation. HCT.shDR4 cells were incubated with 5-FU

(200 μ M, 48 h) and the apoptosis rates measured, where the silencing of DR4 did not impede apoptosis rates, in fact there was an increase in apoptosis observed (**Fig. 3.1.19 B**) In line with this result, caspase-8 (**Fig. 3.1.19 C**) and caspase-3 (**Fig. 3.1.19 D**) were not affected.

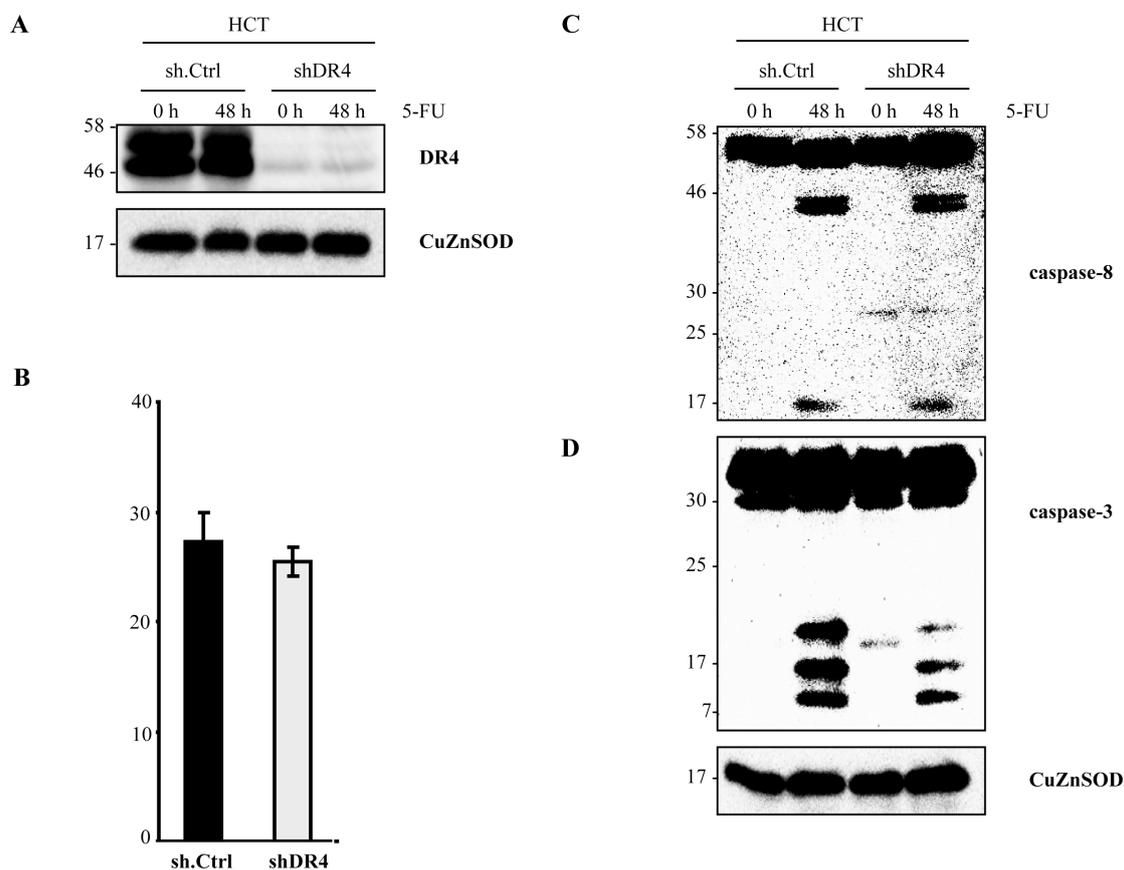


Fig. 3.1.19 The absence of DR4 has no effect on caspase-8 cleavage. Stable DR4 knock down clones were generated in HCT116 cells, termed HCT.shDR4. The HCT.shDR4 cells were stimulated with 5-FU (200 μ M) for 48 h, or untreated as control. Cellular lysates were analysed by Western blotting for (**A**) DR4 to demonstrate their knock down, (**B**) caspase-8 (CS) and (**C**) caspase-3. CuZnSOD served as a loading control and molecular weight markers are indicated. Representative of n = 3 experiments. Note: apoptosis data generated by Dr. Andrea Mohr.

The HCT.shDR5 clone exhibited a complete knockdown of DR5 levels which could not be up-regulated in the HCT.shDR5 clone compared to the HCT.shctrl clone upon 5-FU stimulation (**Fig. 3.1.20 A**). However in contrast to the HCT.shDR4 clone the apoptosis in the HCT.shDR5 clone was approximately halved (**Fig. 3.1.20 B**). In addition, caspase-8 processing to its intermediates was largely unaffected (**Fig. 3.1.20 C**). Interestingly, caspase-3 cleavage was abrogated compared to the HCT.shctrl (**Fig. 3.1.20 D**). This would indicate that while caspase-8 cleavage to its intermediate

fragments is unaffected, the apoptosis pathway is attenuated downstream, when DR5 is silenced which prevents the full cleavage of caspase-3 and impedes the overall apoptosis induced by 5-FU in HCT116 cells. This result led us to investigate the role of DR5 in 5-FU apoptosis in more detail within our group and it was discovered that a second DR5-dependent pathway is occurring in parallel and is independent of caspase-8 and appears to serve to expedite the rate of apoptosis in HCT116 cells. This second pathway and its role in 5-FU-induced apoptosis in HCT116 cells was beyond the scope of this project and was investigated individually as a separate study within our group and was found to involve the c-Jun NH2-Terminal Kinase (JNK) signal transduction pathway (data not shown).

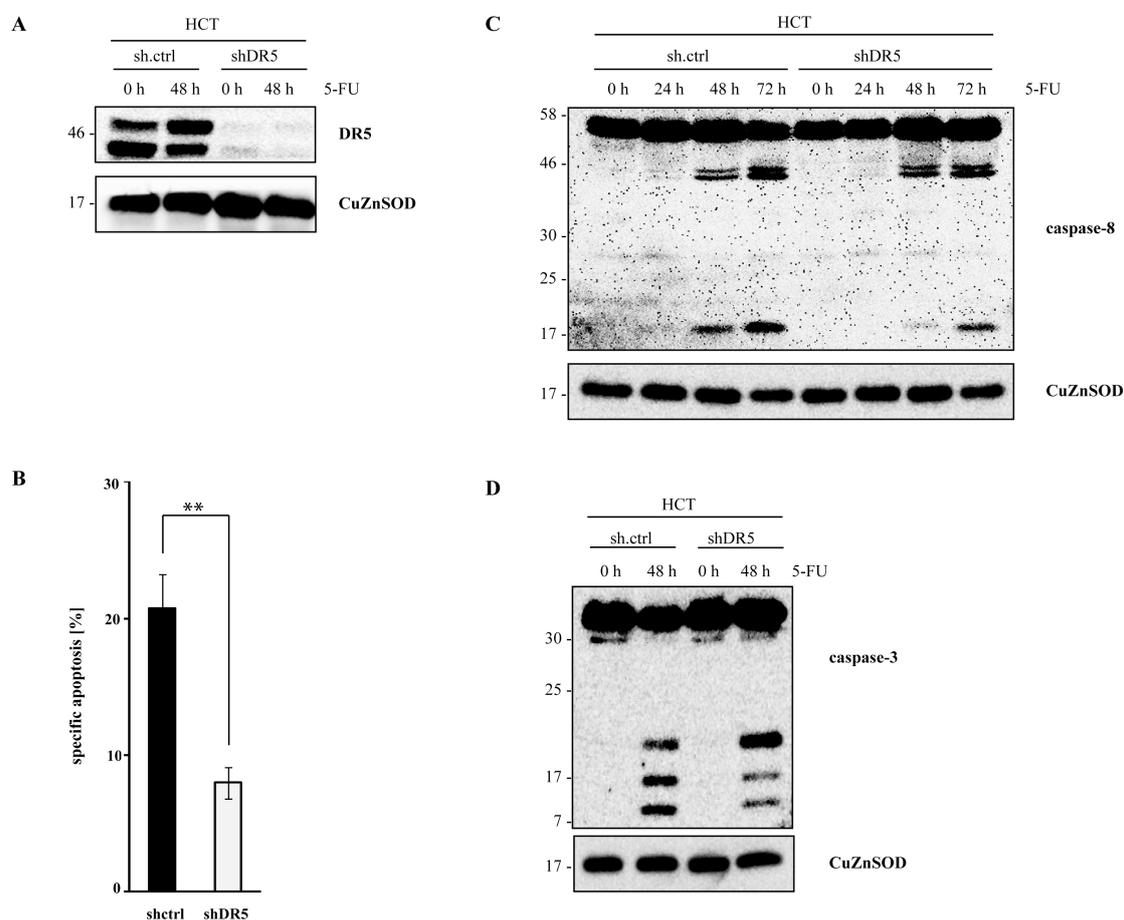


Fig. 3.1.20 Knockdown of DR5 abrogates caspase-3 cleavage downstream of caspase-8 and reduces apoptosis (A) Stable DR5 knock down clones were generated in HCT116 cells, termed HCT.shDR5. The HCT.shDR5 cells were stimulated with 5-FU (200 μ M) for 48 h, or untreated as control. Cellular lysates were analysed by Western blotting for DR5 to demonstrate their level of knock down. **(B)** Cell death was characterised by a Nicoletti Assay as described in Chapter 2. The HCT.shDR5 cells were stimulated with 5-FU (200 μ M) for 24, 48 and 72 h. The cellular lysates were then analysed by Western blot for **(C)** caspase-8 (CS) and **(D)** caspase-3. CuZnSOD served as a loading control and molecular weight markers are indicated. ** = $P < 0.001$. Representative of $n > 4$ experiments.

Further to the apoptosis and caspase-8 and -3 cleavage studies neither DR4 (**Fig. 3.1.21 A**) nor DR5 (**Fig. 3.1.21 B**) co-sedimented with caspase-8 to the high-density region of the sucrose gradient following 5-FU (400 μ M, 15 h), but were clearly up-regulated when compared to their corresponding untreated control gradients. We also included a CD95 Western blot of the sucrose gradient fractionation, as CD95 is also largely up-regulated following 5-FU treatment in HCT116 cells (**Fig. 3.1.18 C**) and while CD95 was largely upregulated following 5-FU in the sucrose gradient when compared to the untreated control gradient (**Fig. 3.1.21 C**), it did not migrate toward the higher density fractions of the gradient. This is in line with previous studies within the group, where an Ad.shCD95 vector did not affect apoptosis rates or caspase-8 and -3 cleavage in HCT116 cells (*Mohr et al., unpublished data*).

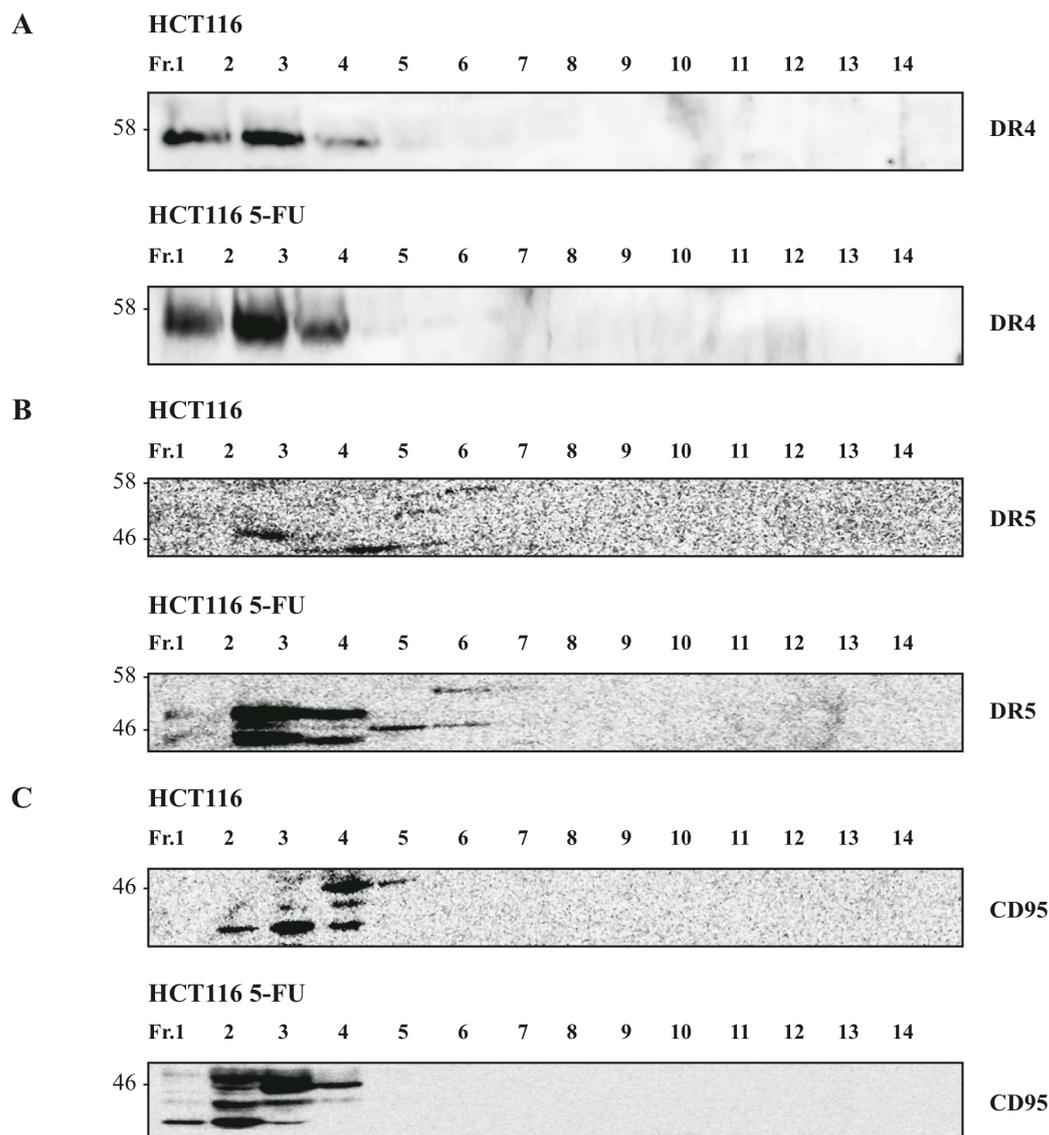


Fig. 3.1.21 Death receptors such as DR4, DR5 and CD95 do not sediment as part of the high molecular weight complex following 5-FU. HCT116 cells were either stimulated with 5-FU (400 μ M) for 15 h or unstimulated as control. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for (A) DR4, (B) DR5 and (C) CD95 respectively. Molecular weight markers are indicated. Representative of n = 1 experiments.

In addition we utilised our HCT.shDR5 stable clone to investigate the effect of silencing DR5 on our caspase-8 complex formation, and found that while there was a small delay in the kinetics of the complex movement, the complex was still capable of forming despite the lack of DR5, whereby both caspase-8 (**Fig. 3.1.22 A**) and FADD (**Fig. 3.1.22 B**) co-sedimented to Fraction 14.

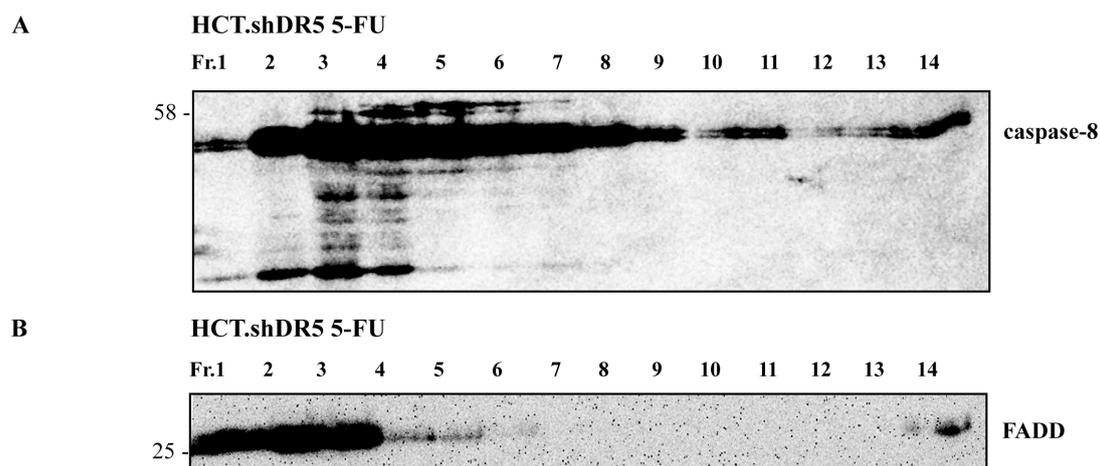


Fig. 3.1.22 DR5 knockdown cannot inhibit complex formation. HCT.shDR5 cells were stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for (A) caspase-8 (BD) and (B) FADD respectively. Molecular weight markers are indicated. Representative of $n = 3$ experiments.

Finally we established a DR5 immunoprecipitation in HEK293 cells. FADD-Myc/Flag was overexpressed in HEK293 cells, which were then treated with 5-FU (400 μ M, 15 h) or left untreated as control. The lysates were subsequently immunoprecipitated for DR5 and then Western blotted for Myc. There was no FADD-Myc/Flag detected following 5-FU (**Fig. 3.1.23 A**). This blot was reprobed for DR5 to demonstrate the DR5 immunoprecipitation was successful. Therefore, in order to rule out any possible interference between the Myc/Flag tag we subsequently overexpressed FL FADD in HEK293 cells treated with 5-FU (400 μ M, 15 h) or left untreated as control. We subjected the lysates to a DR5 immunoprecipitation and Western blots for caspase-8 and FADD (**Fig. 3.1.23 B**) and neither caspase-8 nor FADD were successfully co-immunoprecipitated. This blot was then reprobed with DR5 to demonstrate the DR5 immunoprecipitation was successful. A third higher molecular weight band was observed in the immunoprecipiated DR5. This antibody is reported to detect both precursor and mature forms of DR5 (40, 48 kDa), therefore this appears to be an enrichment of a 60 kDa modified form of DR5 as it is present in the input control, but to a much lesser extent. We can rule out that it is detecting IgGs as the secondary anti-rabbit TrueBlot® was employed to prevent the native IgG being recognized. These results, taking into consideration with the HCT.shDR4/HCT.shDR5 caspase cleavage studies, the sucrose gradient analyses and the HCT.shDR5 sucrose gradient analyses, all point to a complex forming

independent of these death receptors.

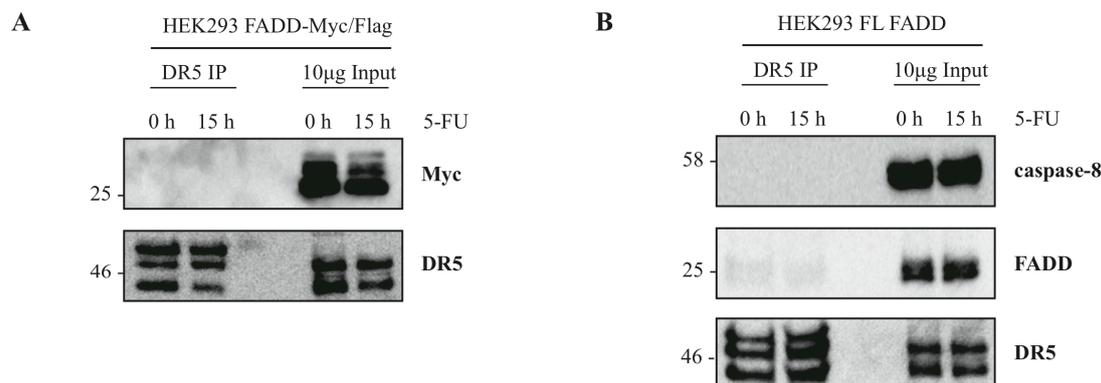


Fig. 3.1.23 DR5 and FADD do not co-immunoprecipitate following 5-FU (A) HEK293 cells were transfected with a plasmid containing Flag/Myc-tagged FADD. 4-6 h post-transfection the cells were stimulated with 5-FU (400 μ M) for 15 h, before subjecting them to a DR5 immunoprecipitation and subsequently analysing them by Western blotting for FADD. This Western blot was reprobbed with DR5 as control. (B) HEK293 cells were transfected with a plasmid containing full-length FADD. 4-6 h post-transfection these cells were stimulated with 5-FU (400 μ M) for 15 h, prior to subjecting them to a DR5 immunoprecipitation and subsequently analysing them by Western blotting for caspase-8 and FADD. This Western blot was reprobbed with DR5 as control. Molecular weight markers are indicated. Representative of n = 2 experiments.

3.1.2.11 *c-FLIP* over expression does not affect caspase-8 cleavage or apoptosis

As previously discussed, apoptosis transduced via the death receptor pathway is mediated by caspase-8 and FADD, and can be inhibited by cytoplasmic factors, most notably *c-FLIP*, which is thought to bind to FADD within the DISC and inhibit caspase-8 activation (Krueger et al. 2001). *C-FLIP* over expression has been found to inhibit death ligand-induced apoptosis in a number of *in vitro* studies. Interestingly, *c-FLIP* has been demonstrated in the past to be over expressed in a high percentage of colonic and gastric carcinomas. To attempt to further rule out death-receptor involvement in the caspase-8 cleavage we transiently over expressed an AD.*FLIP* vector in HCT116 cells to high levels (Fig. 3.1.24 A) and subsequently treated with 5-FU (200 μ M, 48 h), or untreated as control and found that in comparison to control Ad.DsRed over expressing HCT116 cells, apoptosis rates (Fig. 3.1.24 B) and caspase-8 (Fig. 3.1.24 C) and -3 cleavage were not affected (Fig. 3.1.24 D), further pointing to a complex unique to the DISC and not containing death receptors. Furthermore, endogenous *c-FLIP* did not co-sediment with caspase-8 and FADD following 5-FU

(400 μ M, 15 h) as part of the complex in the sucrose gradient (**Fig. 3.1.24 E**). The same lysates were run on a second Western blot and probed for caspase-8 as a control for the lack of FLIP movement, and in contrast to FLIP, caspase-8 clearly sedimented to the high density region of the gradient (**Fig. 3.1.24 F**). This also rules out c-FLIP as an effector of the 5-FU-induced caspase-8 complex.

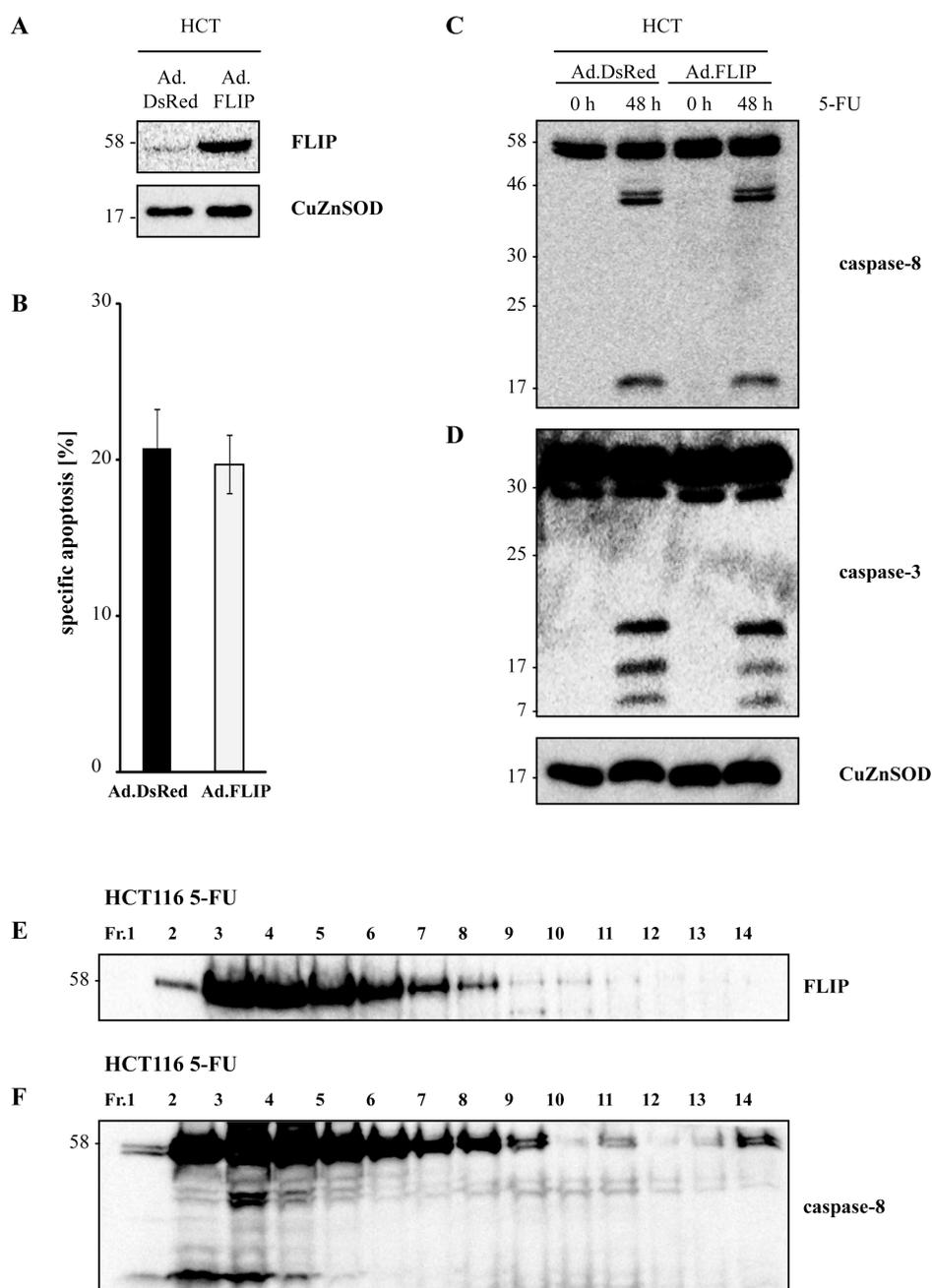


Fig. 3.1.24 c-FLIP over expression does not affect caspase-8 cleavage or apoptosis. (A) HCT116 cells were transduced with an adenoviral vector containing c-FLIP or DsRed as control. 48 h post-transduction cells were analysed by Western blotting to demonstrate c-FLIP overexpression. (B) Cell death was characterised by a Nicoletti Assay as described in Chapter 2. (C) HCT116 cells were transduced with an adenoviral vector containing c-FLIP or DsRed as control. 48 h post-transduction cells

were stimulated with 5-FU (200 μ M) for 48 h. The cellular lysates were analysed by Western blotting for (C) caspase-8 (CS) and (D) caspase-3. CuZnSOD served as a loading control. (E) HCT116 cells were stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for c-FLIP and (F) caspase-8 (B.D.). Molecular weight markers are indicated. Representative of n = 2 experiments.

3.1.2.12 *Over expression of antiapoptotic member Bcl-2 or absence of the proapoptotic member Bax blocks apoptosis downstream of caspase-8*

We then wanted to investigate the role of the Bcl-2 family members in the apoptotic response of 5-FU in HCT116 cells and to determine if their effects were downstream of caspase-8 cleavage. Activation of the intrinsic apoptotic pathway is regulated by the Bcl-2 family of proteins, and although these proteins share some homology, some Bcl-2 family members, such as Bax and Bid, promote apoptosis whereas others, such as Bcl-x_L and Bcl-2 itself, are antiapoptotic (Lindsten et al. 2000; Cheng et al. 2001; Letai et al. 2002). In the case of Bcl-2, we over expressed Bcl-2 using Ad.Bcl-2 (**Fig. 3.1.25 A**). HCT.AdDsRed served as an overexpression control. These cells were treated with 5-FU (200 μ M, 48 h) or untreated as control and HCT.AdBcl-2 resulted in inhibition of apoptosis when compared to control HCT.Ad.DsRed from 22% to 8% (**Fig. 3.1.25 B**). This inhibition of apoptosis did however still permit caspase-8 to be cleaved to its intermediates (**Fig. 3.1.25 C**), further illuminating the apical role of caspase-8 cleavage in this pathway. Caspase-3 was also cleaved to its first intermediate but was not fully processed (**Fig. 3.1.25 D**).

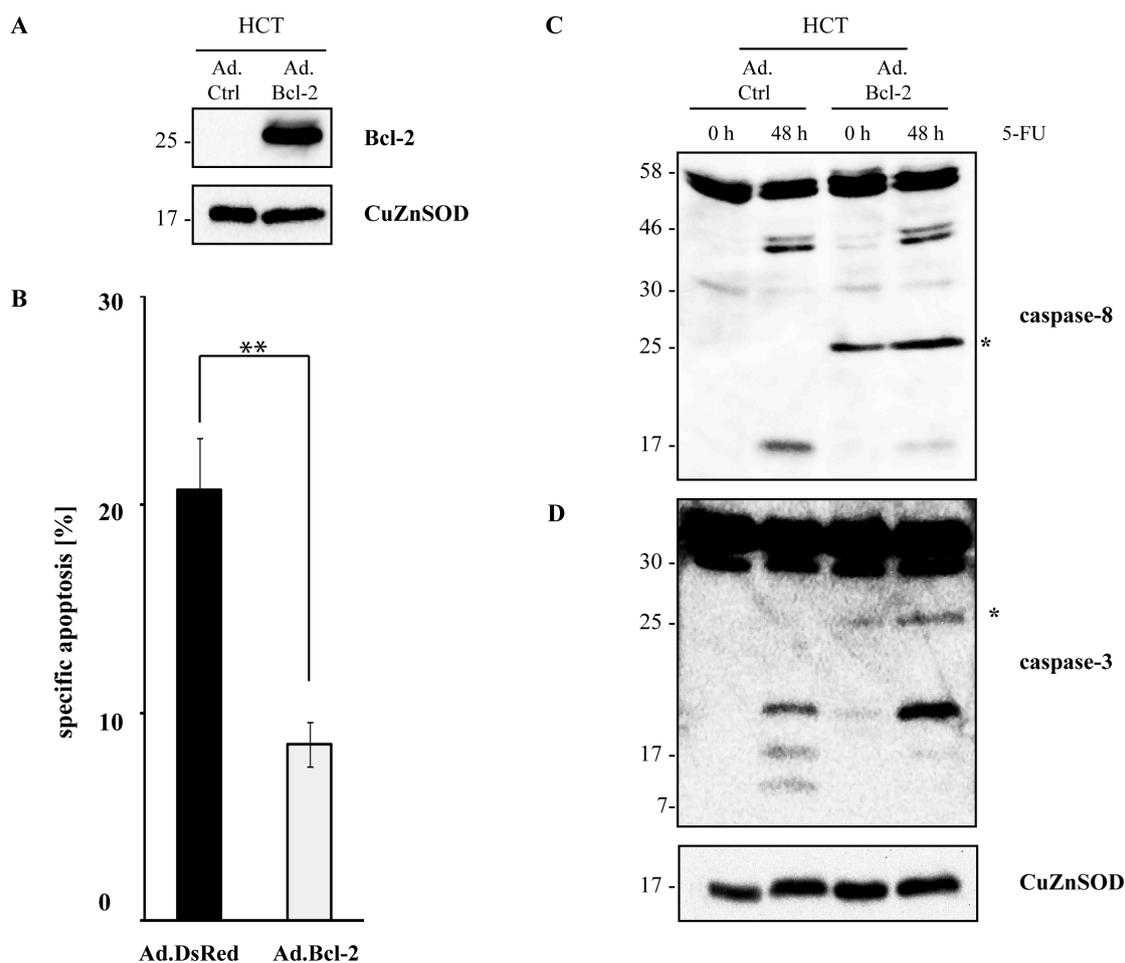


Fig. 3.1.25 Overexpression of Ad.Bcl-2 inhibits 5-FU-induced apoptosis downstream of caspase-8 cleavage (A) HCT116 cells were transduced with adenoviral vector containing Bcl-2 or DsRed as control. 48 h post-transduction cells were analysed by Western blotting to demonstrate the overexpression of Bcl-2. (B) Cell death was characterised by a Nicoletti Assay as described in Chapter 2. (C) HCT116 cells were transduced with adenoviral vector containing Bcl-2 or DsRed as control. 48 h post-transduction cells were stimulated with 5-FU (200 μ M) for 48 h. The cellular lysates were then analysed by Western blotting for caspase-8 (CS) and (D) caspase-3. CuZnSOD served as a loading control and molecular weight markers are indicated. ** = $P < 0.001$. Representative of $n = 2$ experiments.

We then generated a stable Bcl-2 overexpressing clone in HCT116 cells termed HCT.Bcl-2 (Fig. 3.1.26 A). Apoptosis rates were also impeded by this stable overexpression of Bcl-2 from 21% to 7% (Fig. 3.1.26 B).

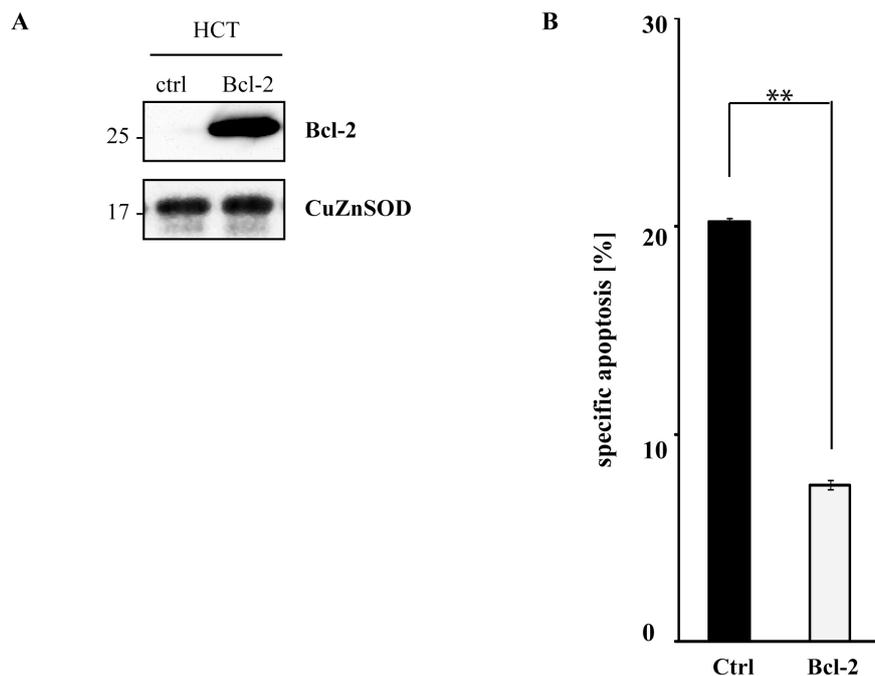


Fig. 3.1.26 Stable Bcl-2 overexpression does not inhibit complex formation (A) Stable clones overexpressing Bcl-2 were generated in HCT116 cells, termed HCT.Bcl-2. Negative clones served as a control. The cellular lysates were analysed by Western blot to demonstrate the high level of Bcl-2 overexpression. CuZnSOD served as a loading control. (B) Cell death was characterised by a Nicoletti Assay as described in Chapter 2. (C) HCT.Bcl-2 cells were stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for caspase-8 (BD). Molecular weight markers are indicated. ** = $P < 0.001$. Representative of $n = 3$ experiments.

We next studied the effects of knocking down Bax in HCT116 cells by generating HCT.shBax stable clones that knocked down Bax to undetectable levels as demonstrated by Western blot (Fig. 3.1.27 A). Apoptosis rates were measured and after 72 h there was a reduction by approximately half from 60% apoptosis to 30% (Fig. 3.1.27 B). We found that 48 h post 5-FU, caspase-8 was processed to its intermediates but full cleavage was impeded (Fig. 3.1.27 C), and caspase-3 cleavage was inhibited (Fig. 3.1.27 E).

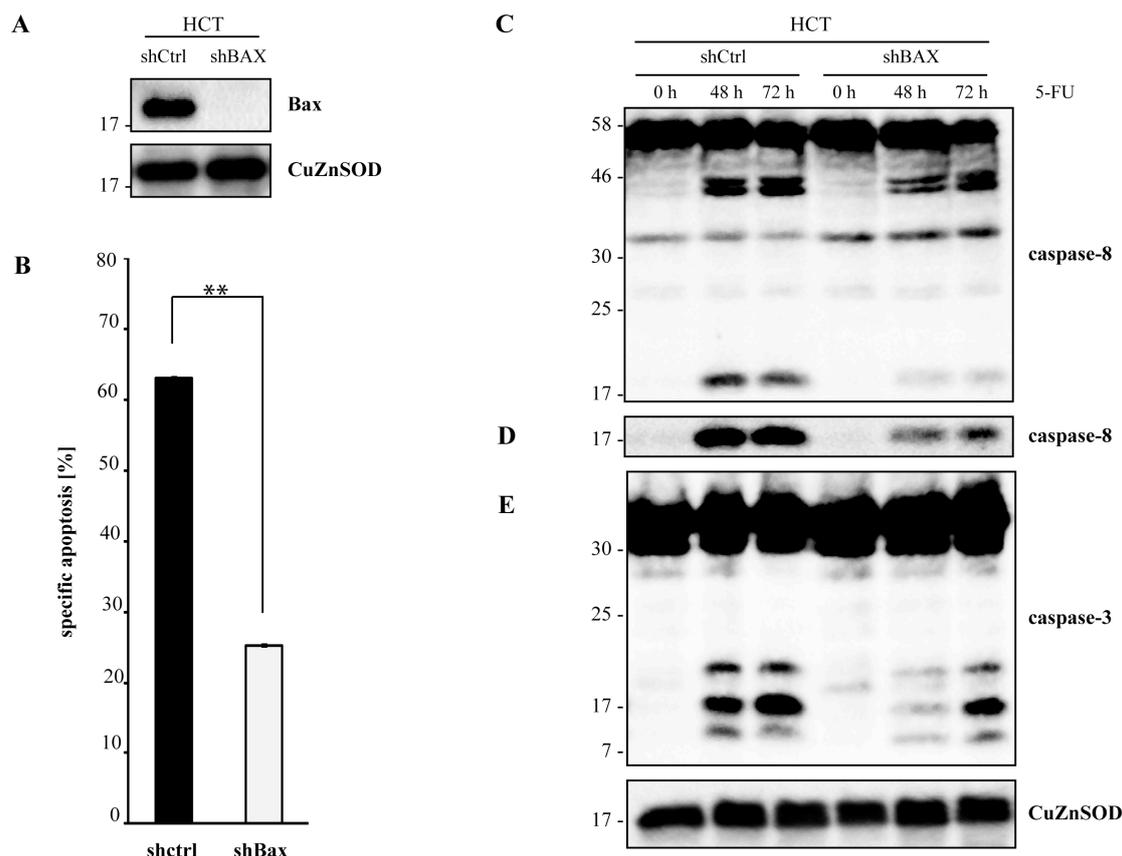


Fig. 3.1.27 shBax reduced apoptosis in HCT cells downstream of caspase-8 (A) Stable clones knocking down Bax were generated in HCT116 cells, termed HCT.shBax. Negative clones served as a control. The cellular lysates were analysed by Western blot to demonstrate the knockdown of Bax. (B) Cell death was characterised by a Nicoletti Assay as described in Chapter 2. (C) HCT.shBax cells were stimulated with 5-FU (200 μ M) for 48 h. The cellular lysates were then analysed by Western blotting for caspase-8 (CS) (D) A longer exposure demonstrates the difference between the 48 and 72 h caspase-8 p18 fragment) and (E) caspase-3. CuZnSOD served as a loading control and molecular weight markers are indicated. ** = $P < 0.001$. Representative of $n = 2$ experiments.

3.1.2.13 Silencing of BID confers resistance to 5-FU.

Bid, a “BH3-only” pro-apoptotic member of the Bcl-2 family plays an important role in connecting death signals initiated from death receptors to the intrinsic pathway (Li et al. 1998; Luo et al. 1998). In a normal cell, Bid is predominantly located in the cytosol. After receiving the stimuli, Bid can be proteolytically cleaved by caspase-8, facilitating its translocation to the mitochondrial outer membrane. After being cleaved Bid can yield a p15 c-terminal truncated fragment (tBid) that can translocate to the mitochondria, where it induces the conformational change of Bax, and leads to the release of mitochondrial proteins. Due to its pivotal role in the intrinsic pathway we next investigated the role of Bid. Generating HCT.shBid knockdown stable clones

(Fig. 3.1.28 A), we observed that Bid protein levels were almost undetectable. Following 5-FU (200 μ M, 48 h) apoptosis was almost completely inhibited, from 26% to 5% (Fig. 3.1.28 B). Similar to the Bcl-2 overexpression, caspase-8 was cleaved to its intermediates (Fig. 3.1.28 C), reemphasising that caspase-8 cleavage is upstream of the mitochondria in this system. Caspase-3 cleavage was also blocked at its intermediate fragment (Fig. 3.1.28 D).

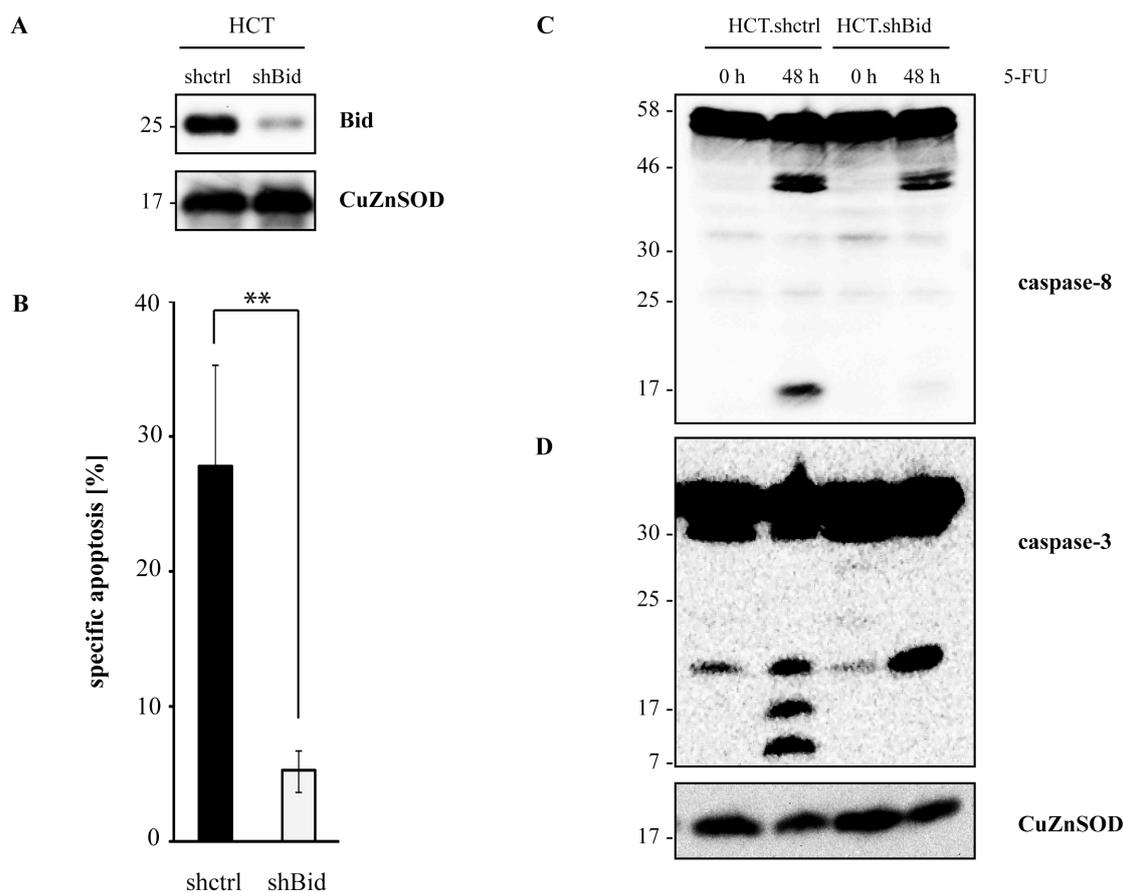


Fig. 3.1.28 Silencing of BID confers resistance to 5-FU (A) Stable clones knocking down Bid were generated in HCT116 cells, termed HCT.shBid. Negative clones served as a control. The cellular lysates were analysed by Western blot to demonstrate the level of Bid knockdown. (B) Cell death was characterised by a Nicoletti Assay as described in Chapter 2. (C) HCT.shBid cells were stimulated with 5-FU (200 μ M) for 48 h. The cellular lysates were then analysed by Western blotting for caspase-8 (CS) and (D) caspase-3. CuZnSOD served as a loading control and molecular weight markers are indicated. ** = $P < 0.001$. Representative of $n = 3$ experiments.

The inhibitor of apoptosis proteins (IAP) family of proteins has 8 mammalian family members, including XIAP that can negatively regulate apoptosis by inhibiting caspase activity directly (Deveraux et al. 1997; Roy et al. 1997; Deveraux et al. 1998). Among these IAP family members, XIAP is the most potent direct caspase inhibitor and the

full cleavage of caspase-3 by caspase-8 can be blocked by XIAP through interaction between XIAP and partially processed caspase-3 (Deveraux et al. 1998). One of the proteins released from the mitochondria in the presence of Bid is Smac/DIABLO, which can bind to and neutralise the inhibitory activity of XIAP (Wu et al. 2000). Since the lack of Bid is blocking the mitochondrial pathway of apoptosis, we hypothesised that knocking down of XIAP would restore apoptotic potential of the HCT.shBID clone. To mimic the function of Smac/DIABLO, we knocked down XIAP by using DNA-directed RNA interference (RNAi) via adenoviral (Ad.shXIAP)-mediated expression of small hairpin RNAs (shRNA) directed at XIAP. 48 h post viral transduction, XIAP levels were assessed by Western blot and found to be non-detectable levels (**Fig. 3.1.29 A**). To account for any adenoviral or shRNA expression induced side effects an adenoviral vector (Ad.shEGFP) containing an shRNA construct against EGFP was used as a control. Cells were then transduced with Ad.shXIAP or Ad.shEGFP, and 48 h after transduction, cells were treated with 5-FU (200 μ M) for a further 48 h, and tested caspase-8 (**Fig. 3.1.29 B**) and -3 (**Fig. 3.1.29 C**) cleavage and found that caspase-8 and -3 processing was fully restored in the absence of XIAP. Therefore in the absence of Bid the observed resistance can be overcome by circumventing the role of Bid and allowing cleaved caspase-8 to directly cleave caspase-3.

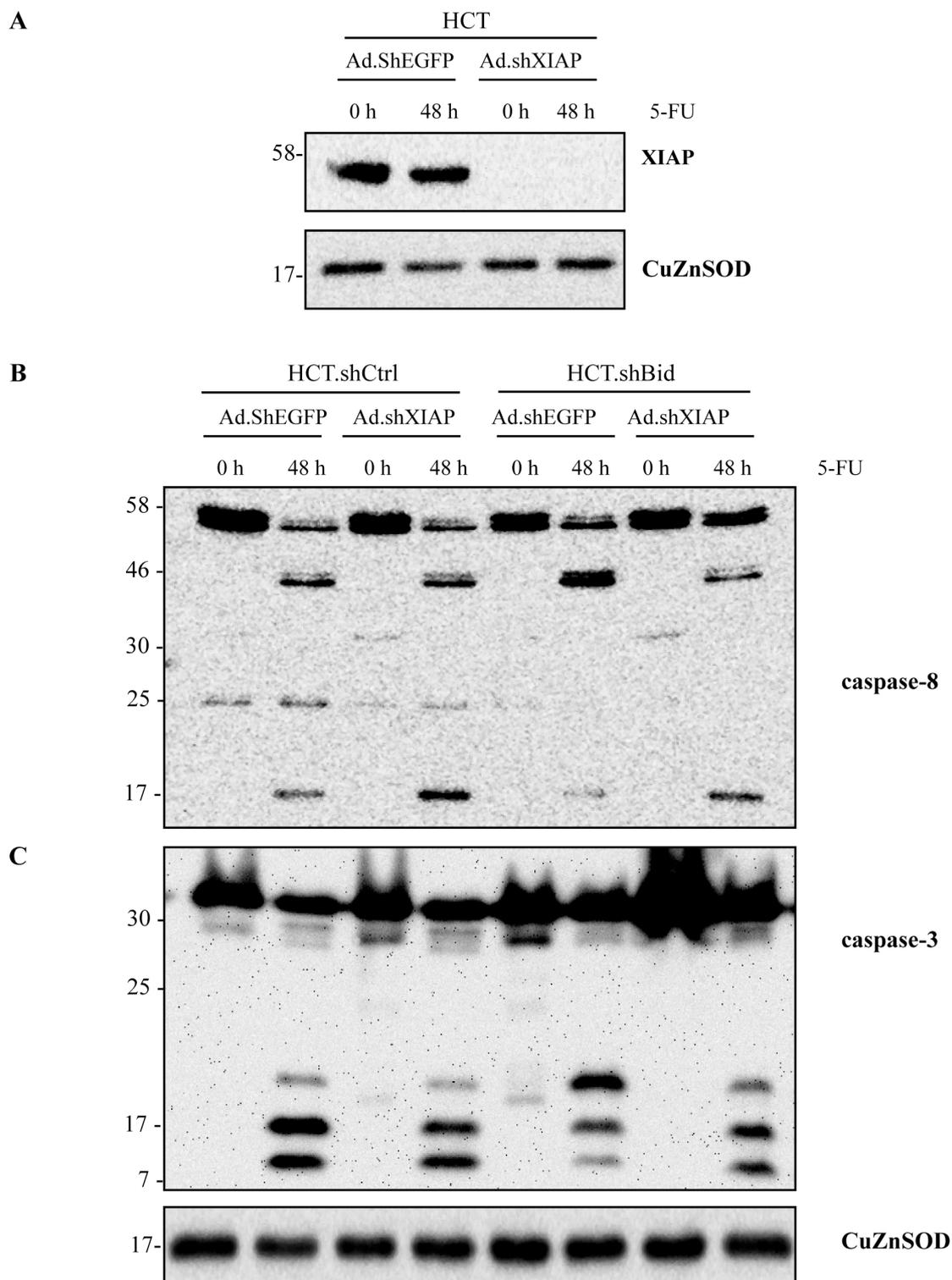


Fig. 3.1.29 Removal of XIAP block restores apoptotic potential of shBid clone (A) HCT.shBid and HCT.shctrl cells were transduced with adenoviral vector containing shXIAP or shEGFP as control. 48 h post-transduction cells were analysed by Western blotting to demonstrate the knockdown of XIAP. (B) HCT.shBid and HCT.shctrl cells were transduced with adenoviral vector containing shXIAP or shEGFP as control. 48 h post-transduction cells were stimulated with 5-FU (200 μ M) for 48 h. The cell lysates were analysed by Western blotting for caspase-8 and (D) caspase-3. CuZnSOD served as a loading control and molecular weight markers are indicated. Representative of n = 2 experiments.

We next analysed whether Bid interacted with the 5-FU-induced caspase-8/FADD complex by Western blotting 5-FU-treated HCT sucrose gradient fractions for Bid. We observed that Bid co-sedimented to Fraction 14 with caspase-8 and FADD (**Fig. 3.1.30 A**) in contrast to the untreated sucrose gradient. In the absence of caspase-8 (**Fig. 3.1.30 B**) and FADD (**Fig. 3.1.30 C**), Bid did not sediment to Fr.14 of the gradient. In addition we subjected the HCT.shBid stable clone to 5-FU treatment (400 μ M, 15 h) and subsequently ran it on the sucrose gradient. In the absence of Bid, caspase-8 and FADD (**Fig. 3.1.30 D**) remained capable of forming a complex sedimenting to the high-density region of the sucrose gradient. Therefore, while Bid may interact with the complex potentially to be cleaved by caspase-8 and act as the intermediate step between the complex and the downstream mitochondrial pathway, it does appear unlikely that Bid is a core component of the complex and the complex forms independently of Bid. Furthermore we Western blotted the HCT.shDR5 5-FU treated sucrose gradient for Bid and found that in the absence of DR5, Bid was not prevented from sedimenting with caspase-8 and FADD (**Fig. 3.1.30 E**).

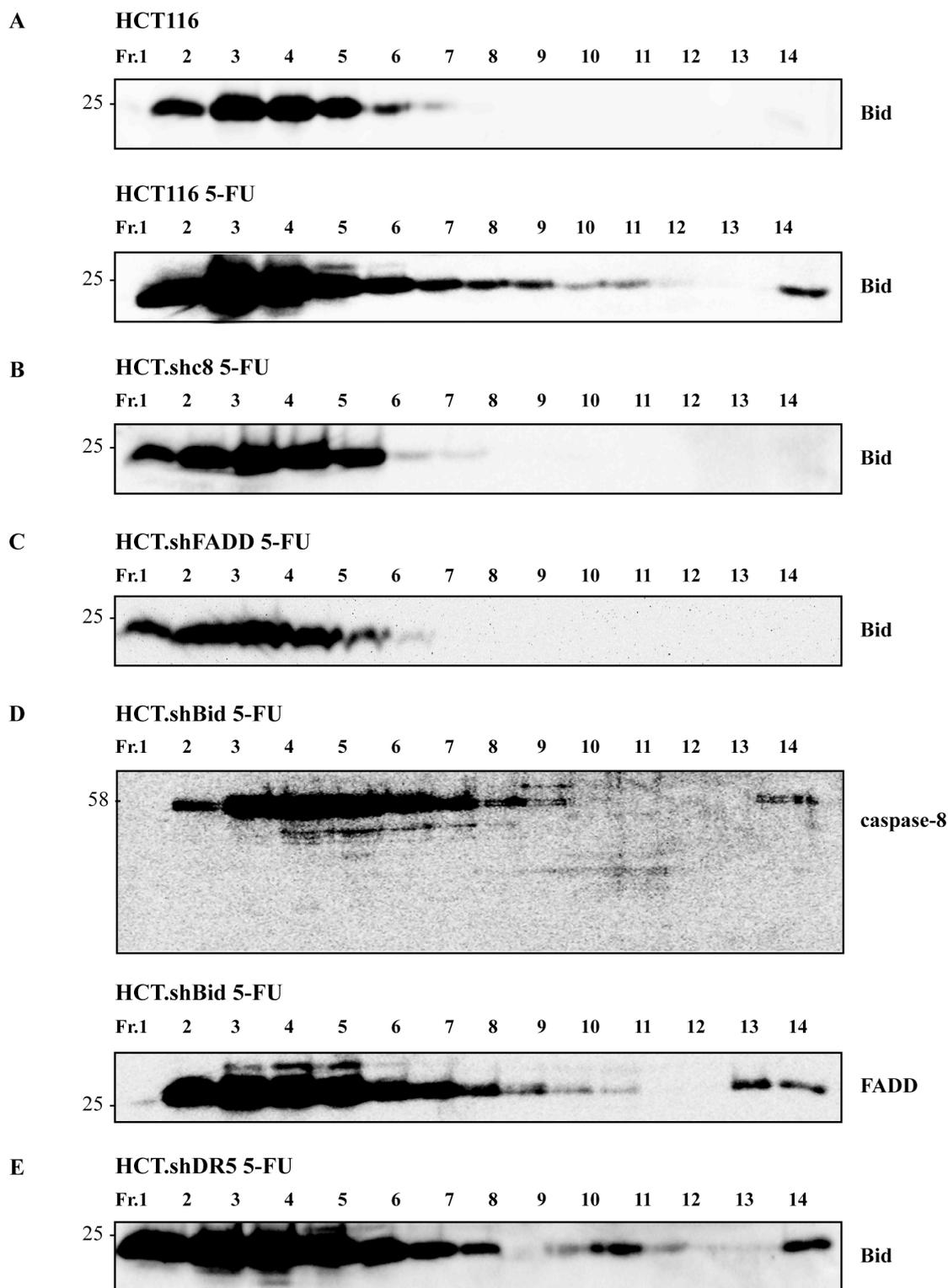


Fig. 3.1.30 Bid co-sediments with the caspase-8 complex but is not required for complex formation (A) HCT116 cells were stimulated with 5-FU (400 μ M) for 15 h or unstimulated as control. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for Bid. (B) HCT.shc-8 cells were stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for Bid. (C) HCT.shFADD cells were stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for Bid. (D) HCT.shBid cells were stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for caspase-8

(BD) and FADD respectively. (E) HCT.shDR5 cells were stimulated with 5-FU (400 μ M) for 15 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for Bid. Molecular weight markers are indicated. Representative of n = 2 experiments.

3.1.2.14 *RIP1 is a fourth putative complex member, found to co-sediment within the sucrose gradient and physically bind to FADD following 5-FU*

While testing for a panel of relevant apoptosis-related proteins in our gradient, we found that RIP1 co-sedimented to fractions 13 and 14 following 5-FU treatment with the other complex members but not in the untreated gradient (**Fig.3.1.31 A**). In the absence of caspase-8 (**Fig.3.1.31 B**) and FADD (**Fig. 3.1.31 C**), RIP1 was not present in the high-density fractions, which suggests it could potentially be a member of this complex. We also tested for RIP1 movement in the shBID (**Fig.3.1.31 D**) and shDR5 (**Fig. 3.1.31 D**) and found that, similar to caspase-8 and FADD, RIP1 sedimented to fractions 13 and 14 despite the absence of Bid or DR5.

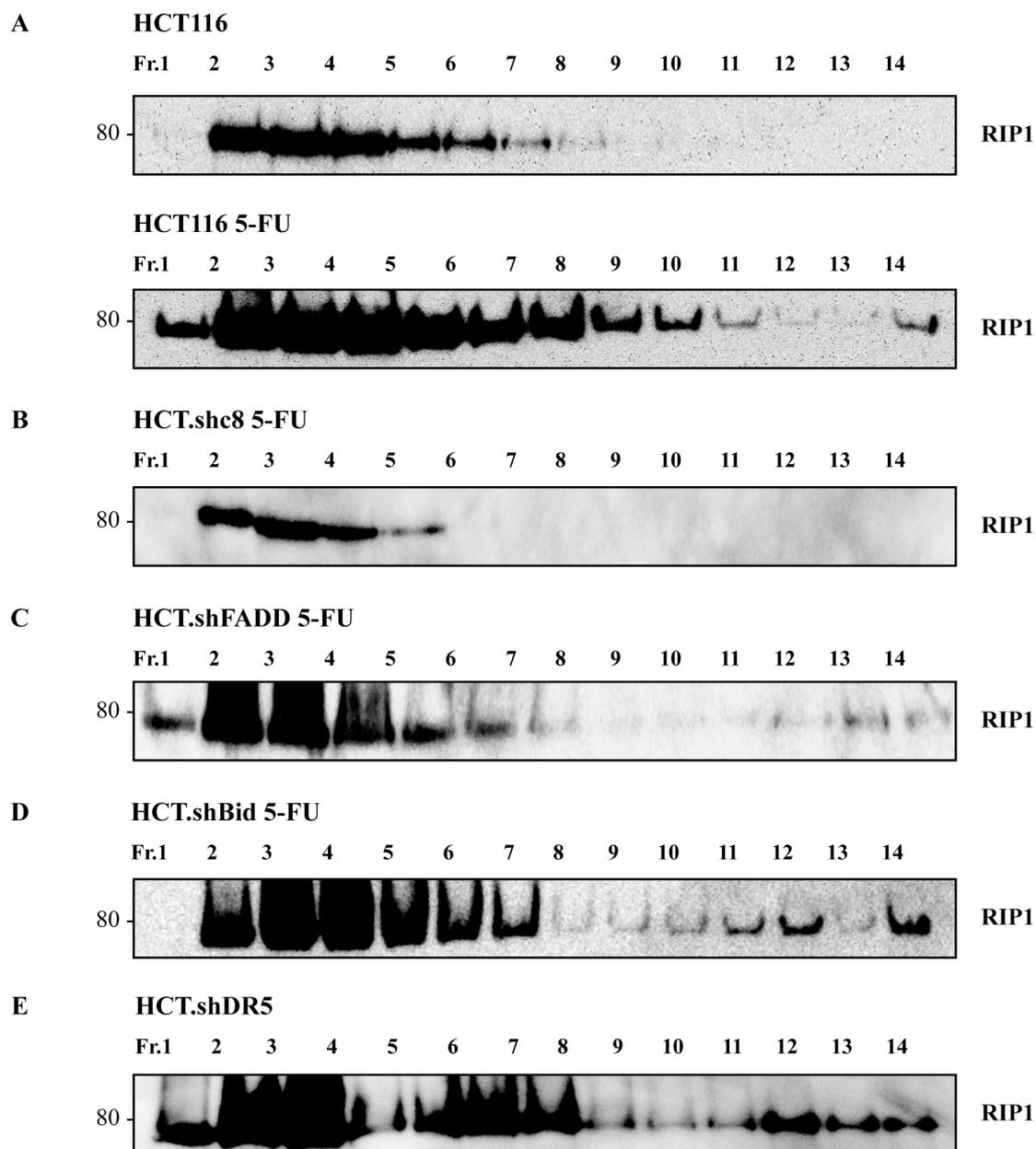


Fig. 3.1.31 RIP1 is a fourth putative complex member, found to co-sediment with caspase-8 and FADD (A) HCT116 cells were stimulated with 5-FU (400 μ M) for 15 h or untreated as control. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for RIP1. This was repeated using (B) HCT.shc-8 cells, (C) HCT.shFADD cells, (D) HCT.shBid cells and (E) HCT.shDR5 cells. Molecular weight markers are indicated. Representative of n = 2 experiments.

Recent reports have shown that caspase-8 and RIP1 can form a protein complex following cytotoxic drugs such as etoposide. We began to further investigate the potential complex member RIP1. To this end, we examined the physical interaction between RIP1 and the important adaptor molecule FADD, as their interaction has been previously reported (Zhang et al. 2011a). We transiently transfected FADD-

Myc/Flag in our HEK293 cell model, as HEK293 cells expressed relevant amounts of RIP1 compared to HCT116 cells (**Fig.3.1.12 A**). We then either treated with 5-FU (400 μ M, 15 h) 24 h post-transfection, or left untreated as a control. The lysates were immunoprecipitated for Myc and then subjected to Western Blotting for RIP1. We observed that following 5-FU treatment, RIP1 was co-immunoprecipitated with FADD (**Fig.3.1.32**). This blot was re-probed with FADD to control for total protein input. These results allow us to postulate that RIP1 is a component of the 5-FU-induced protein complex in HCT116 cells however further investigation into its exact role is required. The over expression of RIP1 and generation of HCT.shRIP clones may give further insight into the importance of RIP1 in this cascade. However these experiments were beyond the scope of the present study.

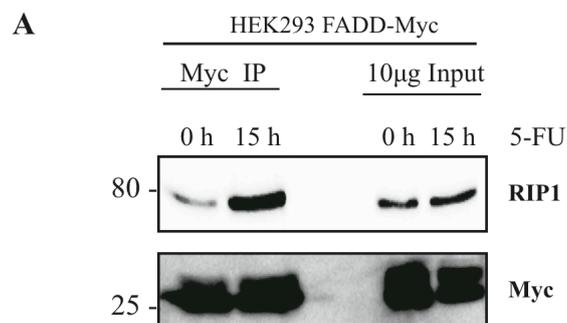


Fig. 3.1.32 FADD and RIP1 physically interact following 5-FU stimulation (A) HEK293 cells were transfected with a plasmid containing Flag/Myc-tagged FADD. 4-6 h post-transfection the cells were stimulated with 5-FU (400 μ M) for 15 h. The cellular lysates were then subjected to a Myc IP and subsequently analysed by Western blot for RIP1. This blot was re-probed with Myc as control. Molecular weight markers are indicated. Representative of n = 3 experiments.

3.1.2.15 5-FU induces RNA stress in HCT116 cells and addition of Uridine can rescue the cells from apoptosis

It has been reported that the 5-FU metabolite FUTP is incorporated into RNA to a large extent, resulting in a disruption of RNA processing and function. This has been found to closely correlate with the loss of clonogenic potential in various cell lines, including those of colon cancer. This result was mirrored in several studies, which led to detrimental effects on cellular metabolism and viability (Longley & Johnston, 2007). Previously, the damage caused by 5-FU was thought to be caused by DNA damage however reports have demonstrated that the effects of 5-FU are mainly

mediated through RNA stress.

In the present study, we sought to identify the role of RNA stress in our complex formation. To this end we co-treated HCT116 cells with 200 μ M 5-FU and 200 μ M Uridine or Thymidine for 48 h. We employed untreated cells, and cells treated with either 5-FU or Uridine or Thymidine alone as control. We found that apoptosis caused by 5-FU after 48 h reached levels of up to 35%, which was reduced to less than 5% when Uridine was added in addition (**Fig. 3.1.33 A**). In the samples treated with Thymidine, the DNA base, we observed that addition of Thymidine could not rescue the cells from apoptosis. These results suggest the cells are dying mainly through an RNA stress-induced mechanism. When we examined caspase-8 and -3 cleavage following 5-FU plus Uridine or Thymidine via Western blot we found that caspase-8 and -3 cleavage were markedly reduced (**Fig. 3.1.33 B**). We also found that addition of Uridine prevented complex formation within the sucrose gradient (*Mohr et al. unpublished data*). These results demonstrate that Uridine, but not Thymidine can rescue 5-FU treated HCT116 cells from apoptosis, and that the effects of 5-FU are mainly induced through RNA-damage. These findings are in line with previous reports (Pritchard et al. 1997), and in addition we could demonstrate that Uridine rescues the cells by inhibition of caspase-8 cleavage and complex formation.

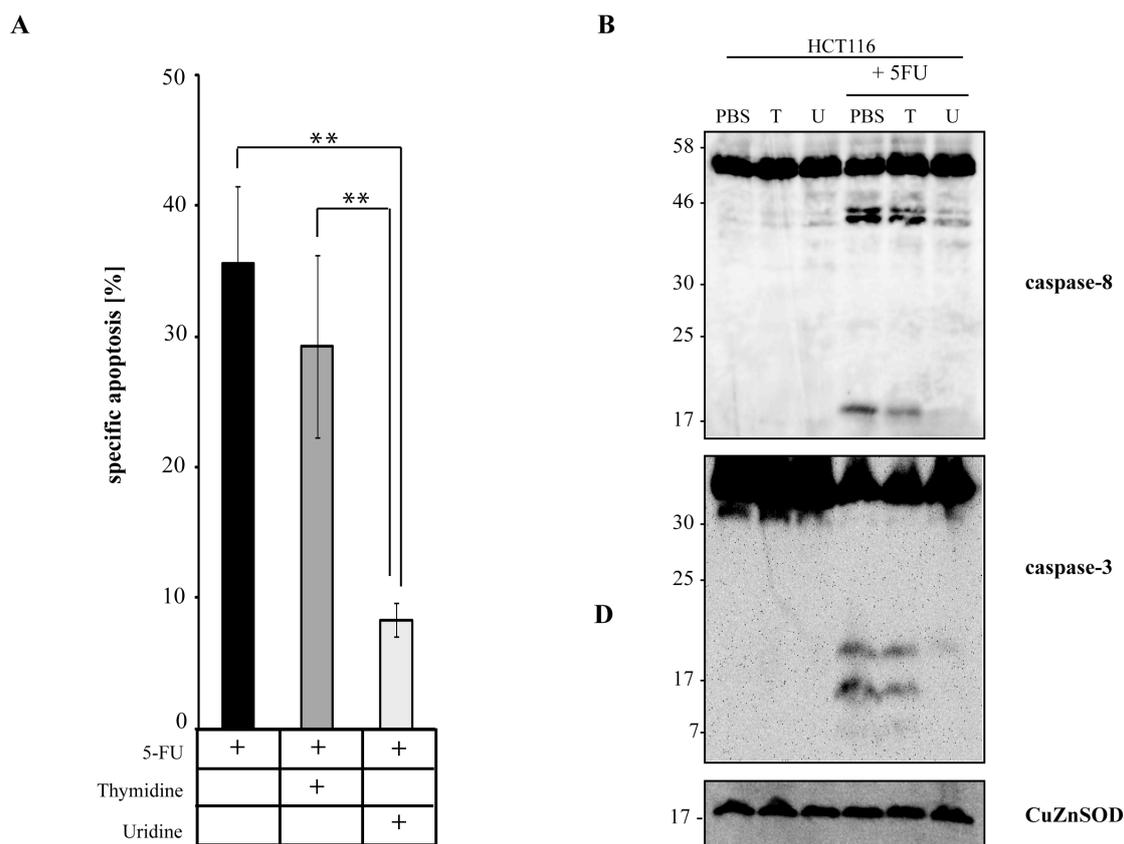


Fig. 3.1.33 5-FU induces RNA damage-induced stress in HCT116 cells and surplus Uridine can rescue the cells from apoptosis. (A) HCT116 cells were stimulated with 5-FU (200 μ M) alone, 5-FU (200 μ M) in the presence of Thymidine (200 μ M) or 5-FU (200 μ M) in the presence of Uridine (200 μ M) for 48 h. Cell death was characterised by a Nicoletti Assay as described in Chapter 2. (B) HCT116 cells were stimulated with 5-FU (200 μ M) alone, or 5-FU (200 μ M) in the presence of Uridine (200 μ M) for 48 h. Cell lysates were analysed by Western blot for caspase-8 (CS) and (D) caspase-3. ** = $P < 0.001$. Representative of $n = 2$ experiments. **Note:** Western blot for caspase-8 and -3 were generated by Dr. Andrea Mohr.

3.1.2.16 The role of p53 in 5-FU induced caspase-8 cleavage and complex formation

A final avenue to be explored in investigating the molecular mechanism of action of 5-FU was that of the diversely functioning tumour suppressor protein p53. There is a critical balance between cell-cycle arrest and cell death following certain environmental and genotoxic stresses such as chemotherapy, and p53 plays a central role in regulation of both (Vogelstein et al. 2000). The gene encoding p53, TP53, is among the most commonly observed mutations in human cancers, where approximately 50% of all tumours estimated to carry a mutation or an altered form of p53 (Ju et al. 2007; Levine 2000). It is expressed at relatively low basal levels within the cell, however in response to genotoxic insults such as treatment with anti-cancer

agents, p53 is rapidly induced and functions as a transcriptional activator. DNA or RNA damage results in activation of upstream kinases such as ATM, ATR, and DNA-PK, which can directly or indirectly activate p53 (Ljungman, 2000). However, depending on cellular context, p53 can trigger elimination of damaged cells by promoting apoptosis through the up-regulation of proapoptotic genes such as Bax, TRAIL-R2, and CD95 (Miyashita et al. 1994; Petak et al. 2000; Schuler & Green 2001; Yu et al. 1999). In addition it has been demonstrated that p53 and p53-target genes are activated in response to RNA-directed 5-FU cytotoxicity (Longley et al. 2002). Since we and others have already demonstrated that it is mainly RNA damage which is responsible for the apoptosis induced by 5-FU in HCT116 cells, we sought to clarify the role of p53 in more detail.

Therefore we took advantage of the HCT116 p53^{-/-} clone (Bunz et al., 1999) to investigate the role of p53 in 5-FU-induced caspase-8 cleavage and complex formation. We treated HCT116 p53^{-/-} cells and HCT116 wild-type cells with 5-FU (200 μ M, 48 h) or left untreated as a control. **Fig. 3.1.34 A** demonstrates that the HCT116 p53^{-/-} clone harbours no detectable levels of p53 protein which cannot be upregulated by 5-FU stimulation, in contrast to the HCT116 wild-type counterpart. In addition we measured apoptosis and we observed that apoptosis was halved in comparison to HCT116 wild type cells (**Fig. 3.1.34 B**). This finding is in line with other *in vitro* studies which reported that loss of p53 function reduced chemosensitivity to 5-FU (Longley et al. 2002; Bunz et al. 1999). Despite the reduction in apoptosis, there is clearly an alternative p53-independent pathway of cell death present, which is mediating the remaining apoptosis. To investigate our complex in the context of p53-independent cell death we treated HCT116 WT and p53^{-/-} cells with 5-FU (200 μ M) for 6, 15, 24 and 48 h and subsequently Western blotted for caspase-8 (**Fig. 3.1.34 C**) and -3 (**Fig. 3.1.34 D**) respectively. We observed that there was little or no difference in the caspase-8 intermediate fragment between WT and p53^{-/-} cells. However, similar to blocking the mitochondrial pathway, we noted a decrease in the p18 fragment of caspase-8. Investigations into the p53-dependant pathway within our lab demonstrated that in the absence of p53, DR5 and WIP1 upregulation are impeded, resulting in abrogation of Bid phosphorylation and subsequent mitochondrial pathway inhibition. Therefore we hypothesised that the caspase-8 complex would still be intact in a p53^{-/-} setting.

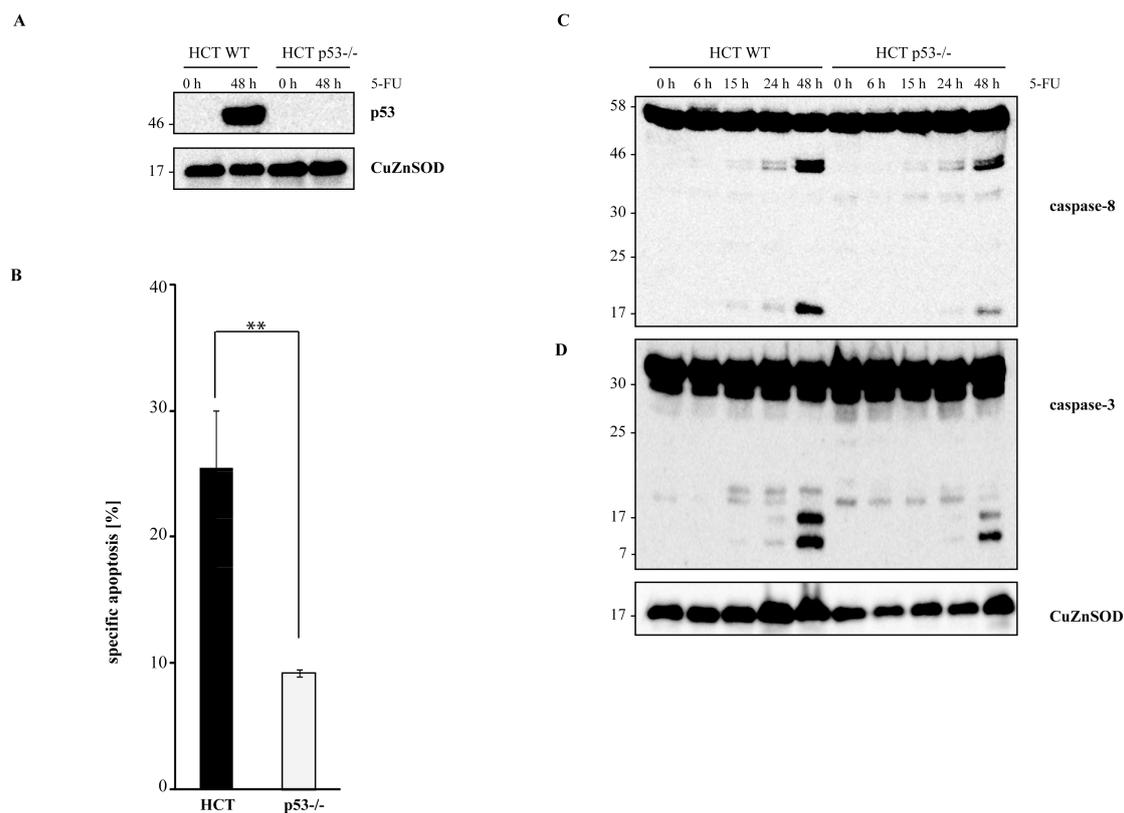


Fig. 3.1.34 P53 modulates the level of apoptosis (A) HCT116 and HCT116 p53^{-/-} cells were stimulated with 5-FU (200 μ M) or untreated as control for 48 h. Cellular lysates were analysed by Western blotting for p53. (B) Cell death was characterised by a Nicoletti Assay as described in Chapter 2. (C) HCT116 and HCT116 p53^{-/-} cells were stimulated with 5-FU (200 μ M) for 6, 15, 24 and 48 h. Cell lysates were analysed by Western blot for caspase-8 (CS) and (D) caspase-3. CuZnSOD served as a loading control and molecular weight markers are indicated. ** = $P < 0.001$. Representative of $n > 4$ experiments.

We next analysed a panel of apoptotic proteins to determine differences between HCT WT and p53^{-/-} cells. We treated HCT WT and p53^{-/-} cells with 5-FU (200 μ M, 48 h) and subsequently Western blotted for p53, CD95, DR5, caspase-9 and caspase-2 (Fig. 3.1.35). We demonstrated that there was a complete inhibition of CD95 upregulation in p53^{-/-} cells, again demonstrating that CD95 is not responsible for the 5-FU-induced apoptosis. Interestingly, DR5 upregulation was delayed by 24 h, however was not inhibited completely, pointing to a p53-independent upregulation of DR5 in HCT116 cells. Caspase-9 cleavage was delayed in the p53^{-/-} cells indicating the block is at the level of the mitochondria. Overall, these results pointed to a p53-independent regulation of caspase-8 cleavage following 5-FU stimulation.

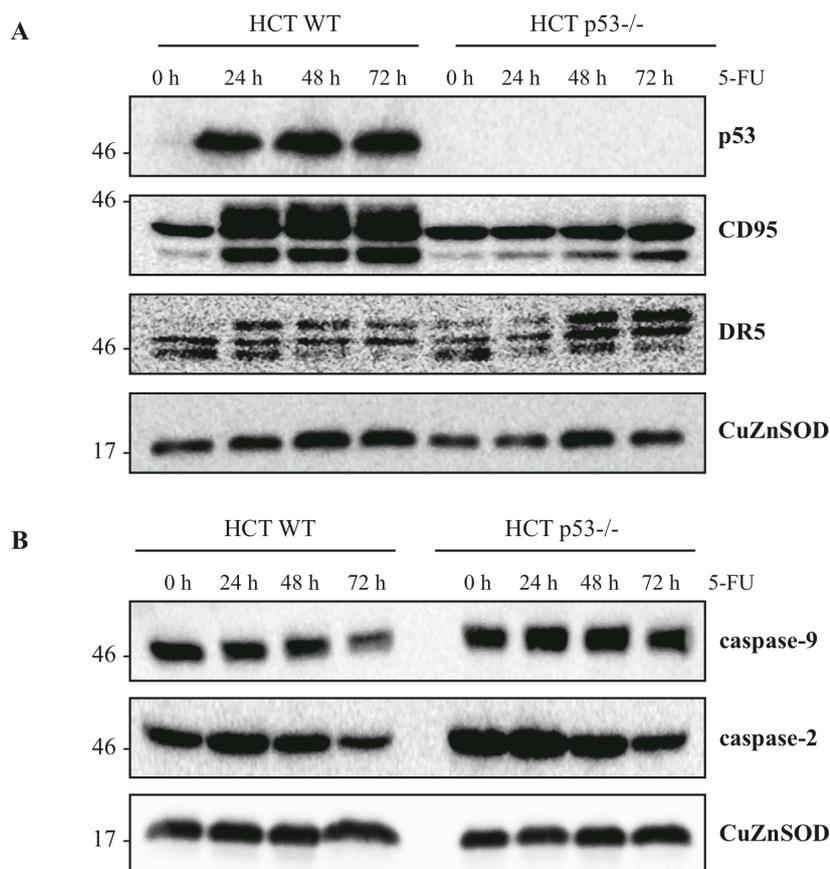


Fig. 3.1. 35 The absence of p53 prevents CD95 upregulation and delays DR5 upregulation following 5-FU (A) HCT116 and HCT116 p53^{-/-} cells were stimulated with 5-FU (200 μ M) or untreated as control for 24, 48 and 72h. Cellular lysates were analysed by Western blotting for p53, CD95, DR5 and (B) caspase-9 and caspase-2. CuZnSOD served as a loading control and molecular weight markers are indicated. Representative of n = 2 experiments.

Therefore, we then analysed our complex formation in the context of p53-independent cell death. We treated HCT116 p53^{-/-} cells with 5-FU (400 μ M, 15 h) and analysed the collected fractions by Western blotting for caspase-8, FADD, RIP1 and Bid (**Fig. 3.1.36 A**). The complex was capable of forming in a p53-independent manner.

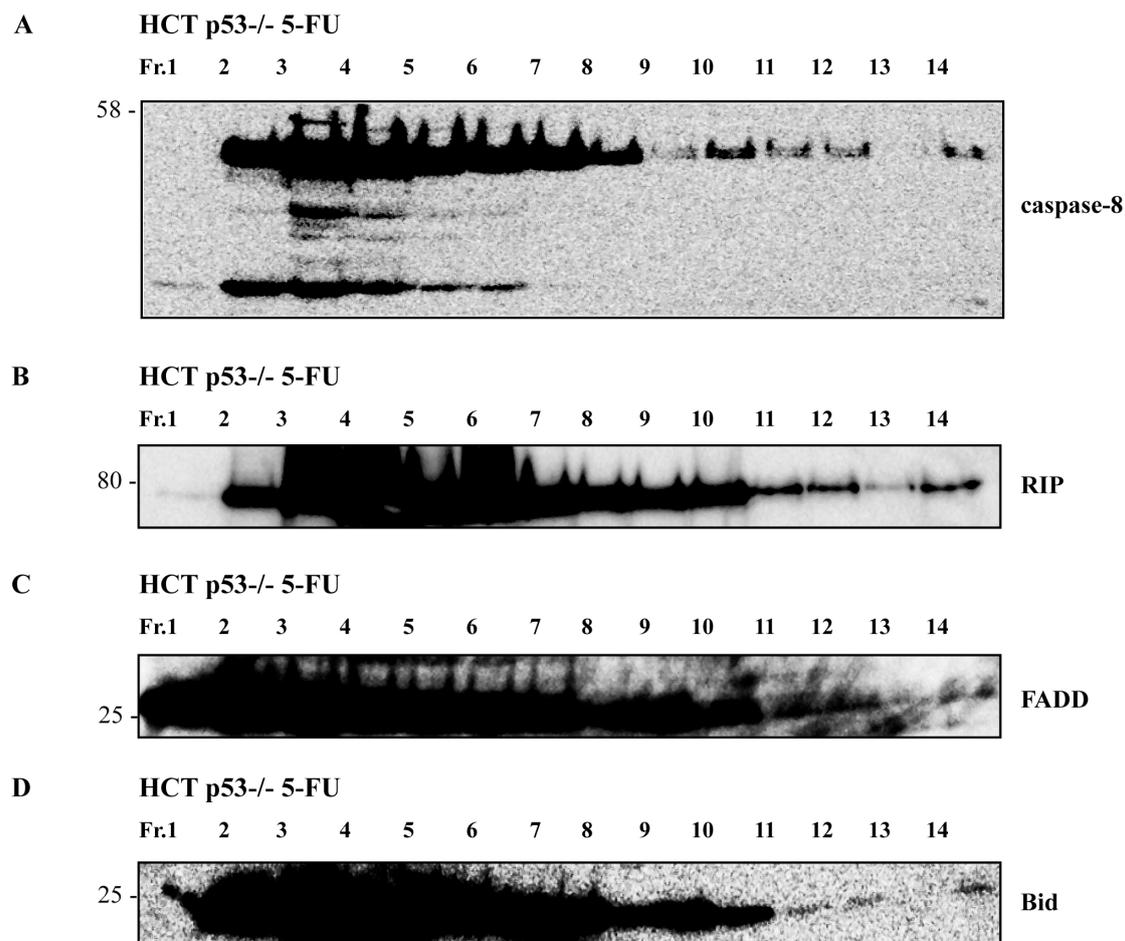


Fig. 3.1.36 The complex is capable of forming in the absence of p53 (A) HCT116 p53^{-/-} cells were stimulated with 5-FU (400 μ M) 24 h. Cellular lysates were used for sucrose gradient analysis. Numbers indicate fractions collected. Fractions 1-14 were analysed by Western blot for caspase-8 (BD), (B) FADD, (C) RIP1 and (D) Bid. Molecular weight markers are indicated. ** = $P < 0.001$. Representative of $n = 3$ experiments (A) and $n = 2$ experiments (B, C, D).

These results indicate that p53 is indeed involved in the overall effect of 5-FU-induced apoptosis by expediting the process however it does not appear to be accountable for the formation of the caspase-8 complex.

3.1.3 DISCUSSION

In the present study we investigated the molecular apoptotic events activated in cytotoxic drug-induced apoptosis by using 5-FU as an exemplary cytotoxic drug in our experiments. We employed the use of the colon cancer cell line, HCT116, as it is a widely used cell line, sensitive to a number of apoptosis-inducing agents (Yamaguchi et al. 2003; Galligan et al. 2005) and the availability of the HCT116 p53^{-/-} cell line, allowed us to easily study the effects of the absence of p53 within our system. The cell death pathway induced by 5-FU in HCT116 cells has been studied in recent years to some extent by various reports (Olsson et al. 2009). However while different proteins have been associated with the pathway, the exact mechanism has yet to be elucidated. The importance of this study lies with the understanding of the mechanism of action behind such chemotherapies as 5-FU. Only by elucidating their molecular mechanism, can we exploit this knowledge by implementing much needed improvements to our currently available cancer therapies. By exploring the proteins responsible for the cell death induced by drugs such as 5-FU we can better design strategies to target these molecules in the case of resistance.

Firstly, we set out to demonstrate that the cell death induced by 5-FU is caspase-dependent. We employed the pan caspase inhibitor Z-VAD-FMK. Apoptosis was completely inhibited on the addition of this compound (**Fig. 3.1.1 A**). Once we had established that the cell death was indeed an apoptotic caspase-dependent process, we wanted to investigate not only the signalling pathway that ensues but also more importantly the initial protein complex formed in this system, responsible for the apoptotic cascade. Therefore we continued to dissect the molecular pathway of apoptosis to determine, not only the initial caspase activated but also to identify putative complex members in this cascade.

We firstly demonstrated the cleavage of the three initiator caspases, -2, -8 and -9 following 5-FU (**Fig. 3.1.1 B**) and furthermore sought to identify which of the three caspases was the initial caspase cleaved and thereby responsible for the subsequent cleavage of the remaining caspases. Previous studies have placed caspase-2 activation upstream of the mitochondria, as a regulatory enzyme able to trigger cytochrome c release (Vakifahmetoglu et al. 2006). However caspase-2 is a controversial initiator caspase candidate for a number of reasons. While caspase-2 is considered to be a

bona fide initiator caspase due to its long prodomain, similar to caspase-8 and -9 (Samraj et al. 2007), the sequence of caspase-2 is also thought to more closely resemble a proinflammatory rather than proapoptotic initiator caspase (Kitevska et al. 2009). It is also difficult to assign a function to caspase-2 as a regulatory or downstream protease as the cleavage specificity is more related to effector caspases (Thornberry et al. 1997) and the lack of an overt phenotype of caspase-2 knock-out mice (Bergeron et al. 1998; O'Reilly et al. 2002). Therefore there have been conflicting studies regarding the role of caspase-2. Various biochemical studies using RNAi or antisense strategies have found caspase-2 activation to be upstream of mitochondria and cytochrome c release (Lassus et al. 2002; Robertson et al. 2002), and demonstrated that caspase-2 might act as an apical protease in stress or death receptor mediated apoptosis. However, in conflicting studies it was demonstrated that caspase-2 is activated downstream of Bax and Bak and cannot bypass the apoptosome. Previous experimental results in our lab using Ad.shcaspase-2 in HCT116 cells found that knocking down caspase-2 had no bearing on caspase-8 cleavage or on apoptosis rates following 5-FU treatment (Mohr et al., unpublished data). We also observed that there was no movement of caspase-2 as part of a large molecular weight protein complex, to the high sucrose density region of our sucrose gradient, following 5-FU treatment (**Fig. 3.1.8 D**). This is in stark contrast to the movement of the other complex members (discussed in more detail below). Therefore we concluded that caspase-2 did not appear to be pertinent to this study, as there was no evidence to suggest it to be a putative partner in our protein complex and could also be ruled out as an initiator caspase. Therefore we did not investigate it further at this stage.

In addition to caspase-2 cleavage following 5-FU treatment in HCT116 cells, we also identified cleaved forms of caspase-8 and -9 (**Fig. 3.1.1 B**). Caspase-9 has long been considered to be the initiator caspase of drug/genotoxic stress-induced apoptosis, whereas caspase-8 has been affiliated with death receptor-mediated apoptosis. In favour of caspase-9 as the initiator caspase, numerous reports have demonstrated its role. The initial characterisation of caspase-9 and Apaf-1 null fibroblasts or embryonic stem cells, were found to be resistant to a broad range of cytotoxic agents (Cecconi et al. 1998; Kuida et al. 1998; Yoshida et al. 1998). Following these studies, in 2007, Samraj *et al.* identified a caspase-9 deficient Jurkat clone, which was

resistant to genotoxic stress-induced apoptosis, and no caspases were activated in response to DNA damage. However following caspase-9 restoration, apoptosis was functional once more. It was also reported in this study that caspase-9 could induce processing of caspase-2 and -8 in some experimental systems (Samraj et al. 2007).

However, subsequent studies hinted at the possibility of the existence of additional context-specific pathways that could operate without caspase-9 or apaf-1 (Marsden et al. 2002). We demonstrated that the down-regulation of caspase-9 by stable knockdown in HCT116 cells had no impact on caspase-8 or -3 cleavage (**Fig. 3.1.4**). This finding was further confirmed by repeating the experiment using transient transduction of Ad.shcaspase-9 in HCT116 cells (**Fig. 3.1.2**), and furthermore, overexpressing an Ad.DN-caspase-9 construct (**Fig. 3.1.3**).

The previous studies (Samraj et al. 2007; Olsson et al. 2009), and the results of the present study, suggested that caspase-2 and -9 could be ruled out as the apical caspases in this cascade. Caspase-8 remained to be examined, therefore we generated a stable knockdown clone of caspase-8 in HCT116 cells (**Fig. 3.1.6 A**) and observed an inhibition of apoptosis (**Fig. 3.1.6 B**) and caspase-3 cleavage (**Fig. 3.1.6 C**) in this clone. Furthermore we demonstrated similar results by transiently expressing Ad.shcaspase-8 in HCT116 cells (**Fig. 3.1.5**). From these findings we could conclude that caspase-8 is the most apical caspase cleaved in response to 5-FU in HCT116 cells, and it is crucial to this pathway, in contrast to caspase-2 and -9.

Previous studies within our lab had identified caspase-8 as a member of an unidentified high molecular weight protein interaction complex induced by 5-FU in HCT116 cells using gel filtration analysis (Mohr *et al.*, unpublished data). Therefore in addition to caspase-8 being the initial caspase in this cascade, we further investigated caspase-8 as a member of this undescribed complex. We employed the technique of sucrose gradient fractionation as described by Feig *et al.* (2007) to easily visualise complex member movement/sedimentation from the fractions correlating to low molecular weight proteins to the high molecular weight fractions containing the high density sucrose concentrations, and identified the presence of caspase-8 in the high density fraction “14” upon 5-FU induction (**Fig. 3.1.7 B**). There was no visible caspase-8 present in fraction 14 in the unstimulated control (**Fig. 3.1.7 A**). As an additional control to verify the movement of caspase-8 in our sucrose gradient we employed Z-VAD-FMK. As previously mentioned, the addition of Z-VAD-FMK

completely inhibited the apoptosis induced by 5-FU, due to the binding of Z-VAD-FMK with caspase-8 preventing caspase-8 forming a complex with FADD. When Z-VAD-FMK (5 µg/ml) was added to the cells at the same time as 5-FU (400 µM) caspase-8 movement was abolished (**Fig. 3.1.7 C**). As a control, a second gradient was run where 15 h post-5-FU, Z-VAD-FMK was added 15 min prior to harvesting. In this case caspase-8 movement was unhampered (**Fig. 3.1.7 D**). Therefore we could conclude that the complex formation/movement was dependent on the interaction between caspase-8 and FADD. In addition to demonstrating that the high molecular weight complex was caspase-8-dependent, we also questioned whether the complex resided in the cytosolic or the membrane-bound fractions. Therefore prior to loading the 5-FU-treated lysate to the sucrose gradient, the lysate was separated into its cytosolic and membrane fractions. These were subsequently run on two separate gradients, and Western blotted for caspase-8. In **Fig. 3.1.8 A** we demonstrated that there was no complex movement visible within the membrane fraction, however there was monomeric forms of caspase-8 present. In contrast when we looked at the cytosolic fraction, we observed full complex movement therefore it appears from this preliminary result that the complex is mainly located in the cytosol of the cell. Before we could continue to investigate additional members of this complex we sought to establish a caspase-8 immunoprecipitation. This would act to enrich the caspase-8 protein amounts aiding us in visualising co-immunoprecipitated partners. **Fig. 3.1.9** demonstrated (**A**) the protein amount optimisation, (**B**) the optimal time of immunoprecipitation and (**C**) the immunoprecipitations were then validated for specificity using the HCT.shc8 clone. We also employed bead only and IgG controls to demonstrate the binding of caspase-8 was specific (**D**).

Once we established the caspase-8 immunoprecipitation conditions and were satisfied that the caspase-8 movement was genuine and caspase-8 was a crucial mediator of 5-FU-induced apoptosis and a protein member of the 5-FU-induced complex, we continued to investigate other putative complex members and their role in the apoptotic cascade.

A second protein, FADD, was identified in the early gel filtration experiment within our group and FADD is a common adapter molecule, and protein partner associated with caspase-8 in death-receptor mediated apoptosis. Therefore we dissected the potential role of FADD in our pathway. The prominent role of the FADD molecule

was initially highlighted by generation of FADD mutant null mice (Yeh et al. 1998; Zhang et al. 1998). Similar to knocking out of caspase-8, FADD knockout mice were not viable, and FADD null embryos died in utero at day 12.5 of development, due to underdevelopment, abdominal haemorrhage and cardiac failure (Tourneur et al. 2005). These results indicated that in addition to its well-known role in cell death, FADD was implicated in survival and proliferation of some cell types (Tourneur et al. 2005). FADD is the main signal transducing intermediate adaptor molecule of several death receptors including Fas, TNF-R1, DR3, TRAIL-R1 and TRAIL-R2 (Chinnaiyan et al. 1995; Yeh et al. 1998; Zhang et al. 1998; Kuang et al. 2000). All of these receptors possess in their intra-cytoplasmic tail, a DD homologous to the DD of FADD, allowing FADD recruitment to the activated receptor. FADD can be recruited directly to Fas and TRAIL Receptors, or indirectly to TNFR1 via another DD-containing adaptor molecule TRADD (Tourneur et al. 2005). FADD then recruits DED-containing initiator procaspase-8 or -10 through DED/DED interactions (Nagata 1999; Kischkel et al. 2001; Wang et al. 2001) thus forming the DISC (Kischkel et al. 1995). Auto processing of the initiator procaspase leads to activation of effector caspases causing the apoptotic death of the cell (Muzio et al. 1996; Martin et al. 1998).

In terms of what is already known about the role of FADD in cytotoxic drug-induced apoptosis, its over-expression was demonstrated to enhance 5-FU-induced apoptosis in colorectal adenocarcinoma cells (Yin et al. 2010), and also Micheau *et al.* investigated the role of FADD in cytotoxic drug-induced apoptosis and found that FADD over expression sensitised the colon cancer cell line HT-29 to cisplatin-induced cytotoxicity. Furthermore, etoposide and cisplatin chemotherapeutic drug-induced apoptosis in addition to taxol pre-treatment, was inhibited by over expression of an unphosphorylatable FADD mutant (Shimada et al. 2004), therefore tumour cells that express the Ser194 FADD mutant (incapable of being phosphorylated) or unable to phosphorylate FADD are expected to resist apoptosis induced by anticancer drugs that induce G2/M arrest, and to be insensitive to the synergistic effects of chemotherapy (Tourneur et al. 2005). It would be conceivable therefore, that a complete lack of FADD expression could have the same consequences and we demonstrated that this was indeed the case in the present study.

Another study to examine the important role of FADD and the consequences of its

absence was undertaken by Tourneur *et al.* who used a mouse model of thyroid adenoma/adenocarcinoma to show spontaneous disappearance of FADD protein expression during the course of tumour development, raising the possibility that loss of FADD could be an additional event contributing to tumourigenesis. Following this study, in 2004, the same group demonstrated that absence of the FADD protein expression in cancer cells is also a relevant phenomenon in human malignancies by looking at FADD expression in human acute myeloid leukemia (AML) cells, and found 2/3 patients at diagnosis expressed low or no FADD protein (Tourneur *et al.* 2004). This is an important finding as leukemic cells of most AML patients are resistant to Fas receptor (Iijima *et al.* 1997) and/or Fas Ligand (Buzyn *et al.* 1999). These reports highlighted the pivotal role FADD plays in apoptosis sensitivity of cancer cells.

To investigate the role of this important molecule in 5-FU-mediated cell death in HCT116 cells, we generated shFADD clones (**Fig. 3.1.10 A**), and found that apoptosis was almost completely abolished (**Fig. 3.1.10 B**) and caspase-8 (**Fig. 3.1.10 C**) and -3 (**Fig. 3.1.10 D**) cleavage was inhibited. Notably caspase-8 intermediates were also almost completely absent. These findings were further confirmed in HCT116 cells and HT-29 cells using transient expression of adenoviral shFADD plasmid (data not shown). These findings were in line with the study by Micheau *et al.* who demonstrated that antisense FADD expression was able to protect HT-29 cells from cisplatin-induced apoptosis (Micheau *et al.* 1999). Furthermore we detected the presence of FADD in the same high-density fraction of our sucrose gradient as caspase-8 (**Fig. 3.1.11 B**). This correlated closely with the initial gel filtration chromatography data obtained previously within our lab (Mohr *et al.*, *unpublished data*). To confirm that FADD was indeed bound to caspase-8 as part of a protein complex following 5-FU treatment, we employed the HCT.shFADD clone. 5-FU treatment of these cells demonstrated that in the absence of FADD, caspase-8 did not sediment to the high-density fraction of the gradient (**Fig. 3.1.11 C**), suggesting the entire complex requires the presence of FADD to form. Furthermore, when utilising our HCT.shcaspase-8 clone, we found that FADD movement in the sucrose gradient following 5-FU was prevented by a lack of caspase-8 (**Fig. 3.1.11 D**). A further proof of the protein-protein interaction of caspase-8 and FADD following 5-FU could be demonstrated by the caspase-8/FADD co-immunoprecipitation (**Fig. 3.1.13**). This

experiment demonstrated that the two molecules physically interact following 5-FU treatment in a HEK293 cell model overexpressing Full Length-FADD. Caspase-8 and FADD have previously been demonstrated to be common binding partners and their mode of interaction has been demonstrated in detail, whereby the molecular model for the interaction was demonstrated to be a physical interaction between the FADD DED and, one, but not both, of the DEDs resident in the prodomain of caspase-8 (Carrington et al. 2006). In addition very recently two independent groups identified a 2MDa complex, termed the Ripoptosome (Tenev et al. 2011; Feoktistova et al. 2011). The Ripoptosome, similar in ways to the complex we have identified in the present study, contains the core molecules caspase-8 and FADD, which they identified by co-immunoprecipitation and gel filtration analyses following etoposide treatment. Therefore there is already ample evidence to suggest that FADD and caspase-8 form part of a high molecular weight complex induced by genotoxic stress. Due to the effect that loss of FADD expression could have on the chemotherapeutic action of 5-FU, research on how to counteract this phenomenon in cancer therapy may be clinically relevant. One potential method of restoring 5-FU sensitivity in the absence of FADD expression is the administration of Carboplatin. Carboplatin (a derivative of cisplatin) is a platinum chemotherapeutic compound used mainly in the treatment of ovarian cancer. It causes inter- and intrastrand crosslinks which covalently link DNA, preventing transcription and regulation (Dhillon et al. 2011). It is also a drug which is potentially effective at re-establishing functional FADD protein expression as demonstrated by a study by Mishima *et al.* who found that treatment with carboplatin enhanced FADD expression in human tongue carcinoma cell lines which expressed low levels of FADD, and rendered them sensitive to Fas-mediated apoptosis (Mishima et al. 2003).

Following on from identifying FADD as an important mediator of the 5-FU-induced complex formation, we sought to identify the event that is responsible for complex assembly upon 5-FU stimulation. FADD recruits caspase-8 to its DED, therefore we questioned whether FADD could form a platform which then enables the recruitment of caspase-8 and subsequent complex members. To examine the event that could mediate this platform assembly we speculated that two or more FADD molecules may interact, forming the structural basis for complex assembly. Since the procaspases are reported to be recruited to the 'DISC' as a preformed dimer (Boatright et al. 2003),

the building blocks of the DISC could potentially require more than one molecule of FADD, one for each molecule of procaspase. Thus Carrington *et al.* speculated that a FADD homodimer forms, at, or between, activated death receptors to create the structural context necessary for procaspase recruitment and activation (Carrington *et al.* 2006). However, recent developments have highlighted a very different mechanism of FADD and caspase-8 binding, whereby one molecule of FADD can recruit up to 9-fold more caspase-8 molecules (Dickens *et al.* 2012). Therefore the stoichiometry of this interaction would require further investigation.

To investigate the possibility of 5-FU-induced FADD interaction we generated Myc/Flag and V5-tagged FADD plasmids and co-expressed them in HEK 293 cells. Using either a Myc/Flag (**Fig. 3.1.16 C & D**) or V5-immunoprecipitation (**Fig. 3.1.16 E**), with and without 5-FU treatment, we Western blotted for the opposite antibody in each case and found that following 5-FU, both tagged-FADDs were present. Whether the putative FADD/FADD interaction event takes place prior to caspase-8 recruitment or as a result of it is yet to be confirmed using the caspase-8 null background of the HCT.shcaspase-8 clone and these experiments are ongoing within the lab (*Mohr et al., unpublished data*).

In addition to the hypothesis that FADD/FADD interaction is possibly an initial event that acts as a platform for complex formation, we also wanted to investigate what event could stimulate the interaction of FADD molecules in this model. One such possibility could be a post-translational modification of FADD, such as glycosylation or phosphorylation. We chose to investigate the role of FADD phosphorylation in greater depth as previous studies have also suggested that the phosphorylation of FADD is crucial for anticancer drug-induced chemosensitivity (Shimada *et al.* 2004). Shimada *et al.* demonstrated that FADD was phosphorylated on treatment with paclitaxel (Shimada *et al.* 2004). This study found synergistic effects of etoposide or cisplatin with paclitaxel were significantly reduced in cells expressing a S194A mutant indicating phospho-FADD is closely related to chemosensitivity in prostate cancer cells. However apoptosis could be induced by lethal doses of etoposide or cisplatin alone even in S194A expressing stable clones, suggesting phosphorylation status of FADD is closely related to chemosensitisation but not to resistance to apoptosis induced by these two agents (Shimada *et al.* 2004). Our initial experiment could not find any correlation between 5-FU treatment and phospho-FADD up

regulation (**Fig. 3.1.14 F**). Previous reports raised the possibility that phosphorylation of FADD at ser194 is closely associated with sensitisation to chemotherapy-induced apoptosis (Gagandeep et al. 1999; Tishler et al. 1992; Geard et al. 1993). To investigate the phosphorylation that may aid in the FADD/FADD interaction, we generated a FADD mutant (FADD-V5-S194A), to establish the effect on the dimer formation. We had already demonstrated that phospho-FADD is present in the complex (**Fig. 3.1.17 A**) and that it is also present as part of the FADD/FADD interaction (**Fig. 3.1.17 B,**) but we have not demonstrated whether this is essential to the complex. We found that over-expression of the FADD-V5 Ser194A mutant did not appear to inhibit FADD interaction (**Fig. 3.1.17 C**) but we have yet to complete the experiments using both phospho-dead mutants simultaneously. We then hope to express them in the FADD^{-/-} background of our HCT.shFADD clones to further investigate their effect in HCT116 cell setting. In summary, FADD is a member of the protein complex induced by 5-FU in HCT116 cells, as in its absence the complex cannot form. It is an inducible binding partner of caspase-8 and it appears to form an inducible FADD/FADD interaction following 5-FU, which at the very least *contains* phospho-FADD, the necessity of which requires further investigation.

Due to the prominent role of caspase-8 and FADD in 5-FU-induced apoptosis in HCT116 cells, we subsequently investigated those death receptors not only known to commonly interact with FADD, but also those upregulated upon 5-FU stimulation. A mechanism of 5-FU-induced apoptosis has been reported in previous studies to be transduced via the Fas-mediated pathway, or that CD95 is an important modulator of the cell death induced by this drug (Borrallho et al. 2007). One reason behind this assumption is that CD95 protein levels are highly up regulated upon 5-FU treatment, due to the p53 up regulation, as CD95 is a p53 target gene (Müller et al. 1998). In line with this assumption, blocking of the CD95 pathway by silencing of CD95 using RNAi has been reported to decrease sensitivity to 5-FU in HCT116 cells (Borrallho et al. 2007). The molecule, FADD-DN has also been employed to demonstrate that the pathway is dependent on CD95 (Borrallho et al. 2007) however, FADD is a common adaptor molecule, one which has been demonstrated to be involved in two alternative 5-FU-induced apoptotic pathways in our lab (Mohr *et al.*, *unpublished data*), and therefore this cannot be used to exclusively point to blocking of the CD95 pathway.

In the present study we found that CD95 protein levels were significantly up regulated

following 5-FU treatment (**Fig. 3.1.18 C**) in a completely p53-dependent manner (**Fig. 3.1.35 A**). In a p53 null background of the HCT116 p53^{-/-} cells, the CD95 protein expression is maintained at a basal level, even after 72 h 5-FU (200 μ M). Despite this lack of CD95 up regulation, apoptosis levels in a p53^{-/-} HCT116 clone are only halved in comparison to their p53 wild-type counterpart (**Fig. 3.1.34 B**). This evidence alone leaves many discrepancies in the hypothesis that CD95 up regulation modulates 5-FU-induced apoptosis, and it appears that CD95 up regulation is simply a bystander effect of the induction of p53 protein. Furthermore, in our lab an adenoviral vector, Ad.shCD95, was used to demonstrate that knocking-down of CD95 in these cells had no effect of apoptosis levels, or on caspase-8 or -3 cleavage following 5-FU (*Mohr et al., unpublished data*). Further to this experiment, an antibody neutralising CD95L had no effect on 5-FU-induced apoptosis, but was capable of inhibiting CD95L-induced apoptosis under the same conditions (*Mohr et al., unpublished data*). This finding is in line with recent studies that demonstrated similar results neutralising CD95 activity (Tenev et al. 2011; Feoktistova et al. 2011).

Therefore, our investigations turned to another receptor, which is up regulated upon 5-FU treatment, via p53 transactivation, TRAIL receptor-2/KILLER/DR5. In HCT116 cells we observed DR5 to be highly up regulated at the protein level (**Fig. 3.1.18 B**) and also in a previous study in our lab, on the cell surface (Deedigan *et al.*, 2013; *Mohr et al., unpublished data*). DR4 was also up regulated on the cell surface but to a much lesser extent (Deedigan *et al.*, 2013; *Mohr et al., unpublished data*).

DR5 up regulation via chemotherapeutic agents has been demonstrated in a wide array of studies (Nimmanapalli *et al.* 2001; Shigano *et al.*, 2003; Nagane *et al.*, 2000; Bertaki *et al.*, 2007). In 2004, Wang and El-deiry demonstrated that silencing of this receptor increased tumour growth in a murine model *in vivo* and that silencing of this receptor rendered HCT116 cells more resistant to 5-FU by halving apoptosis rates (Wang & El-Deiry 2004).

In the present study, our results correlated closely with the results by Wang *et al.* We generated HCT.shDR5 and as a control HCT.shDR4 stable clones and observed HCT.shDR4 apoptosis rates were not affected (**Fig. 3.1.19 B**) and resulted in a more fully processed caspase-3 cleavage (**Fig. 3.1.19 D**). However caspase-8 cleavage was not attenuated by silencing of DR4 (**Fig. 3.1.19 C**). In contrast to this finding, HCT.shDR5 exhibited a reduction in apoptosis rates (**Fig. 3.1.20 B**). Caspase-8

cleavage to intermediates was nonetheless almost fully intact (**Fig. 3.1.20 C**), but caspase-3 processing was abrogated (**Fig. 3.1.20 D**).

Due to the fact that caspase-8, a known constituent of our complex, was largely unaffected by the knocking-down of DR5, and that there was still 50% of the apoptosis intact, yet caspase-3 cleavage was abrogated, we hypothesised that there was at least a second pathway occurring concurrently with our caspase-8-mediated pathway. Though investigation of this second pathway was beyond the scope of the present study, it was analysed further within our lab. To summarise the principle findings of this study, this second pathway was demonstrated to be p53-regulated. Through the p53-mediated up regulation of DR5, WIP1 was subsequently up regulated, and in turn activated the JNK signal transduction pathway. Both the DR5-dependent JNK-mediated pathway, and the caspase-8-dependent pathway were found to be able to occur independently of one another. In the absence of caspase-8, while there were no detectable levels of apoptosis, the JNK pathway was still activated (*Mohr et al., unpublished data*). Likewise, as evidenced by the findings in the present study, in the absence of DR5, the apoptosis was halved, but the caspase-8 cleavage and complex formation was still permitted. Both pathways converge at the level of Bid, and the DR5-dependent pathway acts as an apoptotic amplifier in 5-FU-induced apoptosis (*Mohr et al., unpublished data*).

In addition CD95, DR5 and DR4 were all tested in our sucrose gradient fractionation, and neither of the three receptors cosedimented with caspase-8 or FADD as part of our complex following 5-FU. However they were distinctly up regulated in comparison to the untreated control gradients (**Fig. 3.1.21 A, B & C**). To rule out that our caspase-8 complex was simply a form of complex II as described by Micheau *et al.*, and had originated from a receptor-dependent complex, we employed our HCT.shDR5 clone. When treated with 5-FU, the HCT.shDR5 clone was unable to prevent the movement of caspase-8 and FADD to the high-density region (**Fig. 3.1.22 A & B**). In addition to the caspase cleavage and sucrose gradient fractionation studies, we also established a DR5 immunoprecipitation, and overexpressed both Full-Length FADD and FADD-Myc/Flag in HEK 293 cells. However we were unable to co-immunoprecipitate FADD or caspase-8 with DR5 following 5-FU treatment in contrast to our caspase-8/FADD co-immunoprecipitations under similar conditions. In line with the findings of the present study, recent reports demonstrated the formation

of a protein complex termed the ripoptosome, composed of caspase-8 and FADD, following cytotoxic drug treatment, to form and to be independent of death receptors but to be upstream of the mitochondria, by employing the use of neutralising antibodies (Feoktistova et al. 2011; Tenev et al. 2011). Therefore we could rule out the involvement of death receptors, more specifically DR5, in our initiator caspase-8-dependent complex formation. However we did identify that a second DR5-dependent pathway exists and amplifies or contributes to the overall apoptotic signal induced by 5-FU in HCT116 cells.

To further rule out involvement of the death receptor dependent DISC mediated pathway of apoptosis, our focus then turned to FLIP. When bound by their natural ligands, death receptors (Fas/TRAIL/TNF) can trigger death signals within the cell (Griffith et al. 1998; Nagata 1999). This binding of receptor to its cognate ligand results in the recruitment of adaptor molecules, FADD being the most common example, which can in turn recruit procaspase-8 zymogens to the DISC (Chinnaiyan et al. 1995). A key inhibitor of this signaling pathway is c-FLIP, which inhibits caspase-8 recruitment and processing at the DISC (Krueger et al. 2001). Differential splicing gives rise to eleven known c-FLIP isoforms, the long (c-FLIP_L) and short (c-FLIP_S) forms, both of which are prominently expressed in cultured tumour cells (Budd et al. 2006). Both c-FLIP variants are thought to bind to FADD within the DISC. C-FLIP_S directly inhibits caspase-8 activation at the DISC, whereas c-FLIP_L is first processed to a truncated p43 form by caspase-8, that in turn inhibits the complete processing of caspase-8 to its active subunits (Longley & Johnston 2005).

The importance of c-FLIP as a regulator of apoptosis in cancer cells has been intensely investigated over the years. In a study by Ganten *et al.* the down-regulation of c-FLIP was identified as the mechanism of 5-FU-mediated sensitisation to rhTRAIL in hepatocellular carcinoma cells (Ganten et al. 2004). Then in 2006, Longley *et al.* observed RNAi targeting of c-FLIP dramatically sensitised a panel of colon cancer cell lines to 5-FU without any additional treatment with a death ligand (Longley et al. 2006). These studies suggested that c-FLIP plays an important role in regulating cancer cell chemo-sensitivity. Interestingly, c-FLIP is found over-expressed in a high percentage of colonic and gastric carcinomas (Ryu et al. 2001; Zhou et al. 2004). Numerous studies demonstrated that c-FLIP over-expression confers resistance to death-receptor mediated apoptosis (Irmler et al. 1997; Scaffidi et

al. 1997; Nam et al. 2003), and in addition, there were a number of groups who had analysed the effects of c-FLIP on chemotherapy-induced apoptosis. Micheau *et al.* found over-expressing viral forms (MC159 and E8) inhibited cisplatin-induced apoptosis in HT-29 cells (Micheau et al. 1999). Other groups found that c-FLIP protected against apoptosis by a range of chemotherapies (Matta et al. 2002; Conticello et al. 2004). However, in contrast to these reports, Kataoka *et al.* observed that c-FLIP could not protect Jurkat T cells (Kataoka et al. 1998).

In the present study, we employed adenoviral transient over-expression of c-FLIP (**Fig. 3.1.24 A**) to evaluate its role in 5-FU-induced apoptosis and more specifically, caspase-8 cleavage in HCT116 cells. We did not observe any inhibition of apoptosis (**Fig. 3.1.24 B**) or of subsequent caspase-8/-3 cleavage (**Fig. 3.1.24 C & D**). In addition, when we Western blotted for endogenous c-FLIP using 5-FU treated HCT116 cell sucrose gradient fractions, we could not detect endogenous c-FLIP co-sedimenting with our complex members (caspase-8, FADD, Bid or RIP1) (**Fig. 3.1.24 E**) demonstrating that c-FLIP is not part of our complex.

Having thoroughly investigated the molecular events upstream of the mitochondria, and ruling out the involvement of the common death receptors in the formation of this complex, we continued to investigate downstream of caspase-8 cleavage by concentrating on the important mediators of the mitochondrial pathway. It has long since been demonstrated that Bcl-2 family proteins have been involved in chemotherapy-induced apoptosis (Harris 1996; Nita et al. 1998). Such members as Bcl-2 and Bcl-xl have the ability to block cell death and in contrast over-expression of Bax, Bak and Bad, among others, have been shown to induce apoptosis (Strobel et al. 1996).

Bcl-2, as a potent inhibitor of apoptosis, has the potential to render cancer cells resistant to traditional cytotoxic chemotherapy, radiotherapy and monoclonal antibodies, and hence elevated levels of Bcl-2 protein have been correlated with poor response to chemotherapy in a variety of cancer cell types, for example Non-Hodgkin's Lymphoma, acute myelogenous leukemia, and prostate cancer (Herbst & Frankel 2004). In line with these studies, we demonstrated that when we over-expressed Bcl-2 in HCT116 cells, either via transient adenoviral vector delivery of the plasmid (**Fig. 3.1.25 A**) or through generation of the stable HCT.Bcl-2 clone (**Fig. 3.1.26 A**), that it conferred complete 5-FU resistance to the cells (**Fig. 3.1.25 B & Fig.**

3.1.26 B). This resistance was located downstream of caspase-8 cleavage at the mitochondrial level as evidenced by caspase-8 cleavage to its intermediates and a block in full caspase-3 cleavage (**Fig. 3.1.25 C & D**). Therefore the mechanism of action of 5-FU could potentially be ineffective in the presence of high cellular Bcl-2 levels. One way in which this could be circumvented is by the use of such therapies as the drug ABT 737. This compound is a BH3 mimetic that mimics the physiological antagonist of Bcl-2. It selectively targets Bcl-2 and has been shown to synergise with cytotoxic drugs in preclinical models (Mason et al., 2009). Therefore this could be one method of restoring 5-FU sensitivity to resistant tumours that exhibit high levels of Bcl-2 expression.

Overexpression of the proapoptotic protein Bax has also been found to sensitise cancer cells to 5-FU-induced apoptosis (Xu et al, 2002). It has also been demonstrated that loss of Bax expression decreases sensitivity to 5-FU (Zhang et al. 2000). Nita *et al.* also stated that some members of the Bcl-2 family of proteins in human colon cancer cell lines, are modulated by 5-FU and that the ratio of Bcl-x_L to Bax may be related to chemosensitivity of 5-FU, however according to Bunz *et al.* Bax-deficient cells remain sensitive to 5-FU (Bunz et al. 1999; Zhang et al. 2000). In line with these reports, and in contrast to the study by Bunz *et al.* we demonstrated that depletion of Bax in our HCT.shBax stable clone resulted in reduction of sensitivity to the cytotoxic effects of 5-FU, however it was unable to completely inhibit apoptosis (**Fig. 3.1.27 B**). This may be due to the action of Bak aiding in the role of Bax as these two proteins can act in a similar manner (Ruiz-Vela et al. 2005). Similar to the overexpression of Bcl-2, the removal of Bax resulted in cleavage of caspase-8 to its intermediate fragment, (again downstream of caspase-8 initial cleavage), but prevented full cleavage of caspase-8 and caspase-3 (**Fig. 3.1.27 C & D**).

In addition to our investigations of Bcl-2 and Bax, we carried out another study investigating another proapoptotic protein, Bid. In the past studies have documented the importance of Bid in 5-FU induced apoptosis using Bid null mouse embryonic fibroblasts, and found them to be more resistant to 5-FU, and furthermore Bid overexpression sensitised hepatocellular carcinoma cells to 5-FU (Miao et al. 2004; Sax et al. 2002). In the present study we also investigated the role of Bid in the apoptosis cascade induced by 5-FU. We found that removal of Bid rendered HCT116 cells completely resistant to 5-FU (**Fig. 3.1.28 B**), however caspase-8 remained processed

to its intermediate fragments (**Fig. 3.1.28 C**) therefore its cleavage remains upstream of Bid. Due to the inability of caspase-8 to cleave Bid, it resulted in a similar effect as Bcl-2 over-expression, by blocking the mitochondrial pathway as observed by abrogation of the caspase-3 cleavage (**Fig. 3.1.28 D**). As discussed in chapter 1, XIAP is the most potent direct caspase inhibitor and the cleavage of caspase-3 by caspase-8 can be blocked through interaction of XIAP with partially processed caspase-3. Since the lack of Bid is blocking the mitochondrial pathway, we hypothesised that knocking down XIAP in addition to Bid would restore the apoptotic potential of our HCT.shBid clone. The sensitivity of this clone to 5-FU was considerably increased, and caspase-8 and -3 cleavage was fully restored (**3.1.29 B & C**) in the absence of detectable levels of XIAP (**Fig. 3.1.29 A**). Therefore, the absence of Bid can be overcome by circumventing its role by allowing caspase-8 to directly cleave caspase-3 by mimicking the function of Smac/DIABLO, which is released from the mitochondria and neutralises XIAP. For clinical significance, to target this region of the apoptotic pathway in the scenario of 5-FU resistance, a number of IAP antagonist compounds have been designed to inhibit XIAP to kill tumour cells (Nikolovska-Coleska et al. 2008; Sun et al. 2007; Gao et al. 2007). Therefore a combination of XIAP inhibition plus chemotherapeutic drugs such as 5-FU could overcome 5-FU resistance at this level. Due to the pivotal role Bid plays in the apoptotic signalling, and its interaction with caspase-8, we hypothesised that Bid could potentially be a protein complex interaction partner in this pathway. To test this hypothesis we analysed the presence of Bid within our sucrose gradient. We found Bid co-sedimented with caspase-8 and FADD to the high-density region of the gradient following 5-FU (**Fig. 3.1.30 A**). Bid did not sediment in the absence of both caspase-8 (**Fig. 3.1.30 B**) and/or FADD (**Fig. 3.1.30 C**). Interestingly in the absence of Bid using our HCT.shBid stable clone, we demonstrated that caspase-8 and FADD are still capable of forming the 5-FU-induced high molecular weight complex (**Fig. 3.1.30 D**). Furthermore, as an additional experiment we tested the presence of Bid in the 5-FU treated HCT.shDR5 sucrose gradient and found it to co-sediment with caspase-8 and FADD (**Fig. 3.1.30 E**). Therefore due to the lack of effect on complex formation observed in the absence of Bid the results suggest that while Bid is clearly crucial to the sensitivity of HCT116 cells to 5-FU, it does not appear to be a core member of the protein complex, but rather a downstream effector molecule within this cascade.

After we had established the roles of caspase-8, FADD and Bid within the complex and their potential effects of the sensitivity of HCT116 cells to 5-FU, we then screened a panel of common apoptotic proteins within our sucrose gradient and identified a fourth putative member, RIP1. RIP1 has been recently identified to be a member of the death receptor independent protein complexes formed in response to etoposide (Tenev et al. 2011; Feoktistova et al. 2011). RIP1 has also been demonstrated to be cleaved by caspase-8 (Lin et al., 1998) and be capable of binding to FADD (Zhang et al. 2011a). Therefore it was an appealing candidate protein complex partner. We first tested the presence of RIP1 within our sucrose gradients and found monomeric forms of RIP1 present in the low-density region of the untreated gradient which sedimented to the high-density region following 5-FU (**Fig. 3.1.31 A**). This movement was specific to our complex as in the absence of caspase-8 (**Fig. 3.1.31 B**) and FADD (**Fig. 3.1.31 C**) the movement of RIP1 was prevented. Also similar to caspase-8 and FADD, we identified RIP1 movement in the absence of Bid (**Fig. 3.1.31 D**) and when DR5 was silenced (**Fig. 3.1.31 E**). Once we were satisfied with the possibility that RIP1 was co-sedimenting as part of the complex, we next confirmed its physical interaction with FADD by means of FADD-Myc/Flag overexpression in HEK 293 cells followed by a Myc immunoprecipitation. The immunoprecipitation was subsequently Western blotted for RIP1, which was co-immunoprecipitated with FADD following 5-FU (**Fig. 3.1.32 A**). These results were very promising however more experiments are required to decipher the exact role of RIP1 within this pathway more clearly. Future work will include generating an HCT.shRIP stable clone and overexpression of RIP1 and assessing its effect on complex assembly and caspase cleavage. These experiments are ongoing within the laboratory and preliminary results suggest that while this complex contains the same components as the recently described ripoptosome (Tenev et al. 2011; Feoktistova et al. 2011), knocking down of RIP1 in the HCT116 system did not have any impact on 5-FU-induced apoptosis therefore the role of RIP1 requires closer examination (*Mohr et al., unpublished data*).

Following complex member identification, we briefly re-confirmed that 5-FU induces apoptosis mainly through activating an RNA stress response within the cells as opposed to DNA damage, although DNA damage does indeed occur. In general reports investigating the mechanism of action underlying the therapeutic affect of 5-

FU are unclear. The drug is known to be an inhibitor of the enzyme thymidylate synthase (TS), which catalyses the methylation of deoxyuridylate to thymidylate, a DNA precursor (Parker & Cheng 1990). Additionally, 5-FU has been shown previously to be misincorporated into both DNA and RNA, with consequent effects on the structure and function of these nucleic acids.

To address whether the effects of 5-FU were the result of its effects on DNA synthesis or structure, or indeed RNA, the DNA base thymidine and the RNA base uridine were added at the same time as 5-FU addition to the cells. Thymidine had little effect on the apoptosis observed in these cultures when compared to the HCT116 cells treated with 5-FU. In contrast, inclusion of excess uridine almost completely blocked induction of apoptosis (**Fig. 3.1.33 A**). This is in line with a study by Johnston *et al.* where it was shown that 5-FU was incorporated *in vitro* into RNA but not DNA from HCT116 cells. Incubation of HCT116 cells with uridine inhibited the incorporation of 5-FU into RNA and relieved toxicity, whereas thymidine had no effect on 5-FU toxicity (Johnston *et al.* 1994). This was further demonstrated by caspase-8 and caspase-3 Western blot whereby cleavage by 5-FU was inhibited in the presence of Uridine (**Fig. 3.1.33 B & C**). As previous studies have indicated (Bunz *et al.* 1999), and in line with the results of the present study, the results suggest the impairment of thymidylate generation through inhibition of TS is not the crucial factor for the 5-FU sensitivity in this system, and that the main caspase-8 dependent pathway of apoptosis is induced upon RNA stress. After demonstrating that the RNA stress appears to be responsible for the complex formation/caspase-8 cleavage, we finally analysed the role of the DNA/RNA damage response protein, p53 in the apoptotic response to 5-FU in HCT116 cells. Research to understand the molecular mechanisms that mediate responsiveness to 5-FU has focused on selected genes involved in pathways targeted by 5-FU. The two most studied molecules involved in 5-FU-mediated apoptosis are TS and p53 (Zhang *et al.* 2003). In the case of p53, there are many complexities and interconnecting pathways due to the modulation of multiple genes by p53. Wild type p53 has been generally associated with 5-FU sensitivity (Zhang *et al.* 2003; O'Connor *et al.* 1997) and is induced in response to a host of genotoxic and environmental stresses, including γ and U.V. irradiation, chemotherapeutic agents, hypoxia or alterations in intracellular nucleotide pools (Levine 1997; Ko & Prives 1996; Vogelstein & Kinzler 1992; Linke *et al.* 1996). Once p53 is induced, a host of target

genes are then transcriptionally activated including p21 and bax. Induction of p21, in turn, leads to cell cycle arrest at both G1 and G2 checkpoints (Ju et al. 2007). In contrast to wild-type p53, mutated p53 is generally associated with resistance (Zhang et al. 2003; O'Connor et al. 1997). Pugachera *et al.* revealed a novel gain of function activity conferred by certain p53 mutants that directly link expression of p53 activity with resistance to chemotherapeutic drugs of the fluoro-pyrimidine group (Pugacheva et al. 2002). Furthermore, clinical studies have revealed higher resistance to fluoro-pyrimidine therapy of tumours expressing p53 mutants (Cabelguenne et al. 2000; Zheng et al. 1999). Western blotting of cell lysates showed that 5-FU causes increases in p53 protein levels as shown in previous studies (Bunz et al. 1999). In the study by Bunz *et al.* they found a marked difference in xenografts response to 5-FU treatment, where tumours with intact p53 regressed during treatment whereas deleted p53 genes continued to grow. Also a study by Pritchard *et al.* found that the p53-dependent apoptosis induced *in vivo* was very significantly reduced by administration of uridine but not thymidine, strongly supporting the idea that cell death in intestinal epithelia requires 5-FU metabolites to be incorporated into RNA and this death is by p53-dependent apoptosis (Pritchard et al. 1997).

In the present study, we found that using the p53^{-/-} clone of HCT116 cells that in the absence of p53, apoptosis was reduced by approximately 50% (**Fig.3.1.34 B**). When we Western blotted for caspase-8 and -3, we also noted that these caspases were cleaved, but full processing was attenuated, visible by a slight delay in the extent of cleavage in comparison to wild-type HCT116 cells (**Fig. 3.1.34 C & D**). In our sucrose gradients of HCT116 p53^{-/-} cells, our complex members were still able to migrate to the higher density region. Therefore from these results we could conclude, while p53 is not crucial to caspase-8 complex formation, it does play a very important role of modulating the pace of apoptosis in these cells following 5-FU, and without p53, the overall rates of apoptosis are impeded, thereby reducing the cytotoxic effects of 5-FU in the absence of p53.

3.1.4 CONCLUDING REMARKS

In the present study we have characterised the intracellular signalling platform containing caspase-8, FADD, RIP1 and Bid. We demonstrate that loss of caspase-8,

or FADD inhibits 5-FU-induced apoptosis in HCT116 cells. This finding is important as the initiator caspase of drug-induced apoptosis is classically thought to be caspase-9. This apoptotic signal is initiated by caspase-8 at this large molecular weight complex distinct from TNF-complex II. We demonstrated the physical interaction of caspase-8 with FADD, and with FADD and RIP1. In the case of p53 mutations, which as discussed are extremely common in tumours, we can see from the present study and the other reports described above that in the absence of p53, 5-FU sensitivity is greatly reduced. However, we discovered that the apoptosis initiating caspase-8 cleavage is independent of p53. This, and the discovery of the caspase-8 complex and the various proteins that play important roles downstream of its assembly provide new insights into the fundamental mechanisms through which cancer cells respond to chemotherapeutic drugs, and might help us to better develop more targeted strategies in the future.

CHAPTER 3
RESULTS & DISCUSSION
PART II

**5-FU-Induced Sensitisation to Mesenchymal Stem Cell-
delivered sTRAIL In Vitro**

3.2.1 BACKGROUND TO STUDY RATIONALE

The receptors for the apoptosis-inducing cytokine TRAIL are widely expressed on tumour cells and TRAIL itself has a relatively specific anti-tumour action (Nesterov et al. 2004; Klefstrom et al. 2002) and safety profile (Ashkenazi & Dixit 1998; Ashkenazi et al. 2008; Herbst et al. 2006). This suggests TRAIL receptor (TRAIL-R) targeting is an excellent strategy and clinical trials with TRAIL and TRAIL-R agonistic antibodies Mapatumab (targets DR4) and Lexatumumab (targets DR5) have already been initiated (Fesik, 2005; Rowinsky, 2005). The pathway of TRAIL-induced apoptosis is mediated through the extrinsic/death receptor pathway described in detail in Chapter 1. In brief, TRAIL binds DR4 and/or DR5. Through their cytoplasmic tail containing a DD, DR4 and DR5 then form a protein complex known as the death inducing signalling complex or DISC. The adaptor molecule FADD is then recruited to this platform, and in turn FADD recruits the initiator caspase-8/-10 through their respective death effector domain (DED) (Bodmer et al. 2000; LeBlanc et al. 2002). Caspase-8 is then thought to dimerise and proteolytically cleave and activate itself (Sprick et al. 2000), leading in turn to the activation of downstream executioner caspases, culminating in the morphological and biochemical characteristics associated with apoptosis (Bratton et al. 2000).

Despite the attractive properties of TRAIL, there are a number of disadvantages to its clinical use. The recombinant TRAIL protein has a limited bioavailability possibly

owing to its short half-life in the bloodstream and while agonistic antibodies have a longer half-life (Duiker et al., 2006), their efficacy is limited due to their bivalent nature that does not optimally mimic the trimeric ligand (Allen et al., 2012). Furthermore there is difficulty in delivering TRAIL directly to the tumour site. To tackle these issues we, and others, have employed Mesenchymal Stem Cells (MSCs), which have shown potential as transgene delivery vehicles (Nakamizo et al. 2005; Mohr et al. 2008) and specifically for delivery of TRAIL to the tumour site (Mohr et al. 2010). These studies have improved the model of TRAIL-based therapy, but unfortunately resistance in a high proportion of cancer cell types persists (Zhang et al. 2006). To this end, the use of TRAIL sensitisers has been examined and has been shown to involve TRAIL receptor upregulation (Micheau et al. 1997; Ivanov & Hei 2006; Frese et al. 2006; Hetschko et al. 2008; Nagane et al. 2000; Sung et al. 2010), c-FLIP downregulation (Galligan et al. 2005; Kim et al. 2008), restoration of caspase-8 expression (Fulda & Debatin 2006a; Fulda et al. 2001), enhanced DISC formation (Ducoroy et al. 2003; Ganten et al. 2004; Lacour et al. 2003) or deregulation of cell survival pathway proteins such as Bcl-2 (Fandy et al. 2007; Siegelin et al. 2009). Among the large array of compounds currently being investigated in this context, an important group are cancer chemotherapies already widely used in clinical oncology (Grávalos et al. 2009; Chang et al. 2011). Indeed some of them are currently undergoing clinical trials as TRAIL sensitisers. Having explored the molecular mechanism of 5-FU-induced apoptosis in detail in Chapter 3.1 we set out to investigate the potential of 5-FU as a TRAIL sensitiser. We had already demonstrated that 5-FU is capable of upregulating DR5 in HCT116 cells. Therefore we hypothesised that this upregulation of DR5 could prime HCT116 cells for TRAIL-mediated apoptosis. We also wanted to explore 5-FU in combination with MSC-delivered soluble (s) TRAIL. We sought to understand at the molecular level how this sensitisation was achieved in order to aid in the future development of improved and more patient-specific therapies. We found that 5-FU sensitised the colon cancer cell line HCT116 to MSC-mediated TRAIL-induced apoptosis via the up-regulation of DR5. The sensitisation effect was successful both *in vitro* and *in vivo* (Deedigan et al., 2013). Therefore this could be a potential therapy targeting TRAIL-resistant cancer in the future, provided that the outcome of the clinical trials evaluating chemotherapeutic TRAIL sensitisers continues to be as positive as they have been to date (Leong et al.

2009; Soria et al. 2010; Mom et al. 2009; Sandler et al. 2006).

3.2.2 RESULTS

3.2.2.1 *Subapoptotic damage sensitises HCT116 cells to subapoptotic low-dose recombinant TRAIL (rhTRAIL)*

While TRAIL has been identified as a safe and specific chemotherapeutic agent, resistance remains in a large proportion of cancer cell types. We had previously demonstrated in Chapter 3.1 that 5-FU upregulated the TRAIL receptor DR5 therefore we continued to dissect the role of 5-FU as a TRAIL sensitiser and hypothesised that it was mediated through this mechanism. To this end we examined the cytotoxic effects of 5-FU alone or in combination with rhTRAIL in HCT116 cells.

To evaluate a putative sensitisation, 5-FU was added to HCT116 cells at increasing concentrations of 10, 50, 100, 200 and 400 μM for 48 h (**Fig. 3.2.1 A**). The concentration we chose to continue with was 10 μM , which represented a concentration well below significant apoptosis levels of 10% specific apoptosis 48 h post-treatment (**Fig.3.2.1 A**). We then determined the rhTRAIL concentration by adding it to HCT116 cells at increasing concentrations of 0.25, 0.5, 1, 2.5 and 5 ng/ml for 24 h (**Fig. 3.2.1 B**). Concentrations at 1 ng or below gave rise to less than 10% specific apoptosis and were deemed relevant for the purposes of this study. This confirmed, firstly, that the chosen concentrations of 5-FU represented subapoptotic concentrations, and secondly, that rhTRAIL (at this concentration of 1 ng/ml) alone was not able to induce significant levels of apoptosis in HCT116 cells. However, pretreatment of the HCT116 cells with 5-FU for 24 h hours prior to addition of rhTRAIL for an additional 24 h resulted in the occurrence of significant levels of apoptosis, beyond the expected additive levels of 15-20%. We observed levels approaching 40% (38%) apoptosis, compared to 3% and 9% apoptosis respectively for the single treatments (**Fig 3.2.1 C**). These data demonstrate that 5-FU-induced subapoptotic damage pre-sensitises low dose rhTRAIL-treated HCT116 cells to TRAIL-induced apoptosis.

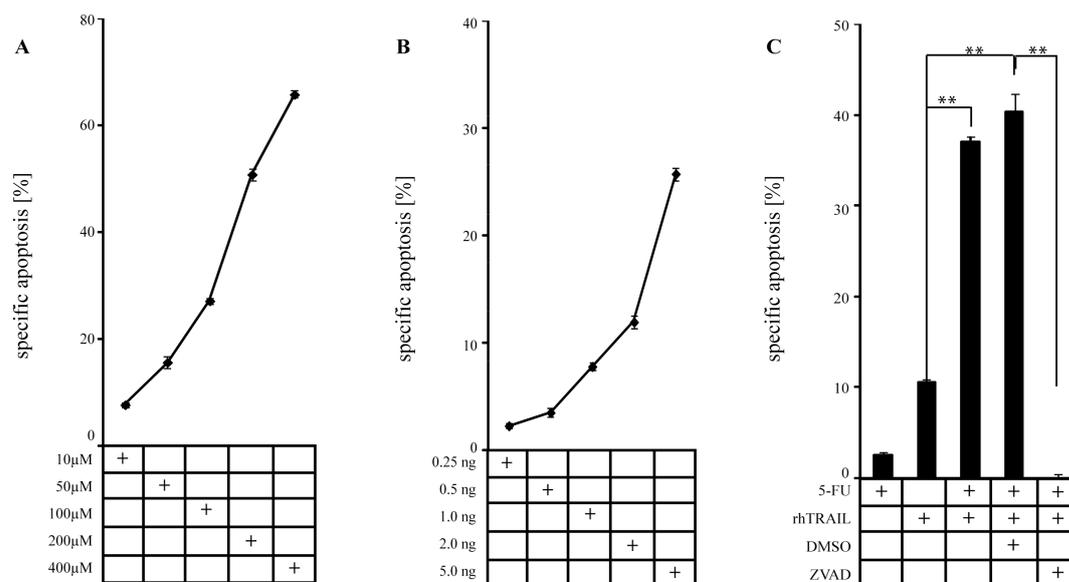


Figure 3.2.1 5-FU sensitises HCT116 cells to TRAIL-mediated apoptosis and this effect is caspase-dependent. **(A)** 5-FU dose-response plot of HCT116 cells. Cells were treated for 48 h with the respective doses of 5-FU. Cell death was characterised by a Nicoletti Assay as described in Chapter 2. **(B)** rhTRAIL dose-response plot of HCT116 cells. Cells were treated for 24 h with the respective doses of rhTRAIL. Cell death was characterised by a Nicoletti Assay as described in Chapter 2. **(C)** Addition of Z-VAD-FMK directly to the HCT116 cells prior to 5-FU pre-treatment and again prior to rhTRAIL treatment inhibited apoptosis by 100%. ** = $P < 0.001$. Representative of $n = 3$ experiments.

3.2.2.2 Apoptosis induced by 5-FU plus rhTRAIL is inhibited by Z-VAD-FMK

We next investigated the role that caspases play in the 5-FU-mediated sensitisation. We hypothesised that the sensitisation would be prevented by the addition of the pan-caspase inhibitor Z-VAD-FMK. To this end we added Z-VAD-FMK directly to HCT116 cells prior to the addition of 5-FU and again before the addition of rhTRAIL. As shown in **Fig 3.2.1 C**, Z-VAD-FMK inhibited the apoptosis induced by the combined treatment of 5-FU and rhTRAIL. As a control for Z-VAD-FMK, the carrier DMSO was added. In contrast, the addition of DMSO had no effect on the apoptosis levels (**Fig. 3.2.1 C**). These results indicated that the observed mode of cell death was a caspase-dependent apoptotic pathway.

3.2.2.3 Silencing of caspase-8 renders HCT116 cells completely resistant to the sensitisation effect

In order to further improve TRAIL-based combination treatments, we wanted a better understanding of the underlying molecular mechanism of the observed sensitisation.

First, we wanted to further examine the role of caspases by investigating caspase-8, the initiator caspase of the TRAIL-mediated apoptosis pathway, in this sensitisation. As caspase-8 activation is one of the initial molecular events in this pathway, we analysed caspase-8 cleavage following the single and combined treatments of 5-FU and rhTRAIL in HCT116 cells. We observed that caspase-8 cleavage is markedly increased in cells exposed to the combined treatment as compared to cells treated with either 5-FU or rhTRAIL alone (**Fig. 3.2.2 A**). In order to demonstrate that caspase-8 is actively involved in the sensitisation and is not just cleaved as a bystander, we generated stable HCT.shcaspase-8 clones. As shown in **Fig. 3.2.2 B**, when we pre-treated these cells with 5-FU (10 μ M) for 24 h followed by rhTRAIL (1 ng/mL) for 24 h we found that there was an almost complete inhibition of apoptosis, with a reduction from 50% to less than 10%. Silencing of caspase-8 rescues the apoptosis induced by 5-FU plus rhTRAIL. Together, these results show that the sensitisation mechanism must be at the level of caspase-8 or above (e.g. TRAIL receptors), as caspase-8 cleavage was increased, and that the sensitisation was signalled through the canonical extrinsic apoptosis pathway. However, while these results show that caspase-8 is required for the 5-FU/TRAIL-triggered apoptosis, we could not detect any indication that this low concentration of 5-FU gave rise to elevated levels of caspase-8 (**Fig. 3.2.3**), thereby making a role as the molecular driver of the 5-FU sensitisation effect unlikely.

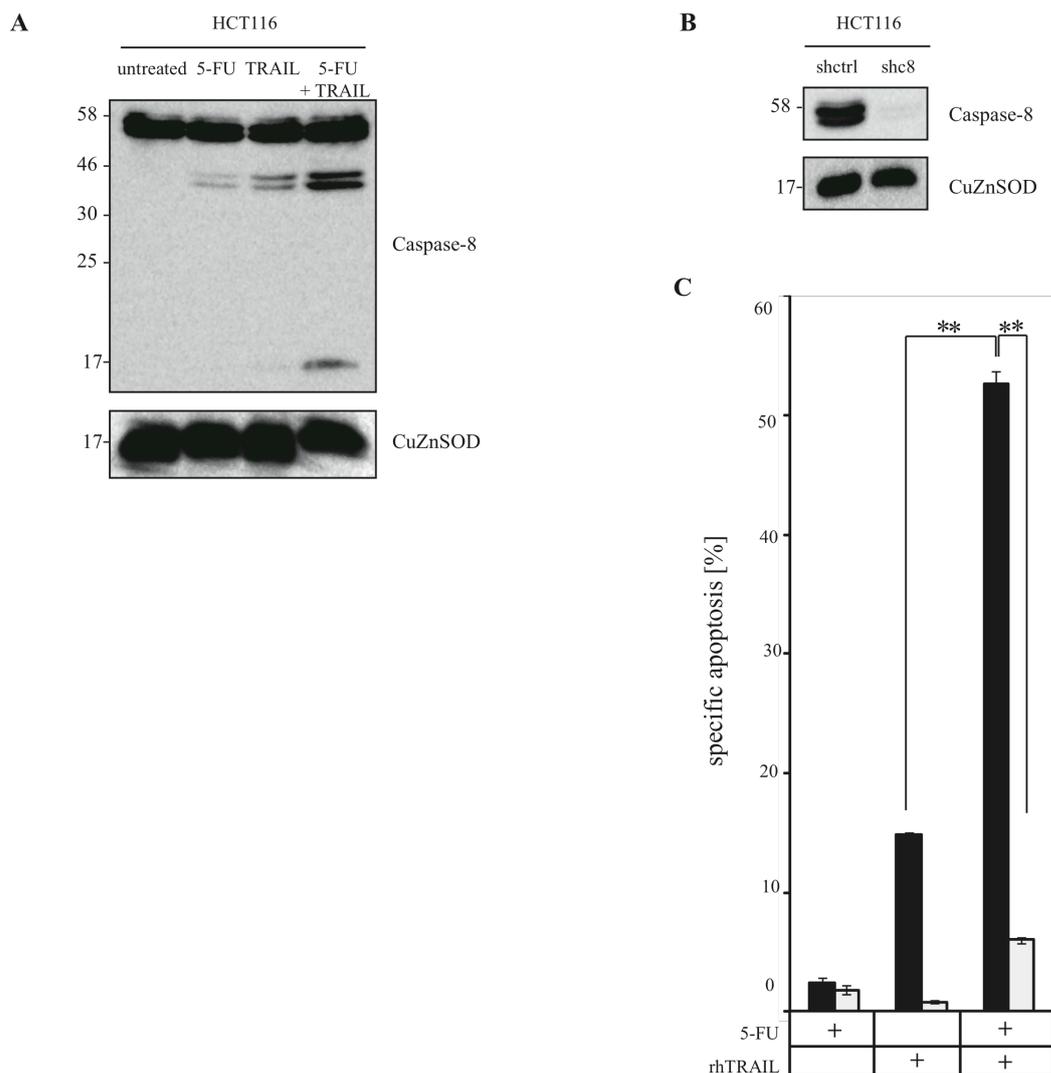


Figure 3.2.2 Stable knockdown of caspase-8 prevents apoptosis in HCT116 cells following the concomitant treatment of 5-FU/rhTRAIL. (A) Western blot for caspase-8 (BD) of parental HCT116 cells treated with 5-FU alone (10 μ M) for 24 h, rhTRAIL (1 ng/mL) for 24 h or 5-FU alone (10 μ M) for 24 h followed by rhTRAIL (1 ng/mL) for a further 24 h. (B) Western blot for caspase-8 of parental cells and HCT116 cells where caspase-8 has been stably knocked down. A CuZnSOD blot served as loading control. (C) HCT.shCtrl (black bars) and HCT.shcaspase-8 (white bars) were pre-treated with 5-FU (10 μ M) for 24 h and then rhTRAIL (1 ng/mL) for 24 h and apoptosis was measured revealing a diminished sensitisation to less than 10% apoptosis in the combined treatment. ** = $P < 0.001$. Representative of $n = 3$ experiments.

To attempt to identify the molecular driver, our group began to investigate other likely candidates in the TRAIL-induced apoptotic cascade. One such protein was c-FLIP, which has been implicated by others to mediate the 5-FU sensitisation effect. However, while decreased levels of c-FLIP protein after 5-FU have been reported to enhance caspase-8 activation (Ganten et al., 2004), we were unable to detect any decreases in c-FLIP levels (**Fig. 3.2.3**) following 5-FU.

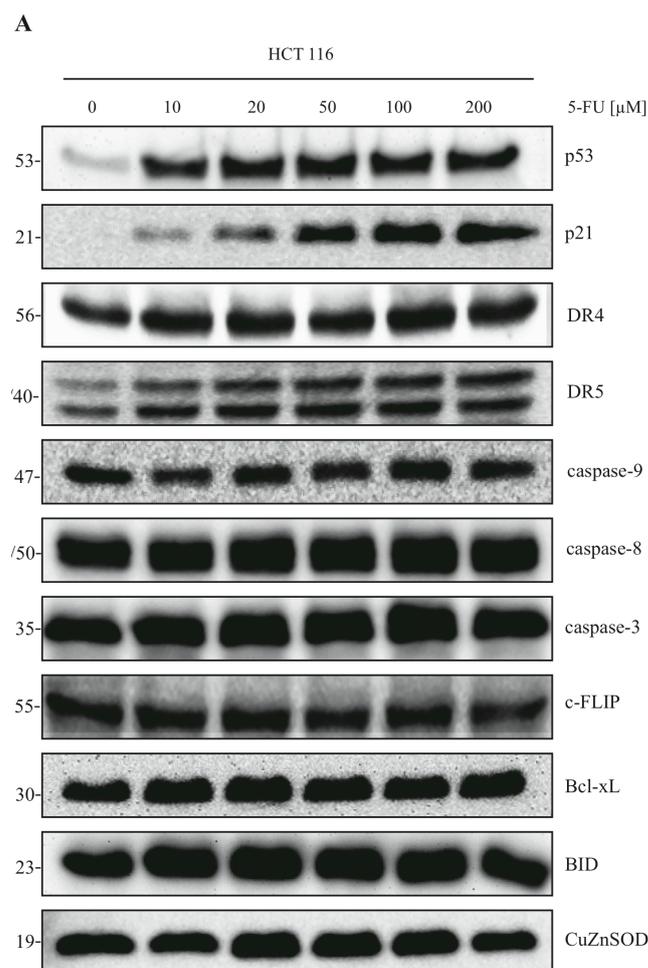


Figure 3.2.3 5-FU treatment results in DR5 upregulation. (A) Western blots for p53, p21, DR4, DR5, caspase-9, caspase-8 (BD), caspase-3, c-FLIP_L, Bcl-x_L, and Bid of protein lysates generated from HCT116 cells treated with 5-FU (10, 20, 50, 100, or 200 μ M) for 24 h or untreated as control, resulted in dose-dependent DR5 upregulation. CuZnSOD served as loading control and molecular weight markers are indicated. Representative of n = 2 experiments.

3.2.2.4 DR5 is up regulated following low-dose 5-FU and removal of DR5 but not DR4 abolished the sensitisation effect

It is well established that TRAIL interacts with the death receptors DR4 and DR5 to trigger apoptotic signalling and caspase-8 plays a central role in the apoptosis mediated by death receptors, especially DR5. Increased TRAIL receptor expression could lead to a higher caspase-8 activation and apoptosis in response to TRAIL. In particular, the modulation of DR5 upregulation by 5-FU has been demonstrated to be via a mechanism that is believed to be p53 dependent (Kuribayashi et al., 2011). We

treated HCT116 cells with increasing doses of 5-FU, from 10 to 200 μ M for 24 h and observed that 5-FU induced upregulation of DR5 protein levels. The protein levels of DR4 were also upregulated but to a lesser extent (**Fig. 3.2.3**).

We also compared the expression levels of other major apoptotic proteins such as FADD, caspase-8, BID, caspase-2, caspase-9, and caspase-3 as well as c-FLIP and Bcl-XL, detected by Western blotting. The results revealed no differences in their expression levels following 5-FU (**Fig. 3.2.3**). To clarify the functional role of DR5 in stimulation of TRAIL-induced apoptosis by 5-FU, we examined the effect of silencing DR5 on 5-FU/TRAIL-induced apoptosis. To this end we generated stable DR5 knockdown clones in the HCT116 cells. These clones were termed HCT.shDR5, and were shown to harbour no detectable DR5 levels by western blot (**Fig 3.2.4 A**). When we pre-treated the HCT.shDR5 clone with 5-FU and then rhTRAIL, we found apoptosis was halved in comparison to the control cells, suggesting the cells may still die via DR4 (**Fig. 3.2.4 B**). These results support the idea that the 5-FU-induced up regulation of DR5 is critical for the achievement of TRAIL sensitivity in HCT116 cells.

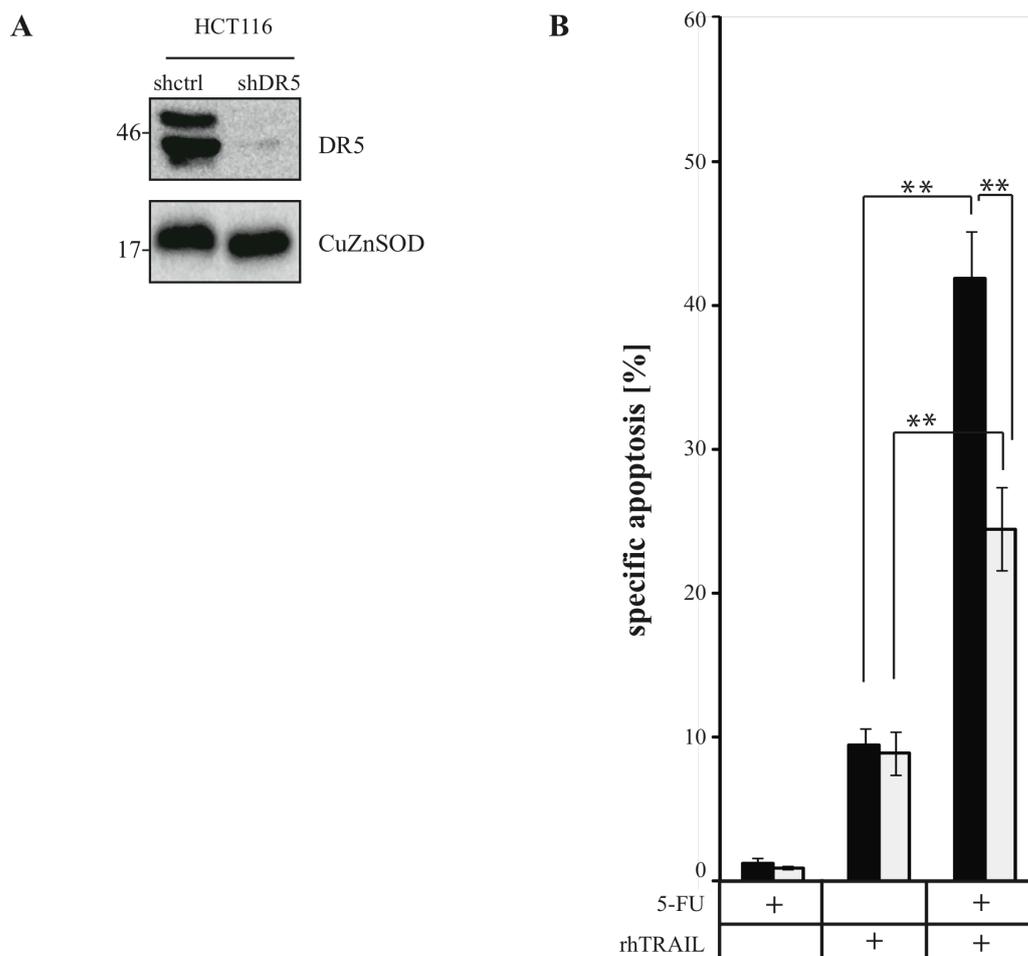
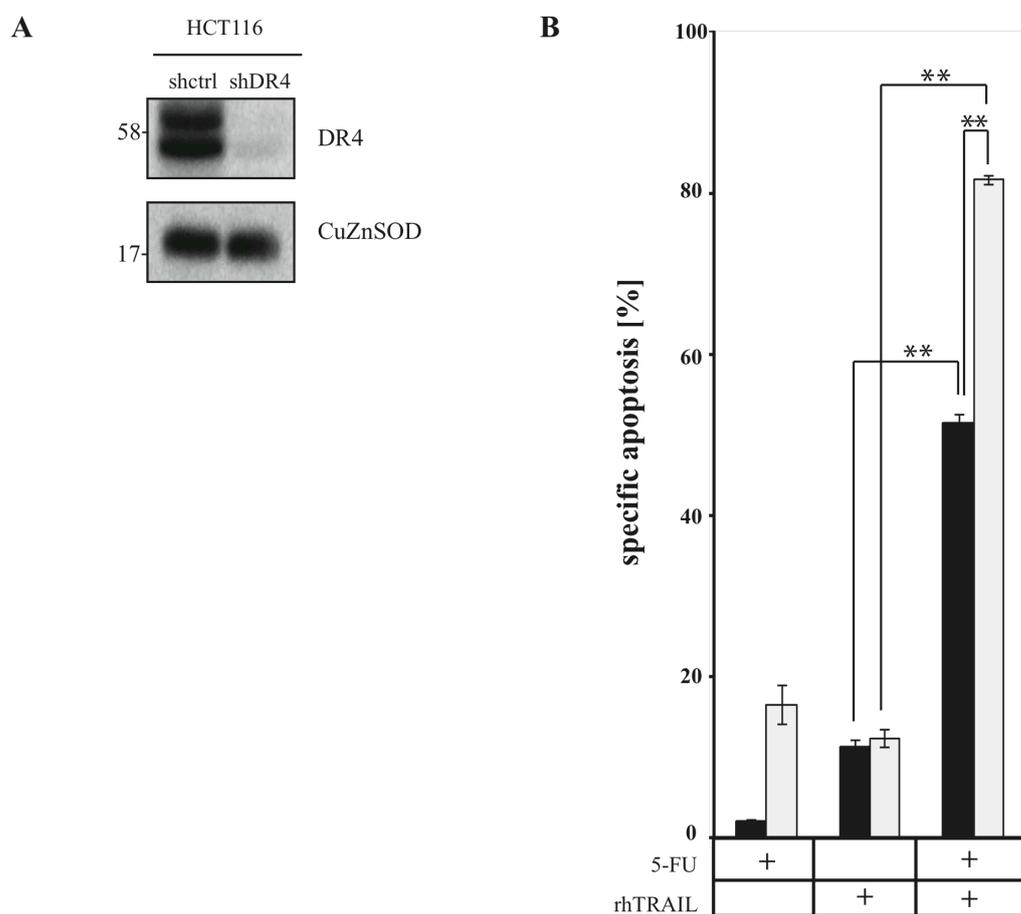


Fig. 3.2.4 DR5 knockdown inhibits the 5-FU-mediated sensitisation (A) Western blot for DR5 of HCT116 parental cells and clones in which DR5 was stably knocked down. A CuZnSOD served as loading control and molecular weight markers are indicated (B) HCT.Ctrl (black bars) and HCT.shDR5 (white bars) were pre-treated with 5-FU (10 μ M) for 24 h then rhTRAIL (1 ng/mL) for 24 h and apoptosis was measured revealing a diminished sensitisation by 50% in the combined treatment. ** = $P < 0.001$. Representative of $n = 3$ experiments.

Next we generated stable DR4 knockdown clones in HCT116 cells (**Fig. 3.2.5 A**) as a control for the HCT.shDR5 cells. These were termed HCT.shDR4 and exhibited a marked increase in apoptosis despite the lack of DR4 pointing to a possible hindrance caused by the presence of DR4 and DR5 heterotrimers in normal HCT116 cells. In contrast, in DR4 silenced cells only DR5 homotrimers can be formed that are more effective in mediating apoptotic signals than heterotrimers. This is particularly the case in cells, such as HCT116 cells, that prefer DR5 signalling over DR4 and this is in line with the finding by Kelley et al. who generally demonstrated a greater contribution of DR5 than DR4 to apoptosis signalling (Kelley et al., 2005). It appears

that HCT116 cells have a preference for DR5-mediated apoptosis and that DR5 up regulation is the molecular driver for the 5-FU-induced TRAIL sensitivity in these cells. In correlation with the results in the present study, additional work within our group demonstrated that HCT116 cells die preferentially via DR5 rather than DR4 by using TRAIL receptor specific variants (Deedigan et al., 2013). Therefore, when DR5 is removed, the cells are forced to die via DR4, with a reduction in apoptosis by approximately 50%, whereas when DR4 is removed, the cells may die completely via the preferred route of DR5, increasing the apoptosis to 80% (Fig. 3.2.5 B).



Figure

3.2.5 shDR4 actually enhances the 5-FU/rhTRAIL apoptosis (A) Western blot for DR4 of HCT116 parental cells and clones in which DR4 was stably knocked down. A CuZnSOD served as loading control and molecular weight markers are indicated **(B)** HCT.Ctrl (black bars) and HCT.shDR4 (white bars) were pre-treated with 5-FU (10 μ M) for 24 h followed by rhTRAIL (1 ng/mL) for 24 h and apoptosis was measured revealing increased sensitisation from 5-15% in the HCT.shDR4 to 5-FU alone, and a large increase in apoptosis for the combined treatment of 50-80%. ** = $P < 0.001$. Representative of $n = 3$ experiments.

3.2.2.5 *Effective apoptosis induction of MSC.sTRAIL in HCT116 cells in vitro by concomitant presence of MSC.sTRAIL and 5-FU pretreatment*

A problem of considerable importance in the context of the clinical use of TRAIL is effective delivery and sufficient bioavailability at the tumour site of the recombinant protein or agonistic TRAIL-receptor antibodies (Ashkenazi et al. 1999). To attempt to circumvent this issue, we, and others, have exploited the tumour infiltrating properties of MSCs to deliver sTRAIL (Kim et al. 2008; Loebinger et al. 2009; Mohr et al. 2010).

Therefore we then wanted to confirm the results we obtained with rhTRAIL using sTRAIL-expressing MSCs *in vitro*. We employed adenoviral vectors, (generated in our group previously (Mohr et al., 2010) and re-amplified for the purpose of this study), containing the ectodomain of TRAIL (aa114 – aa289) fused to a secretory signal peptide from the human fibrillin-1 gene (hFIB), a Furin Cleavage Site (Furin CS) and an Isoleucine zipper (ILZ) domain under the control of the CMV promoter/Enhancer element. This adenoviral vector, termed Ad.sTRAIL was used to transduce MSCs, which have been characterised by their capacity for adipogenic, osteogenic and chondrogenic differentiation as well as FACS analysis with an array of surface marker antibodies (Deedigan et al. 2013). The resulting MSCs expressing sTRAIL were termed MSC.sTRAIL. The levels of sTRAIL expression were measured by Western blot (**Fig. 3.2.6 A**). We chose the lowest amount of Ad.sTRAIL that gave rise to a high level of sTRAIL expression, which did not cause any visible toxicity to the MSCs.

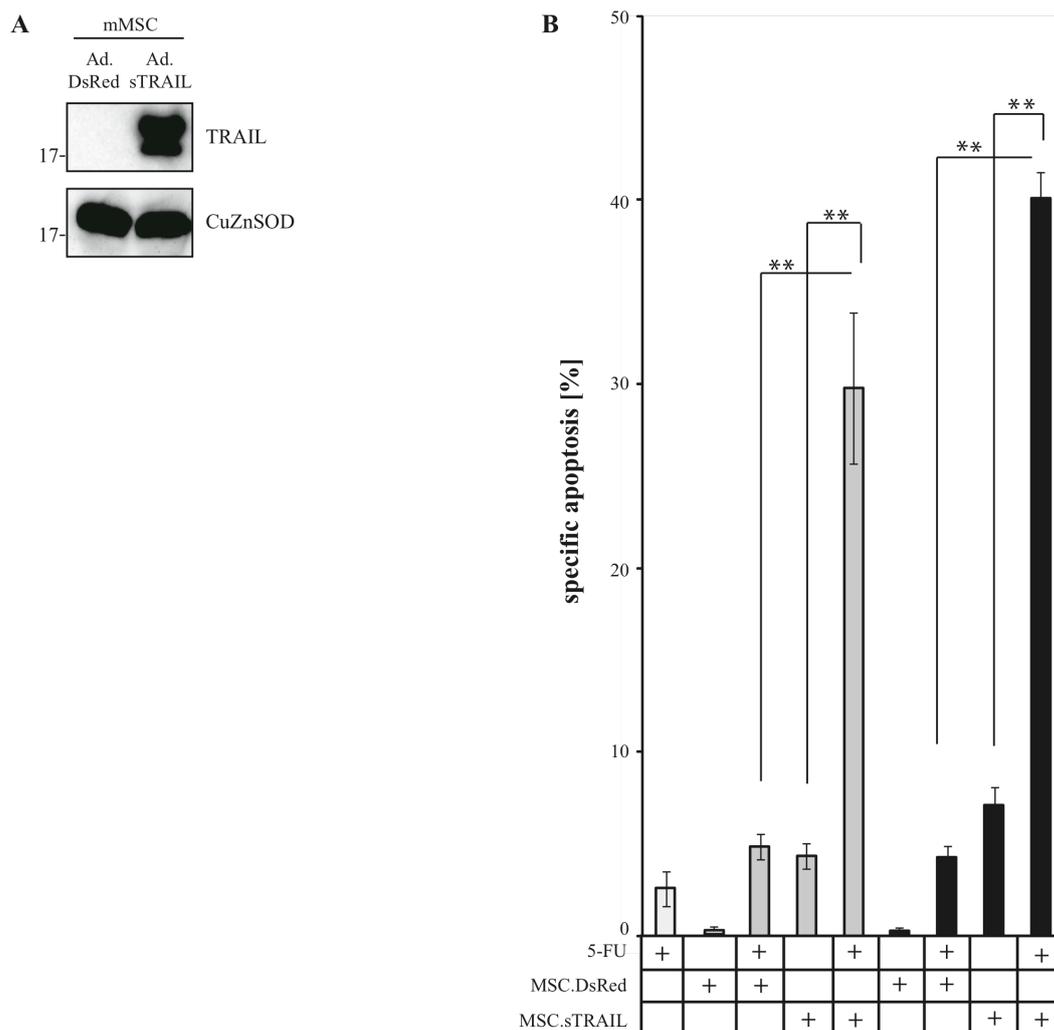


Figure 3.2.6 Medium supernatant from MSCs expressing Ad.sTRAIL transferred onto 5-FU pre-treated HCT116 cells leads to a substantial increase in apoptosis. (A) Western blot for TRAIL of protein lysates from MSCs transduced with Ad.sTRAIL. CuZnSOD blot served as loading control and molecular weight markers are indicated (B) MSC.DsRed and MSC.sTRAIL supernatant transfer (1:10 dilution = grey bars, 1:5 dilution = black bars) onto HCT116 cells alone (white bar), or pre-treated with 10 μ M 5-FU for 24 h and subsequent apoptosis measurement revealed sensitisation of MSC.sTRAIL supernatant co-treated with 5-FU treated HCT116 cells. ** = $P < 0.001$. Representative of $n = 3$ experiments.

When the medium supernatants (1:10 and 1:5 dilution) of MSC.sTRAIL were transferred 48 h after transduction to 5-FU (10 μ M 5-FU 24 h) pre-treated HCT116 cells, they showed significant levels of apoptosis, in comparison to the addition of supernatant of MSC.DsRed and untreated HCT116 cells (+/- 5-FU), which served as controls (Fig. 3.2.6 B).

This demonstrated that the effect we observed was indeed exerted by secreted TRAIL and not via direct MSC-cancer cell interactions. Furthermore, mixing of MSC.sTRAIL with HCT116 cells at a cellular ratio of 1:10, demonstrated that 5-FU pre-treatment of HCT116 cells prior to mixing gave rise to significant TRAIL

sensitisation (**Fig. 3.2.7**). This analysis was performed in direct co culture of MSC.sTRAIL cells with HCT116 cells concomitantly treated with sub apoptotic 5-FU doses. In contrast to these results, untreated co cultures with MSC.sTRAIL as well as 5-FU-treated and untreated co cultures of HCT116 cells with MSC.DsRed served as controls and showed insignificant levels of apoptosis (**Fig. 3.2.7**).

These data show that the simultaneous presence of MSC.sTRAIL and low dose 5-FU can effectively sensitise HCT116 cells to sTRAIL *in vitro*. Based on this observation, we hypothesised that a combination of 5-FU pre-treatment and MSC.sTRAIL can inhibit the xenograft growth of HCT116 cells in a murine model. This combination approach successfully resulted in complete tumour remission as compared to tumour regression seen with sTRAIL alone (Deedigan et al. 2013).

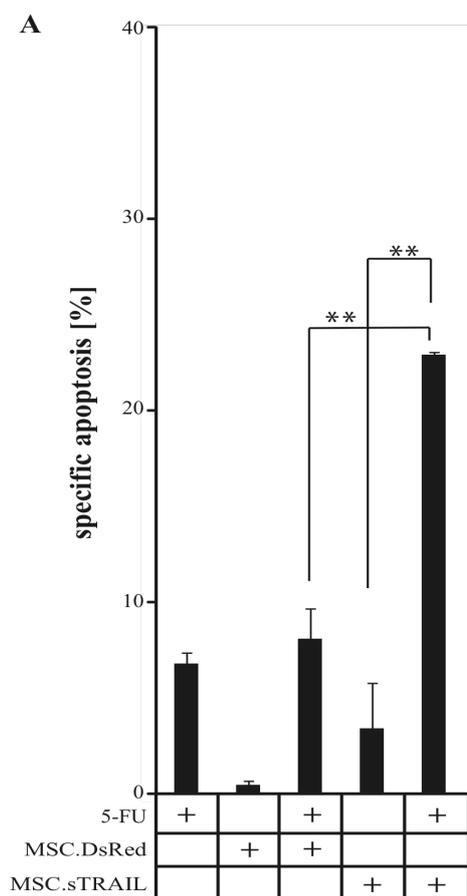


Figure 3.2.7 Mixing of MSC-sTRAIL with 5-FU pretreated HCT116 results in high levels of apoptosis (A) Mixing of HCT116 cells and HCT116 cells pre-treated with 5-FU (10 μ M) with MSC.DsRed or MSC.sTRAIL at a ratio of 10:1 shows that MSC.sTRAIL exerted an increased apoptosis effect on HCT116 cells compared to MSC.DsRed and a significant increase when pre-treated with 5-FU. ** = $P < 0.001$. Representative of $n = 3$ experiments.

3.2.2 DISCUSSION

Despite much advancement in the field of cancer research over the past number of years, there remains an urgent requirement for improvement to therapies as cancer cells continue to evade and acquire resistance to cell death. Furthermore, current modalities are not optimal in terms of their specificity/safety, as non-malignant cells are not differentiated from tumour cells by the chemotherapeutics in regular use. Therefore there is an ongoing search for more tumour-specific and safe anti-cancer treatments, which has led to the investigation of the therapeutic potential of death ligands in chemotherapy: the idea being to specifically trigger apoptosis in tumour cells by targeting their death receptors, which are directly connected to the death machinery of the cell. However in the case of the prototypic death ligands, CD95L or TNF- α , they were found to be unsuitable due to severe toxic side effects. Administration of TNF- α or CD95L caused a severe inflammatory response syndrome or massive liver-cell apoptosis, respectively (Ashkenazi 2002; Lawrence et al. 2001). These findings led to the discovery of the many superior benefits of Tumour-Related Apoptosis Inducing Ligand or TRAIL. TRAIL is a very promising candidate as in particular its optimised recombinant soluble form, (closely resembling the endogenous soluble ligand (Hymowitz et al. 2000; Bodmer et al. 2000)) has been shown to be able to induce apoptosis in a wide variety of cancer cell types (Ashkenazi 2002; Ashkenazi et al. 1999) and to be relatively safe in normal cell types (Walczak et al. 1999; Lawrence et al. 2001; Qin et al. 2001). However, despite its relative safety and tumour- specificity, resistance persists in certain cancer cell types (Todaro et al. 2008; MacFarlane et al. 2002; Dyer et al. 2007; Siegelin et al. 2009) and has indeed been documented in colon cancer (Camidge 2008; Trarbach et al. 2010). In an effort to combat this resistance, investigations into methods of sensitising tumour cells to the effects of TRAIL have taken place, encompassing a broad range of compounds. These agents have successfully sensitised various cancer cell types to TRAIL-induced apoptosis. In a recent review, an array of such TRAIL-sensitising compounds were discussed in detail with a seemingly single and common mechanism of action: up regulation of the TRAIL receptor, DR5 (Yoshida et al. 2010). These agents synergistically induced apoptosis with TRAIL in malignant tumour cells, and included Histone deacetylase inhibitors (HDACIs), such as sodium butyrate (Nakata

et al. 2004), food components including luteolin (Horinaka et al. 2005) and Adigenin (Horinaka et al. 2006) and also cartinoids such as Halocynthiaxanthin and penidin in combination with TRAIL were found to strongly induce apoptosis in human colon cancer cells (Yoshida et al. 2010).

In addition to these natural compounds, another group of important chemotherapies that are widely used in the treatment of cancer are cytotoxic agents (Grávalos et al. 2009; Chang et al. 2011). They have also been extensively studied as TRAIL sensitisers with good success, both *in vitro* and *in vivo* in a large variety of cancer models (Yamanaka et al. 2000; Vignati et al. 2002; Jin et al. 2004; Galligan et al. 2005). Chemotherapeutics were the first group of sensitisers tested in TRAIL-based clinical trials and are also the largest group of sensitisers (Zhao et al. 2012). Among these cytotoxic drugs, already in clinical use are Oxaliplatin and 5-Fluorouracil (5-FU), both of which have been demonstrated to sensitise to TRAIL-induced apoptosis in colon cancer cell lines (Toscano et al. 2008; Naka et al. 2002). 5-FU is already widely used in the clinical treatment of various tumours and an early study demonstrated that one mechanism behind the 5-FU sensitisation was via profoundly activated p53 and up-regulated caspase-8 leading to marked sensitisation of TRAIL-resistant leukemia cells to TRAIL-induced apoptosis (Ehrhardt et al. 2008). A second mechanism of 5-FU sensitisation was demonstrated by Naka et al. where they found that 5-FU could enhance TRAIL-induced tumour suppression in nude mice through up regulation of DR5 (Naka et al. 2002). Following on from these studies, in 2004 it was further demonstrated that several chemotherapies, including 5-FU, can overcome TRAIL resistance in colon cancer cells and enhanced tumour suppression in tumours established from TRAIL-resistant colon cancer cells (Zhu et al. 2004). Subsequently, the FOLFOX regimen (Leucovorin, Fluorouracil and Oxaliplatin) and the FOLFIRI regimen (Irinotecan, Fluorouracil and Leucovorin), current common chemotherapeutic regimens, are being tested as sensitisers to TRAIL or agonistic antibodies in several ongoing clinical trials (Zhao et al. 2012). One ongoing Phase 2 trial in particular is being conducted by Amgen, and is exploring the safety and efficacy of FOLFIRI in combination with AMG 655 vs FOLFIRI in KRAS-mutant metastatic colorectal carcinoma. This trial is studying patients who have failed first line fluoropyrimidine and Oxaliplatin-based regimens, and is expected to end by February 2014. This trial is evaluating AMG 655, a fully human monoclonal antibody

that specifically binds to DR5, in combination with the FOLFIRI regimen (Zhao et al., 2012). As this family of cytotoxic drugs are being tested in clinical trials to sensitise TRAIL, it is of relevance to understand how the effect is induced at the molecular level in order to develop and improve TRAIL-based therapies in the future. Therefore in the present study, we investigated the molecular mechanism behind the ability of 5-FU to enhance TRAIL-induced apoptosis in our model of colon cancer: HCT116 cells. Initially we confirmed that subtoxic concentrations of 5-FU followed by sublethal rhTRAIL amounts could lead to an increased TRAIL-induced apoptosis in HCT116 cells. We observed apoptosis rates of 38% compared to 3% and 9% of the single agents 5-FU and rhTRAIL alone (**Fig 3.2.1 C**).

This low-dose sensitisation has been reported previously with chemotherapeutic drugs, such as 5-FU, and TRAIL (Mueller et al. 2011) but the molecular mechanism was not dissected in more detail in that particular study. This is an attractive approach as drugs such as 5-FU, at high doses, can harm healthy cells in addition to malignant cells; therefore lowering the dose while maintaining an increase in the overall apoptosis is a much-desired result. When we dissected this sensitisation at the molecular level, we began by examining whether this sensitisation was caspase-dependent, and whether the sensitisation is transduced through the prototypical caspase-8-mediated pathway or through a different, as yet unknown pathway. In the present study we found that the combined treatment of 5-FU and rhTRAIL induced increased caspase-8 cleavage (**Fig. 3.2.2 A**), in contrast to minimal caspase-8 cleavage in the single treatments. The presence of Z-VAD-FMK caspase inhibitor (**Fig. 3.2.1 C**) and silencing of caspase-8 itself (**Fig. 3.2.2 A & B**) abrogated the apoptosis induced by the combined treatment of 5-FU and rhTRAIL, indicating that it is a caspase-dependent process and caspase-8 is required for the apoptosis induced by the 5-FU/rhTRAIL combination in HCT116 cells. However, in contrast to the study by Erhardt et al., we did not observe any remarkable caspase-8 up regulation (**Fig. 3.2.3**) and therefore investigated alternative molecular drivers (Erhardt et al. 2008). A second potential protein reported to be responsible for TRAIL sensitisation is c-FLIP, a negative regulator of TRAIL-induced apoptosis, which is a potent inhibitor of caspase-8 (Bagnoli et al. 2010). The downregulation of c-FLIP has been demonstrated to be the cause of 5-FU-induced TRAIL sensitisation in previous studies (Galligan et al. 2005; Stagni et al. 2010). However, after 5-FU treatment we did not observe any

change in c-FLIP protein levels, or in a panel of several other key apoptotic cascade proteins (**Fig. 3.2.3**). However we observed, in line with several other reports, that 5-FU upregulated DR5 expression (**Fig. 3.2.3**), and silencing of this protein with shRNA halved the apoptosis levels obtained in the control cells using the combined treatment of 5-FU and rhTRAIL induced apoptosis (**Fig. 3.2.4**), which shows that DR5 is required for 5-FU/rhTRAIL-induced apoptosis and the up regulation of DR5 by 5-FU pre-treatment is at least one of the molecular drivers for the sensitisation. DR4 was upregulated by 5-FU but to a lesser extent than DR5 (**Fig. 3.2.3**). When DR4 was silenced, apoptosis reached new levels of 80% as compared to 50% in control cells (**Fig. 3.2.5**). Our hypothesis is that in HCT116 cells TRAIL-mediated apoptosis is preferentially transduced via DR5, and furthermore that DR5 homotrimers more efficiently transduce TRAIL-mediated apoptosis. Therefore in HCT.WT cells, 5-FU upregulates DR5 receptor levels and subsequent TRAIL-induced apoptosis (through a mixture of DR4 and DR5 heterotrimers, DR4 homotrimers and DR5 homotrimers) is transduced to a high level (**Fig. 3.2.8**).

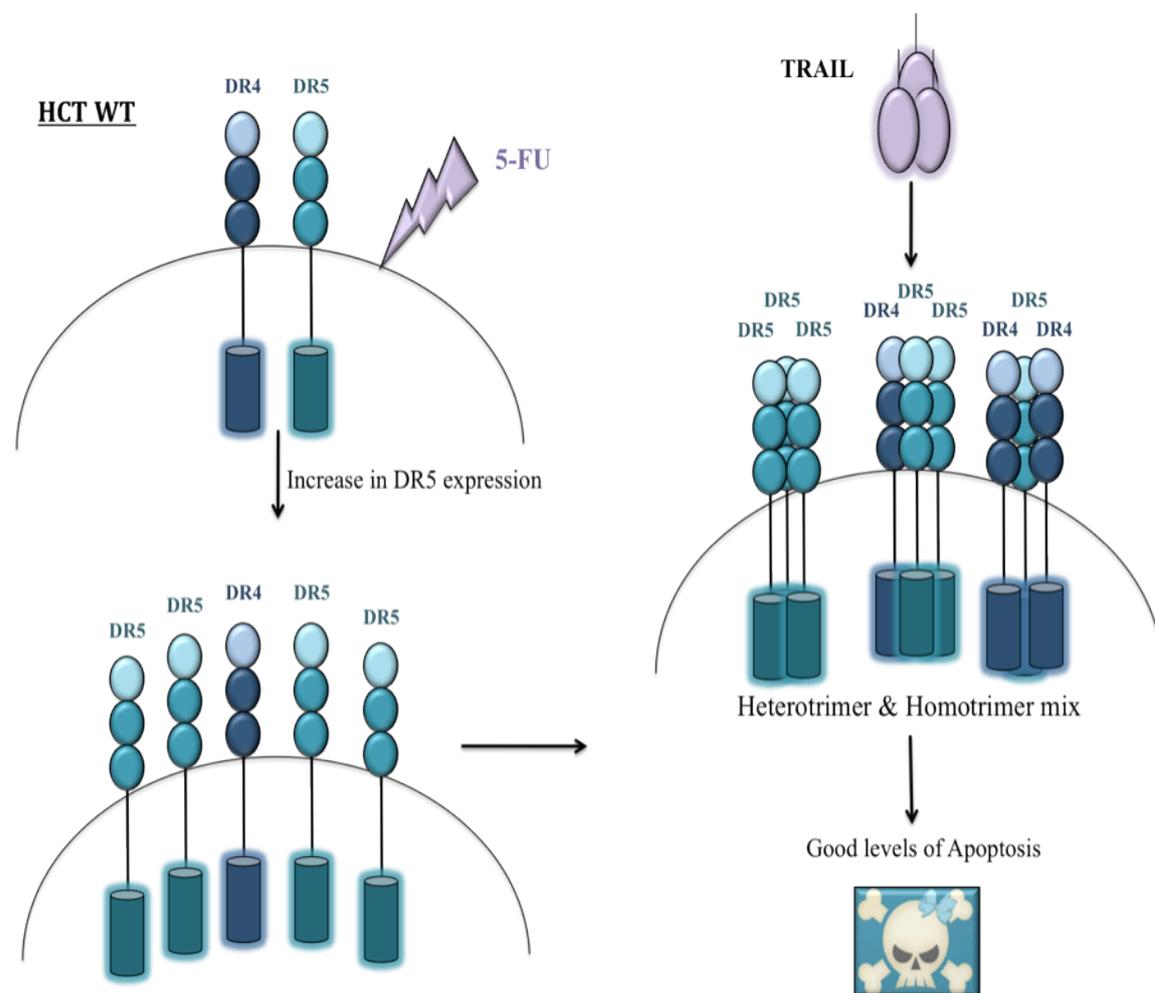


Fig. 3.2.8 Schematic of TRAIL-induced apoptosis in 5-FU pre-treated HCT116 cells.

In the HCT.shDR4 clone, 5-FU still upregulates DR5 expression levels. Due to the increased DR5 receptors present this is the first way in which apoptosis is increased through merely by the increased presence of TRAIL receptors. In addition, due to the lack of DR4 molecules present, apoptosis is transduced solely through DR5 homodimers, and DR5 is the preferred route of apoptosis transduction in HCT116 cells. This leads to a large increase in the overall apoptosis compared to the upregulated DR5 in the HCT.WT setting which while reaching high levels, is hindered somewhat by the interfering DR4 molecules forming heterotrimers with DR5 (see Fig. 3.2.9).

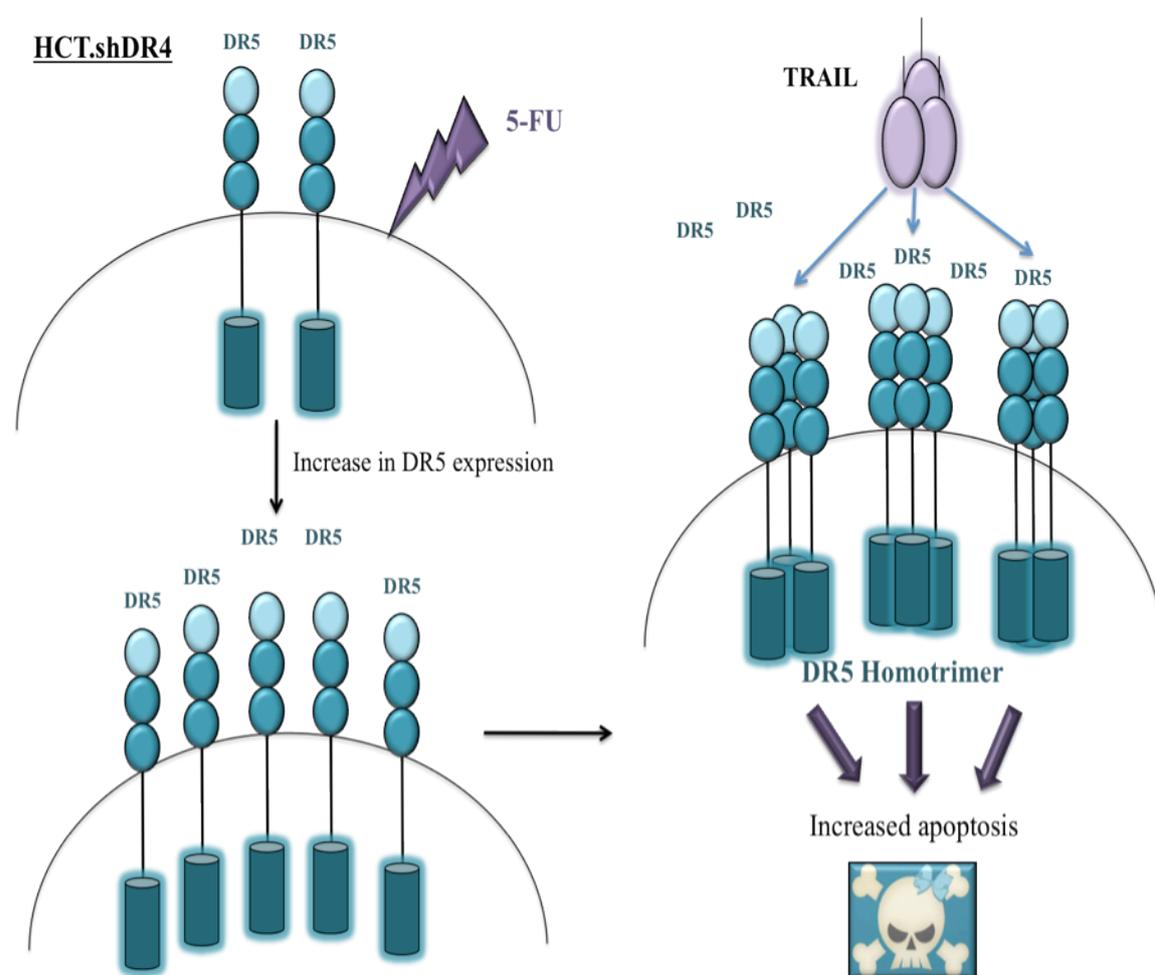


Fig. 3.2.9 Schematic of TRAIL-induced apoptosis in 5-FU pre-treated HCT.shDR4 cells.

In the HCT.shDR5 clone, however, there is no DR5 present to be upregulated by 5-FU, therefore the sensitisation effect is not possible. There are only DR4 homotrimers

formed and as HCT116 cells die preferentially via DR5, this also adds to the reduction in apoptosis (see Fig. 3.2.10).

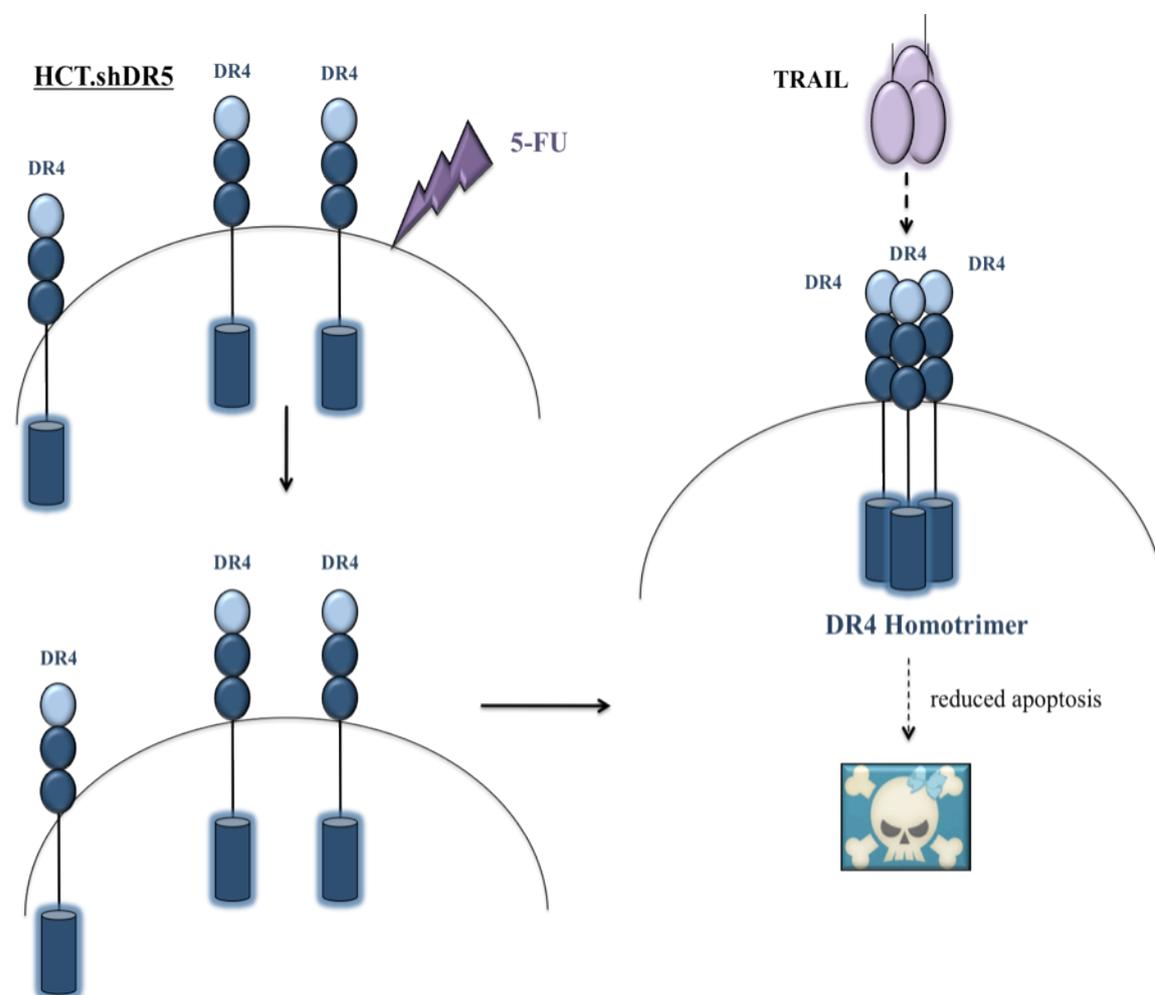


Fig. 3.2.10 Schematic of TRAIL-induced apoptosis in 5-FU pre-treated HCT.shDR5 cells.

In line with this hypothesis, it has been reported that DR5 may play a more prominent role than DR4 in mediating TRAIL-induced apoptosis in cells that express both receptors (Kelley et al. 2005). Furthermore, additional experiments within our lab found when using TRAIL-specific variants specific for DR5 or DR4 in HCT116 cells, apoptosis was observed to be higher using the DR5-specific variant as compared to the DR4-specific variant (Deedigan et al. 2013). However, in other cells such as HeLa cells, the DR4-specific variant proved to be more efficacious (Deedigan et al., 2013). One limitation to the above approach is that the DR5 upregulation is reported to be largely p53-dependent, which would pose a major hurdle as 50% of tumours are said to contain mutated p53 and cancers containing a p53 mutation appear to be less

responsive to therapeutics (Zhao et al. 2012). In addition to the results presented in this study, a further investigation within our group into the p53-dependency of the DR5 up regulation demonstrated that DR5 can be unregulated in an p53-independent manner in the HCT116 p53^{-/-} derivative cell line, albeit with a 24 h delay (Deedigan et al., 2013). This p53-independent up regulation of DR5 following the addition of 5-FU could be modulated by a variety of other factors. Independent to p53, DR5 can be upregulated by CHOP (Sung et al. 2010; Goda et al. 2008; Prasad et al. 2011), JNK (Prasad et al. 2011; Tazzari et al. 2008; Zou et al. 2004), ERK (Yodkeeree et al. 2009), p38 MAPK (Yodkeeree et al. 2009), and NF- κ B (Chen et al. 2008). Therefore further investigation into the molecular mechanism behind the p53-independent DR5 upregulation is required in this model.

In addition to optimisation of the 5-FU and rhTRAIL amounts, and deciphering the molecular mechanism of the sensitisation, we also wanted to further improve the therapeutic effect by overcoming a second important obstacle to TRAIL-based therapy, that of effective TRAIL delivery to, and bioavailability at, the tumour site. Insufficient bioavailability can be attributed to the short half-life of TRAIL protein (Ashkenazi et al. 1999). To this end, MSCs have been identified as potential delivery vehicles. MSCs are a pluripotent cell population, usually isolated from the bone marrow, that are capable of differentiating across lineage barriers into adipocytes, osteocytes and chondrocytes (Barry & Murphy 2004). MSCs exhibit a number of attractive properties outlined in the study by Mohr *et al.* (2008), such as they are immune-privileged and immunosuppressive owing to the fact that they do not express certain co-stimulatory molecules (Di Nicola et al. 2002; Majumdar et al. 2003; Tse et al. 2003).

Additionally, MSCs have been shown to be capable of homing to the site of a tumour in response to certain chemokines expressed on the tumour cell surface (Schichor et al. 2006; Son et al. 2006) and are also capable of being transduced to high levels with Adenovirus or lentivirus (McMahon et al. 2006). Therefore, in terms of clinical use, MSCs can afford protection of the therapeutic protein/transgene they are carrying from a cellular immune response (seen with the use of adenovirus-based therapies (Yang et al. 1995)), home to the site of the tumour and continue to express it, increasing the bioavailability. This is further increased by the use of the soluble secretable form of TRAIL. A number of studies have investigated MSCs as

therapeutic delivery vehicles with good success (Kumar et al. 2008; Kucerova et al. 2007; Studeny et al. 2004; Studeny et al. 2002) and it has also been demonstrated that MSCs expressing TRAIL can lead to tumour growth inhibition or remission in a variety of cancer models (Mohr et al. 2010; Mueller et al. 2010; Luetzkendorf et al. 2010; Mohr et al. 2008).

In the present study we utilised our optimised Ad.sTRAIL-transduced MSCs (MSC.sTRAIL) in combination with 5-FU, as the improved model of colon cancer cell treatment and tested it *in vitro*. We found that 5-FU sensitised HCT116 cells to MSC.sTRAIL, using the medium supernatant transferred from MSC.sTRAIL onto 5-FU pre-treated HCT116 cells (**Fig. 3.2.6**). Directly co-culturing 5-FU pre-treated HCT116 cells with the MSC.sTRAIL cells (**Fig. 3.2.7**) also gave rise to significant levels of apoptosis as compared to untreated and 5-FU-treated MSC.DsRed controls. In addition to these *in vitro* data, the *in vivo* efficacy of this model was explored within our group, and it was demonstrated that 5-FU in combination with MSC.sTRAIL resulted in full tumour remission in a HCT116 xenograft murine model (Deedigan et al., 2013). These results are clinically relevant as MSCs as a cell-based therapy are currently being studied in phase I/II and III clinical trials showing positive results in regards to their safety (Salem & Thiernemann 2010). The information generated from these clinical trials, in addition to our findings, has the potential to establish the foundation for a novel treatment approach in colorectal as well as other cancer types.

3.2.3 CONCLUSIONS

In conclusion, 5-FU enhances TRAIL-induced apoptosis via DR5 up regulation in HCT116 cells and this sensitisation requires the presence of caspase-8. We could demonstrate the use of MSC.sTRAIL in combination with sub toxic doses of 5-FU sensitises HCT116 cells *in vitro* and furthermore could demonstrate in a murine xenograft model of colon cancer, that MSC.sTRAIL in combination with 5-FU resulted in complete tumour remission. This approach may provide an effective, tumour-targeted and specific therapeutic strategy for safely treating human colorectal cancer, as even in the common scenario of p53 mutation, we could demonstrate that 5-FU pretreatment could sensitise HCT116 p53^{-/-} cells to TRAIL-induced apoptosis (Deedigan et al. 2013).

CHAPTER 4

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

4.1 CONCLUSIONS

Despite many advancements in the treatment of colorectal cancer, resistance to chemotherapy, namely 5-FU, remains prevalent. Successful nonsurgical elimination of cancer cells ultimately involves apoptosis, usually not only of malignant cells but also of normal cells, manifested by undesired toxicity. Understanding of how 5-FU exerts its apoptotic effects can help us to devise improved strategies that can directly and specifically induce apoptosis in cancer cells.

This study investigated in Chapter 3.1 how the apoptotic signal is transduced by 5-FU in the colorectal cancer cell line HCT116. This was addressed using a variety of methods including caspase cleavage studies, RNAi techniques, sucrose density gradient analyses and co-immunoprecipitation assays.

We demonstrated in the present study that 5-FU, through RNA stress, induces caspase-dependent cell death in HCT116 cells. Upon RNA stress, a protein interaction complex forms, containing the core components caspase-8, FADD, RIP1 and Bid. This complex formation was abolished when caspase-8 or FADD were knocked down however the absence of Bid or DR5 could not prevent its formation.

The hypothesised molecular mechanism of 5-FU-induced cell death in HCT116 cells is initiated by the assembly of a large protein complex comprised of caspase-8, FADD and RIP1 molecules with interaction from Bid. We hypothesise that this complex assembles spontaneously, independent of death receptors CD95, DR4 and DR5. The complex formation is potentially initiated by FADD dimerisation as a protein complex assembly platform, however this requires further investigation. Caspase-8 molecules then may be recruited along with RIP1. Bid is then cleaved to tBid by caspase-8 and migrates to the mitochondria. Once activated the mitochondria releases smac/DIABLO, which neutralises XIAP, resulting in full caspase-3 activation and apoptosis (See **Fig. 4.1**). Using the stable HCT.shBid and Ad.XIAP study in Chapter

3.1 we demonstrate that in the scenario of a mitochondrial block, the mitochondria can be circumvented by the silencing of XIAP. This allows caspase-8 to directly cleave caspase-3.

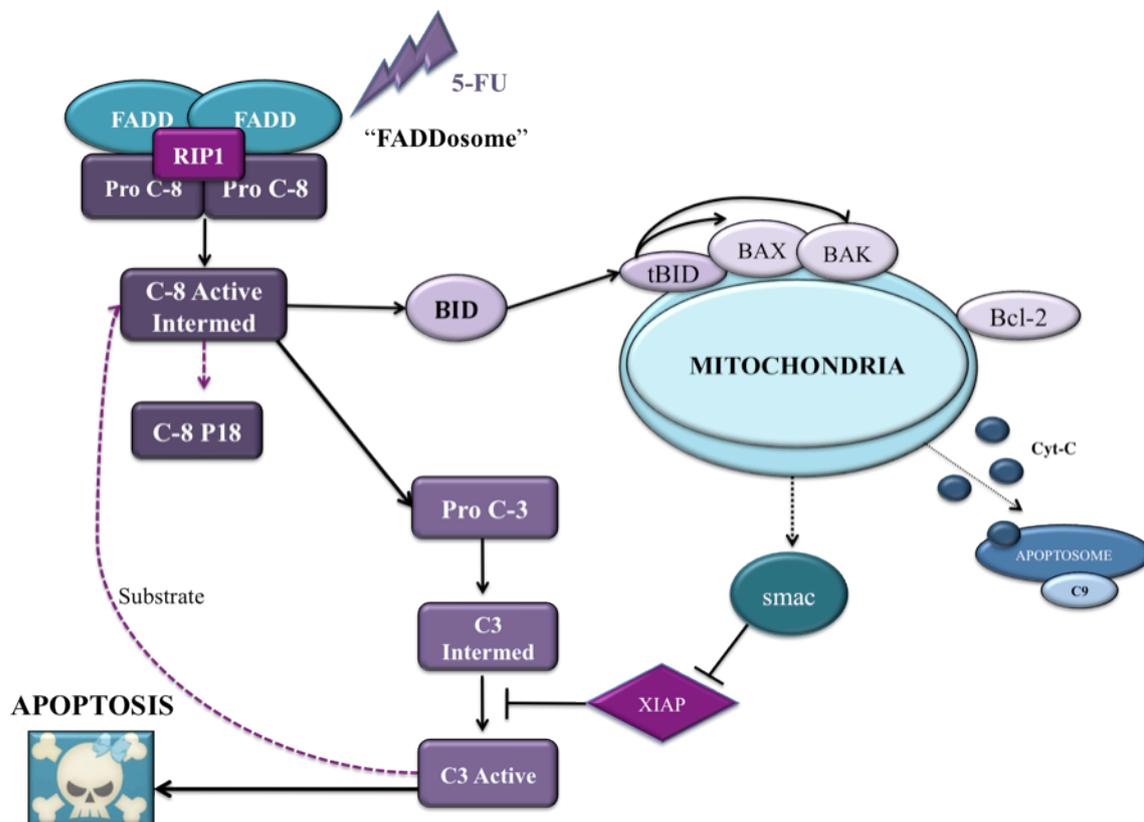


Fig. 4.1 Proposed Pathway of 5-FU-Induced Apoptosis in HCT116 cells. Upon 5-FU stimulation in HCT116 cells, a large (~1-2 MDa) protein complex assembles, putatively called the “FADDosome”, comprising of procaspase-8, FADD and RIP1 molecules. Caspase-8 is cleaved to its intermediate active fragment, which then can both directly cleave procaspase-3 to caspase-3 intermediates and in addition can cleave Bid at the complex to tBid. tBid then moves to the mitochondria and activates the mitochondrial pathway resulting in smac/DIABLO release. This results in XIAP neutralisation, allowing full caspase-3 cleavage and ultimately ending in apoptosis. Cytochrome-c is also released but the caspase-9 studies reveal that the apoptosome does not play a central role in this case. The fully processed caspase-3 fragment then cleaves intermediate caspase-8 as a substrate to its fully cleaved p18 fragment.

The findings of the present study shed some light on the improvement of current treatment regimens for colorectal cancer patients. Patients found to express high levels of Bcl-2 may not benefit from 5-FU therapy without using such compounds, as ABT 737 to inhibit the Bcl-2 expression first. However, importantly the findings of

the present study demonstrate that instead of caspase-9, caspase-8 cleavage is the apical event therefore, even in the case of a Bcl-2/mitochondrial block one could employ XIAP inhibitors which would allow bypassing of the mitochondrial pathway entirely as demonstrated with our HCT.shBid Ad.shXIAP experiment. In the scenario of low caspase-8, FADD or Bid expression, 5-FU-induced apoptosis would also be diminished and a different treatment strategy may be required, instead of using 5-FU as a first line therapy causing unnecessary toxicity and cost for the patient.

Furthermore, in Chapter 3.2 we questioned the molecular mechanism of 5-FU sensitisation to TRAIL-induced apoptosis in HCT116 cells. Unlike its family members TNF and FasL, TRAIL is not associated with induction of sepsis-like effects and hepatotoxicity respectively. Based on preclinical toxicity and activity data and the results described in this thesis showing expression of the pro-apoptotic TRAIL receptors and their upregulation via 5-FU, in colorectal cancer cell, recombinant (rh) TRAIL is considered of great interest for clinical use. Clinical trials incorporating agonist TRAIL receptor antibodies are underway and results from these studies will indicate the value of these agents as anticancer therapies (Zhao et al., 2012).

Futhermore combination regimens including rhTRAIL were explored in this study and in the clinical trial setting with promising results. Findings from several reports indicate that the combination of rhTRAIL with certain chemotherapeutic drugs, NSAIDs, proteosome inhibitors and interferon- γ all induce apoptosis in an additive or synergistic fashion. Such combination strategies potentially reduce the dose of not only TRAIL but also of the cytotoxic drug required for anti-tumour activity and were demonstrated with good effects in the present study. By dissecting the molecular mechanism of this sensitisation we demonstrate that it is the upregulation of DR5 that may be the crucial aspect for TRAIL-sensitivity. For example, patients found to be p53 mutated, may benefit from a combination therapy of 5-FU with TRAIL, but may require a longer 5-FU pretreatment prior to the TRAIL as demonstrated by the 24 h delay observed DR5 up regulation. Future treatment regimens should include molecular analysis of the patients protein expression profiles to allow for a more patient-tailored approach to chemotherapy.

In conclusion, new treatment options for CRC have been explored based on insights into mechanisms involved in the regulation of apoptosis. The potential clinical applications of combination therapies using 5-FU and rhTRAIL in the treatment of

colorectal cancer should be further explored. Results from the phase-1 clinical studies with chemotherapeutic drugs and TRAIL combination therapies should guide future therapeutic strategies. Ultimately there may not be one ideal anti-cancer agent but an optimal combination of agents targeting different intracellular pathways that will result in the killing of colorectal tumour cells.

4.2 FUTURE WORK

Following the investigations presented in this thesis, there remains a number of open questions to be addressed. Future work would include establishing whether RIP1 plays an important role in 5-FU-induced apoptosis in HCT116 cells, using RNAi stable clone generation and/or adenoviral knockdown of RIP1, followed by assessment of apoptosis and caspase cleavage after 5-FU stimulation.

To determine the mechanism of FADD dimerisation by transfecting the FADD constructs into the HCT.shc8 stable clone to demonstrate whether the dimerisation formed independently of caspase-8 molecules or if this was just a result of two caspase-8 molecules bringing together two FADD molecules.

To further purify the complex using the sucrose gradient analyses to finally identify the remaining (if any) complex members. This work was already begun prior to completing this thesis. This was achieved by combining fraction 14 of 6 gradients using HCT untreated, HCT 5-FU treated and HCT.shc8 5-FU treated cells. These gradients were all subsequently ran on a second purification step gradient of 35-60% and the fractions 9, 10 and 11 (containing caspase-8/FADD/RIP1) were combined and sent for 2D-DIGE and mass spectrometry analysis. Unfortunately the results were inconclusive as we were unable to detect the confirmed proteins present as the levels were insufficient. Future work would involve scaling up of the sucrose gradient 10 fold (which was already established prior to completion of thesis) and performing dialysis on the sucrose fraction, with a subsequent immunoprecipitation to purify the sample further. This would hopefully allow us to identify the complex members investigated in this thesis and in addition, novel putative members.

The next step would be to examine the role of any novel putative partners identified in

the 2D-DIGE and mass spec in the caspase-8 complex.

Overall it would be interesting to design and develop a protein array/chip that contains all essential factors for 5-FU-induced apoptosis. Such arrays could be used with individual tumour samples and inform therapeutic regimens.

CHAPTER 5

LIST OF CO-AUTHORED PUBLICATIONS

Deedigan L^{*}, Yu R^{*}, Albarenque SM, Mohr A, Zwacka RM. “Delivery of sTRAIL variants by MSCs in combination with cytotoxic drug treatment leads to p53-independent enhanced anti-tumour effects” *Cell Death and Disease*, 2013 manuscript accepted. ^{*} Both authors contributed equally.

Büneker C, Yu R, Deedigan L, Mohr A, Zwacka R. “IFN- γ combined with targeting of XIAP leads to increased apoptosis-sensitization of TRAIL-resistant pancreatic carcinoma cells.” *Cancer Lett.* 2012 Mar 28; 316(2): 168-77.

Mohr A, Albarenque SM, Deedigan L, Yu R, Reidy M, Fulda S, Zwacka RM. “Targeting of XIAP combined with systemic mesenchymal stem cell-mediated delivery of sTRAIL ligand inhibits metastatic growth of pancreatic carcinoma cells.” *Stem Cells.* 2010 Nov; 28(11): 2109-20.

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CHAPTER 6

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