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Regulation of cytotoxic drug-induced apoptosis by death receptor 5 in human colon cancer cells

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ABSTRACT

5-Fluorouracil (5-FU) is a cytotoxic drug that is widely used for the treatment of colon cancer. However, 5-FU-resistance is still a common issue which needs to be overcome. Understanding the molecular pathway of 5-FU-induced apoptosis will help designing more effective cancer treatments. Chemotherapeutic drugs such as 5-FU are thought to trigger cell death via the intrinsic apoptosis pathway which involves caspase-9 activation as one of the initiating molecular events. However, our group recently found the new apoptotic pathways which are involved in 5-FU-induced apoptosis in which caspase-8 is an essential factor for 5-FU-induced cell death with caspase-9 being dispensable for this process. Aside from caspase-8, the death receptor TRAIL-R2 (DR5), which is usually involved in the extrinsic pathway of apoptosis, was found to be required for the execution of 5-FU-triggered cell death. Moreover, following 5-FU treatment, DR5 is not responsible for caspase-8 activation, but for the activation of c-Jun N-terminal kinases (JNK) without the involvement of its natural ligand TRAIL. In the current study, factors associated with DR5 that are involved in JNK activation are identified. Also, the involvement of TRAF2, FADD, caspase-8, caspase-10 and RIP1 in JNK activation were examined. It was found that JNK is activated in HCT116, but not in DR5, caspase-8 and FADD knockdown cells after 5-FU treatment. Moreover, the absence of caspase-10, TRAF2 and RIP1 does not affect JNK activation. Also it was discovered that similar to 5-FU, when cells are treated with etoposide, another cytotoxic drug , JNK is also activated and initiated by DR5 and mediated by FADD and caspase-8. Thus, in the present study, the factors that are involved in activating JNK after 5-FU and etoposide treatment have been identified. The obtained results further clarify the mechanism of cell death in colon cancer cells after 5-FU and etoposide treatment which constitutes a novel regulating pathway of apoptosis.

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LIST OF ABBREVIATIONS

5-FU	Fluoropyrimidine 5-flourouracil
ABIN	A20-binding Inhibitor of Nuclear Factor-B
ANOVA	analysis of variance
APAF-1	Apoptotic Protease Activating Factor-1
APS	Ammonium persulfate
ASK1	Apoptosis Signal-Regulating Kinase 1
BID	BH3 interacting-domain death
BIM	Bcl-2 Interacting Mediator
BMF	Bcl-2 modifying Factor
BSA	Bovine Serum Albumin
CAD	Caspase-activated DNase
CBP	CREB-binding protein
CC	coiled-coil
CCC	C-terminal coiled-coil region

cFLICE	Cellular FLICE-inhibitory Protein
CML	chronic myelogenous leukaemia
DD	Death Domain
DED	Death Effector Domain
DISC	Death-inducing Signalling Complex
DNA	Deoxyribonucleic acid
DR	Death Receptor
ERK	Extracellular Signal-regulated Kinase
ETO	Etoposide
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated Death Domain
IKK	IκB Kinase
IMDM	Iscove's Modified Dulbecco's Medium
JIST	JNK-inducing signal transduction
JNK	c-Jun-terminal kinase
MAPK	Mitogen Activated Protein Kinase

MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MEKK	MAP and ERK Kinase Kinase
MLK	mixed-lineage protein kinase
MOMP	Mitochondrial Outer Membrane Permeabilization
NEMO	NF-κB essential modulator
OPG	Osteoprotegerin
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate-Buffered Saline
RHD	Relhomology domain
RIP	Receptor-interacting Protein
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SODD	Silencer of Death Domain
TBD	TRX-binding domain
TBS	Tris-Buffered Saline

TBST	TRIS Buffered Saline Tween 20
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
TNFRSF	TNF receptor superfamily
TRADD	TNFR1-associated Death Domain
TRAF	TNF receptor–associated Factor
TRAIL	TNF-related Apoptosis-inducing Ligand
TRX	Thioredoxin
TS	thymidylate synthase
XIAP	x-linked Inhibitor of Apoptosis Protein

LIST OF PUBLICATIONS

MOHR, A., DEEDIGAN, L., JENCZ, S., MEHRABADI, Y., HOULDEN, L., ALBARENQUE, S.-M. & ZWACKA, R. M. 2017. Caspase-10: a molecular switch from cell-autonomous apoptosis to communal cell death in response to chemotherapeutic drug treatment. Cell Death and Differentiation, 25, 340-352.

Du, S., Kendall, K., Toloueinia, P., Mehrabadi, Y., Gupta, G. and Newton, J., 2012. Aggregation and adhesion of gold nanoparticles in phosphate buffered saline. Journal of nanoparticle research, 14(3), p.758.

Chapter 1

LITERATURE REVIEW

1.1 Introduction

Cancer is one of the major public health problems in the world, caused by environmental factors that mutate genes which encode critical cell-regulatory proteins. The resulting abnormal cell behaviour leads to extensive masses of abnormal cells that destroy surrounding normal tissues and can spread to vital organs to develop disseminated tumours.

The majority of anti-cancer agents such as chemotherapy, radiotherapy, immunotherapy or suicide gene therapy induce apoptotic pathways to remove cancer cells (Makin and Hickman, 2000). However, resistance to apoptosis is one of the hallmarks of human cancers. As such, a better understanding and subsequent therapeutic interference in apoptosis pathways have the potential to help to overcome the clinical problems of drug resistance and improve therapeutic strategies to fight cancer.

1.2 Apoptosis

Apoptosis (from the Greek word for leaves falling from a tree) is a distinct mode of "programmed" cell death, resulting in eliminating of unwanted cells. Normally, apoptosis happens during development and aging. Apoptosis happens as a homeostatic mechanism to retain cell population. It also happens as a defence mechanism in immune reactions or when cells are damaged by diseases, irradiation and cancer therapy drugs (Kerr et al., 1972, Kerr, 2002). Inhibition and dysregulation of apoptosis lead to cancer and inflammatory diseases.

Although dysfunctional apoptosis causes severe human diseases such as cancer, excessive apoptosis also causes major diseases such as Alzheimer disease, Huntington disease, stroke, heart disease and AIDS (Alison et al., 2011). During apoptosis, a series of morphological features occur. These features include cell shrinkage, extensive plasma membrane blebbing, DNA fragmentation, cytoskeleton protein degradation and formation of apoptotic bodies during a process named "budding". Apoptotic bodies contain cytoplasm with tightly packed organelles either with or without a nucleus (Häcker, 2000, Hengartner, 2000, Kerr et al., 1972).

The mechanism of apoptosis is very complex; it involves an energy-dependent cascade of molecular events. There are two main apoptotic pathways: the extrinsic (death receptor or receptor-mediated) pathway and the intrinsic (stress-induced or mitochondrial) pathway. However, several studies have shown that there is a cross-talk between the extrinsic and intrinsic pathways and molecules from one pathway can affect the other one (Bold et al., 1997).

The key mediators of apoptosis are caspases (cysteine-dependent aspartate-specific proteases). Caspases are expressed in an in-active proenzyme form in majority of cells and when activated can stimulate other procaspases. Some procaspases can be auto-activated as well. Caspases have proteolytic activities meaning that they can cleave proteins at aspartic acid residues. To date, in mammals, ten main caspases have been identified and classified upon their role in apoptosis as follows: initiator (caspase-2, -8, -9, -10), effector (caspase-3, -6, -7) and inflammatory caspases (caspase-1, -4, -5) (Cohen, 1997, Ola et al., 2011).

1.3 Apoptotic pathways

In the extrinsic pathway of apoptosis, the death ligands such as Fas/APO-1/CD95 and Apo2L/TRAIL bind to their death receptors and create receptor-ligand interactions. Death receptors are members of the tumour necrosis factor (TNF) receptor gene superfamily comprising 20 proteins. Death receptors can regulate many biological functions such as cell death and cell survival (Locksley et al., 2001, Ashkenazi and Dixit, 1998). TNF is a member of the cytokines family which include TNF, CD95 (Fas) ligand (FasL, CD95L), CD40 ligand (CD40L) and TNF-related apoptosis-inducing ligand (TRAIL). Most of the TNF family members induce signalling pathways which lead to the activation of nuclear transcription factor κB (NF-κB), and some of these ligands can initiate apoptosis by binding to death receptors. So far, tumour necrosis factor receptors 1 (TNF-R1); Fas; Apo3 (DR3); TRAIL-R1 (DR4); TRAIL-R2 (DR5) and DR6 have been identified as death receptors which can initiate apoptosis. These receptors contain amino-terminal cysteine-rich extracellular domains (CRDs) (Pitti et al., 1996). These death receptors also share a unique cytoplasmic domain of about 70-80 amino acids named the "death domain" (DD). The death domain of death receptors plays a fundamental role in transferring the death signal from the surface of the cell to the intercellular signalling pathways (Ashkenazi and Dixit, 1998, Lavrik et al., 2005). In the death receptor signalling pathway, binding of a death receptor such as Fas with its ligand (FasL) mediates binding of the Fas-associated death domain (FADD) which is an adaptor molecule to the death receptor. Similarly, binding of TNF receptor to its ligand results in binding of TNFR1-associated death domain protein (TRADD) (Wajant, 2002). Then, FADD binds to TRADD and recruits procaspase-8 via its death effector domain. At this stage, a death-inducing

signalling complex (DISC) is formed, in which caspase-8 becomes auto-activated. After caspase-8 activation, the execution phase of apoptosis is activated via downstream effector caspases such as caspase-3 (Kischkel et al., 2001, Wajant, 2002, Gonzalvez and Ashkenazi, 2010) (Figure 1.1). Apart from adaptor molecules and caspases, cellular FLICE-inhibitory protein (cFLIP) also plays an important role in apoptosis. It interacts and binds to FADD, caspase-8 or caspase-10 and prevents DISC formation and thus supress caspase-8 or -10 activation (Bagnoli et al., 2010, He and He, 2013).

The intrinsic or mitochondrial pathway of apoptosis is regulated by the Bcl-2 family of proteins. This pathway is initiated through mitochondrial permeability and later the release of cytochrome c into the cytosol. Release of cytochrome c mediates activation of caspase-9 at a complex named the apoptosome (Figure 1.1). One of the key regulators of this apoptosis pathway is binding of apoptotic protease activating factor-1(APAF-1) to caspase-9 which executes apoptosis.

Apart from classical pathways of apoptosis (i.e., extrinsic and intrinsic pathways), novel apoptosis-inducing complexes such as the Ripoptosome have been identified. The Ripoptosome signalling complex consists of RIP1, FADD and caspase-8 and its formation is independent of death receptor and mitochondrial pathway. Formation of the Ripoptosome complex simulates caspase-8 activation. This complex requires kinase activity of RIP1 and it regulates caspase-8-mediated apoptosis (Tenev et al., 2011). Our group has recently found a new apoptosis inducing complex termed FADDosome. This complex is regulated by ATR and is caspase-10 but not caspase-9 dependent. The FADDosome complex is also independent of death receptor and ligand ligation. Essential

members of FADDosome complex are caspase-10, caspase-8, ATR, FADD, TNF receptor–associated factor 2 (TRAF2) and RIP1. One of the hallmarks of this complex is degradation of $cFLIP_L$. TRAF2 adaptor protein acts as a ubiquitination ligase and it mediates ubiquitination and degradation of $cFLIP_L$. Both RIP1 and caspase-10 are required for recruitment of TRAF2. Lack of caspase-10, TRAF2 and ATR switches the formation of FADDosome to FLIPosome formation. In the FLIPosome complex, caspase-8 mediates cleavage of $cFLIP_L$ to $cFLIP_{43}$ and TNF-α production (Mohr et al., 2017).

Although formation of FADDosome and FLIPosome are both p53-independent, significant reduction of apoptosis in p53-negative HCT116 cells was observed (Mohr et al., 2017). In the extrinsic pathway of apoptosis, p53 regulates TNF receptor family transcriptionally including Fas and DR5 death receptors (Amaral et al., 2010). While most death receptors only activate the canonical (caspase-8) apoptosis pathway, the TNF receptors (TNFR) can activate non-canonical apoptosis pathways as well. In this study, inspired by the TNFR signalling pathway, it was hypothesised that p53 is also involved in another pathway in 5-FU induced HCT116 cells.

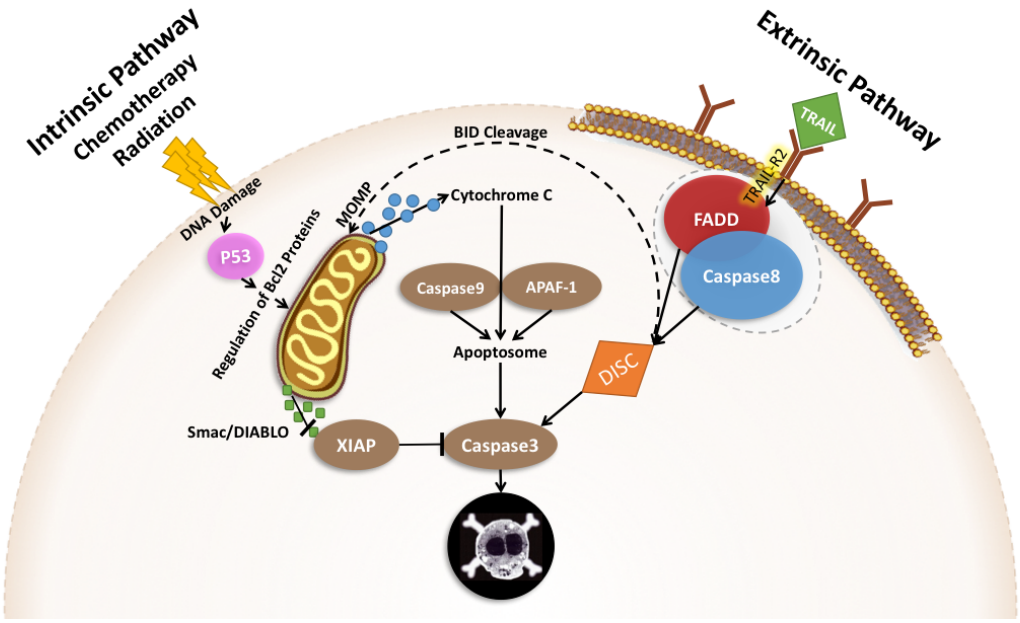

Figure 1.1. Pathways of apoptosis

According to conventional models, apoptosis is triggered either by the extrinsic or intrinsic pathway. The extrinsic (receptor-mediated) pathway is started by binding of a death receptor to its ligand and subsequent recruitment of adaptor molecules such as (FADD) and formation of a death-inducing complex (DISC) that will activate caspase-8. The active initiator caspase-8 will activate executioner caspases including caspase-3. The intrinsic or stress-activated pathway of apoptosis is principally regulated by Bcl-2 family proteins, which control the permeability of the mitochondrial membrane. Pro-apoptotic Bcl-2 family members such as Bid, Bax and Bak induce the release of cytochrome c into the cytoplasm. Then, cytochrome c binds to Apaf-1 and caspase-9 and activates caspase-9 (APOPTOSOME). Then, the Apoptosome cleaves caspase-3 that will eventually lead to DNA fragmentation and cell death. Smac/DIABLO is also released into the cytoplasm, where it inhibits the anti-apoptotic protein named x-linked inhibitor of apoptosis protein (XIAP), thereby releasing the molecular brake it exerts on caspase-3 and caspase-9.

1.3.1 TNF receptor-induced signalling pathway

Tumour Necrosis Factor (TNF) is a 26KDa cytokine (cell signalling protein) which stimulates various responses at cellular and systemic level such as control of cell proliferation, differentiation and apoptosis (Falvo et al., 2010).

Both soluble and membrane TNF can bind to and signals via its two transmembrane receptor molecules: TNF receptor 1 (TNF-R1), also known as p55/p60, containing a death domain (DD) and TNF receptor 2 (TNF-R2), also known as p75/p80. TNF-R1 is widely and ubiquitously expressed on most of cells of the human body, whereas TNF-R2 is more limited and is normally found in the immune system cells (Tartaglia et al., 1991).

TNF-induced apoptosis is mediated through TNF-R1 (Naudé et al., 2011). Release of an intracellular TNF inhibitor, the silencer of death domain (SODD) protein is necessary in this pathway. Upon TNFR interaction, it permits both SODD release and recruitment of DISC complex proteins such as TRADD, FADD and TNF-R-associated factor 1 or 2 (TRAF1 or TRAF2). These proteins create a scaffold which promotes the recruitment and activation of pro-caspase-8. The activated caspase-8 leads to activation of effector caspases such as caspase -3, -6, -7 and finally apoptotic cell death (Sedger and McDermott, 2014).

In TNF-induced signalling pathway, negative inhibitor protein FLICE (cFLIP) regulates the protease activity of caspase-8. The death effector domain (DED) of cFLIP allows it to interact with pro-caspase-8 and other DED containing proteins (Sedger and McDermott, 2014). In addition to cFLIP, the inhibitor of apoptosis proteins (IAPs) also

interact with TRAF2 and regulate TNFR-induced apoptosis (Vince et al., 2009). Apart from activation of canonical pathway of apoptosis, TNFR signalling can also activate non-canonical pathways such as NF-κB, p38 mitogen activated protein kinase (MAPK) and c-Jun-terminal kinase (JNK) (Mak and Yeh, 2002). Inspired by TNFR signalling pathway mechanism, it was hypothesized that p53 also activates non-canonical pathways such as NF-κB, JNK or p38.

1.3.2 NF-κB signalling pathway and its regulation

NF-κB is a transcription factor for large number of genes involved in different pathways including inflammation, stress response, cell differentiation and apoptosis. The NF-κB family is comprised of five transcription factors: p105 (NF-κB1), p100 (NF-κB2), RelA (p56), RelB and c-Rel. the later three contain C-terminal transcriptional domain (TADs) which enable them to promote activation of target gene expression. These transcription factors share around 300 amino acid long amino-terminal called Rel homology domain (RHD). The RHD provides a platform in which family members can regulate their expression (Hayden and Ghosh, 2012, Aggarwal, 2003).

Regulation of NF-κB pathway is mediated by its interaction with inhibitor of κB (IκB) proteins. IκB proteins inhibit 'resting' state cytosolic NF-κB dimers from binding target sites. In fact, IκB causes inhibition of DNA binding in cytosolic NF-κB and it keeps NF-κB in the cytoplasm. Currently, eight members of IκB proteins have been identified: IκBα, IκBβ, IκBε, IκBγ, BCL-3, IκBδ and the precursor proteins p105 and p100 (Ghosh et al., 1998).

The NF-κB signalling pathway can be categorised to classical (canonical) and alternative (non-canonical) signalling pathway. Activation of an IκB kinase (IKK) complex is a regulator step in both cascades. This complex is consisted of two IκB kinases (IKK): IKKα (IKK1) and/or IKKβ (IKK2) along with a regulatory subunit named NEMO (NF-κB essential modulator) which also known as IKKγ (Hayden et al., 2006). Dimers of NF-κB are triggered by IKK-mediated phosphorylation of IκB that mediates proteasomal IκB activation. This permits translocation of active NF-κB transcription factor to the nucleus and initiates gene transcription (Hayden et al., 2006) (Figure 1.2).

In the canonical signalling pathway, binding of ligand, tumour necrosis factor α (TNFα), to a cell surface receptor, TNFR1, leads to recruitment of molecule adaptor such as TRAF2 to the cytoplasmic domain of the receptor. Then, TRAF2 or other adaptor molecules recruit the IKK complex which mediates the phosphorylation and degradation of the IκB proteins. The classical pathway triggers NF-κB dimers containing RelA, c-Rel, RelB and p50 (Hayden et al., 2006).

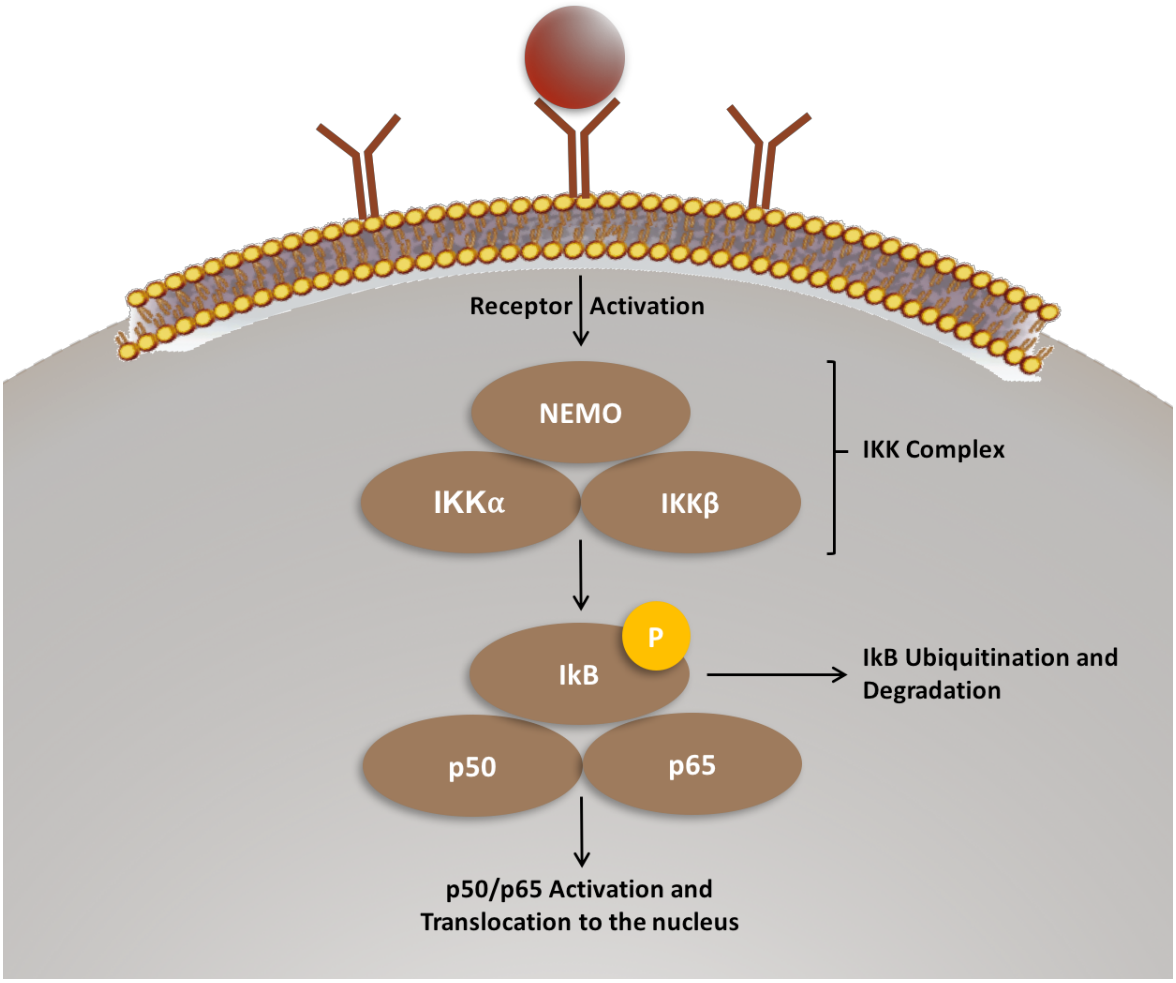

Figure 1.2. The canonical pathway of NF-κB

Ligation of ligand (TNFα) with cell receptor (TNFR1) results in activation of an IKK complex containing IKKα and IKKβ. activation these complex results in KB degradation. Translocation of p50 and p65 to nucleus results in activation of target genes.

The non-canonical pathway of NF-κB is responsible for activation of p100 and RelB complexes. The alternative signalling pathway includes an IKK complex which have two IKKα subunits. Unlike the canonical pathway, NEMO is not involved in this pathway.

In this pathway, ligation of cell receptor with a ligand triggers NF-κB inducing kinase (NIK) to phosphorylate and activate the IKKα complex. The activated IKKα complex phosphorylates p100 which results in processing and translocation of RelB and p52 into the nucleus (Hayden et al., 2006) (Figure 1.3).

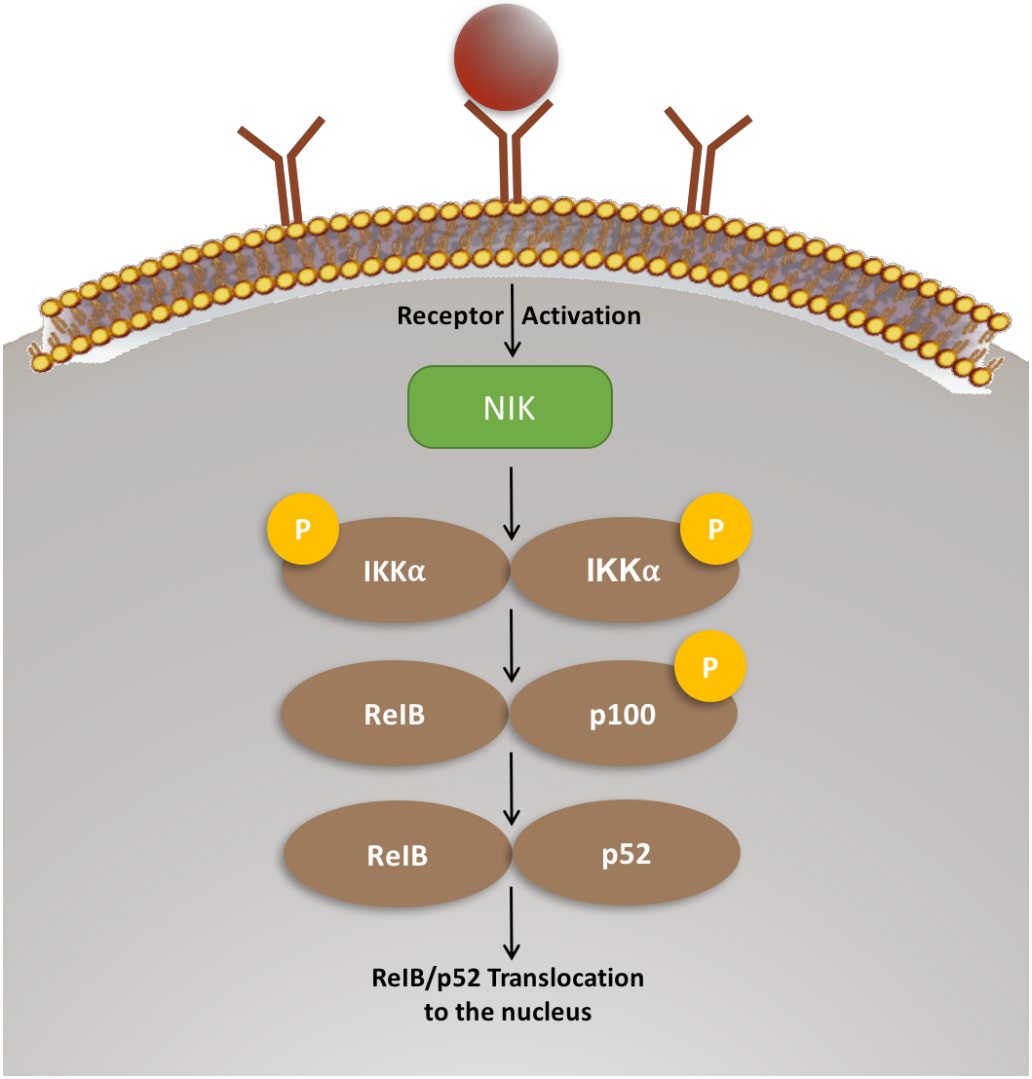

Figure 1.3. The non-canonical pathway of NF-κB

Ligation of cell receptor with a ligand triggers NF-κB inducing kinase (NIK). This pathway includes phosphorylation of IKKα, p100 and p52 which is mediated by NIK. Translocation of RelB/p52 into nucleus results in activation of target genes.

1.3.3 MAPK signalling pathway

Mitogen activated protein kinases (MAPKs) are signalling components which play an important role in converting extracellular stimuli into cellular responses. The MAPK cascade is a signalling system consists of three activated protein kinases which transduce wide range of extra-cellular signals. MAPK signalling pathway controls many extracellular signals which are responsible for cell proliferation, cell differentiation and cell death. Each cascade is initiated by extracellular signals and causes activation of a specific MAPK following successful activation of a MAPK kinase kinase (MAPKKK or MAP3K) and a MAPK Kinase (MAPKK or MAP2K). MAPKs activation requires dual phosphorylation on the Thr-X-Tyr motif by MAP2K. After MAPKs activation, they phosphorylate specific serine and threonine residues of target substrates such as c JUN and p53 (Fang and Richardson, 2005).

The activated MAP kinases convert the external stimuli to the corresponded physiological responses through phosphorylation of downstream substrates such as transcription factors, cytoskeletal proteins and mRNA translation proteins. The MAPKKK is normally initiated by interactions with a small GTPase and/or phosphorylation by cell surface receptors (Keshet and Seger, 2010). The MAPKKK phosphorylates and activates MAPKK, by phosphorylation of Ser and/or Thr residues in the MAPKK T-loop which in turn activates the MAPK by phosphorylation of a pThr-Xaa-pTyr motif located in the MAP kinase T-loop. The sequence of this T-loop motif is a defining feature of MAP kinases: Thr-Glu-Tyr (ERK); Thr-Gly-Tyr (p38); and Thr-Pro-Tyr (JNK). As mentioned, three groups of MAPK kinases have been characterised so far: extracellular signal-regulated kinase

(ERK); JNK; and the p38 MAP kinases. Generally, ERK are initiated via mitogens and differentiation signals, whereas the p38 and JNK are activated via stress stimuli (Pitzschke, 2015, Morrison, 2012). Apoptosis signal-regulating kinase 1 (ASK1) is a MAP3K isoform which activates JNK and p38 MAP kinase via TNF and regulates inflammatory cytokines expression (Tobiume et al., 2001, Nishitoh et al., 1998). The external stimulus can be transformed to correct physiological responses through phosphorylation of downstream substrates such as transcription factors by activated MAP kinases.

1.3.4 ERK signalling pathway

The extracellular signal-regulated kinase-1 (ERK1) and extracellular signal-regulated kinase-2 (ERK2) MAPKs gets activated by mitogens and have shown to be upregulated in human tumours. Both ERK1 and ERK2 are serine/threonine kinases. ERK1 and ERK2 are expressed in most of the mammalian cells. Both ERK1 and ERK2 are activated through MAP2K isoforms: MKK1 and MKK2. Activation of both MKK1 and MKK2 is regulated by Tumour Progression Locus2 (TPL2) which is a MAP3K isoform (Das et al., 2005). The ERK cascade acts in cellular proliferation, differentiation and survival. TPL2 together with A20-binding inhibitor of nuclear factor-κB (ABIN) and NF-κB1(p105) form a complex in resting cells and is not active. Activation of TPL2 requires both the MAP3K isoforms transforming growth factor β (TGFβ)-activated kinase 1 (TAK1) and activation of IκB kinase 2 (IKK2). The phosphorylated/activated IKK2 in turns activate p105 which itself cause degradation of p105/NF-κB1 and release of TPL2 from the ABIN

complex. In the ERK signalling pathway, the role of TAK1 is not very clear as the function of TAK1 is cell type specific (Sabio and Davis, 2014).

1.3.5 p38 signalling pathway

Both JNK and p38 MAPKs signalling pathways can be activated by environmental and genotoxic stresses. They play important roles in inflammation and tissue homeostasis as they can regulate and control cell proliferation, differentiation, survival and migration. Consequently, JNK and p38 MAP kinase signalling pathways are called stress-activated MAP kinase and they have shown to be activated in the cells treated with TNFα. Both p38 and JNK pathways are mediated by MAP3K isoforms; however, different activation of MAP2K isoforms leads to activation of either JNK or p38 MAP kinase. There are four members of the p38 MAP kinase signalling family: p38α , p38β , p38γ and p38δ. MKK3, MKK4 and MKK6 mediate p38 signalling pathway in vitro. However, only MKK3 and MKK6 mediate p38 in vivo. Previous studies on fibroblasts treated with TNFα have indicated that both MKK3 and MKK6 kinases lead to activation of p38α and p38β MAP kinases. Also, MKK3 is the key activator of p38δ MAP kinase and MKK6 is the main activator of p38γ MAP kinase (Brancho et al., 2003, Remy et al., 2010).

1.3.6 JNK signalling pathway

The JNK signalling pathway is a stress-activated MAP kinase signalling cascade, which is activated in the cells treated with TNF. JNK1, JNK2, and JNK3 are three members of

the JNK family. *MAPK8, MAPK9 and MAPK10* are the three genes which codes for JNK1, JNK2 and JNK3 proteins respectively. JNK3 can only be found in brain, cardiac smooth muscle and testis. However, JNK1 and JNK2 are expressed ubiquitously. JNK isoforms play an important role in the brain. JNK1 and JNK2 has shown to have crucial roles in the modulation of immune cell function and in the development of embryonic nervous system. Also, JNK3 has shown to be important in controlling brain function, brain development, memory and learning (Dhanasekaran and Reddy, 2008, Johnson and Nakamura, 2007). One of the major targets of JNK is the transcription factor AP1 which mediates gene expression. JNK's ability to phosphorylate JUN and further to activate AP1 gives them oncogenic function. On the other hand, their pro-apoptotic activities make them able to function as a tumour suppressive agent (Wagner and Nebreda, 2009, Eferl and Wagner, 2003).

JNK signalling activation depends on interaction of the scaffold proteins belonging to the JNK activation complex. The interactions of proteins in this scaffold cause the activation JNK via bi-phosphorylation of various substrate enabling activation of diverse functions. There are various stimuli which can stimulate JNK activation such as nerve growth factor (NGF), DNA damage, oxidative stress, low potassium, excitotoxic, UV irradiation and TNF (Davis, 2000). MKK4 and MKK7 are the MAP2K which activate JNK and phosphorylate JNK on Tyrosine and Threonine respectively (Dhanasekaran and Reddy, 2008).

One study has shown that TNF is an activator of MKK7 but not for MKK4. Thus, MKK7 is an essential MAP2K for TNF-stimulated JNK activation. However, the mechanism of

activation of MKK7 by TNF is still unclear. In another study lack of MKK4 and MKK7 in mice cells prohibited TNFα- induced JNK activation (Wajant et al., 2003). Therefore, it can be concluded that activation of JNK requires combined action of both MKK4 and MKK7. It also has been shown that in response to TNF, MKK7 is a potent activator of JNK and MKK4 leads to extended JNK activation (Tournier et al., 2001).

1.3.6.1 Role of JNK signalling pathway

Most studies about pro-and anti-apoptotic effects of JNKs have been achieved using studies in fibroblasts. Both pro- and anti-apoptotic effect of JNK depend on many factors such as stimuli, strength of stimuli and tissue particularity (Wagner and Nebreda, 2009). JNK1 and JNK2 interact with JUN differently. JNK2 has shown to target JUN for degradation in non-stimulated cells. However, when cells are stimulated, JNK1 phosphorylates/activates JUN which leads to transcriptional activity whereas when cells are not stimulated, JNK2 mostly target JUN for degradation (Fuchs et al., 1996). Also, the pro-apoptotic function of JNK has shown to be involved in mitochondrial pathway. JNKs can phosphorylate and regulate expression of the Bcl2 family of proteins, including Bax and BAD and 14-3-3 proteins (Tournier et al., 2000). Phosphorylation of 14-3-3 proteins by JNKs regulates the release of pro-apoptotic proteins such as BAX and transcription factors such as FOXO (Weston and Davis, 2007). Also, following TNFα JNK mediates cleavage of BH3 interacting-domain death (BID) independent of caspase-8 (Wagner and Nebreda, 2009).

Apart from activation of Bcl-2 protein, the activated JNK can also regulate transcription factors such as c-Jun, c-Fos, ATF-2, activator protein (AP-1) and p53 (Davis, 2000,

Akhtar et al., 2014, Sui et al., 2014). It has previously shown that in response to UV, c-Jun prevents p53 cell cycle arrest; therefore, it promotes p53-dependent apoptosis. c-Jun can act as a direct repressor of p53 (Reyes-Zurita et al., 2011). Moreover, it has been shown that in leukaemia cells, anticancer drugs upsurge p53 phosphorylation and JNK induction (Mertens-Talcott et al., 2005).The main substrate which is activated by JNK phosphorylation is c-Jun which can then interact with JunB, JunD, c-Fos and ATF and form the AP-1 transcription factor leading to regulation of cellular stress-response or regulating the signals leading to activation of the caspases signalling pathway which is also capable of phosphorylating and activating apoptosis regulating proteins such as Bcl-2 interacting mediator (BIM) and Bcl-2 modifying factor (BMF) which both mediate caspases activation (Eferl and Wagner, 2003, Harris and Johnson, 2001). JNK can also phosphorylate anti-apoptotic proteins such as DP5-HRK, Bcl-2 and Bcl-xL and inhibits their function (Yarza et al., 2015).

Activation of both p38 and JNK MAP kinase signalling pathway is initiated by MAP3K protein kinase family such as ASK, MEKK, MLK, TAK1 and TPL2. However, regulation of these pathways is cell type specific (Sabio and Davis, 2014).

1.3.6.2 Regulation of MAPK signalling pathway by ASK1

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAP3K family which activates MKK3/6-p38 and MKK4/7-JNK signalling pathways through activation of downstream MAP2Ks including MKK3, MKK6, MKK4 and MKK7 which results in apoptosis (Ichijo et al., 1997). Many stressors such as oxidative stress, endoplasmic reticulum stress, calcium influx and TNFα can activate ASK1 (Hayakawa et al., 2012).

Also, deregulation in some diseases such as cancer, neurodegeneration and cardiovascular diseases can initiate ASK1 activity. ASK1 activity is highly regulated through interaction with thioredoxin (TRX) , TRAF2 , TRAF6 and the scaffolding protein 14-3-3 (Obsil and Obsilova, 2017) (Figure 1.4).

Human ASK1 contains 1374 amino acids with three domains including the N-terminal TRX-binding domain (ASK1-TBD), the central regulatory region (ASK1-CRR), which includes the TRAF-binding region and the serine/threonine kinase domain (ASK1 / CD), which is located in the centre of the molecule. ASK1 also contains a coiled-coil (CC) region which is at the C-terminus of the ASK1 molecule (Tobiume, 2002).

Apart from ASK1 and ASK2, ASK3 has also been identified as an apoptosis signal regulating kinase in response to cellular stresses. ASK1,2 and 3 have the same structural characters. The function of protein kinases is mediated by two strategies. The first one is mediated through scaffold proteins. In the ASK1 situation, β-arrestin and JNK-interacting proteins (JIPs) are known as scaffold proteins. The other strategy is the increase of kinase activity. Phosphorylation of the activation loop in the kinase domain stimulates a conformational alteration of the N- and C- terminus and therefore upregulates the kinase activity. Amongst ASK family Thr838, Thr806 and Thr808 are the critical residue in the activation loop. Phosphorylation at Thr838 regulates ASK1 activity. ASK2 and ASK3 also contain same two sites for their kinase activities (Nishida et al., 2017). The regulatory region is located between the kinase domain and the TRX binding domain (TBD) of ASK1 which regulates ASK1 positively and negatively (Weijman et al., 2017). The central regulatory region of ASK1 connects TBD and its kinase domain. This linking

regulates a compact arrangement of these regions that reduce the activity of ASK1. However, the central regulatory region of ASK1 also prepares MKK6 which then it stimulates ASK1-MKK6 signalling between family members. Between ASK1,2 and 3, ASK1 is the most well-known one. ASK1 is known to form a homo-oligomer via its C-terminal coiled-coil region (CCC) in non-stressed cells. The arrangement of this oligomer is vital for basal activity of ASK1 which is regulated by transautophophorylation of ASK1. Also, it has been shown that under specific condition (no stimulation) the CCC region regulates a high molecular mass complex formation of ASK1 that is designated the ASK1 signalosome. Apart from ASK1, there are many components which have been identified to establish this signalosome and mediate ASK1 signalling such as Trx and TRAFs family (Nishida et al., 2017).

1.3.6.2.1 Trx and TRAFs family

Trx has been identified as an important negative regulator of ASK1 activity. It binds to the N-terminal region of ASK1 and prevents its kinase activity (Kosek, 2014). While Trx is an inhibitor of ASK activity, TRAF family interaction triggers ASK1. TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6 are known to interact with ASK1 (Figure 1.5). One study has shown that TRAF2, TRAF5 and TRAF6 upsurge its activity (Nishida et al., 2017). Also, it has been shown that TRAF2 and TRAF6 are necessary for ASK1-JNK/p38 activation in response to H_2O_2. TRAF2 and TRAF6 assists the homophilic interaction of ASK1 (Sau et al., 2011, Wu et al., 2006). Also, TRAF1 has also shown to mediate ASK1 signalling pathway in neuronal and hepatic cells (Nishida et al., 2017). These data suggest that the effect of TRAF family members on ASK1 is cell type or situation dependent.

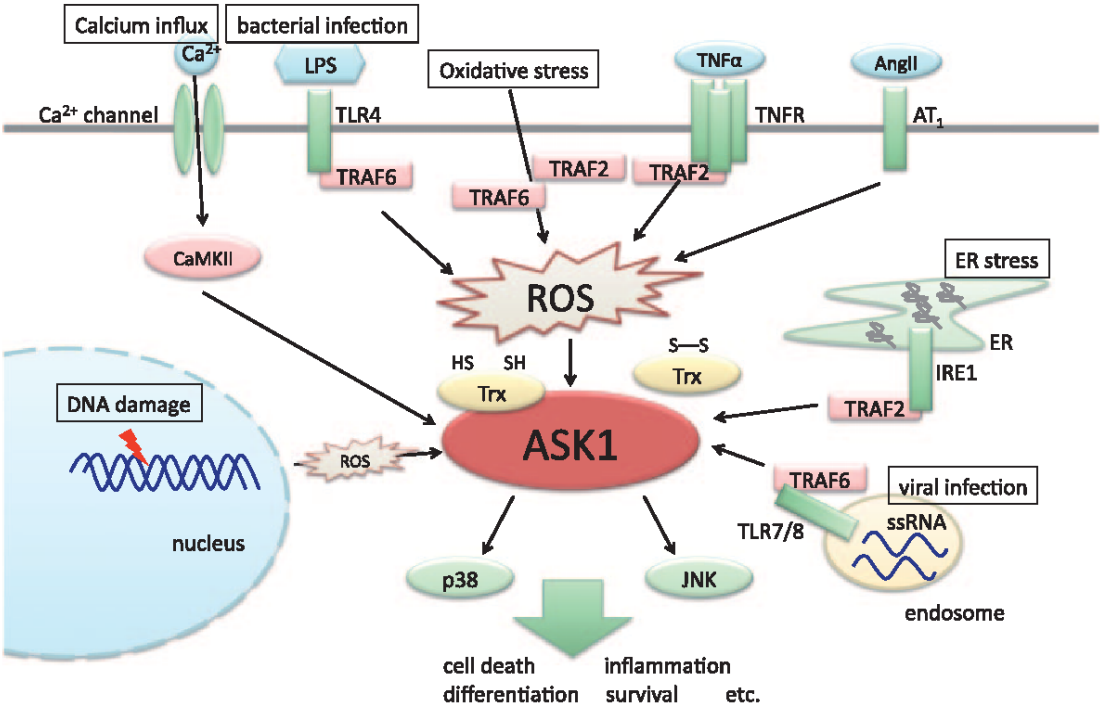

Figure 1.4. Overview of ASK1 pathway.

ASK1 can be activated by different stimuli including oxidative stress, calcium influx, bacterial infection or DNA damage-inducing agents such as anti-cancer drugs. ASK1 can also be activated by TNF signalling pathway through TNF receptor (TNFR) and TNFα. In ASK1 signalling pathway, TRAF2, TRAF6 and CaMKII act as activators of ASK1. Moreover, in this pathway, Thioredoxin (Trx) which is a redox protein alters its structure depending on the cellular redox state (converts to the oxidized form) and dissociate from ASK1 which then activates ASK1. Activated ASK1 stimulates p38 and JNK signalling pathways and will induce many cellular responses such as apoptosis, inflammation and cell survival (figure from (Hayakawa et al., 2012)).

1.3.6.2.2 ASK Signalling pathway

It has been shown that in ASK1- deficient cells ASK1 is necessary for JNK and p38 activation and initiation of apoptosis following TNFα or oxidative stress (Hayakawa et al., 2012). Apart from TNFα , ASK1 also mediates signalling of Fas for ASK1- JNK

pathway which is Daxx , a death domain-associated protein shown to interact with Fas death receptor, dependent (Nishida et al., 2017).

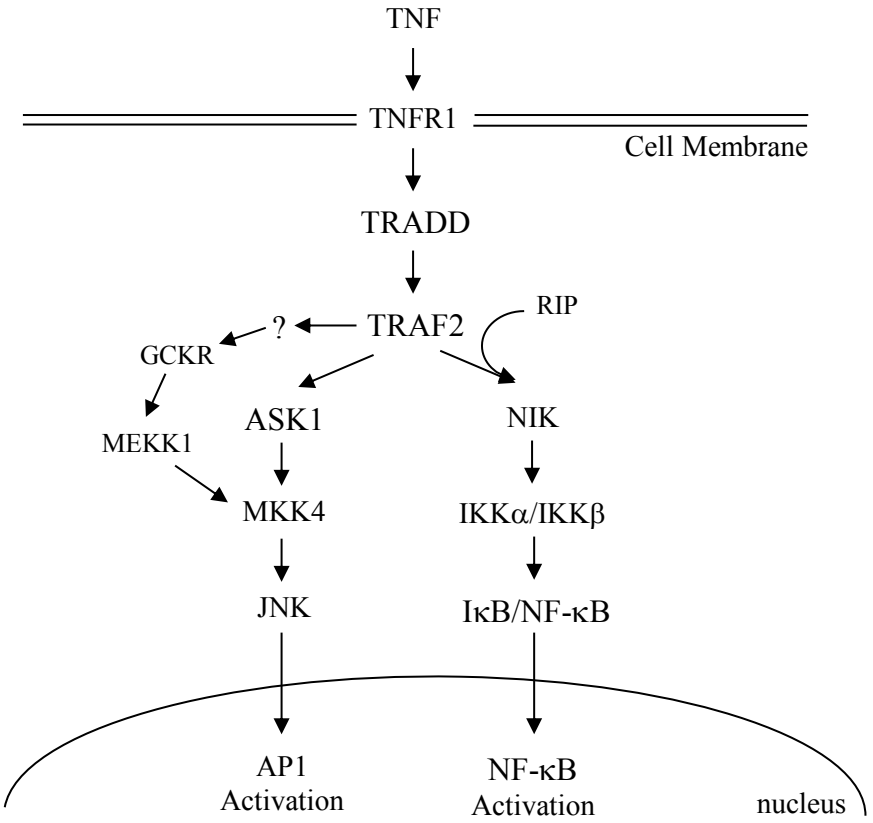

Figure 1.5. Role of ASK1 in TNF signalling pathway.

ASK1 is MAPKKK which is a component of the TNF-induced JNK activation pathway through TRAF-2. ASK-1 is a mediator of TRAF-2-dependent JNK activation that interacts with TRAF-2. Interaction of TRAF-2 with ASK-1 mediates NF-κB and JNK pathway. One pathway is mediated through interaction of TRAF-2 with NIK and RIP1 which stimulates the NF-κB signalling pathway. In the other pathway, upon TNF stimulation, TRAF2 requites ASK1 and activates the JNK pathway (figure from (Nishitoh et al., 1998)).

1.3.6.3 TAK1

Transforming growth factor β-activated protein kinase 1 (TAK1) is another important mediator of JNK (by MKK4 and MKK7) and p38 (by MKK3 and MKK6) MAP signalling pathway. TAK1 also regulates ERK signalling pathway through IKK2/TPL2 pathway. TAK1 also identified as an important mediator of JNK and p38 MAP kinase in response to TNFα. However, several recent studies have shown the partial role of TAK1 in TNF-stimulated activation of stress-activated MAP kinases (Sabio and Davis, 2014, Sakurai, 2012).

1.3.6.4 MEKK

So far, four family members of the MAP and ERK kinase kinase (MEKK) have been identified (Zang et al., 2013). MEKK1 has been reported to be fundamental for JNK activation in response to TNFα (Xia et al., 2000). However, this result has not been confirmed and it is possible that MEKK leads to MAP kinase regulation through TRAIL and CD40L signalling pathways (Sabio and Davis, 2014).

1.3.6.5 MLK

Rac/Cdc42 is a Rho family GTpase which can activate JNK by the mixed-lineage protein kinase (MLK) pathway. There are four members of the MLK family: MLK1, MLK2, MLK3, MLK4 (Gallo and Johnson, 2002). One study in *vivo* showed that interruption of the MLK2 and MLK3 genes decreased activation of both JNK and p38 signalling pathway (Kant et al., 2011).

1.4 TRAIL pathway of apoptosis

TNF-related apoptosis-inducing ligand (TRAIL, Apo2-L), a soluble zinc-coordinated homotrimeric protein, is a member of the TNF superfamily of cytokines which regulates extrinsic pathway of apoptosis. TRAIL is a type II transmembrane protein containing of 281 amino acids. It also has DD domain which contains 70-amino acids (Schneider et al., 1997, Walczak et al., 1997). TRAIL binds to four membrane receptors and one soluble receptor. The human TRAIL receptor can be divided into two types. The first type is full-length intracellular DD-containing pro-apoptotic receptors which are DR4 and DR5 (Schneider et al., 1997, Walczak et al., 1997). These two receptors are capable of inducing apoptosis. The second type which is a called decoy receptor includes TRAIL-R3 (DcR1, TNFRSF10D) (Degli-Esposti et al., 1997b), TRAIL-R4(DcR2, TNFRSF10D) (Degli-Esposti et al., 1997a) and osteoprotegerin (OPG, TNFRSF11B) (Emery et al., 1998). TRAIL-R3 does not have an intracellular domain. TRAIL-R4 has a cytoplasmic domain which can stimulate NF-κB. However, it cannot induce apoptosis as it only contains a truncated DD (Karstedt et al., 2017).

TRAIL is a homotrimeric molecule. Each monomer of TRAIL is composed of two antiparallel β-sheets. A distinctive feature of TRAIL is its central zinc atom at the trimer interface. This zinc ion binds to the cysteine-230 sulfhydryl of each monomer and support maintaining the trimeric structure of the TRAIL. This cysteine-zinc structure is vital for stability, solubility and biological activity of TRAIL. Also, unlike other ligands of TNF the family, TRAIL did not have serious side effects when it was used for cancer therapy. This unique feature of TRAIL which is being able to kill cancer cells selectively led to

make recombinant ligands and agonistic anti-DR4/DR5 antibodies and use them for clinical tests. However, clinical tests utilised recombinant TRAIL and humanized agonistic antibodies had partial anti-tumour outcomes. The limited anti-tumour result could be due to reduced activation of caspase-8 and decrease of expression of pro-apoptotic receptors on the cell surface. Also, it could be due to over expression of anti-apoptotic factors such as cFLIP or IAP and anti-apoptotic members of the Bcl-2 family (Lim et al., 2015, Lemke et al., 2014). TNF, TRAIL and FasL can be discharged from cell the surface by the action of proteases. Consequently, these death ligands can exist as both membrane-bound and soluble proteins. For Fas, the membrane-bound type of the protein can only initiate apoptosis (Hengartner, 2000). Whereas, for TRAIL it is not very clear. However, some studies have shown that liposome-bound TRAIL which act as membrane-bound type is more active in removing cancer cells than the soluble type (Karstedt et al., 2017).

1.4.1 TRAIL death receptors (TRAIL-R)

Comparing with other mammals, expression of DR5 and DR4 receptors in human and monkey cells shows that there are differences between DR4 and DR5 in terms of structure and function. For instance, in pancreatic carcinoma cell, apoptosis is mediated by DR4. Whereas in other cells such as colorectal cells apoptosis is mediated through DR5 (Nahacka et al., 2017). The second mentioned receptor of TRAIL are called ‘decoys’ receptors since through their binding to TRAIL, they will decrease concentration of TRAIL available for DR4 and DR5 to bind; therefore, it will mediate apoptosis negatively.

The only difference between DR4 and DR5 receptor is that there is only one splice variant for DR4; however, for DR5, there are two splice variants (LeBlanc and Ashkenazi, 2003).

1.4.2 Signalling pathway of TRAIL

Like other family members of TNF, TRAIL can also initiate many biological responses in addition to canonical caspase-dependent apoptosis through binding to its death receptors, TRAIL can activate non-canonical cell survival or proliferation pathways by the same cell receptors.

1.4.2.1 Canonical pathway of TRAIL

Upon ligation of DR4 or DR5 by trimeric TRAIL, the intracellular DDs of receptors enables them to recruit DD-binding adaptor proteins such as FADD or TRADD and form multiprotein signalling complex. Adaptor molecules such as FADD and TRADD contain a DED enabling them to recruit initiator caspases (-8 and/or-10).

Interaction of receptors with adaptor molecules and caspases assembles TRAIL DISC. TRAIL-DISC consisting of oligomerized receptor, adaptor molecule such as FADD, initiator caspase such as caspase-10 or caspase-8 and depending on the circumstance, the FLICE- inhibitory protein (c-FLIP). C-FLIP is an inhibitor of the extrinsic pathway of apoptosis which has represented to be a main inhibitor of caspase-8 at the DISC signalling complex. C-FLIP has a structural homology with caspase-8 and caspase-10 (Boatright and Salvesen, 2003). However, c-FLIP deficits a catalytic cysteine residue; therefore, it does not have enzymatic activity. To date, 10 different splice variants of c-FLIP have

been identified. However, three c-FLIP isoforms and two cleavage products are identified in humans: two short variant S which are: c-FLIP$_S$ and c-FLIP$_R$ and one long variant which is c-FLIP$_L$. All c-FLIP isoforms contain two DED domains which can bind to the DISC. The short isoforms of c-FLIP, c-FLIP$_S$ and c-FLIP$_R$ can block caspase-8 and therefore apoptosis. c-FLIP$_L$ can also act as an anti-apoptotic member apoptosis at higher concentrations. However, c-FLIP$_L$ can also act as a pro-apoptotic and regulator of caspase-8 in the company of higher amount of short c-FLIP isoforms such as c-FLIP$_S$ and c-FLIP$_R$. c-FLIP$_S$ has two N-terminal DEDs and a short C-terminal tail which prevents DR-mediated apoptosis by competing with caspase-8 and caspase-10 for linking with FADD. c-FLIP$_L$ has two N-terminal DED and two catalytically inactive caspase-like domains at the C-terminus. The function of c-FLIP$_L$ at DISC is complex. One study has shown that c-FLIP$_L$ overexpression protected cells from apoptosis. However, in another study it was shown that ectopic c-FLIP$_L$ overexpression encouraged activation of caspase-8 and apoptosis. In another study silencing of c-FLIP$_L$ improved activation and recruitment of caspase-8 at DISC (Safa, 2012). These foundlings propose that at larger amount, c-FLIP$_L$ can act as an anti-apoptotic member of DISC complex by competing for replacing caspase-8 and caspase-10. However, at lower amount of c-FLIP$_L$, it can heterodimerize with caspase-8 or caspase-10 at DISC complex and act as a pro-apoptotic member (Safa, 2012).

In the canonical TRAIL pathway of apoptosis, DISC complex members lead to initiator caspases (-8 and/or -10) activation and release of enzymes to cytoplasm. Activated caspase-8 and caspase-10 are discharged into the cytosol. In turn, they can activate

effector caspases such as -3, -6 or -7 and finally apoptosis (Sprick et al., 2002, Kischkel et al., 2000, Kuang et al., 2000).

It is still unclear whether caspase-8 and caspase-10 have redundant or dissimilar roles in the TRAIL pathway of apoptosis. Previously it has been shown that in TRAIL DISC, in the absence of caspase-8, caspase-10 cannot compensate the role of caspase-8 to initiate extrinsic apoptosis (Kischkel et al., 2001). However, two other studies have shown that both caspase-8 and caspase-10 have significant roles in apoptosis induction. Also, in another study, the distinct role of caspase-10 was shown as it could act independently of caspase-8 in initiating extrinsic pathway of apoptosis (Sprick et al., 2002, Wang et al., 2001).

As mentioned previously, based on cell type, the extrinsic pathway of apoptosis can occur dependent or independent of intrinsic apoptosis namely type I and type II cells. One of the possible causes of decision of apoptosis to be type I or type II is c-FLIP. C-FLIP can adjudicate cell to be more reliant on intrinsic pathway through preventing caspase-8 activation at DISC complex. Also, in many cancer cell types such as colorectal carcinoma and pancreatic carcinoma, high level of c-FLIP has been observed. Another factor which can derive apoptosis is XIAP. XIAP gene ablation in mice hepatocytes and B cells leaded apoptosis to happen self-reliantly of Bid. Furthermore, XIAP downregulation avoid recruitment for amplification of mitochondria and type II cells treated with FasL and TRAIL were sensitized to apoptosis. Therefore, the ratios of cFLIP to caspase-8 and XIAP to Smac/DIABLO may control the response of cell to apoptosis and whether to be a type I or type II kind (Rudner et al., 2004).

1.4.2.2 Non-canonical pathway of TRAIL

Apart from the induction of extrinsic apoptosis through DISC formation and caspases, binding of TRAIL to DR4, DR5 and TRAIL-R4 can also promote kinase pathways such as IKK, JNK and p38 (Schneider et al., 1997). However, studies with overexpression of DR4 and DR5 have shown that TRAIL is less effective than TNFα for NF-κB activation. The details of activation of convictional caspase-activated apoptosis through FasL and TRAIL have been well reported and defined. However, there is little known about the exact mechanism of their kinase-activating function. Some studies focused on cell death signalling and did not focus on exact mechanism of kinase pathway (Figure 1.6).

In the non-canonical pathway of TRAIL, it has been shown that upon TRAIL to TRAIL-R ligation, RIP kinase 1 (RIPK1) mediates NF-κB activation through IKK complex activation with IKK-α and IKK-β and NEMO/IKK-γ members (Fulda, 2013). In fact, degradation of inhibitor of κB (I-κB) allows NF-κB transfer to nucleus and finally regulating apoptosis by transcription of genes which regulate apoptosis. In addition, for NF-κB activation, TRAIL recruits RIP1, TRAF2 and NEMO to an extracellular complex named complex II which is down stream of DISC complex. Apart from mentioned members, complex II also contain FADD, TRADD, caspase-8 and caspase-10 (Lin et al., 2000).

Apart from RIPK1, FADD also mediates TRAIL-induced translocation. FLIP which is an anti-apoptotic protein also regulates NF-κB activation through TRAIL but it is not still very clear that how this pathway is regulated (Wajant, 2002, Truneh et al., 2000, Hu et al., 2000). Also, a study has shown that TRAIL can activate kinase pathways through

association of two signalling complex. Both complexes contain FADD and caspase-8. However, the secondary complex also contains RIP1, TRAF2 and NEMO. They also indicated that JNK activation by TRAIL requires RIP1 and TRAF2 whereas IKK activation needs NEMO. Formation of this complex trigger NF-κB, JNK and p38 MAPK pathways (Varfolomeev et al., 2005).

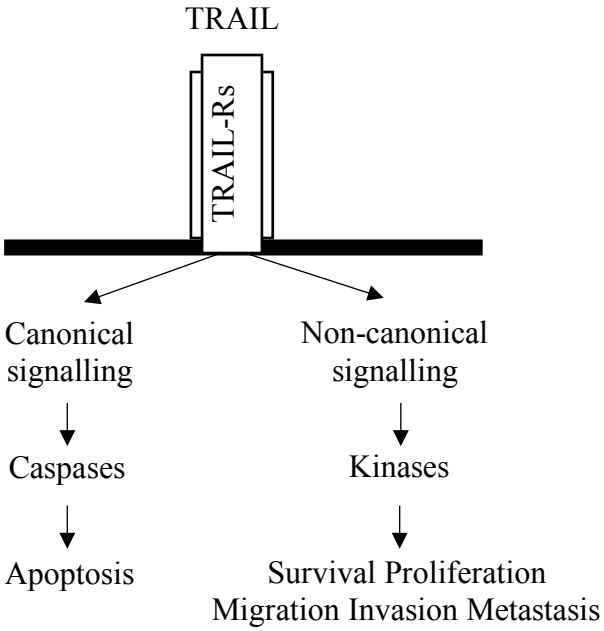

Figure 1.6. Schematic diagram of canonical and non-canonical pathways of TRAIL signalling pathway.

Binding of TRAIL to its receptors activates either canonical or non-canonical pathways. In canonical pathway of apoptosis, upon binding, DISC complex is formed which results in caspase activation. Non-canonical pathway engages cascades of kinases which leads to proliferation, Migration, Invasion and Metastasis (figure from (Fulda, 2013)).

1.5 CD95L (FasL) pathway of apoptosis

Binding of CD95L (FasL) to CD95 (Fas) leads to creation of a receptor complex at the cellular membrane which is called FasL-DISC. The DD of the death receptor interacts with the DD of the FADD. Also, the DED of FADD interacts with the N-terminal tandem DEDs of procaspase-8, -10 and c-FLIP (Lavrik and Krammer, 2012).

Similar to TRAIL pathway of apoptosis, there are also two types of apoptosis signalling pathways: type I (higher activation of caspase-8) and type II (lower activation of caspase-8 and involvement of Bid cleavage by caspase-8 to generate t Bid) (Lavrik and Krammer, 2012). Another study has also shown that upon Fas stimulation, second signalling complex is formed named as complex II. Complex II contains FADD, pro-caspase-8 and c-FLIP$_L$ but not Fas. Formation of this complex leads to activation of caspase-8 (Lavrik et al., 2008).

1.6 Intrinsic pathway of apoptosis

The intrinsic pathway of apoptosis is triggered by non-receptor stimuli which generate intracellular signals that initiate mitochondrial events. These stimuli include, radiation, toxins, stress signals and DNA damage. Changes in the inner mitochondrial membrane is an initial stage of intrinsic apoptosis which cause mitochondrial outer membrane permeabilization (MOMP) mainly controlled by the Bcl-2 family of proteins (Saelens et al., 2004, Cosentino and García-Sáez, 2014). Intrinsic pathway has two main characteristics: first, loss of the mitochondrial transmembrane potential and release of pro-apoptotic proteins such as cytochrome c and Smac/DIABLO proteins from the intermembrane space into cytosol. Second, formation of the "apoptosome" by cytochrome c, Apaf-1 and pro-caspase-9 which later activates caspase-9 (Hill et al., 2004, Chinnaiyan, 1999). In the intrinsic pathway, Smac/DIABLO stimulate apoptosis by inhibition of IAP (inhibitors of apoptosis proteins) activity (Schimmer, 2004) (Figure 1.1).

The Bcl-2 family of proteins cause outer membrane permeability and can be classified into pro- or anti- apoptotic. To date, in the Bcl-2 family, twenty-five genes have been known. Some of the anti-apoptotic proteins are: Bcl-2; Bcl-XL; Bcl-XS; Bcl-w; Bag; Mcl-1 and A1 and some of the pro-apoptotic proteins are: Bax; Bak and the BH3 only proteins, i.e. Bad, Bim, Bid, Bik, Puma, Hrk, Bmf and Noxa. These proteins play essential roles in apoptosis since they decide whether cells commit to apoptosis or aborts it (Kuwana and Newmeyer, 2003). The main role of the Bcl-2 family of proteins such as Bax/Bak is known to be causing putative mitochondrial pores and regulation of cytochrome c from mitochondria (Siddiqui et al., 2015).

In some cell types, such as hepatocytes and many cancer cell lines there is a cross talk between intrinsic and extrinsic pathway of apoptosis where caspase-8 is unable to activate effector caspases due to existence of XIAP (Type II cells). Therefore, in type II cells, instead of activating effector caspases, caspase-8 activate and cleave the BH3-only protein Bid. The cleaved Bid then moves to mitochondria and activates Bax/Bak. Then, Bax/Bak facilitates release of cytochrome c and formation of the apoptosome.

As mentioned, the main regulators of the intrinsic pathway of apoptosis is the Bcl-2 family of proteins which are regulated and controlled via the tumour suppressor protein p53 (Cory and Adams, 2002, Bieging et al., 2014).

1.6.1.1 Regulation of intrinsic pathway of apoptosis by p53

p53, also known as TP53 or tumour suppressor protein is a cellular stress sensor and a significant mediator of transient and permanent cell cycle arrest and cell death in response to different stresses such as, DNA damage, oxidative stress and ribonucleotide depletion (Efeyan and Serrano, 2007).

In response to major stresses, p53 is transferred from its negative regulators Mdm2 and Mdm4 which allows its stabilisation and activation (Hu et al., 2012, Marine et al., 2006). Apart from promoting apoptosis or permanent cell-cycle-arrest, under low-level stress signals, p53 stimulates temporary cell-cycle arrest, DNA repair and anti-oxidant protein generation to repair the damages (Efeyan and Serrano, 2007).

Human p53 has 393 amino acids. p53 protein domains have a size of between 40 and 200 amino acids. To date, four domains of p53 have been identified: transactivation domain

(1-70 residues); the sequence–specific DNA binding domain (94-293 residues) , tetramerization domain (324-355 residues) and a last domain which identifies damaged DNA (Stavridi et al., 2005).

The N-terminus of p53 refers to a transactivation domain containing two transcriptional activation domains (TADs) named TAD1 and TAD2. These domains can individually increase transcription of p53 target genes by using histone-modifying enzymes such as STAGA and mediator (Brady and Attardi, 2010). The transactivation domain of p53 can also regulate p53 function and stability by interacting with other transcription factors including p300, CREB-binding protein (CBP) and Mdm2 (Finnberg et al., 2005, Davison et al., 2001).

The most interesting domain and the central core of p53 is its sequence-specific DNA binding domain which is targeted by most of cancer-associated p53 mutations. This domain is responsible for sequence-specific binding of the protein to p53 response elements in DNA. The majority of cancer-associated p53 mutations are missense mutations in the sequence-specific DNA binding domain (Davison et al., 2001).

p53 mutation usually happens after loss of heterozygosity and results in complete p53 deficiency. This p53 deficiency increase the progression of cancer. Tumours lacking p53 have certain characteristics such as: lack of cellular differentiations, genetic instabilities and metastatic potentials (Olivier and Taniere, 2011, Rivlin et al., 2011). The cancer-associated p53 mutation is either contact mutants which change the residues that are crucial for direct contacts with p53 response elements or structural mutants. R175, G425, R284, R249, R273 and R282 are the most six common p53 amino acids which are

changed in cancer. Apart from disturbing DNA binding, these mutations can also increase invasiveness and metastasis as well (Brosh and Rotter, 2009).

p53 can regulate apoptosis by different mechanisms including transcriptional and non-transcriptional activity. p53 can regulate MOMP by binding and sequestration of Bcl-2 and Bcl-XL to activate Bak and Bax proteins. p53 can also physically interact with anti-apoptotic Bcl-2 and Bcl-XL at DNA binding domains causing their interaction with Bax/Bak proteins and finally release of cytochrome c from mitochondria or by direct interaction with Bak to relieve it from Mcl-1 (anti-apoptotic protein). p53 may activate the expression of Apaf1 as mice cells with caspase-9 or Apaf-1 deficiency were resistant to DNA-damage-induce apoptosis and p53-dependent death stimuli (Schuler and Green, 2001). Also, in response to stress, p53 can activate the transcription of Puma which binds to Bcl-XL and allows p53 to activate Bax (Schuler and Green, 2001).

Apart from mediation of apoptosis through regulation of mitochondrial events (mediation of Bax and Apaf-1 expression) p53 can transcriptionally or non-transcriptionally control the expression of death receptors such as Fas and DR5 in response to DNA damage-induced apoptosis (Amaral et al., 2010).

1.7 Execution pathway

Both extrinsic and intrinsic pathway of apoptosis end at execution phase which is the final stage of apoptosis. Activation of the execution caspases initiates this stage of cell death. These caspases activate cytoplasmic endonuclease that degrades nuclear material. Executioner caspases such as -3, -6 and -7 cleave many substrates such as cytokeratins, poly (ADP-ribose) polymerase (PARP), alpha fodrin (cytoskeletal protein of plasma membrane) and NuMA (nuclear protein) that eventually cause morphological changes in apoptotic cells (Jamil et al., 2015).

One of the most important executioner caspases is caspase-3 which is activated by initiator caspases such as -8, -9, or -10. Caspase-3 in turn activates the caspase-activated DNase (CAD). Finally, CAD degrades chromosomal DNA within nuclei which results result in chromatin condensation and cell death. Caspase-3, also causes cytoskeletal reorganization and fragmentation of the cell into apoptotic bodies (Brentnall et al., 2013, Lalaoui et al., 2015).

1.8 Research motivation

The fluoropyrimidine 5-flourouracil (5-FU) is an antimetabolite drug which is widely used for different cancer types but particularly for colon cancer (Longley et al., 2003). This drug exerts its effects through inhibition of crucial biosynthetic processes and incorporation and inhibition of normal function of DNA and RNA molecules. Although 5-FU can help patients with stage III colon cancer, the response rates of chemotherapy with 5-FU as a first treatment are only 10-15% (Xu et al., 2006, Longley et al., 2003).

5-FU is a key anticancer drug which has been widely used for antitumor activity for more than 50 years (Miura et al., 2010). However, there is an urgent need to identify and elucidate new therapeutic strategies either by finding new intervention points and compounds or by optimising the use of current agents such as 5-FU (Longley and Johnston, 2005).

In the latter approach, understanding of pathways and mechanisms by which chemotherapeutic drugs such as 5-FU induce apoptosis is a significant initial step in finding ways of optimising its use either alone or in combination with other treatments.

Therefore, the aim of this study is to identify the mechanism of action of 5-FU and identify the proteins having key roles in this process using the human colon cancer cell line HCT116 as our main model system. These factors might be therapeutically valuable as they may provide novel molecular targets that can help to overcome drug resistance and decrease the toxic side effects of unnecessary treatments.

Recent results from our laboratory show that absence of caspase-8 or FADD inhibits 5-FU-induced apoptosis in HCT116 cells and that an intracellular signalling platform containing caspase-8, FADD, caspase-10, RIP1 and TRAF2 termed the FADDosome complex is responsible for caspase-8 activation and 5-FU-induced apoptosis (Mohr et al., 2017). As part of the investigation into the mechanisms of 5-FU-induced caspase-8 activation, our group examined the potential involvement of death receptors in it. When DR5 knocked down cells were used, there was a significant drop in apoptosis, which was not the case in DR4- or Fas-silenced cells. Therefore, it was concluded that DR5 might be providing the platform for caspase-8 activation in response to 5-FU. However, in DR5-silenced cells caspase-8 activation in response to 5-FU was unaffected. This left other, noncanonical signalling cascades, namely NF-κB and the various MAPK pathways, that can be triggered by DR5 as potential candidates for the observed apoptosis reduction.

The aim of the current study was to analyse the DR5-associated complex and identify factors that participate in the activation of JNK after 5-FU treatment (Figure 1.7). This model serves as our current working model and is based on our findings as well as on hypothetical complexes (e.g. JIST (JNK-inducing signal transduction)) and pathways, of which most are in turn are based on what is known about cytokine-induced JNK activation (e.g. TRAIL/TRAIL-R and TNFα/TNF-R1) (Figure 1.7).

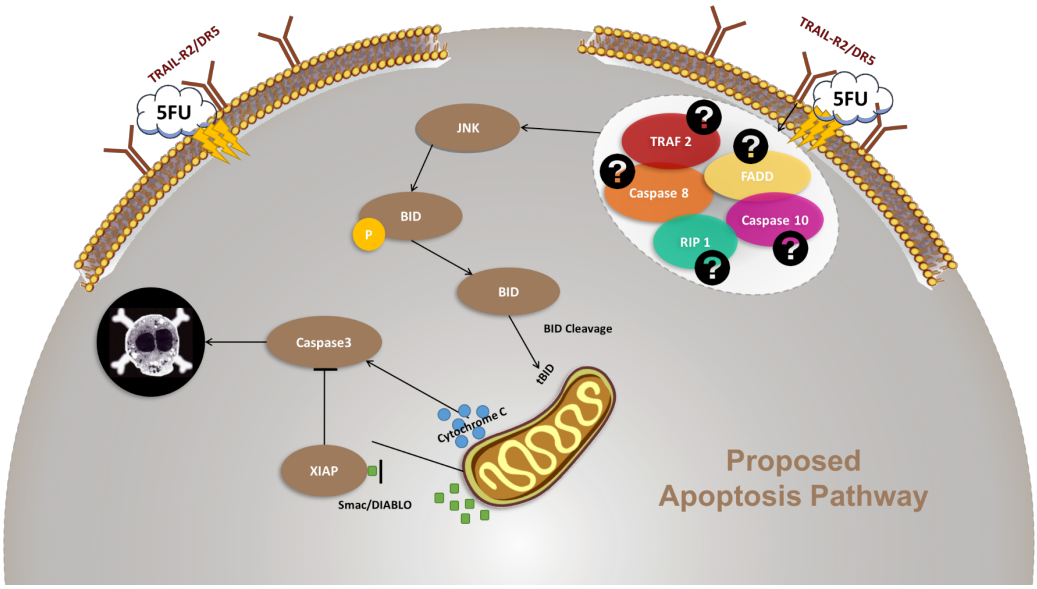

Figure 1.7. The novel 5-FU mechanistic molecular apoptosis model

Apoptosis is induced by 5-FU leading to activation of JNK signalling upon upregulation DR5 death receptor. it is postulated/hypothesised that at the DR5 receptor, a signalling complex forms that leads to JNK activation. This complex is termed JIST (JNK-inducing signal transduction) complex. Some potential molecular constituents of the JIST complex are shown which includes TRAF-2, FADD, caspase-8, caspase-10 and RIP1. In addition, in this model, it is postulated/hypothesised that further downstream apoptosis is mediated by BID cleavage by caspase-8. It was assumed that Bid cleavage to tBid is required for cytochrome c and Smac/DIABLO release form mitochondria into the cytosol. The role of cytosolic Smac/DIABLO is to inhibit XIAP activity. Blockage of XIAP permits proteolytic cleavage and activation of the executioner caspases including caspase-3. This eventually gives rise to apoptosis.

Chapter 2

MATERIALS AND METHODS

2.1 Cell culture

The following cells were used in this study: HAP1 cells, HCT116 human colon cancer cells and stable knockdown of HCT116 cells (HCT.shctrl, HCTp53-/-, HCT116.shDR5, HCT116.shDR4, HCT116.shLamin, HCT116.shCD95, HCT116.shTRAF2, HCT116.shFADD, HCT116.shcaspase-8, HCT116.shcaspase-10, and HCT116.shRIP1). HCT116 Cells were grown in McCoy's 5A medium and HAP1 cells were grown in IMDM. The HCT116 derived stable knockdown cells were available in the Mohr/Zwacka lab.

Upon reaching 70-80% confluence, cells were sub-cultured at appropriate ratio (1:6). Cells were washed with 5 ml of phosphate buffer saline (PBS) and detached from the culture flask upon incubation by 2 ml of trypsin solution for 2-5 minutes. Trypsin was neutralised after adding 12 ml of fresh medium. 2 ml of suspended cells were transferred into new flask and 12 ml of fresh medium was added. Cells were kept at 37° C, 5% CO_2 in an incubator. Sub-culturing of the cells was repeated every 3 days.

2.2 Stable RNAi cell line generation

All the RNAi knock-down constructs were previously generated in the lab. The following small hairpin (sh) RNA motifs were used to knock-down target genes:

- Caspase-10 (shC10) (5'-GCATTGACTCAGAGAACTTAA-3')
- Caspase-8 (shC8) (5'- GGGTCATGCTCTATCAGAT-3')
- FADD (shFADD) (5'- GTGCAGCATTTAACGTCAT-3')
- RIP1 (shRIP1) (5'-GCTGCTAAGTACCAAGCTATC-3')
- TRAF2 (shTRAF2) (Mohr et al., 2017)
- p53 (shp53) (Mohr et al., 2017)
- CD95 (shCD95) (Mohr et al., 2017)
- DR5 (shDR5) (5'- GCTAGAAGGTAATGCAGACTCTGCCATGTC-3')
- DR4 (shDR4) (5'- GCTGTTCTTTGACAAGTTGC-3')
- Lamin A/C (shLamin) (Life Technologies).

Both sense and anti-sense oligos containing the short-hairpin sequence and 5' overhang representing a restricted BbsI site and EcoRI site on the 3' were hybridised to generate the double-stranded DNA fragments. Then, the fragments were cloned into a modified pU6.ENTR plasmid (Life Technologies). Using LR Clonase II system (Life technologies), the resulting pU6.ENTR plasmids were used to generate the pAd.sh plasmids. Moreover, the pU6.ENTR plasmids were utilised to generate the stable knock-downs. Accordingly, the specific pBlockiT.sh plasmids were FuGene HD transfected into cells. After three days, the transfected cells were split into Blasticidin containing selection medium. Arising clones were picked, transferred to 24-well plates and verified for gene silencing. Furthermore, shLamin clones, generated by transfection with the pBlockiT6-GW/U6-lamin.shRNA plasmid, were functioned as RNAi controls.

2.3 Drug Treatment

To induce apoptosis, cells were treated with (I) 5-Fluorouracil (0.1% BSA in PBS) at 50 μM, 100 μM, 200 μM and 400 μM concentration, (II) TRAIL (0.1% BSA in PBS) at of 1-10 ng/ml, (III) TNFα (0.1% BSA in PBS) at 25 ng/ml concentration, and (IV) etoposide (0.1% BSA in DMSO) with 100 μM concentration.

2.4 Cellular protein extraction and Western blotting

To prepare cell extracts, cells were treated with 5-FU (24 h), etoposide (24 h) and TNF (15 min). Cells were harvested after 24h. For this, cells were first rinsed with ice-cold sterile 1XPBS (500 μl). Following washing with PBS, trypsin/EDTA (500 μl) solution was added and cells were incubated for 2-5 min until cells were detached. Then, the cell mixture was centrifuged (5 minutes 1200 rpm (g-force = 105)). Following harvesting, cells pellets were lysed for 20 minutes on ice with gentle agitation (vortexed) with equal amount of Triton X-100 cellular lysis buffer (containing phosphatase inhibitor cocktail). Lysates were clarified via centrifugation (13,000 rpm (g-force = 13226)), 10 minutes, 4°C). Following centrifugation, the clear supernatant was transferred to a fresh microcentrifuge tube and was utilised for protein determination.

According to manufacturer's guidance, protein concentration was measured in a 96-well plate by BCA™ protein assay reagent kit. Dilution of a BSA standard was 2 mg/ml in 1

96-well plate. (Mini-Protean, Bio-Rad) was assembled according to the manufacturer's instructions.

For Western blots, equal amounts of protein (50µg protein) were loaded into the wells of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE gels) with acrylamide concentration of 12% separating gel and 4% stacking gel. Before loading, each sample was mix with 10µl of loading buffer (Total volume ≤ 40µl for loading) and were boiled at 100°C for 5 min. s

The proteins were separated by SDS-PAGE. The SDS-PAGE gels were assembled in Bio-Rad gel rigs according to the manufacturer's guidance. The chamber was filled with 1000 ml of 1X running buffer. Gels were run at 90 V for 30 minutes (until the dye has moved through the stacking gel). Then, voltage was increased to 120 V until the dye reached the bottom of the gel.

SDS-PAGE gels were transferred to PVDF membranes by electrophoretic transfer using Bio-Rad. The blotting sandwich was prepared by filter papers (two pieces of Anode I-, one piece of Anode II and three pieces of Cathode-soaked filter papers), SDS-PAGE gel and PVDF membrane. The transfer was performed at 50 mA constant current for 60 min. Equal loading was checked by Ponceau S solution. Following transfer, the PVDF membranes were blocked with 3% non-fat dry milk in TBST buffer for 1 h at room temperature.

After incubation with milk, the membranes were probed with primary antibodies by diluting anti-human Ab 1:1000 in the antibody dilution buffer (TBS 0.1% Tween, 3%

BSA) at optimum condition (4°C with gentle shaking) overnight. After washing membranes three times in TBST buffer to remove excess of primary antibody, they were incubated with secondary antibodies with the dilution of 1:5000 in the antibody dilution buffer (TBS 0.1% Tween, 3% BSA) for 1-2 h at room temperature, after which the membranes were washed three times in TBST to remove excess secondary antibody. Protein detection was performed by using enhanced chemiluminescence (ECL) Western Blot Chemiluminescence Reagent and a digital imaging system (Fusion-FX Chemiluminescence System (Vilber Lourmat). After washing membranes following incubation in the secondary antibody, membranes were visualised with ECL western blot chemiluminescence regent (Pierce) following manufacturer's instructions (Solution A and B, mixed 1:1). The membranes were exposed automatically with a digital imaging system (A Fusion-FX Chemiluminescence System (Vilber Lourmat). CuZnSOD was used as a loading control.

2.5 DNA Hypodiploidy/Nicoletti Assay (Cell Death Measurement)

As described by (Riccardi and Nicoletti, 2006), Nicoletti assay was used to detect apoptosis in cells using flow cytometry (BD Accuri/C6/FACS cytometry). Upon 48 h of treatment with 5-FU and TRAIL, cells were collected, including the medium and PBS upon trypsinisation and centrifuged at 5000 rpm (g-force = 1817). for 1 minute. Following washing with PBS, 500 µl of Nicoletti buffer was added to cell pellets and they were vortexed for 10 s at medium speed and left on ice (4°C) for 1 h in the dark. The

fluorescence intensity was measured by flow cytometer and analysed by Venturi one software. Specific apoptosis was measured by subtracting basal apoptosis of untreated cells from 5-FU/TRAIL-treated cells. In this assay, cells containing the normal DNA illustrate normal G1-M-G2 cell cycle specific DNA content histograms. Whereas, cells with reduced DNA content (sub-G1) were considered as apoptotic cells. For each sample, 1000 events were measured, and each measurement was repeated for three times.

2.6 Caspase-8 Immunoprecipitation followed by caspase-8 assay

Equal amounts of proteins from cell lysates (1440 ng) were used in caspase-8 immunoprecipitation (IP). Cells were collected after 24 h treatment. Cells were re-suspended and washed in 1ml of PBS and centrifuged at 5000 rpm (g-force = 104.6448). for 1 minute. Following washing with PBS, cells were lysed in NP-40 lysis buffer for 10 minutes on ice. After lysing of cells, sample were centrifuged at 13,000 rpm (g-force = 13225.94) for 10 minutes at 4° C. After centrifugation, supernatants were incubated for 4 h at 4° C with caspase-8 antibody with concentration of 1µg/mg protein and 30µl of protein G (Pierce) magnetic beads. After incubation, the beads were precipitated by magnetic field. Magnetic beads were washed five times with lysis buffer and one time with cold aqua bidest. Beads were resuspended in 100µl of aqua bidest and 110µl of caspase-8 Glo-assay substrate were added to each sample. Luminescent reading was performed, and samples were measured after 1 h incubation.

2.7 Statistical Analyses

Microsoft Excel and MATLAB has been used extensively to implement the statistical analysis presented in this thesis. To achieve highly-trusted results, minimum three independent experiments were performed in triplicate ($N \geq 3$).

Pivot analysis (tables and charts) were used to summarise data and measure several statistical calculations including mean, average, sum, standard deviation and other statistics. Pivot table groups the data together based on these statistics, and adds a value and meaning to the data.

Then Pivot charts were generated based on Pivot tables. These data presented in the Pivot charts then were grouped and presented based on various factors, such as treatment duration, cell lines and cytotoxic drugs. Experimental values were then expressed as the mean value ± standard error of mean (SEM).

To calculate the standard error bars the following formula has been used:

$$\text{SEM} = \pm \frac{SD}{\sqrt{n}} \qquad \text{(eq. 2.1)}$$

Where “SD” is the standard deviation and “n” is the number of samples or data points.

Analysis of variance or ANOVA can be used to compare the means between two or more groups of values.

Furthermore, ANOVA single factor (aka. analysis of variance) and student’s t-test were used between groups for significance analyses and calculation of p value. ANOVA

analysis compares the data groups' mean values. In doing so, the null hypothesis of "equal means of each sample" was used. Then the probability value (i.e. p-value) of the statistical model (when the mentioned null hypothesis is true) was calculated, considering the mean difference between each two groups would be greater than or equal to the observed values.

Finally, $p<0.05$ (*) was considered significant, $p<0.01$ (**) as very significant and $p<0.001$ (***) as highly significant. As it can be seen, the higher the p-value, the lower the significance. This is because it illustrates that the hypothesis may not sufficiently describe the observation.

Chapter 3

IDENTIFICATION AND CHARACTERISATION OF A NOVEL APOPTOSIS-REGULATING PATHWAY IN RESPONSE TO CHEMOTHERAPEUTIC DRUGS

3.1 Introduction and objectives

Although significant progress has been achieved in the treatment of colon cancer, it is still one of the most common types of cancer and cause of cancer-related death worldwide. 5-FU, an antimetabolite drug, is widely used in the treatment of a wide range of cancers, especially for colon cancer. 5-FU partly exerts its effects via inducing p53-dependent cell growth arrest and apoptosis; therefore, mutation or absence of p53 can cause 5-FU resistance (Subbarayan et al., 2010, Huang et al., 2009). Thus, understanding of 5-FU-resistance following mutation or deletion of p53 will be a key point to address and design better therapeutic strategies.

Previously our group have shown that drugs causing single-strand DNA breaks such as 5-FU, raltitrexed or irinotecan cause caspase-8-dependent apoptosis independent of death-receptor signalling (Mohr et al., 2017). Moreover, in response to chemotherapeutic drugs, apoptosis happens through formation of a complex called FADDosome consisting of caspase-8, FADD, caspase-10, TRAF2, and RIP1. Within the FADDosome caspase-8 is activated, and this activation is p53-independent. Furthermore, lacking caspase-10, TRAF2, RIP1 or ATR leads to apoptosis by formation of a FADD- and p53-independent complex termed named FLIPosome (Mohr et al., 2017).

Although the FADDosome and FLIPosome complexes are p53-independent in HCT116 cells, apoptosis is reduced in the absence of p53 when cell death is triggered via the FADDosome in unmodified HCT116 colorectal cancer cells (Mohr et al., 2017). Our previous data suggested that additional pathway may participate in apoptosis.

Therefore, it was hypothesised that an additional p53-dependent pathway modulated the FADDosome-initiated apoptosis signal in HCT116 cells. The aim of this study was to identify and characterise this additional apoptosis pathway in human colon.

3.2 Lack of p53 inhibits TRAIL-R2 (DR5) upregulation

In our previous report our group showed that p53-deficient cells are relatively resistant to apoptosis induced by 5-FU (Mohr et al., 2017). Therefore, in the current study, the aim was to explore the role of p53 in the 5-FU-induced apoptosis in HCT116 cells.

The p53 tumour suppressor has been known to play a significant role in mediating apoptosis by targeting specific genes (Benchimol, 2001). Noxa and Bax are pro-apoptotic members of the Bcl-2 family of proteins which are regulated by p53 and their activation results in release and activation of cytochrome c and later activation of casapase-9 (Oda et al., 2000, Toshiyuki and Reed, 1995). However, our group have discovered that caspase-9 is dispensable for 5-FU-induced apoptosis making an involvement of the above mentioned Bcl-2 family members less likely (Mohr et al., 2017). Instead, caspase-8 was found to be essential, which is usually activated by ligand binding (death ligands) to their cognate receptors such as Fas (Müller et al., 1998) and DR5 (Burns et al., 2001). These death receptors have also been identified as target genes of p53. Thus, first it was checked whether p53 can influence DR5 upregulation in HCT116 cells treated with 5-FU.

As shown in (Figure 3.1), in the absence of p53 (HCTp53-/-), DR5 upregulation is also blocked following 200 μM of 5-FU treatment (24 h). These results suggested that DR5 might be a potential candidate to be involved in the regulation of 5-FU-induced apoptosis.

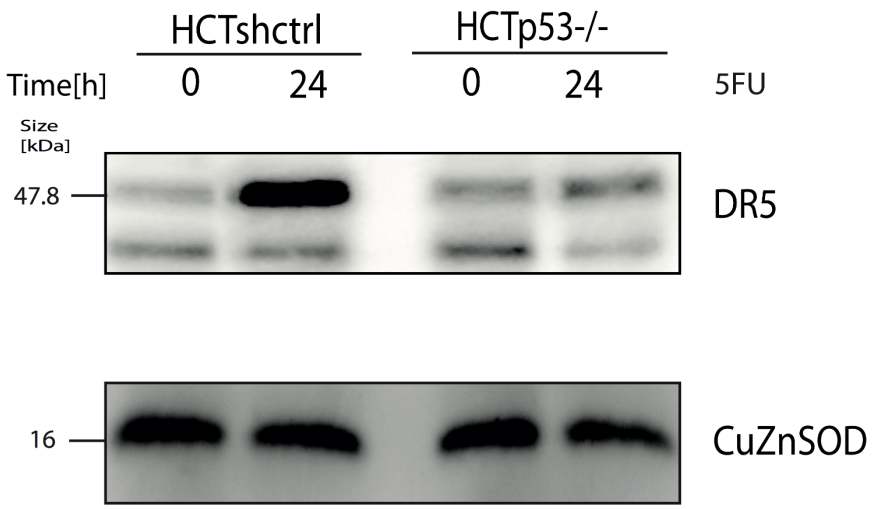

Figure 3.1. Lack of p53 inhibits DR5 upregulation.

HCTshctrl and HCTp53-/- cells were treated with 200 μM of 5-FU (24 h) and DR5 protein levels were analysed by western blotting. CuZnSOD was used as a loading control This experiment were repeated three times (N=3).

3.3 TRAIL-R2 (DR5) is an apoptotic regulator in HCT116 cells in response to 5-FU

In order to study the role of DR5 and other death receptors in 5-FU-induced apoptosis, HCT116 cells lacking CD95, DR4 and DR5 were used. HCT116, HCTshctrl, HCTshLamin, HCTshCD95, HCTshDR4 and HCTshDR5 were induced with 200 μM of 5-FU for 48 h and tested them by DNA hypodiploidy assays for apoptosis measurement. As shown in (Figure 3.2), the levels of apoptosis in HCT116, HCTshctrl, HCTshLamin, HCTshCD95, and HCTshDR4 were about 43.7%, 48.01%, 42.91%, 43.08% and 45.05% respectively after 48 h treatment of 5-FU. However, at the same time point (48 h) the level of apoptosis in HCTshDR5 cells was 18.4% ($P<0.01$).

These results indicate that the levels of specific apoptosis were not affected in the three different control cells (HCT116, HCTshctrl, HCTshLamin) and not in the death-receptor knock-down cell lines (HCTshCD95 and HCTshDR4) but it was significantly reduced in cells lacking DR5 expression (HCTshDR5). Our findings suggested that DR5 may play a key role in mediating the p53-dependent apoptosis regulating pathways, irrespective of its ligand TRAIL, as TRAIL was not produced in response to 5-FU (data not shown).

HCT116, HCTshctrl, HCTshLamin and HCTshDR5 cells were induced with different concentrations of TRAIL (1 ng/ml, 2 ng/ml, 5 ng/ml and 10 ng/ml) for 48 h to analyse the functionality of DR5.

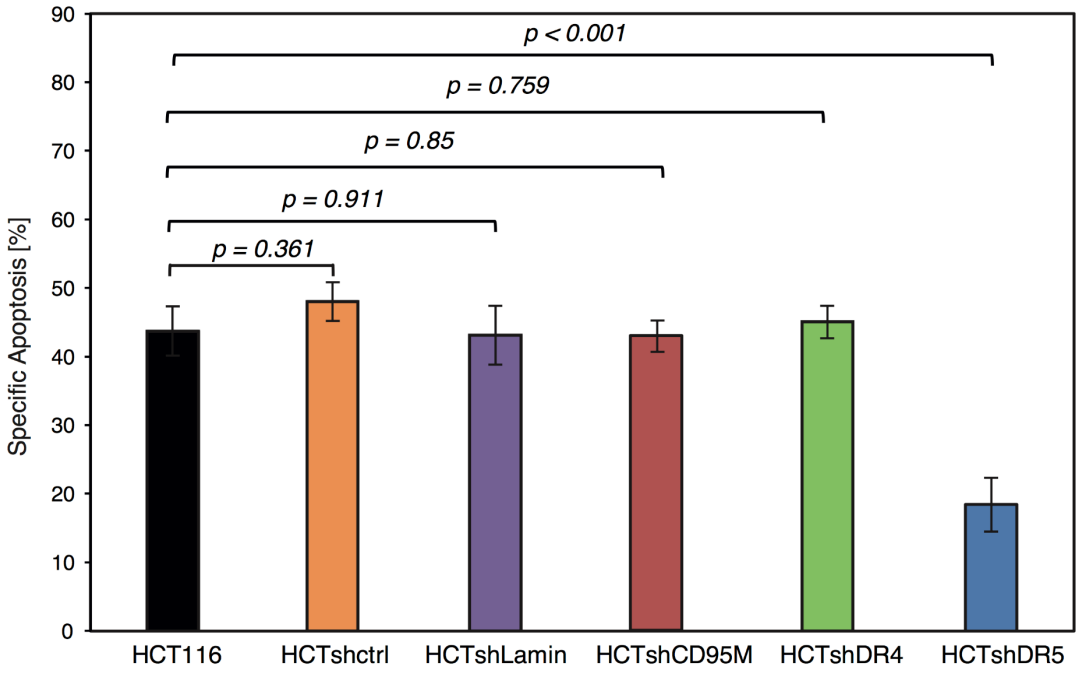

Figure 3.2. HCTshDR5 cells are resistant to 5-FU.

Cell death induced by 200 µM of 5-FU (48 h) was measured by flow cytometry and compared in HCT116, HCTshctrl, HCTshLamin, HCTshCD95, HCTshDR4 and HCTshDR5. The HCT116, HCTshLamin and HCTshctrl were used as control cells. Each experiment was repeated three times. The standard error shows an estimate of the standard deviation of a sampling distribution which was calculated by dividing standard deviation by the square root of number of measurements that make up the mean.

As shown in (Figure 3.3), at lower concentration of TRAIL (1, 2 and 5 ng/ml), HCTshDR5 cells are more resistant to TRAIL than HCT116, HCTshctrl and HCTshLamin cells. The difference decreased with increased TRAIL concentrations can be explained by the fact that DR4 can take over the function of DR5 by binding to TRAIL at higher concentrations leading to comparable levels of cell death in HCTshDR5 cells. Notwithstanding, our results demonstrate the functionality of the DR5 knockdown in HCTshDR5 cells.

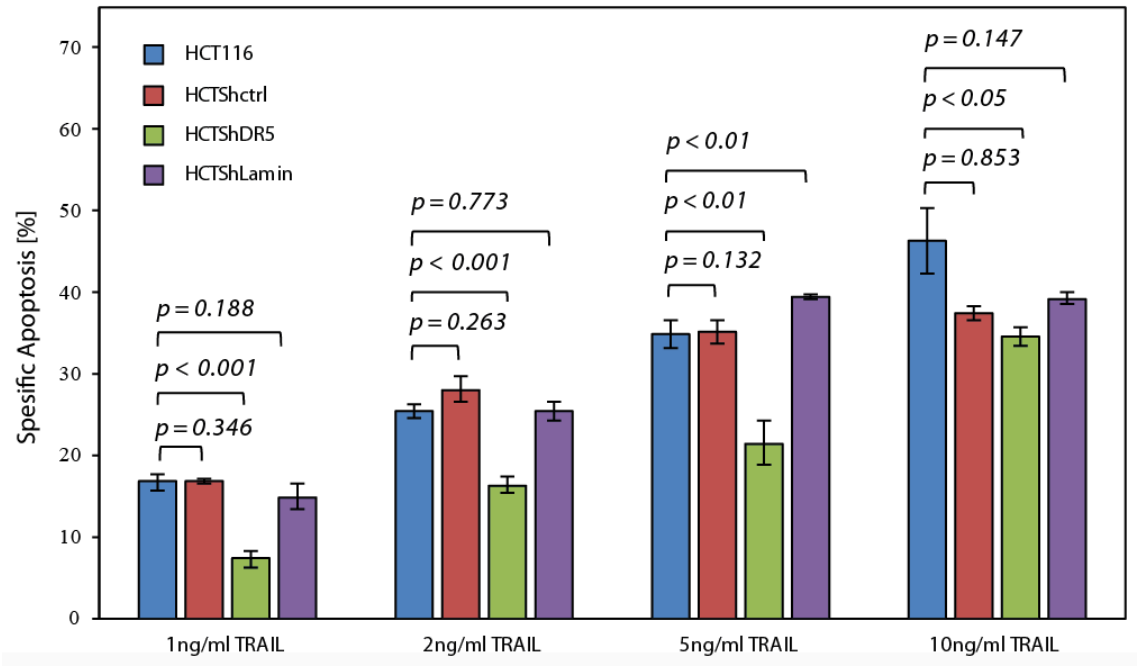

Figure 3.3. HCT116, HCTshctrl, HCTDR5 and HCTshLamin cells were treated with different concentrations of TRAIL (1 ng/ml, 2 ng/ml, 5 ng/ml, 10 ng/ml).

Amount of apoptosis was measured as population of cells at sub-G1 using flow cytometry (DNA hypodiploidy assays). The average of three experiments at different concentrations of TRAIL is illustrated. Each experiment was repeated three times. The standard error shows an estimate of the standard deviation of a sampling distribution which was calculated by dividing standard deviation by the square root of number of measurements that make up the mean.

3.4 TRAIL-R2 (DR5) mediates apoptosis through caspase-8 independent pathway

Caspase-8 is an initiator caspase in the TRAIL and TRAIL-receptor-induced pathway of apoptosis. Our group has recently showed that cell death induced by 5-FU in HCT116 cells is caspase-8 dependent (Mohr et al., 2017). Others studies have also shown that DR5 requires FADD and caspase-8 to assemble the DISC, in which caspase-8 becomes auto-activated leading to apoptosis (Huang et al., 2016). Therefore, it was hypothesised that caspase-8 activation is mediated by DR5.

To determine whether apoptosis reduction in the absence of DR5 results from inhibition of caspase-8 activity, the activation of caspase-8 in HCT116 cells lacking DR5 death receptor (HCT116.shDR5) was checked. Caspase-8 activity assays revealed that the resistance of HCTshDR5 cells to apoptosis is not due to inhibition of caspase-8 activity. As shown in (Figure 4.4), there was not any difference between caspase-8 activity in HCTshctrl and HCT116.shDR5 cells. Therefore, it was hypothesised that DR5-mediated regulation of apoptosis might be due to the activation of non-canonical pathways including NF-κB or JNK MAP kinase pathways (Azijli et al., 2013a).

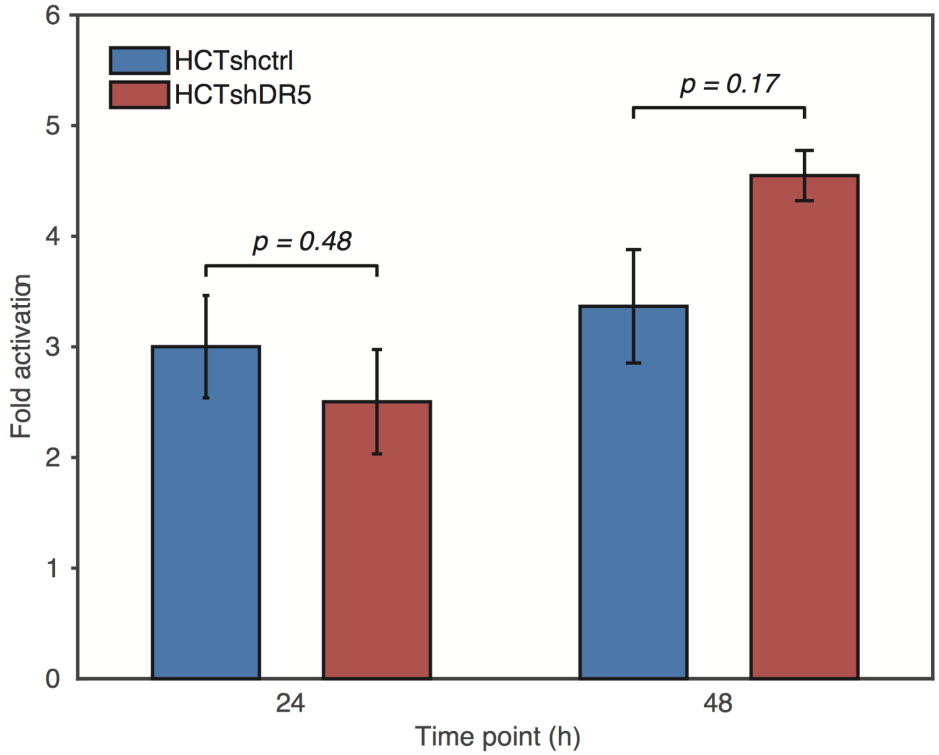

Figure 3.4. DR5 is not required for caspase-8 activation induced by 5-FU.

HCTshctrl and HCTshDR5 cells were treated with 200 μM of 5-FU for 24 h and 48 h. Caspase-8 activity was measured by caspase-8 assay. The level of caspase-8 activity was shown by fold activation. The experiment was repeated three times. The standard error shows an estimate of the standard deviation of a sampling distribution which was calculated by dividing standard deviation by the square root of number of measurements that make up the mean.

3.5 TRAIL-R2 (DR5) mediates apoptosis through a JNK signalling pathway

After ruling out a role of DR5 in caspase-8 activation, the possible involvement of non-canonical pathways induced by death receptors, namely the NF-κB pathway and JNK MAP kinase pathway was analysed. After ruling out NF-κB activation (data not shown), activation of JNK was examined by western blotting using cell lysates of HCTshctrl and HCTshDR5 cells (Figure 3.5a). Both HCTshctrl and HCTshDR5 cells were treated with 200 μM of 5-FU and tested them for JNK activation after 24h treatment (Figure 3.5b). As shown in (Figure 3.5b), following 5-FU treatment, absence of DR5 (HCTshDR5) inhibits JNK activation. Therefore, our results indicate that DR5 transduces its death signals through JNK activation. For confirmation, whether lack of p53 inhibits JNK phosphorylation was tested (Figure 3.6) as it was previously shown that DR5 upregulation was controlled by p53. As expected, JNK activation was also inhibited in HCT116-knock-out p53 cells (HCTp53-/-) when treated with 5-FU for 24 h (Figure 3.6). Interestingly, even the basal levels of JNK phosphorylation, without 5-FU treatment, were markedly reduced.

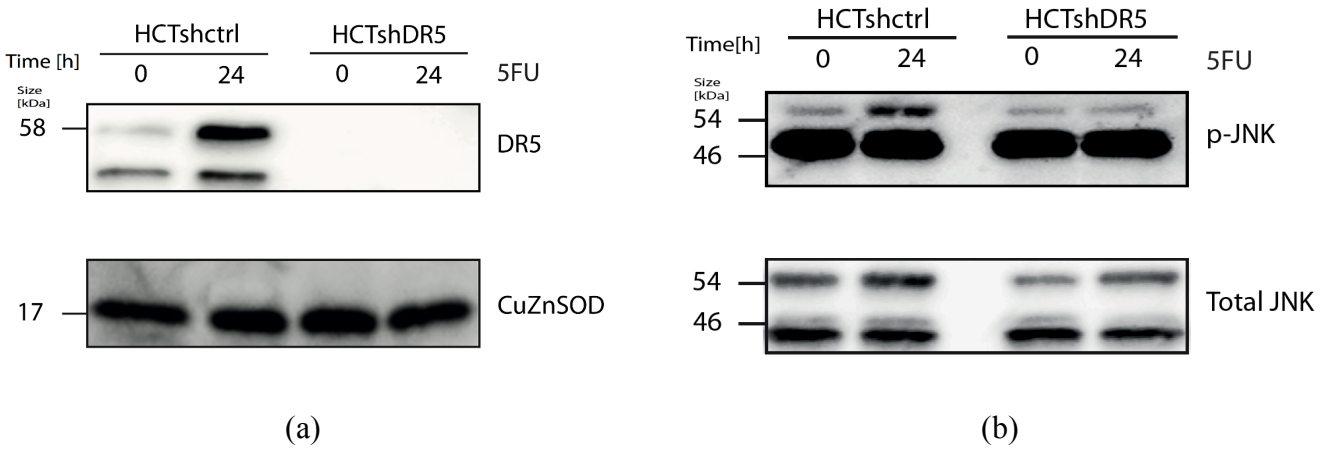

Figure 3.5. TRAIL-R2 (DR5) mediates apoptosis through a JNK signalling pathway.

(a) HCTshctrl and HCTshDR5 cells were treated with 200 μM of 5-FU (24 h) and DR5 protein levels were analysed by using a western blot to demonstrate DR5 is silenced. CuZnSOD was used as a loading control. (b) HCTshctrl and HCTshDR5 cells were treated with 5-FU (200 μM) for 24 h and cellular lysates were analysed by using a western blot for JNK activation, indicated by JNK phosphorylation (p-JNK). Total JNK was used as a control and molecular weight markers are indicated (N≥3).

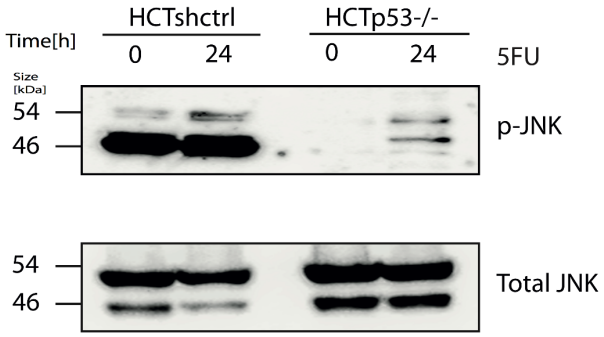

Figure 3.6. HCTshctrl and HCTp53-/- cells were treated with 5-FU (200 μM) for 24 h and cellular lysates were analysed by using a western blot for JNK activation.

Total JNK was used as a loading control and molecular weight markers are indicated (N≥3).

3.6 TRAIL-R2 (DR5) mediates JNK activation through adaptor molecule FADD

In our previous report it was shown that silencing FADD in HCT116 cells , blocked 5-FU induced apoptosis (Mohr et al., 2017). Therefore, the role of the receptor adaptor molecule FADD in activation of JNK was examined. Apart from our former data, previous studies have shown that activation of JNK signalling depends on the presence of DD containing adaptor molecules such as FADD. FADD is known to bind to the DD domains of DR4 and DR5 to mediate apoptosis (Gonzalvez and Ashkenazi, 2010). Therefore, the role of FADD in activation of JNK in response to 5-FU was studied.

It was hypothesized that DR5 transduces its signal through formation of a signalling complex leading to JNK activation. This putative signalling complex was named JNK-inducing-signal transducing (JIST) complex. To examine the contribution of FADD in the JIST complex, HCTshFADD cells that are stable knockdown clones for FADD were used (Figure 4.7a). These cells were treated with 200 μM of 5-FU for 24 h and investigated for JNK activation by western blotting (Figure 4.7b).

As shown in (Figure 4.7b), JNK cannot be activated in the absence of FADD (HCTshFADD). Moreover, DR5 upregulation in HCTshFADD cells was checked. As illustrated in (Figure 4.7c), silencing of FADD does not inhibit DR5 upregulation ruling out an indirect effect of DR5 death receptor.

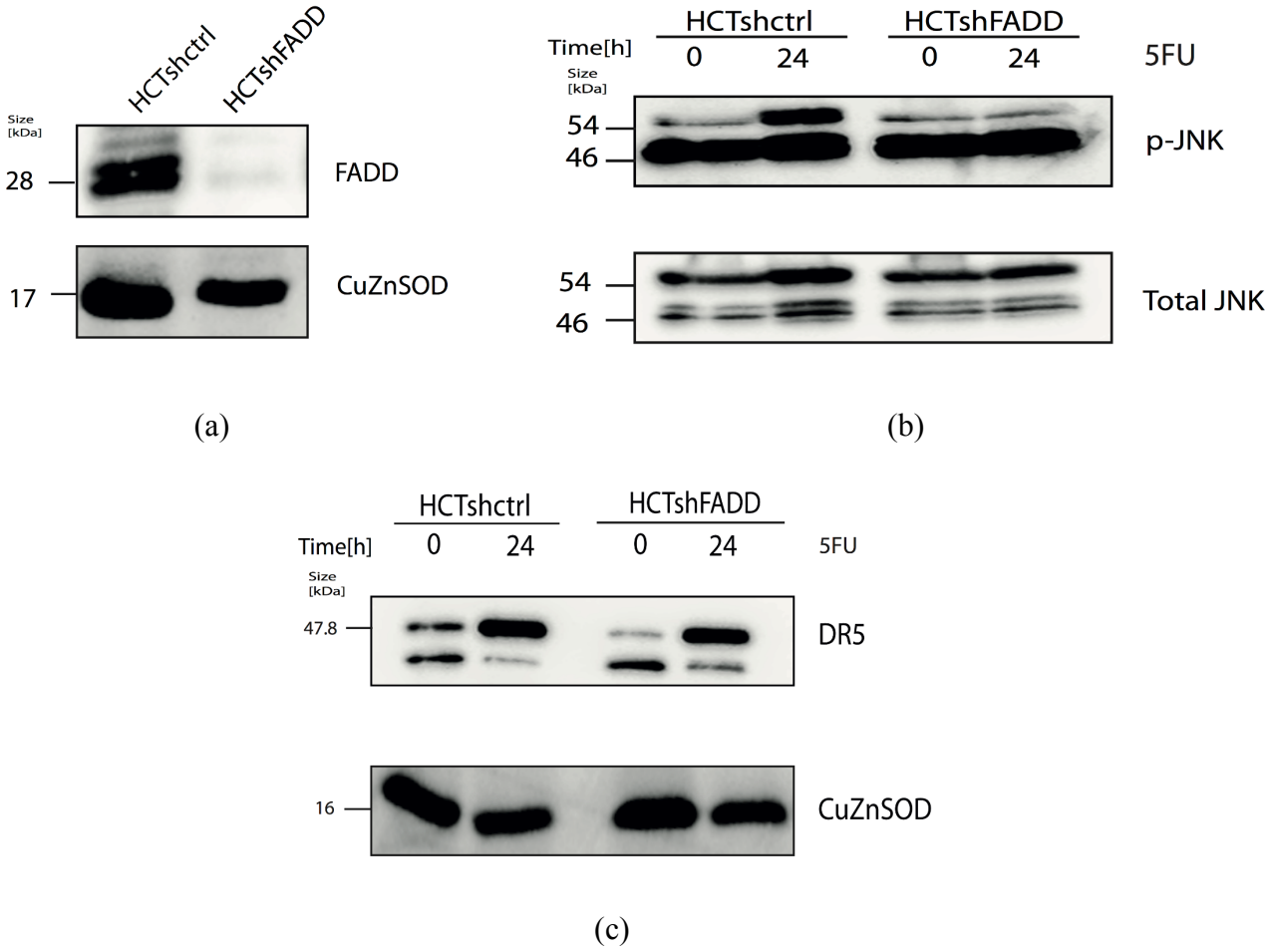

Figure 3.7. FADD plays a key role in JNK activation.

(a) HCTshctrl and HCTshFADD were analysed by western blotting to demonstrate that FADD is silenced. CuZnSOD was used as a loading control. (b) HCTshctrl and HCTshFADD cells were treated with 5-FU (200 μM) for 24 h and cellular lysates were analysed by western blotting for JNK activation. Total JNK was used as a control and molecular weight markers are indicated. (c). HCTshctrl and HCTshFADD cells were treated with 5-FU (200 μM, 24 h) and cellular lysates were analysed for DR5 protein upregulation by western blotting. CuZnSOD was used as a loading control (N=3).

3.7 Lack of caspase-8 also blocks JNK activation

Similar to FADD, our group have previously shown that in response to anti-cancer drugs such as 5-FU apoptosis is mediated through caspase-8 and silencing caspase-8 in HCT116 cells blocked 5-FU-induced apoptosis (Mohr et al., 2017). So, the next question was whether, despite the DR5 independence for its activation, caspase-8 is required in the JIST complex for activation of JNK in response to 5-FU, as caspase-8 is also known to potentially bind to FADD and DR5 in the regular TRAIL- and TRAIL-receptor-induced pathway of apoptosis. Also, another study showed the requirement of caspase-8 for JNK activation through TRAIL (Varfolomeev et al., 2005).

To test our hypothesis, both HCTshctrl and HCTshcaspase-8 cells (Figure 4.8a) were treated with 5-FU (200 μM) and examined for JNK phosphorylation after 24 h (Figure 4.8b). Lack of caspase-8 (HCTshcaspase8) stops JNK activation in HCT116 cells indicating that like FADD, caspase-8 is also important for JNK activation by 5-FU. As shown in (Figure 3.8c), it was confirmed that caspase-8 is not required for DR5 upregulation.

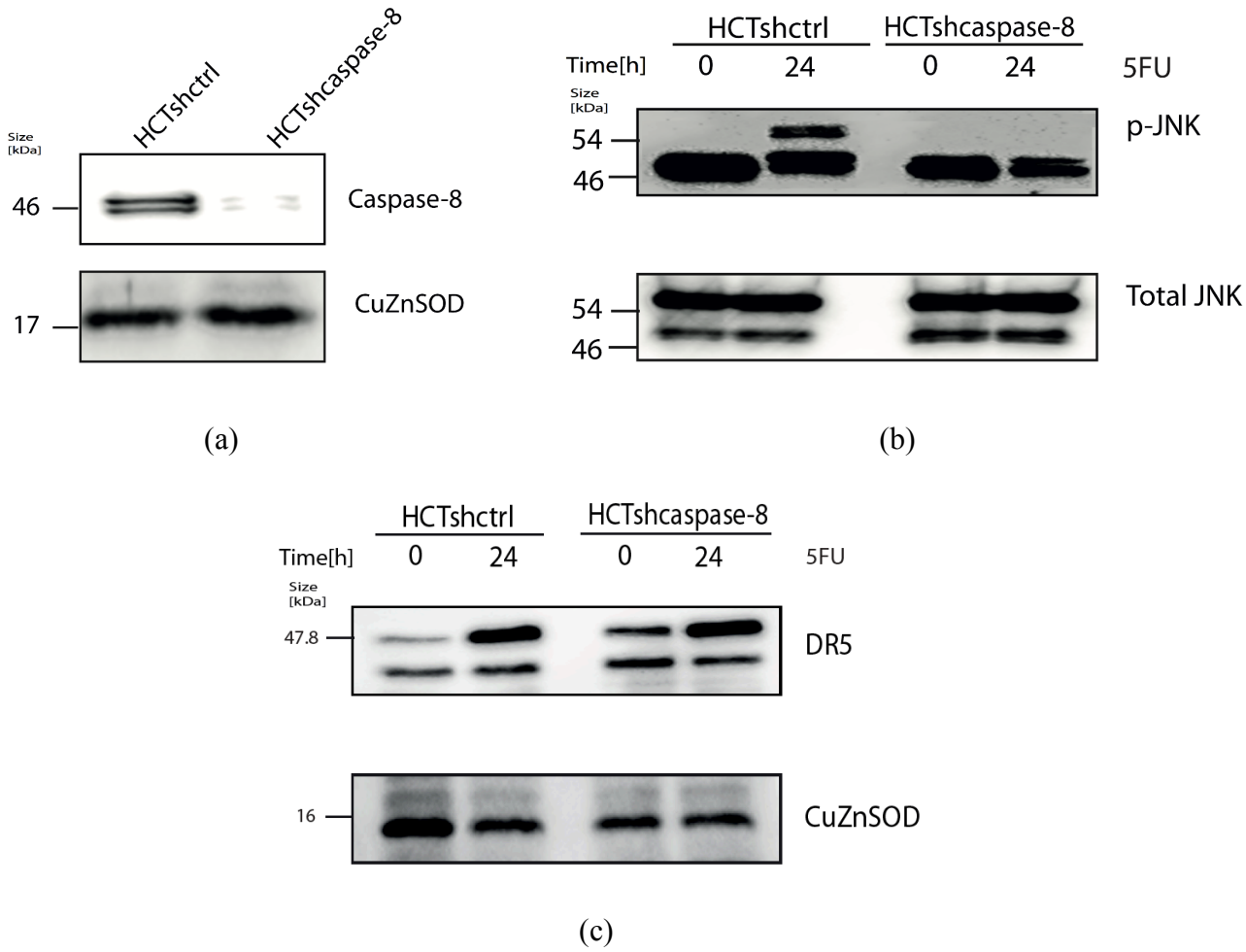

Figure 3.8. Caspase-8 is needed for JNK activation in HCT116 cells.

(a) Cell extracts of HCTshctrl and HCTshcaspase-8 cells were analysed for caspase-8 by western blotting to check the knockdown effect. CuZnSOD was used as loading a control. (b) HCTshctrl and HCTshcaspase-8 cells were treated with 5-FU (200 μM) for 24 h and cellular lysates were analysed by western blotting for JNK activation. Total JNK was used as control and molecular weight markers are indicated. (c) HCTshctrl and HCTshcaspase-8 cells were treated with 5-FU (200 μM, 24 h) and cellular lysates were analysed for DR5 protein upregulation by western blotting (N=3).

3.8 JIST complex is caspase-10-independent

Apart from caspase-8, caspase-10 can also potentially bind to DED of adaptor molecules such as FADD or TRAF2 and be auto-activated and induce cell death. However, our group has recently shown that apoptosis was higher in HCTshcaspase-10 cells when treated with 5-FU (Mohr et al., 2017).

To determine the potential role of caspase-10 in JNK activation, cell lysates of HCTshctrl and HCTshcaspase-10 cells (Figure 3.9a) were used. These cells were treated with 200 µM of 5-FU for 24 h and examined for JNK activation by using a western blot. As shown in (Figure 3.9b), JNK activation was unchanged when caspase-10 was silenced in HCT116 cells meaning that caspase-10 is not an essential member of the JIST complex and does not regulate apoptosis at this level.

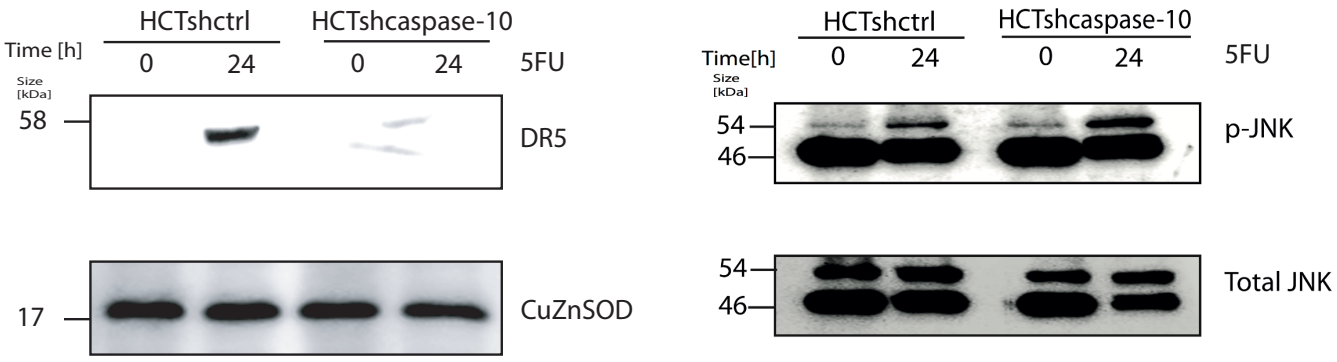

Figure 3.9. Caspase-10 is not needed for JNK activation in HCT116 cells.

(a) Cell extracts of and HCTshcaspase-10 cells were analysed for caspase-10 by western blotting to check the knockdown of caspase-10. CuZnSOD was used as a loading control. (b) Western blot of JNK-phosphorylation in lysates from HCTshctrl and HCTshcaspase-10 cells untreated and treated with 5-FU (200 µM, 24 h). The membrane was probed with anti-phospho JNK (p-JNK) and anti-total JNK antibodies (N=3).

3.9 JIST complex is TRAF2-independent

In addition to FADD, TRAF2 has shown to mediate activation of JNK and NF-κB through receptors from the TNFR super family such as TNF and TRAIL receptors (Hu et al., 1999, Hsu et al., 1996). Our previous data have also discovered that TRAF2 mediated apoptosis signalling in response to 5-FU by formation of FADDosome (Mohr et al., 2017). As it was shown that FADD is a member of the JIST complex, the role of TRAF2 in JNK activation induced by 5-FU was also tested.

To determine the potential role of TRAF2 in JNK activation in 5-FU induced apoptosis, HCT116 cells with silenced TRAF2 (HCTshTRAF2) (Figure 3.10a) were used. Analysing the western blot of HCTshctrl and HCTshTRAF2 cells treated with 200 μM of 5-FU for 24 h using JNK phosphorylation/activation specific antibody (Figure 4.10b) showed that JNK activation is detectable in HCTshTRAF2 cells. These findings exclude the possibility that TRAF2 may be involved in the JIST complex and JNK activation.

The levels of apoptosis in HCTshctrl and HCTshTRAF2 cells were compared in response to 5-FU with different dosages (50μM, 100μM, 200μM and 400μM) incubated for 48 hours (Figure 3.10c). Comparing the results, it was found that HCTshctrl cells are more sensitive to apoptosis than HCTshTRAF2 cells. The increase of apoptosis in HCTshTRAF2 can be due to redundancy, occurring when the same structural components such as TRAF6, TRAF3 can perform similar function (TRAF2) but perform different functions in other conditions.

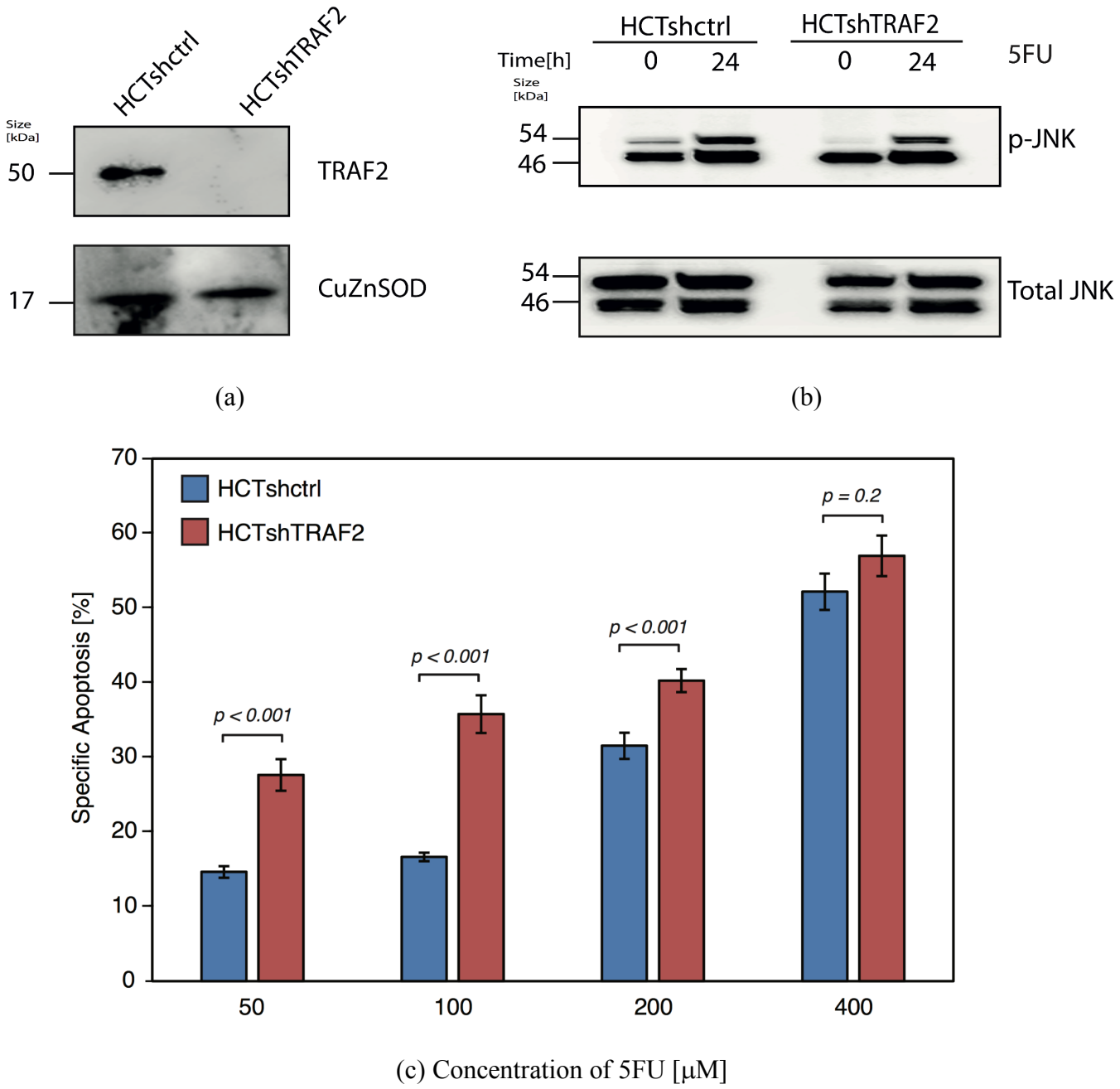

Figure 3.10. JIST complex is TRAF2-independent

(a) Western blot analysis to check HCTshTRAF2 and HCTshctrl with anti-TRAF2 antibodies. CuZnSOD was utilised as a loading control. (b) Western blot of JNK-phosphorylation in lysates from HCTshctrl, HCTshTRAF2 untreated and treated with 5-FU (200 µM, 24 h). The membrane was probed with anti-phospho-JNK(p-JNK) and anti-total JNK antibodies (N=3). (c) Levels of apoptosis in HCTshctrl and HCTshTRAF2 cells. Analysis of apoptosis assay (Nicoletti Assay) by flow cytometry on HCTshctrl and HCTshTRAF2 cells with 50,100,200 and 400 µM of 5-FU treatment for 48h. Each experiment was repeated three times. The standard error shows an estimate of the standard deviation of a sampling distribution, which was calculated by dividing standard deviation by the square root of number of measurements that make up the mean.

3.10 **JIST complex is RIP1-independent**

Finally, Receptor Interacting Protein 1 (RIP1) contributions to the JIST complex was tested, as previous studies have shown that RIP1 is an important protein for the activation of JNK through TRAIL and FasL (Devin et al., 2003). our group has previously shown that apoptosis was regulated by RIP1 as $cFLIP_L$ was ubiquitinated and degraded through RIP1. However, it was found that in the absence of RIP1 the amount of apoptosis was higher when in HCT116 cells (Mohr et al., 2017).

To test the role of RIP1 in activation of JNK, HCTshctrl and HCTshRIP1 cells (Figure 3.11a) were used and checked for JNK activation in response to 5-FU (200µM) after 24 h (Figure 3.11b). As shown in (Figure 3.11b), our data showed the activation of JNK in both HCTshctrl and HCTshRIP1 cells, suggesting that RIP1 is not a member of JIST complex in HCTshctrl cell and RIP1 is not required for JNK activation.

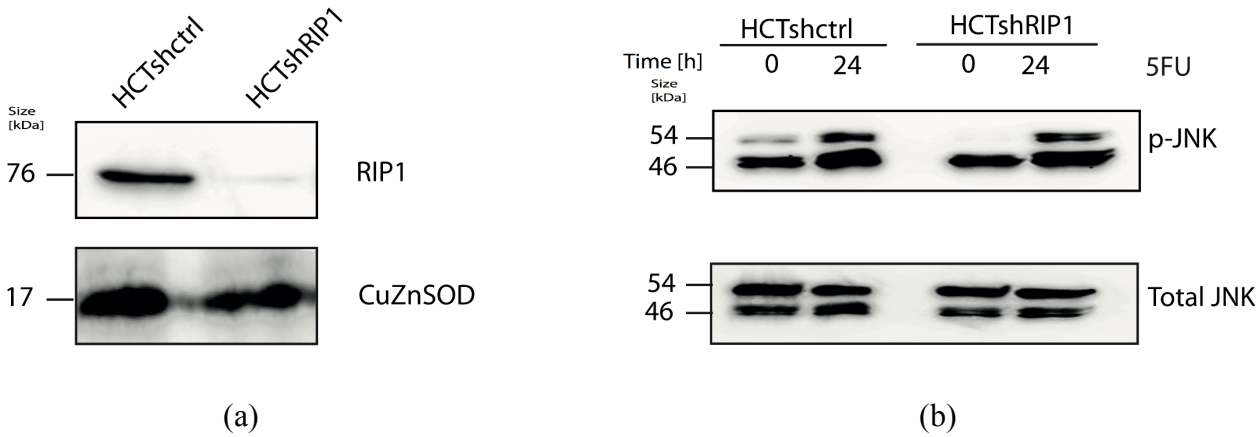

Figure 3.11. RIP1 is not needed for JNK activation in HCT116 cells.

(a) HCTshctrl and HCTshRIP1 cells were analysed by western blotting to illustrate that RIP1 is knock-down with anti-RIP1 antibody. CuZnSOD was utilised as a loading control. (b) Western blot of JNK-phosphorylation in lysates from HCTshctrl and HCTshRIP1 untreated and treated with 5-FU (200 µM, 24 h). The membrane was probed with anti-phospho-JNK (p-JNK) and anti-total JNK antibodies (N=3).

It was discovered that in HCT116 cells treated with 5-FU, activation of JNK through DR5 was FADD- and caspase-8-dependent and JNK phosphorylation occurs independent of TRAF2, caspase-10 and RIP1.

3.11 TNFα-mediated JNK does not require DR5, FADD and caspase-8

To address the concern that HCTshDR5 cells might not be able to activate JNK owing to an additional, unknown issue with the cellular JNK activation machinery the JNK activation in response to TNFα in DR5, FADD and caspase-8 silenced cells was tested. HCTshctrl, HCTshDR5, HCTshFADD and HCTshcaspase-8 cells were treated with 10 ng/ml TNFα for 15 minutes and examined them for JNK activation.

As shown in Figure 3.12, lack of DR5, caspase-8 but not FADD in HCT116 cells does not inhibit JNK activation in response to TNFα. These findings demonstrate that the HCTshDR5 cells have the capacity to activate JNK and that the lack of 5-FU induced JNK activation is specific. Furthermore, it shows that the TNFα-induced JNK activation is different from 5-FU-triggered JNK activation.

3.12 TRAIL-R2 (DR5) plays a similar role in HCT116 cells in response to etoposide

It was found that upon 5-FU treatment, DR5 is upregulated and JNK activation is regulated through formation of the JIST complex. Furthermore, it was shown that FADD and caspase-8 are members of the JIST signalling complex. Next, HCT116 cells were subjected to etoposide in order to test whether the DR5-JNK apoptosis pathway in HCT116 cells is specific for 5-FU or whether DR5-JNK apoptosis pathway is important for other chemotherapeutic drugs. Etoposide is a topoisomerase inhibitor causing damage by forming a complex with topoisomerase II resulting in DNA damage (Van Maanen et al., 1988). It is believed to cause DNA double-strand breaks, in contrast to 5-FU that gives rise to single-strand breaks.

To determine the effect of etoposide on HCT116 cells, both HCTshctrl cells and HCTshDR5 cells were treated with 100 μM of etoposide for 24 h and tested for p53 and DR5 upregulation, as well as JNK activation.

As illustrated in (Figure 3.13), HCTshctrl cells treated with etoposide, shows DR5 upregulation which is p53-dependent. Moreover, it was shown that JNK is activated when HCT116 cells were treated with etoposide for 24 h. Our initial data suggested that in response to etoposide, DR5 upregulation results in JNK activation through formation of the JIST complex with the same protein members i.e. FADD and caspase-8.

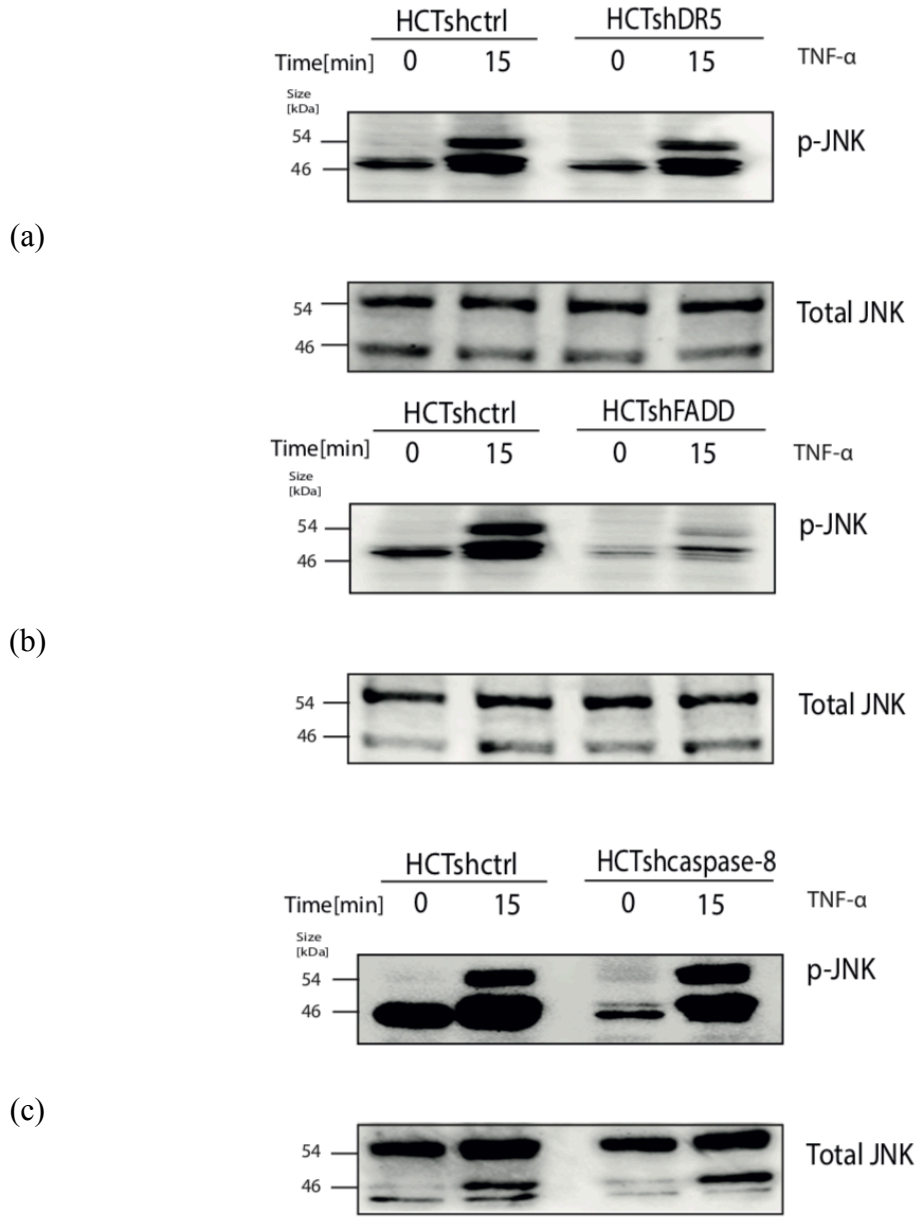

Figure 3.12. TNFα-mediated JNK does not require DR5, FADD and caspase-8

(a) Western blot of JNK-phosphorylation in lysates from HCTshctrl and HCTshDR5 cells untreated and treated with TNFα (10 ng/ml,15 min). The membrane was probed with anti-phospho-JNK (p-JNK) and anti-total JNK antibodies. (b) Western blot of JNK-phosphorylation in lysates from HCTshctrl and HCTshFADD cells untreated and treated with TNFα (10 ng/ml,15 min). The membrane was probed with anti-phospho-JNK (p-JNK) and anti-total JNK antibodies. (c)Western blot of JNK-phosphorylation in lysates from HCTshctrl and HCTshcaspase-8 cells untreated and treated with TNFα (10 ng/ml,15 min). The membrane was probed with anti-phospho-JNK (p-JNK) and anti-total JNK antibodies (N≥3).

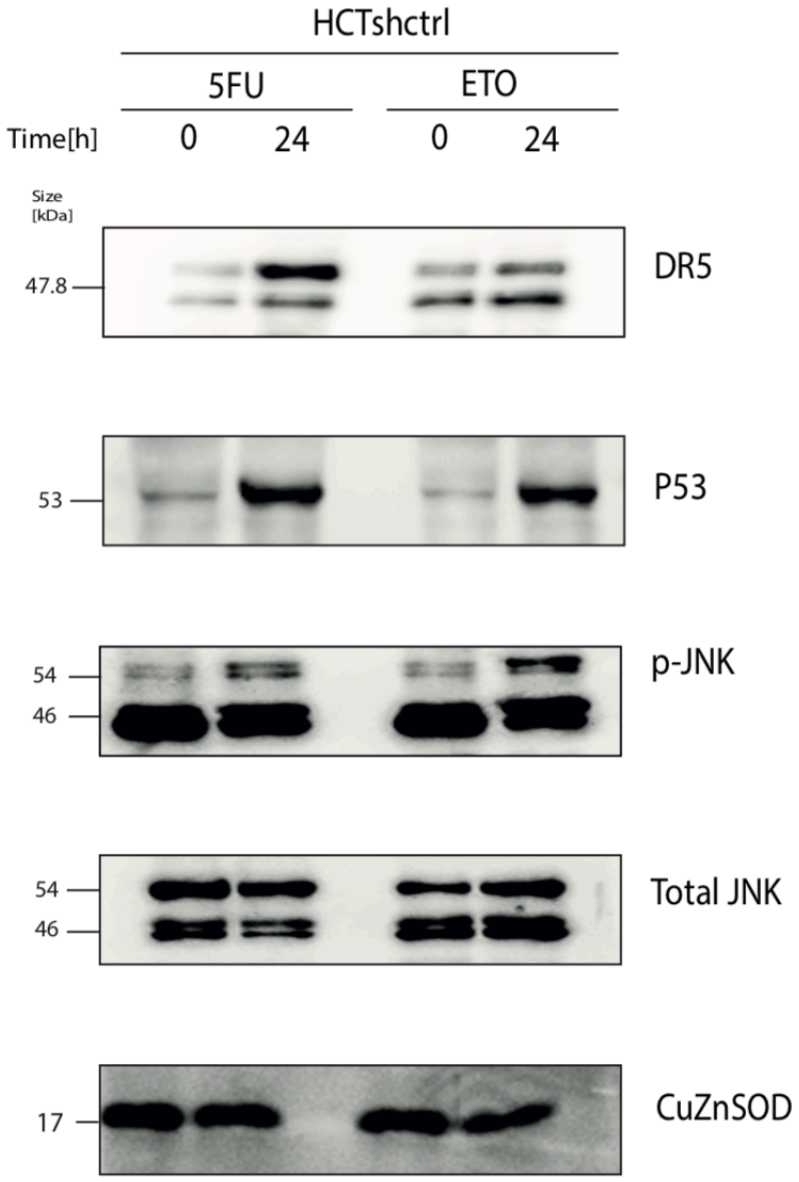

Figure 3.13. HCTshctrl cells were treated with 5-FU and etoposide (200 µM, 24 h) and cellular lysates were analysed for DR5 protein upregulation by western blotting.

HCTshctrl cells were treated with 200 µM of 5-FU and Etoposide (100 µM ,24 h) and p53 protein levels were analysed by western blot. Western blot of JNK-phosphorylation in lysates from HCTshctrl cells untreated and treated with 5-FU and etoposide (100 µM, 24 h). The membrane was probed with anti-phospho-JNK (p-JNK) and anti-total JNK antibodies. CuZnSOD was utilised as a loading control (N≥3).

3.13 Like 5-FU, etoposide-induced JNK activation requires DR5, FADD and caspase-8

Following upregulation of DR5 and activation of JNK in HCT116 cells treated with etoposide, it was hypothesized that upon etoposide treatment, DR5 upregulation leads to JIST complex formation and subsequent JNK activation. Therefore, it was hypothesised that lack of DR5, FADD and caspase-8 also blocks JNK activation in HCT116 cells following Etoposide treatment.

To test this, HCTshctrl, HCTshDR5, HCTshFADD and HCTshcaspase-8 were used to check activation of JNK after 24 h treatment with 100 μM of etoposide.

As illustrated in (Figure 3.14), it was shown that when HCT116 cells were treated with etoposide, like 5-FU, lack of DR5, FADD and caspase-8 inhibits JNK activation, indicating that the mechanism and pathway of apoptosis regulation is the same in HCT116 cells in response to 5-FU and etoposide. These results are consistent with our hypothesis that the mechanism of apoptosis in HCT116 cells is not drug specific and has a general applicability.

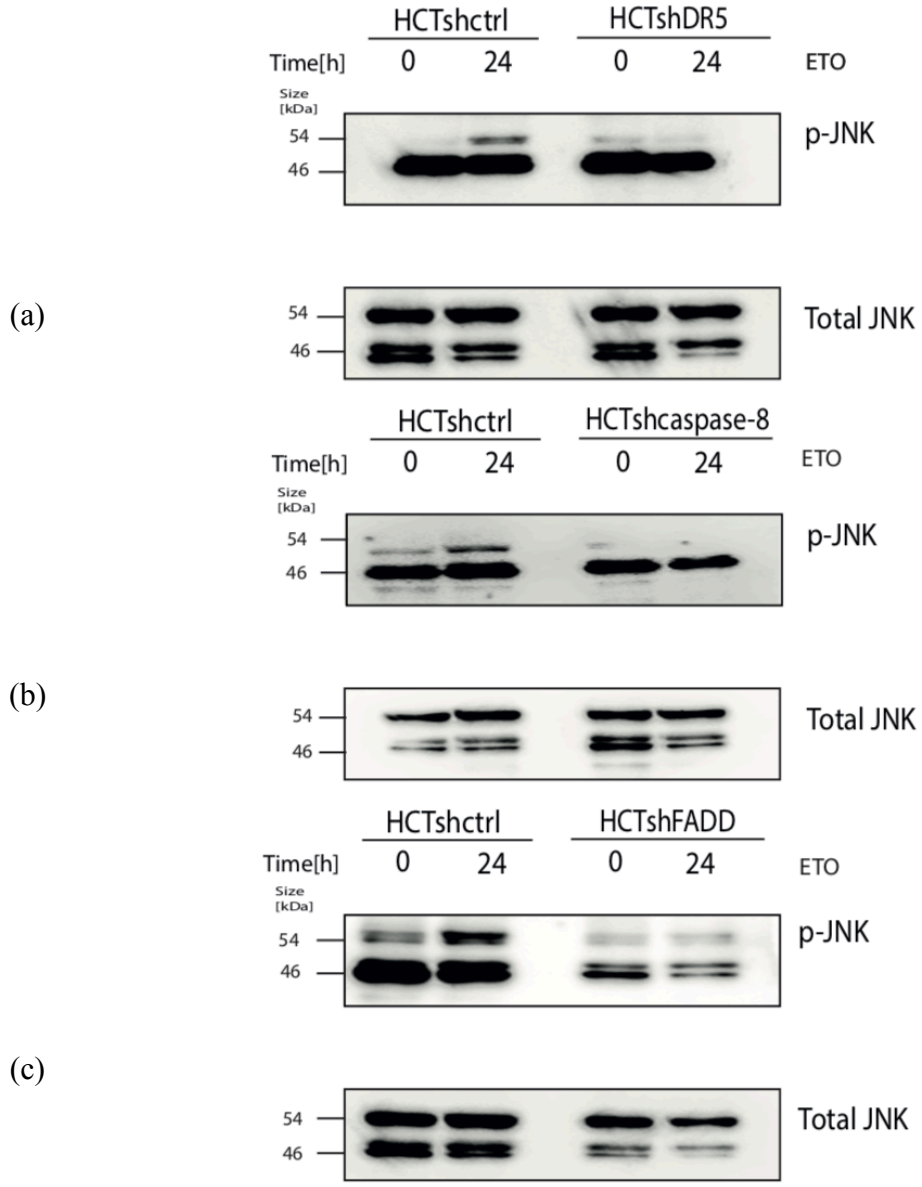

Figure 3.14. Like 5-FU, etoposide-induced JNK activation requires DR5, FADD and caspase-8.

(a) Western blot of JNK-phosphorylation in lysates from HCTshctrl and HCTshDR5 cells untreated and treated with Etoposide (100 μM, 24 h). The membrane was probed with anti-phospho-JNK (p-JNK) and anti-total JNK antibodies. (b) Western blot of JNK-phosphorylation in lysates from HCTshctrl and HCTshcaspase-8 cells untreated and treated with etoposide (100 μM, 24 h). The membrane was probed with anti-phospho-JNK (p-JNK) and anti-total JNK antibodies. (c) Western blot of JNK-phosphorylation in lysates from HCTshctrl and HCTshFADD cells untreated and treated with etoposide (100 μM, 24 h). The membrane was probed with anti-phospho-JNK (p-JNK) and anti-total JNK antibodies (N=3).

3.14 HAP1 cells potentially have the same apoptosis signalling pathway as HCT116 cells

To test whether the 5-FU-induced JNK activation is a unique phenomenon in HCT116 cells, another different cell type, HAP1, was used to check JNK activation in response to 5-FU and TNFα. Specifically, HAP1 cells were used to test whether apoptosis is mediated through JNK activation. HAP1 cells are derived from a chronic myelogenous leukaemia (CML) cell line.

To compare our previous model with another cancer cell line, HAP1 cells were treated with 5-FU and compared the results with HCT116 cells. Also, to check that HAP1 cells might not be able to activate JNK owing to an additional, due to unknown issue with the cellular JNK activation machinery, JNK activation in response to TNFα was also examined.

As shown in (Figure 3.15), in addition to HCT116 cells, JNK activation in HAP1 cells as well was detected. Therefore, it could be possible that the mechanism of apoptosis in HAP1 cells and HCT116 cells is potentially the same as JNK is also activated in response to 5-FU. However, comparing to untreated HACT116 cells, less phosphorylated JNK was detected in HAP1 cell suggesting that HAP1 cells might have different pattern of JNK activation.

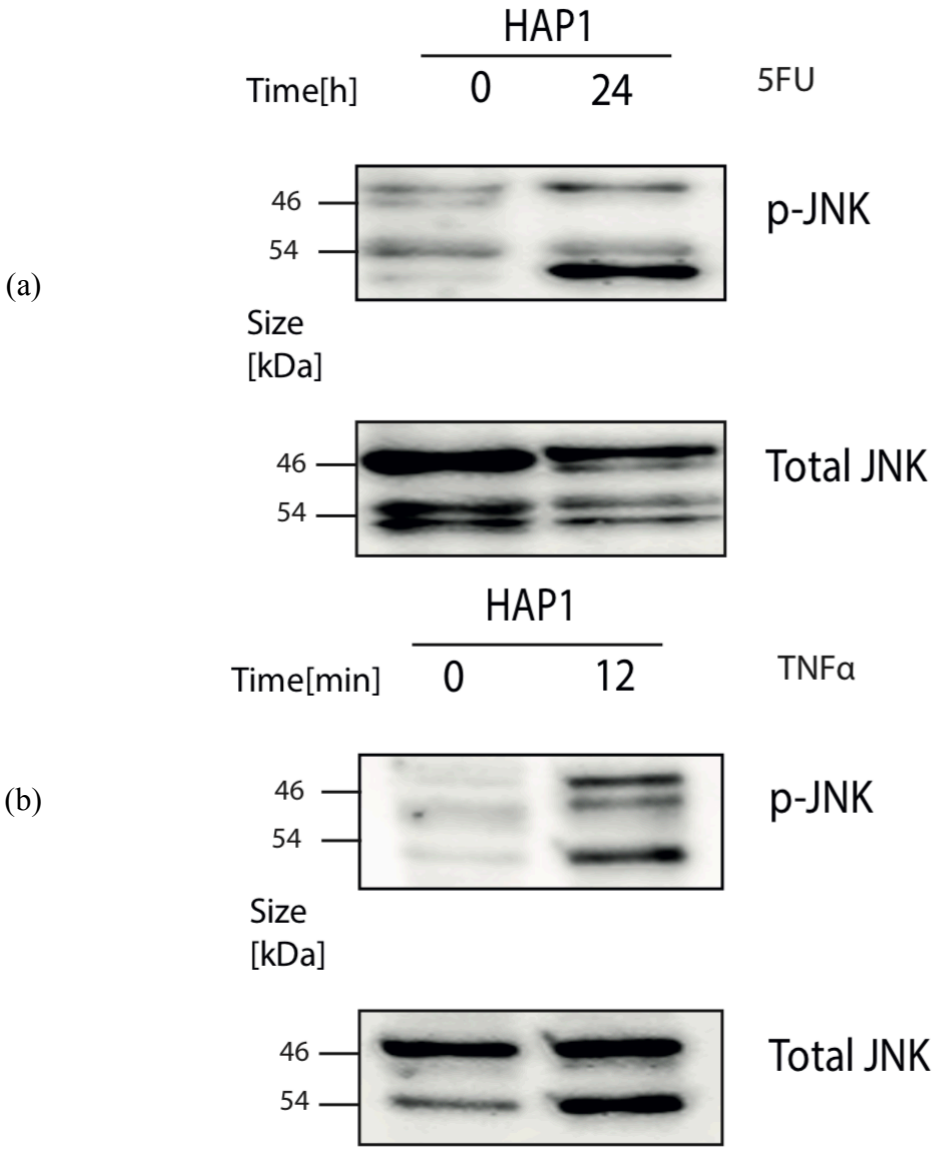

Figure 3.15. HAP1 cells potentially have the same apoptosis signalling pathway as HCT116 cells.

(a) Western blot of JNK-phosphorylation in lysates from HAP1 cells untreated and treated with 5-FU (200 μM, 24 h). The membrane was probed with anti-phospho-JNK (p-JNK) and anti-total JNK antibodies. (b) Western blot of JNK-phosphorylation in lysates from HAP1 cells untreated and treated with TNFα (10 ng/ml,15 min). The membrane was probed with anti-phospho-JNK (p-JNK) and anti-total JNK antibodies (N=3).

Chapter 4

DISCUSSION

4.1 Discussion

Colon cancer is the second most common cause of cancer related deaths. 5-FU is a well-known anti-cancer drug which has been used for colon cancer since 1957 (Grem, 2000). The therapeutic actions of 5-FU are regulated through an active metabolite, 5-fluorodeoxyuridylate (5FdUMP). 5FdUMP causes inhibition of thymidylate synthase (TS). TS is an enzyme which catalyses the conversion of deoxyuridylate to deoxythymidylate which is necessary for DNA replication and repair. FdUMP binds to the nucleotide-binding site of TS and prevents its action This active metabolite also disrupts the synthesis of RNA (Grem, 2000, Longley et al., 2003)

Despite development of strategies which have increased 5-FU antitumor activity over the past 20 years, the overall response to this anti-cancer agent is still limited (Pardini et al., 2011). One of these limitations is the resistance of tumours to 5-FU (Douillard et al., 2000). Anti-cancer drugs target tumour cells through induction of apoptosis. Both extrinsic and intrinsic pathways of apoptosis are triggered in response to anti-cancer agents (Kaufmann and Earnshaw, 2000, Petak and Houghton, 2001). Apart from conventional pathways of apoptosis such as extrinsic and intrinsic pathways of apoptosis, several new pathways have been discovered. Recently a new caspase-8-dependent pathway of apoptosis in connection with compound which inhibit the activity of inhibitor of apoptosis proteins (IAPs) has been discovered (Feoktistova et al., 2011). Moreover, it also has been shown that etoposide (anti-cancer drug) triggers apoptosis with the same mechanism in HeLa cells (Biton and Ashkenazi, 2011). Furthermore, it was discovered that inhibition of IAP on its own or with combination of etoposide results in formation of

complex termed Ripoptosome (Tenev et al., 2011). Therefore, it appears that apart from intrinsic pathway of apoptosis with engage of caspase-9, possibly other apoptosis mechanism which are cell or drug specific exists as well. As a result, a greater understanding of molecular events of apoptosis pathways induced by anti-tumour agents potentially provides better approaches to new chemotherapy drug design to cure cancer and is a critical step towards overcoming drug resistance.

It was previously shown that anti-cancer drugs which are known to induce single- strand DNA breaks such as 5-FU, lead to caspase-8 but not caspase-9 initiated apoptosis (Mohr et al., 2017). It was found that caspase-8 activation was death receptor independent (without the involvement of TNFα, CD95 or TRAIL receptors). Our group has shown that when cancer cells were treated with anti-cancer drugs, ATR identifies the drug induced DNA lesions and stimulates upstream signals which leads to p53-independent upregulation of caspase-10. Upregulation of caspase-10 gives rise to the formation of the FADDosome complex which contains caspase-10, FADD, caspase-8, RIP1 and TRAF2 (Figure 4.1).

In the FADDosome, TRAF2 is responsible for cFLIPL ubiquitination which leads to its degradation (Mohr et al., 2017). Moreover, it was shown that lack of these factors will cause cancer cells to shift to an alternative mechanism and a complex which was termed FLIPosome (Figure 4.1). FLIPosome formation causes NF-κB activation which leads to expression of TNFα and autocrine/paracrine apoptosis that was p53 independent.

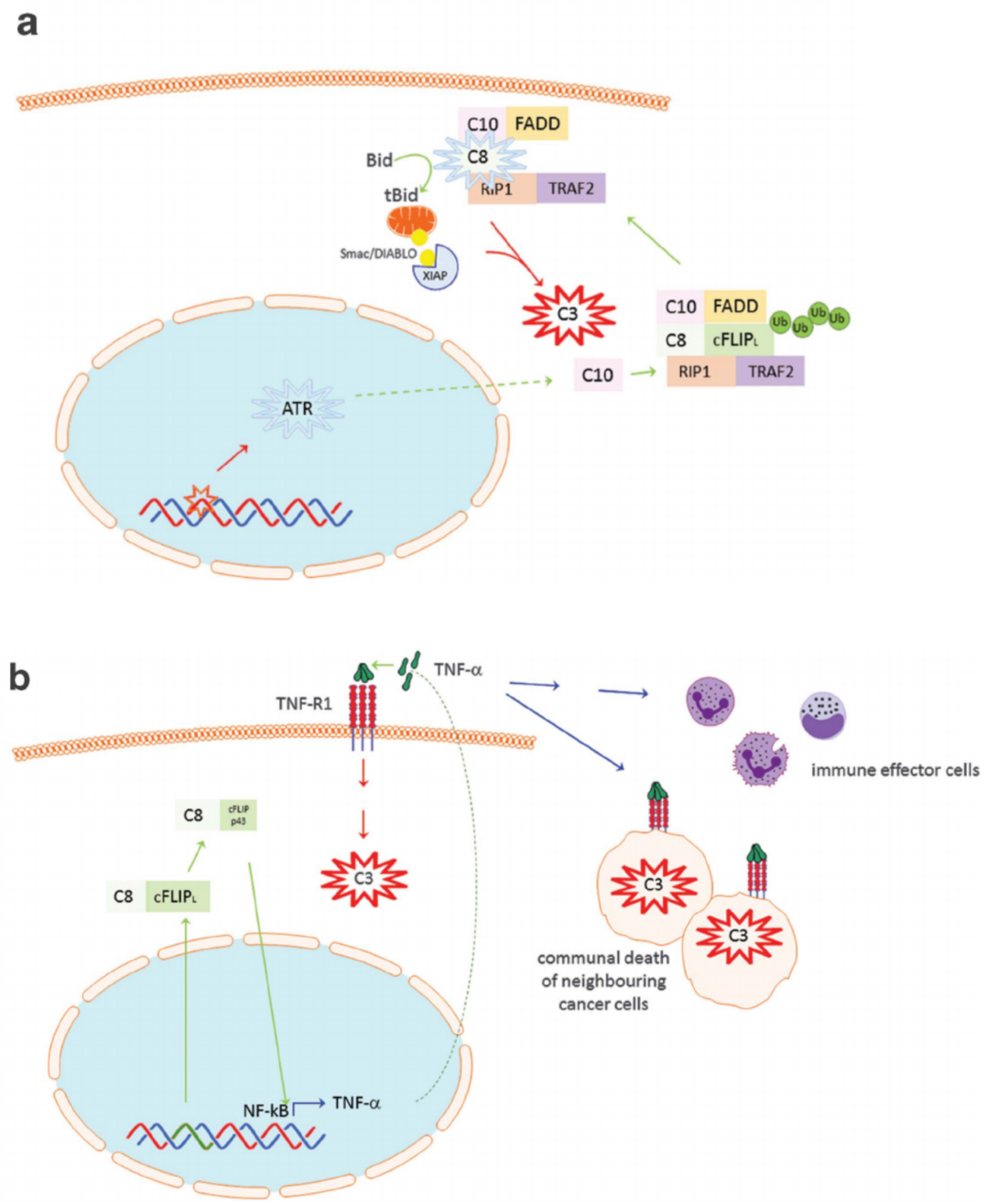

Figure 4.1. Apoptosis model upon anti-cancer treatment.

(a). FADDosome complex is formed in response to5-FU. DNA single strand breaks are shown by star. In response to anti-cancer drugs, ATR identifies the drug induced DNA lesions and stimulates upstream signals which leads to p53-independent upregulation of caspase-10. Upregulation of caspase-10 gives rise to the formation of the FADDosome complex which contains caspase-10, FADD, caspase-8, RIP1 and TRAF2. (b). FLIPosome complex is formed upon 5-FU treatment. Cancer cells lacking caspase-10, TRAF2 or ATR switch from FADDosome to FLIPosome complex which leads to processing of cFLIPL to cFLIPp43 and TNF-α production (figure from (Mohr et al., 2017)).

The raised question was that although caspase-8 activation in the FADDosome was p53-independent, apoptosis was highly reduced in p53 knock-out cells (Mohr et al., 2017). Thus, a second p53-controlled pathway acting downstream of the caspase-8 activation must exist that regulates the overall apoptotic output. Therefore, it was set out to understand the mechanism behind this and to elucidate the molecular details of this pathway.

4.2 5-FU-induced apoptosis is modulated by a p53-regulated TRAIL-R2 axis

Induction of apoptosis stimulated by anti-cancer drugs normally involves p53 activation (Longley et al., 2003). In agreement with previous studies which have shown that the absence of p53 function can reduce cellular sensitivity to 5-F (Bunz et al., 1999, Longley et al., 2002). Our group has recently found that apoptosis is reduced in HCTp53-/- cells ; however, 5-FU induced caspase-8 activation is p53-independent (Mohr et al., 2017). Therefore, it was hypothesised that p53 is involved in 5-FU induced apoptosis via engagement of death receptors such as Fas, DR4 and DR5 since p53 has been shown to initiate apoptosis through up-regulation of pro-apoptotic factors such as receptors from the TNF receptor superfamily (TNFRSF) including Fas and DR5 (Fridman and Lowe, 2003) (Longley et al., 2003). When the level of apoptosis in HCT116 cells with silenced DR5 and CD95 as well as the other TRAIL receptor, DR4, were tested, it was found that inactivation of DR5 but not DR4 and CD95, significantly reduced apoptosis stimulated by 5-FU. Thus, it was hypothesised that mutation or loss of p53 negatively affects 5-FU-

induced apoptosis in HCT116 cells due to a lack of DR5 upregulation. Therefore, DR5 upregulation in HCT116 $p53^{-/-}$ was examined. As expected, lack of p53 inhibited DR5 upregulation. Thus, p53 leads to transcriptional regulation of DR5 in HCT116 cells treated with 5-FU which is in line with previous findings (Akpinar et al., 2015). Our data shows that both DR5 and p53 are fundamental for 5-FU-induced apoptosis.

Aside from p53, DR5 mutations have also been reported in head, neck, lung and breast cancers. Also in lung cancer, several mutations in the death domain were found suggesting that DR5 can act as tumour suppressor gene (Wang and El-Deiry, 2004). It was also reported that silencing of DR5 death receptor in mice increase tumour growth and cause 5-FU resistance (Wang and El-Deiry, 2004).

4.3 JNK regulates 5-FU-induced apoptosis in HCT116 cells

Upon ligation of TRAIL with its receptor DR5, it induces apoptosis through formation of the DISC complex which promotes caspase-8 activation and later, activation of effector caspases. Although silencing DR5 reduced apoptosis in HCT116 cells in response to 5-FU, it was shown that lack of DR5 has no effect on caspase-8 activation meaning that DR5 does not induce the canonical pathway of apoptosis. Our results are also in line with our recent findings that caspase-8 is activated independently of death receptors in a complex termed FADDosome (Mohr et al., 2017). However, apart from activating canonical caspase-dependent pathway of apoptosis, ligation of TRAIL to DR5 can also activate non-canonical pathway of apoptosis. These non-canonical pathways of apoptosis include: NF-κB, JNK and p38 pathways. As it was shown that NF-κB is not affected in the absence of DR5 (data not show), the regulation of JNK by DR5 was checked. JNK is stress-activated member of MAP kinase family which can be activated by TRAIL through both caspase dependent and independent manner (Muhlenbeck et al., 1998). Thus, after ruling out the involvement of NF-κB, regulation/activation of MAPK signalling pathway in HCT116 cells in response to 5-FU was checked. It was found that 5-FU did not trigger the NF-κB pathway but discovered that JNK activation was blocked in DR5 silenced cells. Our result showed that lack of DR5 or p53 can block JNK activation indicating an absolute requirement for JNK in 5-FU-induced cell death in HCT116 cells.

4.4 FADD and caspase-8 but not caspase-10 regulate JNK activation

Previous studies have reported that DR5 mediates JNK activation through formation of a secondary intracellular complex following assembly of the DISC. The secondary complex is composed of the same DISC complex components including FADD and caspase-8 but it also contained other components which are required in JNK activation through TNF including RIP1, TRAF2 and NEMO (Varfolomeev et al., 2005).

It is important to note that the molecular mechanisms of how anticancer drugs, in contrast to cytokines such as TNFα or TRAIL, activate JNKs are still unclear. Therefore, it was turned back to the DR5-mediated JNK activation in response to 5-FU and asked what intracellular factors are essential for this pathway to be triggered.

The primary focus in this study was on proteins that potentially bind to DR5 as predicted by their structure and their previously reported functions. In the agreement with previous studies (Varfolomeev et al., 2005), it was found that DR5 death receptor requires FADD to activate the JNK pathway. As in silenced FADD cells (HCTshFADD) JNK activation was blocked. Therefore, it was hypothesised that a complex, similar to the DISC complex, but without caspase-8 activating function, forms at the intracellular side of DR5 in response to 5-FU. This putative complex was termed JIST complex (JNK inducing signal transduction complex).

Apart from FADD, the question was whether caspase-8 is also involved in mediation of JNK pathway through DR5. Previously (Varfolomeev et al., 2005), a previous study study has shown that caspase-8 is required in the secondary complex for activation of JNK and

p38 by TRAIL. Also, in another study inhibiting caspase-8 activity blocked TRAIL-induced JNK phosphorylation (Azijli et al., 2013b). In order to study the role of caspase-8 in activation of JNK, caspase-8 knock down cells were used. Consistent with previous studies, it was found that apart from FADD, caspase-8 contributes to JNK activation as silencing of caspase-8 inhibits JNK phosphorylation/ activation. In our model, caspase-8 act as a structural protein through interacting with FADD and eventually formation of JIST signalling complex. As DED of caspase-8 and -10 and DD of Fas have shown to interact with each other and both are needed for formation of DISC complex as well (Wajant, 2002).

Similar to caspase-8, caspase-10 was another candidate for the JIST complex since in the extrinsic pathway of apoptosis the signals from DR5 are mediated through recruitment and activation of caspase-8 or caspase-10 at the DISC complex (Varfolomeev and Ashkenazi, 2004). However, JNK activation in HCT116 cells upon 5-FU treatment does not require caspase-10 activation. Since, upon 5-FU treatment, neither the amount of apoptosis nor JNK activation/phosphorylation are affected when caspase-10 is absent, a role for caspase-10 was ruled out. Our findings suggest that caspase-8 in the JIST complex is sufficient to facilitate JNK activation through the DR5 death receptor after 5-FU stimulation.

4.5 JIST activation is TRAF2 and RIP1 independent

TRAF2 is one of the important molecules in TNFR1 signalling. Following TNFR activation, TRADD, an adaptor molecule, recruits TRAF2. Then TRAF2 can in turn mediates the signals induced by TNFR1 for NF-κB and JNK activation (Baud et al., 1999). TRAF2 has been shown to interact with ASK1 through its RING domain. The RING domain is essential for its function and JNK activation (Au and Yeh, 2007). Also, K63 ubiquitination of TRAF2 has found to be important for JNK activation (Habelhah et al., 2004). Despite these findings, it was found that when HCT116 cells lacking TRAF2 (HCT116.shTRAF2) were treated with 5-FU, JNK activation was not inhibited. However, interestingly, TRAF2 depletion sensitized HCT116 cells to cell death by 5-FU independent of JNK activation settings via activation of the FLIPosome (Mohr et al., 2017). It is possible that in contrast to FADDosome-induced apoptosis, FLIPosome-induced apoptosis is not modulated by JNK. Our findings suggest that TRAF2 is not engaged in the activation of JNK through DR5. Another reason for the absence of an effect in relation to JNK activation can be redundancy in the TRAF protein family, as TRAF3, TRAF5 or TRAF6 can perform similar functions while performing different functions in other settings (Lee and Kim, 2003).

It was previously found that RIP1 is not a critical factor for apoptosis in response to anti-cancer drugs; however, it was found that it is a vital molecule for the FADDosome complex and RIP1 deficiency lead to FLIPosome formation (Mohr et al., 2017). The question was whether RIP1 regulates JNK activation as earlier studies have shown that

RIP1 expression is essential for TRAIL-induced JNK activation (Kreuz et al., 2004, Ofengeim and Yuan, 2013). Also, a later study indicated that the early phase of JNK is mediated by RIP1 and the delayed phase is mediated by MEKK1 through a caspase-dependent pathway. However, a recent study showed that RIP1 is not required for JNK activation in response to TRAIL (Grunert et al., 2012). In agreement with the later study, it was found that upon 5-FU treatment, RIP1 is not required for JNK activation as lack of RIP1 did not inhibit JNK phosphorylation. Therefore, JNK activation and modulation of apoptosis in HCT116 cells treated with 5-FU is RIP1 independent.

4.6 Etoposide-induced apoptosis is modulated by a p53-regulated TRAIL-R2/JNK axis

In the current study, it was found that DR5 is not responsible for caspase-8 activation, but for the activation of JNK following 5-FU treatment without the involvement of its natural ligand TRAIL. It was also shown that JNK is activated in HCT116 cells, but not in DR5-, caspase-8- and FADD-knockdown cells after 5-FU treatment. It was also found that the absence of caspase-10, TRAF2 and RIP1 does not affect JNK activation.

In order to obtain better understanding of the role of JIST formation and JNK activation in HCT116 cells in response to anticancer agents, it was decided to compare the effect of a functionally different anticancer agents (etoposide) in HCT116 cells.

Etoposide is a common anti-cancer drug used in the chemotherapy for lung, ovarian and many other types of cancer. Etoposide induces DNA damage through Topoisomerase II inhibition (Burden et al., 1996). The DNA damage will result in cell cycle arrest and DNA repair but eventually cell death if the repair is not successful (Clifford et al., 2003). Previously, it has been shown that etoposide induces cell death via mitochondrial-dependent action of p53 in mouse embryonic fibroblasts (MEFs) (Jamil et al., 2015). Another study has also shown that etoposide-induce apoptosis is regulated by JNK activation and p53 expression in KB-3 cells (Brantley-Finley et al., 2003). Also, it was shown that effects of etoposide on DNA damage depend on concentration of etoposide and whether it is used at higher or lower concentration. In this study, higher concentration of etoposide in MEFs, trigger apoptosis and activate transcription functions of

p53.Whereas, lower concentration of etoposide is still capable to trigger apoptosis but it cannot activate transcription factor of p53 (Jamil et al., 2015). As it was previously shown that 5-FU-induced apoptosis is modulated by p53 through up-regulation of DR5 and JNK activation, it was hypothesised that mechanism of apoptosis is the same in HCT116 cells in response to etoposide. As expected from other studies and our previous findings, when HCT116 cells were treated with etoposide, p53 protein levels were upregulated. As p53 levels were upregulated in HCT116 cells in response to etoposide, it was hypothesized that etoposide-induced apoptosis is regulated through JNK activation in activation in a DR5-and p53-dependent manner. As expected, treatment of HCT116 cells with etoposide also activated JNK. It was previously shown that in HCT116 cells treated with 5-FU, JNK activation requires DR5 upregulation and formation of the JIST complex and; therefore, it was predicated that in HCT116 cells treated with etoposide JNK activation requires the formation of the JIST complex and DR5 up-regulation. In other words, it was examined whether DR5 and JIST complex members i.e., FADD, and caspase-8 play the same role. Indeed, when HCTshDR5, HCTshFADD, HCTshcaspase-8 were treated with etoposide, JNK was not activated.

Our data demonstrate that both 5-FU and etoposide caused JNK activation in HCT116 cells. Therefore, our apoptotic signalling pathway in HCT116 cells is not limited to 5-FU.

4.7 Apoptosis pathway in HAP1 cells can be regulated through JNK activation through DR5 upregulation

As both 5-FU and etoposide anticancer drugs were identified to activate JNK through formation of the JIST complex at the DR5 receptor in a p53-dependent pathway in HCT116 cells, it was wondered whether other cells show the same response or whether the effect is cell type-specific. To check our hypothesis, HAP1 cells were used which is a near-haploid human cell line derived from a male chronic myelogenous leukaemia (CML) cell line. Treating HAP1 cells with 5-FU and TNF also leaded to JNK phosphorylation after 24 hours. These findings suggested that our apoptosis signalling pathway is not cell specific. However, further investigation was needed to clarify our findings.

4.8 Conclusions

5-FU is one of the most common cytotoxic drugs which has been used for the treatment of colon cancer. However, 5-FU still has numerous limitations such as drug resistant which needs to be overcome in order to deliver effective chemotherapy treatments. Understanding the molecular pathway of 5-FU-induced apoptosis will help designing more effective cancer treatments. In the present study, the aim was to study a new apoptosis-inducing mechanism with the long-term goal to design novel diagnostic and therapeutic strategies to defeat cancer. As well as a new 5-FU-induced apoptosis pathway

in HCT116 human colon cancer cells, the proteins involved in this pathway were also studied.

Apoptosis can be triggered via different stimuli such as anti-cancer drugs; growth factor withdrawal or UV and it has been broadly divided into extrinsic and intrinsic pathways. Chemotherapeutic drugs such as 5-FU are thought to trigger cell death via intrinsic apoptosis pathway which typically uses caspase-9 as an initiator caspase. However, our group have recently found novel apoptotic pathways which are involved in 5-FU-induced apoptosis. It was found that caspase-8 is as an essential factor for 5-FU-induced cell death with caspase-9 being dispensable for this process (Mohr et al., 2017). Aside from caspase-8, here it was discovered that the TRAIL receptor, DR5, which is usually involved in the extrinsic pathway of apoptosis, is required for 5-FU-triggered cell death. It was also found that following 5-FU treatment, DR5 is not responsible for caspase-8 activation, but for the activation of JNK without the involvement of its natural ligand TRAIL. Moreover, factors associated with DR5 which are involved in JNK activation were identified. It was examined whether TRAF2, FADD, caspase-8, caspase-10 and RIP1 are involved in JNK activation. It was found that JNK is activated in HCT116, but not in DR5, caspase-8 and FADD knockdown cells after 5-FU treatment. Furthermore, it was found that the absence of caspase-10, TRAF2 and RIP1 does not affect JNK activation. After identification of putative members of the JIST complex in HCT116 cells treated with 5-FU, JNK activation and the possibility of involvement of same the JIST complex members in response to etoposide which is another common anticancer drug were investigated. It was found that like 5-FU, etoposide-induced apoptosis activates JNK which is DR5, caspase-8 and FADD dependent as well. In conclusion, factors that are involved in mediating JNK

activation after 5-FU and etoposide treatment and our results further clarify the mechanism of cell death in colon cancer cells after 5-FU and etoposide treatment which is a novel pathway of apoptosis were identified (Figure 4.2).

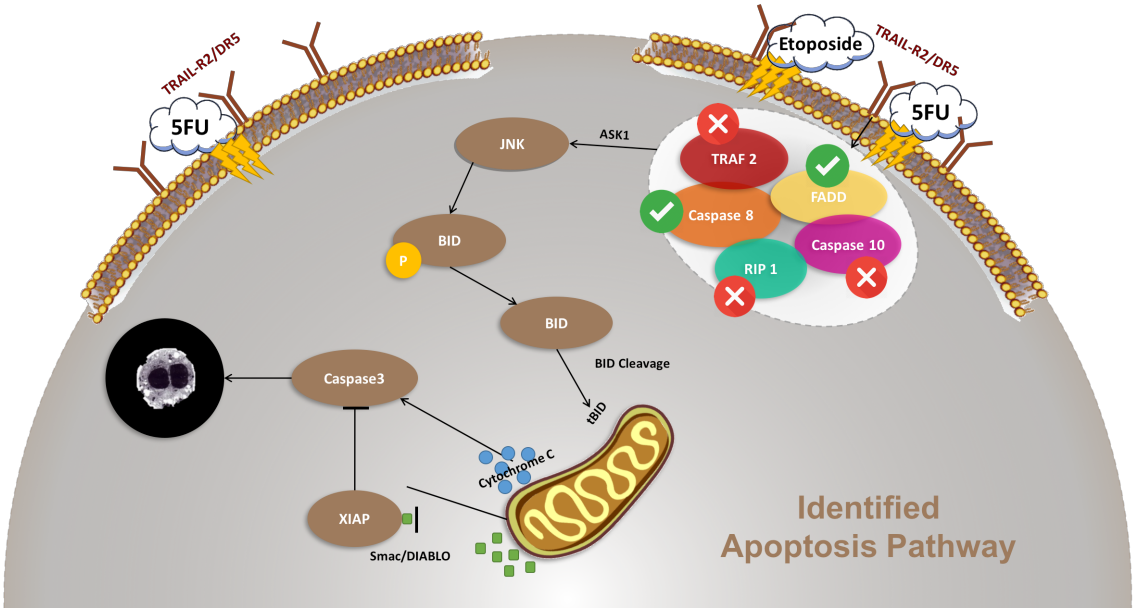

Figure 4.2. New apoptosis pathway in HCT116 cells treated with 5-FU.

In conclusion apoptosis induced by 5-FU is DR5-dependent in HCT116 cells. In this pathway, JNK activation is regulated through upregulation of DR5 death receptor and formation of JIST complex. The JIST complex is composed of FADD and caspase-8. However, RIP1, caspase-10 and TRAF2 proteins appear not to play a role in this pathway. In this model, it was hypothesised that further downstream apoptosis is regulated by BID cleavage through caspase-8. It was assumed that Bid cleavage to tBid is required for cytochrome c and Smac/DIABLO release form mitochondria in order to block XIAP activity. In this pathway, cytochrome c regulates proteolytic cleavage and activation of the caspase-3 which finally leads cell death.

4.9 Future Directions

It was previously shown that 5-FU induced apoptosis in HCT116 cells is caspase-8 dependent. It was also shown that silencing Bid significantly reduced apoptosis in HCT116 cells in response to 5-FU but did not affect caspase-8 activity (Mohr et al., 2017). as it has previously shown that Bid, a pro-apoptotic member of Bcl2 family, plays an important role in 5-FU induced apoptosis specially in mediation of mitochondrial pathway (Mohr et al., 2017). It was previously found that in response to cytotoxic drugs, formation of FADDosome which promotes caspase-8 activation is responsible for regulating apoptosis. In the current study, it was found that in response to anticancer drugs, JNK is activated and regulated by the JIST complex. Bid phosphorylation can be a potential meeting point of these two independent signalling pathways in HCT116 cells.

JNK can mediate both extrinsic and intrinsic pathways of apoptosis through two mechanisms. The first mechanism targets nuclear events. In this pathway, the phosphorylated JNK translocate to the nucleolus and activate c-Jun. Activated c-Jun can regulate transcription of pro-apoptotic genes. The second mechanism is regulating mitochondria signalling of apoptosis. JNK has shown to be required for the release of cytochrome c from mitochondria. Apart from that, JNK regulate Bid through phosphorylation (Dhanasekaran and Reddy, 2008). Therefore, it was hypothesised that Bid phosphorylation is also mediated by JNK. Our hypothesis, regulation of Bid through JNK, can be tested via inhibiting JNK and testing Bid phosphorylation and identifying any potential molecules which can be involved in regulation of Bid via JNK.

In the current study, a signalling complex (JIST) which activates JNK and later mediates apoptosis was identified in HCT116 cells. However, it was not identified which MAP2K is responsible for JNK activation in HCT116 cells in response to 5-FU. Out of 20 MAP3K which have been identified, 14 of them have been shown to be involved in the JNK signalling activation through MKK4 or MKK7 kinases. Among these 14 identified kinases, ASK1 is the most well-known MAP2K which regulates JNK (Tobiume et al., 2001). Identification of the members of MAPK signalling and Bid dephosphorylating mechanism would provide more information about the novel postulated hypothesis apoptosis (JNK activation through JIST complex formation).

Chapter 5

APPENDIX: MATERIALS, CHEMICAL AND REAGENTS

5.1 Primary Antibodies

TABLE 5.1 LIST OF PRIMARY ANTIBODIES

Primary Antibodies	MW (kDa)	Company
Caspase-10	59	MBL Woburn, MA, USA
Caspase-8	55	BD Pharmingen, Franklin Lakes, NJ, USA
CuZnSOD	17	The Binding Site, Birmingham, England
FADD	28	Clone 1F7, Upstate (Millipore), Billerica, MA, USA
p53	53	Biotechnology, Santa Cruz, CA, USA
PHOSPHO-JNK	46 54	Cell Signalling Technology, Beverly, MA, USA
RIP1	76	BD Pharmingen, Franklin Lakes, NJ, USA
TOTAL-JNK	46 54	Cell Signalling Technology, Beverly, MA, USA
TRAF2	50	Cell Signalling Technology, Beverly, MA, USA
TRAIL-R2/DR5	40 (mature) 47.8 (precursor)	Acris Antibodies, Herford, Germany

5.2 Secondary Antibodies

TABLE 5.2 LIST OF SECONDARY ANTIBODIES

Secondary Antibodies	Company	SPECIES
Anti-Mouse IgG	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Mouse
Anti-Rabbit IgG	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Rabbit
Anti-Sheep IgG	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Rabbit
Mouse IgG1	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Mouse

5.3 List of Buffers and Solutions

TABLE 5.3 LIST OF BUFFERS AND SOLUTIONS REQUIRED

Solutions	Recipe
Anode I Buffer	300 mM Trisbase, 20% Methanol
Anode II Buffer	25 mM Trisbase, 20% Methanol
Antibody Dilution Buffer	3% Bovine Serum Albumin (BSA) in TBS-Tween 20
Blocking Buffer	5% (w/v) Semi-Skimmed Dry Milk in TBS-Tween 20
Cathode Buffer	25 mM Trisbase, 40 mM 6-Aminohexanoic acid, 20% Methanol
Nicoletti Buffer	0.1% (w/v) Sodium Citrate; 0.1% (w/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 Inhibitor Cocktail
Phosphate-Buffered Saline (PBS)	5 X Tablets/1000ml dH_2O
Protein loading buffer	Tris-HCl 65 mM [pH 6.8], 10% Glycerol, 4% SDS, 4% β- Mercaptoethanol, 0.2% Bromophenol Blue.
SDS-PAGE 10X Running Buffer	Tris-HCl 25mM, Glycine 192mM, 1% SDS (w/v) in 1l dH_2O
TBS-Tween	Tris-HCl 25 mM [pH 8.0], NaCl 137 mM, dH_2O, 1% Tween- 20
Tris-Buffered saline (TBS)	Tris-HCl 25 mM [pH 8.0] , NaCl 137 mM
Tris-HCl 0.5M pH 6.8	30.3g Tris, 400ml dH_2O, adjust to pH 6.8 with HCl, adjust to 500ml with dH2O
Tris-HCl 1.5M pH 8.8	90.75g Tris, 400ml dH_2O, adjust to pH 8.8 with HCl, adjust to 500ml with dH_2O
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 Cocktail Tablets (Roche, Basel, Switzerland) 1tablet in 50ml

5.4 Reagents

TABLE 5.4 LIST OF REAGENTS

Chemical Reagents	Company
1,2-Bis-(dimethylamino)-Ethane (TEMED)	Sigma, St.Louis,MO,USA
5-Fluorouracil (5-FU)	Sigma, St.Louis,MO,USA
Acrylamide-Bis-Acrylamide	Sigma, St.Louis, MO, USA
Aminohexanoic acid	Sigma, St.Louis, MO, USA
Ammoniumpersulfate (APS)	Sigma, St.Louis, MO, USA
BCA Protein Assay Reagent (bicinchoninic acid)	Pierce/Thermoscientific, Waltham, MA, USA
Bovine Serum Albumin (BSA)	Sigma, St.Louis,MO,USA
Calcium chloride	Sigma, St.Louis,MO,USA
Caspase-8 Glo buffer	Promega, Madison,WN,USA
Enhanced chemiluminescent (ECL) Western Blotting Substrate	Pierce/Thermoscientific, Waltham, MA, USA
Ethanol	Sigma, St.Louis, MO, USA
Etoposide (ETO)	Sigma, St.Louis,MO,USA
Glycerol	Sigma, St.Louis,MO,USA
Glycine	Sigma, St.Louis,MO,USA
Magnesium chloride	Sigma, St.Louis,MO,USA
Methanol	Sigma, St.Louis, MO, USA
PhosStop Phosphatase Inhibitor Cocktail Tablets	Thermo Scientific , USA
Phosphate-Buffered Saline Tablets	Sigma, St.Louis, MO, USA
Ponceau-S Solution	Sigma, St.Louis, MO, USA
Potassium chloride	Sigma, St.Louis,MO,USA
Pre-Stained Protein Marker, Broad Range (7-175KDa)	New England Biolabs, UK
Propidium Iodide solution	Sigma, St.Louis,MO,USA
PVDF membrane	Thermo Scientific, USA
Skimmed milk	Sigma, St.Louis,MO,USA
Sodium chloride	Fisher Scientific, UK
Sodium citrate	Sigma, St.Louis,MO,USA
Sodium dodecyl sulfate (SDS)	Fisher Scientific, UK

Chemical Reagents	Company
TRAIL	Sigma, St.Louis,MO,USA
Triton X-100	Sigma, St.Louis,MO,USA
Trizma Base (Tris/ Trisbase)	Sigma, St.Louis, MO, USA
Trypsin EDTA Solution	Sigma, St.Louis, MO, USA
Tumour Necrosis Factor (TNF)	Sigma, St.Louis,MO,USA
Tween 20	Sigma, St.Louis,MO,USA
Western Blot Chemiluminescence Reagent	SuperSignal West Pico, Thermo Scientific

TABLE 5.5 LIST OF CELL LINES

Cell line	Source	Description
HCT116	Cells line stock of the lab (ATCC)	Human colorectal tumour cells
HCTshDR5	Cells line stock of the lab	Human colorectal tumour cells
HCTshDR4	Cells line stock of the lab	Human colorectal tumour cells
HCTshlamin	Cells line stock of the lab	Human colorectal tumour cells
HCTshFADD	Cells line stock of the lab	Human colorectal tumour cells
HCTshcaspase-8	Cells line stock of the lab	Human colorectal tumour cells
HCTshcaspase-10	Cells line stock of the lab	Human colorectal tumour cells
HCTshRIP1	Cells line stock of the lab	Human colorectal tumour cells
HCT116p53-/-	Cells line stock of the lab (gift from Bert Vogelstein)	Human colorectal tumour cells
HAP1	Cells line stock of the lab (ATCC)	Human Male Chronic Myelogenous Leukaemia cells

TABLE 5.6 CELL CULTURE MEDIA

Cell culture media	Company
McCoy's 5A for HCT116 cells	Sigma, St.Louis,MO,USA
Iscove's Modified Dulbecco's Medium (IMDM) for HAP1 cells	GIBCO

TABLE 5.7 SDS-PAGE

Reagents	12% Separating Gel	4% Stacking Gel (ml)
0.5 MTris, pH 6.8	-	1.89 ml
1.5 M Tris , pH 8.8	2.6 ml	-
10% APS	50 µl	90 µl
10% SDS	100 µl	90 µl
30% Arcylamide	4 ml	1.25 ml
H_2O	3.4 ml	5.7 ml
TEMED	5 µl	9 µl

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