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Novel Role of CHK2 in the Intrinsic and Extrinsic Apoptosis Pathway

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Washington University in St. Louis

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Division of Biology and Biomedical Sciences

Molecular Cell Biology

Dissertation Examination Committee:

Helen Piwnica-Worms, Chair

Robert Mecham

Deborah Novack

Barry Sleckman

Sheila Stewart

Zhongsheng You

Novel Role of CHK2 in the intrinsic and Extrinsic Apoptosis Pathway

By

Anurag Agarwal

A dissertation presented to the

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Table of Contents

erent

List of Figures

Chapter 3

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ABSTRACT OF THE DISSERTATION

Novel Role of CHK2 in the Intrinsic and Extrinsic Apoptosis Pathway

by

Anurag Agarwal

Doctor of Philosophy in Biology and Biomedical Sciences

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Washington University in Saint Louis, 2014

Professor Helen Piwnica-Worms, Chair

In response to genotoxic stress, cells activate DNA damage checkpoint pathways that maintain genetic fidelity, promote cell survival, and prevent malignant transformation. Checkpoint kinase 2 (CHK2) is an evolutionarily conserved serine/threonine protein kinase that is activated in response to DNA double strand breaks. Upon activation by the DNA damage transducer kinase, ATM, CHK2 phosphorylates a wide variety of down stream effectors to promote cell cycle arrest, DNA repair and apoptosis. One of they key downstream substrate of CHK2 is the tumor suppressor protein, p53. CHK2 is considered as a key kinase in DNA damage-induced p53 regulation and thought to mediate most of its downstream effects through p53.

This dissertation has further strengthened the growing pool of evidence that CHK2 is dispensable for p53 regulation but rather signals to the apoptotic machinery through effector proteins other than p53. I have identified cellular Inhibitor of Apoptosis proteins (cIAPs) as novel CHK2 targets and established their role in CHK2-mediated cell death. In response to DNA damage, CHK2 inhibits both cIAP1 and cIAP2 to relieve

their anti-apoptotic effects and drive cells into apoptosis. CHK2 inhibits the E3 ubiquitin ligase activity of cIAP2 in a phosphorylation dependent manner and potentially regulates cIAP1 through its interaction with cIAP2. Furthermore, this work provides preliminary evidence that CHK2 serves as a negative regulator of the NFκB pathway through the inhibition of cIAPs. Upon activation, CHK2 potentiates TNFα Related Apoptosis Inducing Ligand (TRAIL)-induced apoptosis through the regulation of the NFκB pathway.

Overall, my work has discovered a novel role of CHK2 in the DNA damage induced intrinsic apoptotic pathway and uncovered its potential role in death receptor mediated extrinsic apoptotic pathway.

Chapter 1:

Introduction

1.1 Genomic Instability and DNA Damage Response Pathway

Numerous internal and environmental factors pose a constant threat to the genetic information within a cell. These factors include replication fidelity, collapse of replication forks, and reactive oxygen species, and external factors, like ionizing radiation (IR), ultraviolet radiation (UV), and genotoxic chemicals. In order to maintain genomic integrity, cells have developed a complex array of signaling pathways, known as the DNA damage response (DDR) pathway, to sense DNA damage and execute specific cellular responses like cell cycle arrest, i.e. senescence, DNA damage repair and/or cell death. Defects in DDR can render the genome unstable and drive malignant transformation through accumulation of various genetic alterations. For this reason, Hanahan and Weinberg have proposed genomic instability as the most prominent enabling characteristic of tumorogenesis in their seminal description of the hallmarks of cancer (Hanahan and Weinberg et. al., 2011). Therefore, careful understanding of the DDR pathway has provided key insight into the process of transformation and cancer development.

The DDR pathway can be divided into three tiers: *sensors, transducers* and *effectors.* DDR sensors identify the lesion and with the help of mediators and activate the transducers to begin the DDR signaling cascade. Transducers amplify the DNA damage signal from the sensors and activate the effector molecules to execute the appropriate cellular response.

DNA Single Strand Breaks (SSBs) and Double Strand Breaks (DSBs) are two major forms of DNA damage that elicit a DDR response. DNA DSBs are formed in response to either various stimuli like IR or treatment with topoisomerase II inhibitors (VP16 and

doxorubicin) that prevent re-ligation of the DNA strands broken by topoisomerase II. DNA DSBs can also be formed by replicating DNA with specific lesions like DNA single strand breaks (SSBs) or by nucleases in V(D)J and class switch recombination. On the other hand, DNA SSBs are formed in response to treatment with UV, hydroxyurea (HU), DNA alkylating agents, or other chemicals that stall the replication fork. Distinct DDR pathways are activated in response to different forms of DNA damage. While the ATM-CHK2-p53 axis controls the cellular response to DNA DSBs, the ATR-CHK1-Cdc25A axis mediates the effect of DNA SSBs (Smith et al. 2010). Although distinct pathways are activated initially in response to different forms of DNA damage, over a period of time, one form of damage is converted into another form, which leads to the activation of both DDR responses. For example, repair of DNA DSBs requires processing of the DNA ends. As a result, single stranded DNA is exposed and activates the SSB response. Similarly, SSBs are converted into the DSBs during replication. In the next section, I will introduce the cellular response to DNA DSBs in more detail and discuss the signaling cascade that eventually leads to activation of the checkpoint kinase, CHK2. Table 1 summarizes the different components of the DDR response to different forms of DNA damage.

	DNA DSBs	**DNA SSBs**
Sensors	Mre11, Rad50, Nbs1	RPA
Mediators	MDC1, BRCA1, 53BP1, H2AX	ATRIP, Claspin, TopBP1, 9-1-1, Rad17
Transducers	ATM	ATR
Effectors	CHK2, p53, E2F1, MDMX	p53, Cdc25A

Table 1. List of proteins involved in the DDR pathway in response to DNA DSB and SSBs.

1.2 DNA Double Strand Breaks

DNA DSBs are one of the most detrimental forms of DNA damage as a double strand break does not leave behind a complementary strand to recover the lost information. Furthermore, DSBs are pre-requisites for chromosomal rearrangements, especially translocations, which are associated with various malignancies (Mitelman et al., 1997; Rabbitts, 1994). One the one hand, chromosomal translocations can produce a fusion protein composed of two truncated proteins with the fusion protein acquiring unique or altered activity. On the other hand, chromosomal translocations can result in transferring a whole gene downstream to a different regulatory element resulting in altered expression of the native protein.

In 1961 Nowell and Hungerford discovered the first chromosomal translocation, known as Philadelphia Chromosome, in chronic myelogenous leukemia (Nowell, 2007). This translocation results in the fusion of *BCR and ABL1* gene. Abl1 is a tyrosine kinase whose activity is tightly regulated on the plasma membrane. The fusion protein disrupts the native regulation and converts Abl1 into a constitutively active kinase (Ben-Neriah et al., 1986; Van Etten et al., 1989). Soon after, translocation between chromosome 8 and 14 was discovered in Burkitt's lymphoma (Zech et al., 1976) where *c-MYC* gene is juxtaposed to the immunoglobulin heavy-chain genes and results in over-expression of MYC (Deshmukh et al., 1982; Taub et al., 1982). While chromosomal translocations are highly prevalent in the hematological malignancies, translocations have also been implicated in the development of several epithelial and mesenchymal malignancies as well (Rowley, 2001). Therefore, in order to prevent detrimental effects of unresolved

DSBs, cells have developed a sensitive response pathway that detects and resolves such breaks.

1.3 DNA Double Strand Break Sensors

The Mre11-Rad50-Nbs1 (MRN) protein complex serves as the sensor of DNA DSBs. MRN complex rapidly moves to the damage sites and forms a bridge spanning the two edges of the break. Mre11 possesses intrinsic DNA binding property to synapse the DSB termini (Williams et al., 2008). Additionally, exonuclease and endonuclease properties of Mre11 are critical for Non homologous end joining (NHEJ)- and HR-mediated repair of damaged ends (de Jager et al., 2001a; Paull and Gellert, 1998, 1999). Mre11 interacts with Rad50 and recruits it to damage sites. This strengthens the interaction of the complex with the broken ends of the DNA. Rad50 is also capable to mediating long distance interactions like those with sister chromatids (de Jager et al., 2001b; Lamarche et al. 2010). Nbs1, the third component of the MRN complex, contains the FHA and BRCT domains that mediate protein-protein interactions and is critical in recruiting other proteins to the damage site (Lloyd et al., 2009; Williams et al., 2009). Moreover, the nuclear localization signal in Nbs1 keeps the MRN complex in the nucleus (Desai-Mehta et al., 2001).

Defects in MRN complex results in dampened DDR to DSBs and predispose cells to malignant transformation. Hypomorphic mutations in Mre11, Rad50 and Nbs1 results in neurological disorders such as ataxia-telangiectasia like disorder (ATLD), Nijmegen breakage syndrome (NBS) and NBS like disorder (NBSLD) (Carney et al.,

1998; Kobayashi et al., 2004; Taylor et al., 2004; Waltes et al., 2009). This underscores the importance of the MRN complex, as well as DDR, in the nervous system.

1.4 Transducer Kinase: ATM

One of the central roles of MRN complex in DDR is the recruitment and activation of the transducer kinase, ataxia-telangiectasia mutated (ATM). Analysis of ataxia-telangiectasia (AT) patients, who are characterized by cerebral ataxia, immunological dysfunction, sensitivity to ionizing radiation and cancer pre-disposition, gave the first insight into the role of ATM in DNA DSBs. ATM is a serine/threonine protein kinase that belongs to the family of PI3K-like protein kinases (PIKK). ATR and DNA-PK are other two PIKKs that are also involved in the DDR pathway. While ATR is activated in response to SSB (Nam and Cortez), DNA-PK is primarily known for its role in NHEJ-mediated DNA repair (Hill and Lee; Neal and Meek). On the other hand, ATM is preferentially activated by DNA DSBs and plays a central role in cellular response to DSBs. Under normal conditions, ATM exists as an inactive homodimer. Upon DSBs, ATM is rapidly recruited to the DNA DSBs through its interaction with Nbs1, (You et al., 2005) where it undergoes intermolecular phosphorylation at the conserved residue, serine 1981, to transform itself into an active monomer (Bakkenist and Kastan, 2003). In addition to S1981, three other autophosphorylation sites, as well as acetylation of lysine 3016, regulates the kinase activity of ATM (Kozlov et al.; Kozlov et al., 2006; Sun et al., 2005; Sun et al., 2007). Although these modifications facilitate ATM activation, ATM activity is fine tuned by other DDR pathway proteins like MDC1, BRCA1, 53BP1 and MRN complex (Cerosaletti and Concannon, 2004; Lee and Paull, 2004, 2005). Among

these mediators of the DDR pathway, it is noteworthy to discuss the interaction between ATM and MDC1. MDC1 is required for timely activation of ATM and plays a key role in expanding the DNA damage signal around the damage sites. ATM phosphorylates the histone variant H2AX at serine 139, to generate γH2AX (Burma et al., 2001), which is widely used as a marker for DSBs. MDC1 is tethered to the chromatin through its interaction with γH2AX and in parallel binds to ATM (Stucki et al., 2005). This allows ATM to phosphorylate more H2AX molecules resulting in expansion of the DNA damage signal around the sites of damage. Similarly, ATM phosphorylates Nbs1 to form a positive feedback loop that keeps ATM activation for prolonged periods of time (Gatei et al., 2000; Zhao et al., 2000).

Upon activation, ATM phosphorylates numerous effector proteins to mediate various cellular responses like cell cycle arrest, DNA repair and/or apoptosis. Like other PIKK family members, ATM preferentially phosphorylates the serine or threonine residues that precede glutamine residue (SQ/TQ) (Kastan and Lim, 2000; Kim et al., 1999; O'Neill et al., 2000). Among various downstream effectors of ATM, I will focus on three specific proteins – p53, CHK2 and NFκB.

One of the first well-characterized targets of ATM was p53. p53 is a transcription factor that is most commonly mutated in cancers and contributes to the development of more than half of all human cancers. p53 regulates a wide variety of cellular functions like cell cycle arrest, apoptosis, DNA repair, autophagy and many more (Choi et al.; Rashi-Elkeles et al.; Sullivan et al.). The first evidence of ATM-mediated regulation of p53 came from the observation that p53 levels were dysregulated in cells lacking ATM (Canman et al., 1994; Siliciano et al., 1997). It was later found that ATM phosphorylates

p53 on Ser-15 that promotes its interaction with another transcription factor, p300, which is essential for transcriptional activity of p53 (Banin et al., 1998; Canman et al., 1998; Dumaz and Meek, 1999). Moreover, ATM phosphorylates and inhibits MDM2 and MDMX to promote p53 stabilization in response to DSBs (Chen et al., 2005; Cheng et al., 2009; Cheng et al.; Khosravi et al., 1999; Maya et al., 2001). ATM is also able to regulate p53 through activation of CHK2 (discussed later in section 1.5.2). Another key downstream effector of ATM is NFκB. NFκB is a transcription factor that is sequestered in the nucleus by IκBα. In response to various stimuli, including DNA damage (Miyamoto), an upstream kinase IKK phosphorylates IκBα and targets it for proteosomal degradation (Perkins, 2007; Piret et al., 1999; Wu et al., 2006). As a result the NFκB transcription factor moves into the nucleus and transcribes a wide variety of genes that support cell survival. ATM is believed to play a key role in NFκB activation in response to DNA damage by phosphorylating the regulatory subunit of IKK, NEMO (Piret et al., 1999; Wu et al., 2006; Wu et al.; Yang et al.). ATM has also been shown to regulate the NFκB pathway independent of its effect on NEMO (Wu et al.; Yang et al.). Overall, ATM plays a central role in mediating the DDR to DSBs through amplification of DNA damage signaling and transmitting the signal downstream to effector proteins.

1.5 Effector Kinase: CHK2

CHK2 is a serine/threonine protein kinase that is activated in response to DNA DSBs in an ATM-dependent manner. CHK2 serves as a tumor suppressor by inducing cell cycle arrest or apoptosis in cells with DNA damage. Heterozygous germline mutations in *CHK2* are associated with a p53-independent variant form of the Li-Fraumeni syndrome

(Bell et al., 1999). Additionally, CHK2 mutations are also found in sporadic cancers (Dong et al., 2003; Schutte et al., 2003; Sodha et al., 2002) and down regulation of CHK2 protein has been reported in several cancers (Bartkova et al., 2004; Sullivan et al., 2002; Tort et al., 2002). The role of CHK2 mutations in different forms of familial and sporadic cancers will be discussed in detail later (Section 1.6).

1.5.1 CHK2: Structure

The *CHK2* gene on chromosome 22 (22q12.1) encodes a 68kD protein that consists of three distinct functional domains; an SQ/TQ cluster domain (SCD, residues 19 - 69), a forkhead-association (FHA) domain (112- 175), and a Ser/Thr kinase domain (residues 220 – 486). The SCD contains five serine (S19, S27, S33, S35, and S50) and two threonine (T36, and T68) residues that precede a glautamine (Q). Serine or threonine residues followed by glutamine (SQ/TQ) provides a substrate recognition motif that is phosphorylated by ATM in response to DNA damage. Moreover, residues surrounding Thr68, S-T_{68}-Q-E (residues 67 – 70) closely resemble the refined substrate motif (LSQE) of ATM (O'Neill et al., 2000). ATM phopshorylates CHK2 at T68 to initiate its activation (Ahn et al., 2000a). In addition to T68, other SQ/TQ sites have been shown to be phosphorylated by ATM (Matsuoka, 1998; Matsuoka et al., 2000). Downstream of SCD is the FHA domain that works primarily in *trans* to modulate protein-protein interactions. The FHA domain can also work in *cis* to affect other functional domains within a protein (Ahn et al., 2004). FHA domains are 65 – 100 amino acid long phosphopeptide binding domains that were first identified in a family of forkhead transcription factors (Hofmann and Bucher, 1995). The FHA domain of Rad53, a CHK2

10

homolog in *Saccharomyces cerevisiae,* is essential for its interaction with *Rad9* (human homolog of *Rad9* remains to be identified) (Sun et al., 1998b). Both, biochemical analysis and the crystal structure of the FHA domain of CHK2 indicate that the FHA domain is crucial for CHK2 dimerization and activation in response to DNA damage (Cai et al., 2009; Schwarz et al., 2003a; Sun et al., 1998a). The C-terminus of CHK2 is occupied by the kinase domain. It is characterized by a glycine rich region at the vicinity of lysine residue in the N-terminal and an aspartic acid as a catalytic residue in the active site. Like other kinases, the activation loop prevents ATP binding and keeps the kinase in an inactive conformation. Upon phosphorylation, the activation loop is displaced from the catalytic center to activate kinase activity (Oliver et al., 2006).

1.5.2 CHK2: Activation

CHK2 remains in an inactive state throughout the mammalian cell cycle (Lukas et al., 2001). However, upon DNA damage, CHK2 is rapidly phosphorylated and activated in an ATM-dependent manner (Figure 1). Like ATM, CHK2 is preferentially activated in response to DNA DSBs which are caused by IR, chemotherapeutic agents like etoposide (VP16) and doxorubicin. Upon DNA DSBs, ATM phosphorylates CHK2 at Thr68 within the SCD, a key step in CHK2 activation. There are several key lines of evidence that underscore a role for ATM in phosphorylating CHK2. First, CHK2 activation and phosphorylation at Thr68 is abrogated in cells lacking ATM. However, CHK2 activation can be restored through exogenous expression of WT ATM (Brown et al., 1999; Chaturvedi et al., 1999; Matsuoka, 1998). IR-mediated CHK2 activation is

impaired in cell lines derived from AT patients that lack functional ATM (Zhou et al., 2000). Furthermore, ATM directly phosphorylates CHK2 *in vitro* at several sites including Thr68 (Ahn et al., 2000b; Matsuoka et al., 2000; Melchionna et al., 2000). In addition to ATM, DNA-PK, one of the PIKK family members, and PLK1 are also able to phosphorylate CHK2 at Thr68 *in vitro* (Li and Stern, 2005; Tsvetkov et al., 2003).

Phosphorylation of Thr68 plays a key role in CHK2 activation by promoting oligomerization-induced auto- and trans-phosphorylation. In fact, complete activation of CHK2 requires *cis*- and *trans*-phosphorylation of several residues, including Thr383 and Thr387 within the activation loop (Schwarz et al., 2003b). In the model proposed by Schwarz *et. al. (2003)*, a molecule of CHK2 phosphorylated at Thr-68 binds to the FHA domain of another CHK2 molecule. Such dimerization drives several auto-phosphorylation events that are required for CHK2 activation (Schwarz et al., 2003b). This model is supported by numerous independent studies. First, substituting alanine for threonine at residue 68 abolishes the interaction of CHK2 with recombinant FHA domain of CHK2 and impairs CHK2 activation. (Ahn et al., 2002; Li et al., 2002). Additionally, mutation of FHA domain that affects oligomerization impairs the ability of CHK2 to be activated after DNA damage (Cai et al., 2009; Oliver et al., 2006). Furthermore, forced dimerization of CHK2 is sufficient to activate its kinase activity. Overproduction of CHK2 in HEK293 cells or bacteria can induce CHK2 dimerization, Thr68 phosphorylation, and activation in the absence of DNA damage (Schwarz et al., 2003b; Xu et al., 2002). Additionally, CHK2 can be forced to oligomerize, and become activated, by the addition of a modified FKBP epitope and a synthetic ligand. Forced oligomerization of CHK2 also results in the phosphorylation of Thr68 (Xu et al., 2002).

This suggests that, in addition to ATM, CHK2 can auto-phosphorylate Thr68 and drive its own activation. In addition to interaction with other CHK2 molecules, CHK2 phosphorylation on Thr68 mediates CHK2 interaction with another DDR pathway protein, MDC1. The FHA domain of MDC1 interacts with Thr68 phosphorylated CHK2 both *in vitro* and *in vivo* (Lou et al., 2003)*.* This interaction is critical for DNA repair and apoptosis in cells with DNA DSBs.

As discussed above, oligomerization-induced autophosphorylation of CHK2 is essential for its activation. Among numerous auto-phosphorylation sites, Thr383 and Thr387 are most critical to CHK2 kinase activity. Thr383 and Thr387 are present in the activation loop and mutation of these residues to alanine abrogates CHK2 activity. (Lee and Chung, 2001). The a ctivation loop is a 20 – 30 amino acid long protein segment that blocks substrate access to the ATP binding pocket of protein kinases (Hanks and Hunter, 1995). Upon phosphorylation, the activation loop is tethered away from the ATP pocket allowing substrate access and transfer of the phosphate. Similarly, phosphorylation of Thr383 and Thr387 within the activation loop opens the active site of CHK2 and allows it to phosphorylate its substrates.

CHK2 has also been shown to interact with two different phosphatases, PP2A and Wip1 that dephosphorylate CHK2 on various residues. A yeast two-hybrid screen identified the B' regulatory subunit of PP2A as a CHK2 partner. PP2A was capable of dephosphorylating CHK2 *in vitro* and countered adramycin-induced CHK2 activation (Dozier et al., 2004). Two separate groups demonstrated that Wip1 can dephosphorylate CHK2 at Thr68 and antagonize its downstream function (Fujimoto et al., 2005; Oliva-Trastoy et al., 2007). Fujimoto *et. al.* 2005 found that CHK2 associates

with Wip1 both *in vitro* and *in vivo*. Moreover, Wip1 is able to dephosphorylate CHK2 on Ser19, Ser33/35, Thr68, and Thr432 but not on Thr387 or Ser516. Furthermore, overexpression of Wip1 inhibits Thr68 phosphorylation *in vivo.* On the other hand, Oliva-Trastoy *et. al.* 2007 demonstrated that overexpression of Wip1 in HCT15 cells abrogates CHK2-mediated G2 arrest in response to IR.

1.5.3 CHK2: Regulation

Upon oligomerization, CHK2 can auto-and trans phosphorylate on several serine and threonine residues. Thus, various studies have focused on identifying these phosphorylation sites and their role in CHK2 function (Kass et al., 2007; King et al., 2007; King et al., 2006). In this section, I will introduce how CHK2 auto/trans-phosphorylation sites regulate CHK2 ubiquitination. Ubiquitin is a small protein that is covalently attached to proteins through E3 ligases. Ubiquitination of proteins can target them for proteosomal degradation or alter their activity and downstream signaling. Cellular protein levels are tightly regulated through a balance between ubiquitination by E3 ligases and deubiquitination by deubiquitining enzymes. In mammalian cells, CHK2 is a stable protein and is expressed throughout the cell cycle. However, low levels of CHK2 are observed in a subset of breast cancer (Sullivan et al., 2002; Vahteristo et al., 2002), testicular cancer (Bartkova et al., 2001) and lymphomas (Tort et al., 2002). First evidence of post-translational modification of CHK2 other than phosphorylation came from the study by Lovly *et. al.* (2008). This study identified Ser379 phosphorylation as a positive regulator of CHK2 ubiquitination (Lovly et al., 2008). Ectopically expressed wild-type CHK2, but not the S37A mutant of CHK2, underwent ubiquitination in HEK293

cells. Interestingly, this ubiquitination did not alter CHK2 turnover in the cells but rather contributed to the apoptotic pathway downstream of CHK2 (Lovly et al., 2008). Ubiquitination of CHK2 is balanced by deubiquitinating enzyme USP28. USP28 promotes CHK2 stabilization (Zhang et al., 2006) and is also found in the same complex as CHK2 and Pirh2. Overall, several proteins have been shown to regulate CHK2 levels in response to DNA damage; however, further studies would be needed to understand the physiological role of such regulation and if these pathways are dysregulated in certain cancers.

1.5.4 CHK2: Downstream Effectors

CHK2 plays a central role in cellular response to DNA DSBs. Once activated, CHK2 phosphorylates a wide-variety of substrates, which in turn regulate cell cycle arrest, DNA repair and apoptosis (Figure 2). In this section, I will introduce various downstream effectors of CHK2 and their effects on cells with DNA damage.

BRCA1

BRCA1 has been shown to be a CHK2 substrate. It plays multiple roles in the DDR pathway, including activation of the intra-S and G2/M cell cycle checkpoints, DNA repair through homologous recombination (HR) NHEJ and modulating transcription of DNA damage responsive genes, 14-3-3σ and GADD45 (Ting and Lee, 2004). BRCA1 mutations pre-dispose patients to breast and ovarian cancers. In response to DNA damage, BRCA1 is hyper-phosphorylated by various DDR kinases including ATM (Cortez et al., 1999) and CHK2 (Lee et al., 2000). BRCA1 contains a CHK2 consensus

phosphorylation motif (L X R X X S/T) (O'Neill et al., 2002) and is phosphorylated at Ser988. Studies have reported that unlike the wild type protein, expression of the S988A mutant in BRCA1 deficient cells fails to increase survival and restore HR (Zhang et al., 2004a). Similarly overexpression of kinase-dead CHK2 (acts as a dominant negative mutant), blocks Ser988 phosphorylation of BRCA1 and abolishes DSB repair in response to IR. In addition to HR, Ser988 phosphorylation of BRCA1 is critical for error-free NHEJ in response to DNA damage (Zhuang et al., 2006).

E2F-1

In response to VP16, CHK2 has been reported to phosphorylate E2F-1 at Ser364, a CHK2 consensus motif (L X R X X S). E2F-1 is a member of E2F family of transcription factors that play a key role in G1- to S- cell cycle transition. E2F-1 activity is regulated during cell cycle through association with the tumor suppressor protein, retinoblastoma (Rb) (Flemington et al., 1993; Helin et al., 1993). Under normal conditions, cyclin D/cdk4, 6 (G1 phase) and cyclin E/cdk2 (S phase) hyperphosphorylate Rb, which results in the dissociation of E2F-1/Rb complex (Knudsen and Wang, 1997). In turn, E2F-1 promotes the transcription of various genes critical for cell cycle progression. The ability of E2F-1 to induce apoptosis in response to DNA damage differentiates it from other members of the E2F family. E2F-1 is best known for targeting the promoters of the pro-apoptotic genes, p73 and Arf (Bates et al., 1998; Irwin et al., 2000). CHK2 has been reported to phosphorylate E2F-1 *in vitro* at Ser364. Phosphorylation of E2F-1 at Ser264 stabilizes the E2F-1 protein by increasing its half-life (Stevens et al., 2003) and localizes the phosphorylated protein to DNA damage sites. Overexpression of dominant negative

CHK2 can prevent E2F-1 induced apoptosis in U2OS cells. Furthermore, CHK2, as well as CHK1, stabilize E2F-1 to drive p73 expression and induce p53-independent apoptosis in cells with DNA damage (Urist et al., 2004). Interestingly, E2F-1 has been shown to induce ATM-mediated phosphorylation of CHK2 at Thr68, suggesting a potential positive feedback loop (Powers et al., 2004).

PML

Another pro-apoptotic substrate of CHK2 is PML. PML is a tumor suppressor protein that is translocated in most promyelocytic leukemias. PML nuclear bodies are dynamic sensors of DNA damage and their numbers rapidly increase in response to DNA DSBs. PML has been shown to play a role in multiple apoptotic pathways, including in response to DNA damage (Quignon et al., 1998; Wang et al., 1998). Yang *et. al. (2002)* reported that CHK2 co-localizes at nuclear foci and co-immunoprecipitates with PML in non-irradiated cells. After IR, CHK2 phosphorylates PML at Ser117, which in turn, promotes the dissociation of CHK2 and PML (Yang et al., 2002). Mutation of Ser117 to Alanine impairs IR-induced apoptosis indicating a critical role of Ser117 phosphorylation in PML-induced apoptosis. Furthermore, it has been suggested that PML forms the scaffold protein for CHK2 and p53 and all the three proteins co-localize to nuclear bodies. Moreover, like CHK2 null MEFs (Hirao et al., 2002; Hirao et al., 2000; Takai et al., 2002), PML null MEFs fail to stabilize p53, phosphorylate p53 at Ser20 and induce apoptosis in response to IR (Louria-Hayon et al., 2003).

Che-1

CHK2 and ATM have both been shown to interact with, phosphorylate and promote the accumulation of Che-1 in response to DNA damage (Bruno et al., 2006). Che-1 is a highly conserved RNA polymerase II (pol II) binding protein that is important for normal gene transcription and cell proliferation (Bruno et al., 2002; Fanciulli et al., 2000; Lindfors et al., 2000). In response to DNA damage, Che-1 is phosphorylated on multiple sites resulting in its accumulation. Che-1 stabilization is reduced in cells lacking functional CHK2 and can be restored with reconstituted wild-type but not kinase-dead CHK2. Furthermore, accumulated Che-1 is recruited to the *TP53* promoter resulting in upregulation of p53 expression, suggesting another mechanism by which ATM and CHK2 kinases regulate p53-dependent apoptosis in response to DNA damage (Bruno et al., 2006).

FoxM1

FoxM1 is a member of Forkhead family of transcription factors that share a common winged helix DNA binding domain (Clark et al., 1993). FoxM1 is involved in regulating the expression of cell cycle genes that are crucial for progression through DNA replication and mitosis (Wang et al., 2005; Wang et al., 2002). CHK2 has been reported to phosphorylate FoxM1 on Ser361, which results in stabilization of the FoxM1 protein. In turn, FoxM1 transcribes XRCC and BRCA2 genes that are involved in DNA repair. These finidings provides additional insight into how CHK2 might regulate DNA repair in cells with DNA damage.

Survivin

Survivin is the smallest member of Inhibitor of Apoptosis protein (IAP) family, which have the characteristic N-terminal Zn^{2+}-binding baculoviral IAP repeat (BIR) domain. Survivin is primarily known for its role in mitosis and apoptosis. Survivin is a part of the chromosomal passenger complex and is required for activation of Aurora Kinase B in mitosis (Bolton et al., 2002; Kelly et al.). Survivin also regulates apoptosis by cooperating with XIAP and interfering with caspase-3 and caspase-9 (Shin et al., 2001; Tamm et al., 1998). Unlike other members of the IAP family, survivin is expressed in a variety of neoplasms but not in differentiated tissues. Moreover, its expression is associated with poor prognosis in oral, breast and colorectal cancer patients (Altieri, 2008a, b; Kanwar et al.). Ghosh *et. al. (*2006) showed that following DNA damage, survivin is released from the mitochondria in a CHK2-dependent manner to exert its anti-apoptotic effect. This is in contrast to the general belief that CHK2 primarily plays a pro-apoptotic role. Overexpression of kinase-dead CHK2, or knockdown of CHK2 using siRNA, prevented release of Survivin from mitochondria and increased apoptosis in response to DNA damage (Ghosh et al., 2006). The same group went further to show that CHK2 phosphorylates multiple residues of a splice variant form of Survivin, Survivin ΔEx3 (Lopergolo et al.). Through bioinformatic analysis, Lopergolo *et. al.* 2012 showed that Survivin ΔEx3 is associated with aggressive cancer and is primarily localized to the nucleus where it becomes a CHK2 substrate. Mutation of CHK2 target sites on survivin (Thr79, Ser98, and Thr127) to alanine enhanced Survivin ΔEx3 ability, abrogated DNA sensing, reducing the appearance of γH2AX (marker for DNA DSBs) and impaired long-term clonogenic tumor cell survival. In summary, these studies show that CHK2 positively regulates Survivin to promote cell survival to drive tumorogenesis.

p53, MDM2 and MDMX

One of the most important and widely studied substrate of CHK2 is p53. p53 is a

transcription factor that has been coined the "guardian of the genome" as it is

responsible for maintaining genomic stability (Lane, 1992a, b). It is mutated in more

than half-of all human cancers with more than 18,000 characterized mutations (Olivier

et al., 2004). Under unstressed conditions, p53 is expressed at low levels and has a

very short half-life. It is rapidly targeted for ubiquitin-mediated proteosomal degradation

by its E3 ligase Mdm2 (Honda et al., 1997; Kastan et al., 1991; Momand et al., 1992). In

response to DNA damage, p53 dissociates from MDM2 and accumulates within the cell.

In turn, p53 tertramerizes and induces transcription of various genes involved in cell

cycle arrest (*p21 (el-Deiry et al., 1993; Harper et al., 1993), 14-3-3σ (Hermeking et al.,*

1997)), and apoptosis (*BAX (Miyashita and Reed, 1995; Reed et al., 1998), PUMA (Yu*

et al., 2001a), NOXA (Oda et al., 2000)). Additionally, p53 also transcribes its own

negative-regulator, *MDM2* (Barak et al., 1994; Barak et al., 1993).

The relation between p53 and CHK2 is complex. Traditionally it was believed that

CHK2 is the sole kinase responsible for phosphorylating p53 on Ser20. Phosphorylation

of p53 on Ser20 disrupts its interaction with MDM2, which in turn, results in p53

accumulation and cell cycle arrest and apoptosis (Chehab et al., 2000; Chehab et al.,

1999; Hirao et al., 2000). CHK2 was shown to phosphorylate p53 *in vitro* Ser15, Thr18,

Ser20 and Ser37 (Shieh et al., 2000). Analysis of various deletion mutants of p53

revealed that CHK2 was only able to phosphorylate the terameric form of p53, and not

the monomeric form, at Ser15 and Ser20. Ectopic expression of dominant negative

CHK2 abrogated p53 stabilization and Ser20 phosphorylation in IR-treated U2OS cells. Conversely, overexpression of CHK2 induced p53 stabilization and G1 cell cycle arrest (Chehab et al., 2000).

Analysis of two different CHK2 null mouse lines further strengthened the role of CHK2 in p53 phosphorylation. First, Hirao *et. al.* (2000) showed the CD4+ and CD8+ thymocytes, developing neurons and hair follicular cells isolated from CHK2 null mice were unable to undergo apoptosis in response to whole body IR and these mice developed tumors more rapidly when compared to CHK2 wild-type mice (Hirao et al., 2002). More importantly, IR-induced p53 stabilization and activation of its target genes, p21 and BAX, were impaired in thymocytes isolated from CHK2 null mice. Similar observations were made in a CHK2 null MEFs (Hirao et al., 2000; Jack et al., 2002). p53 stabilization and Ser20 phosphorylation were rescued by reintroduction of wild-type CHK2 and completely abrogated by introduction of dominant negative p53. This provided further support that CHK2 acts upstream of p53 to phosphoryate it at Ser20 and regulate its stabilization in response to DNA damage.

Takai *et. al.* (2002) generated another line of CHK2 null mice and showed that multiple tissues of these mice were more resistant to the IR-induced apoptosis and failed to induce G1 cell cycle arrest. This is consistent with the role of CHK2 in p53-mediated apoptosis and cell cycle arrest (Takai et al., 2002). However, Takai and colleagues observed a 30 – 50% reduction in p53 levels in CHK2 null MEFs compared to wild-type control MEFs. Interestingly, despite marginal reduction in p53 accumulation, absence of CHK2 completely abolished p53-mediated transcription of *p21, BAX, NOXA,*

Cyclin G1 and MDM2. Absence of transcriptional activity of p53 would explain the defects in apoptosis and cell cycle arrest observed in CHK2 null mouse.

In addition to regulating p53 directly, CHK2 has also been shown to regulate p53 through the regulation of MDMX. MDMX is a MDM2 homolog that is capable of binding p53 and inhibiting its transcriptional activation (Shvarts et al., 1996). Unlike MDM2, MDMX cannot directly ubiquitinate and degrade p53 (Stad et al., 2001), but rather coordinates with MDM2 to promote p53 destruction (Gu et al., 2002). The role of MDMX in p53 regulation is further supported by the fact that the embryonic lethality of MDMX null mice is rescued by p53 loss (Migliorini et al., 2002; Parant et al., 2001). CHK2 has been shown to phosphorylate MDMX at Ser342 and Ser367 *in vitro* and *in vivo (Chen et al., 2005; Pereg et al., 2006)*. Mutation of Ser367 to alanine was sufficient in stabilizing MDMX and reducing its ubiquitination. Numerous studies have shown that MDM2 can induce MDMX degradation in response to DNA damage (de Graaf et al., 2003; Kawai et al., 2003; Pan and Chen, 2003). MDMX degradation was significantly reduced in CHK2 null HCT116 cells further supporting a role for CHK2 in MDMX degradation. However, in both parental and CHK2 null HCT116 cells, MDM2 was successful in degrading ectopically expressed MDMX. Taken together this suggests that CHK2 can facilitate MDMX degradation, and thereby, promote p53 accumulation and its downstream function (Okamoto et al., 2005). However, it still remains unknown how CHK2 phosphorylation increases association between MDM2 and MDMX. A couple of recent studies have indicated that Ser367 phosphorylation of MDM2 creates a 14-3-3 binding site and can result in nuclear translocation of MDMX where it associates with MDM2 (LeBron et al., 2006). On the other hand, 14-3-3 binding might displace the

deubiquitinating enzyme, HAUSP, and promote degradation of MDMX (Meulmeester et al., 2005).

It has been recently challenged that CHK2 is the sole kinase responsible for p53 accumulation and Ser20 phosphorylation. MEFs made from the CHK2 null mice by Hirao and colleagues (Hirao et al., 2002) have an intact IR-induced G1 checkpoint and p21 accumulates normally (Jack et al., 2002). Studies in mice have also shown that mutation of the murine equivalent of human Ser20, (Ser23), did not alter p53 stabilization in response to DNA damage (Wu et al., 2002). Second, siRNA-mediated knockdown of CHK2 in various p53-proficient cancer cell lines failed to alter IR-induced p53 stabilization or activation of p53 transcriptional targets (Ahn et al., 2003). Ahn *et. al. (2003)* went on to show that there was no difference in CHK2-mediated p53 phosphorylation when CHK2 when isolated from cells with or without damage. Moreover, Similarly, CHK2 null HCT116 did not alter p53 phosphorylation at Ser-20 when compared to the isogenic parental HCT116 cells (Jallepalli et al., 2003). To summarize, CHK2 is able to phosphorylate p53 and induce its stabilization. However, cells have evolved alternate ways to regulate p53, rendering CHK2 dispensable under certain conditions.

1.6 *CHK2* Mutations in Cancers

CHK2 inactivating mutations have been found in various human cancers. Figure 3 outlines a few mutations and their locations have been *CHK2* germline mutations have been linked to increased risk towards familial breast, colon and prostate cancer. Somatic mutations in *CHK2* have also been identified in breast, colon, lung, prostate

and lung cancers. Although in mice, loss of CHK2 is not sufficient to induce transformation, its loss can predispose cells to malignant transformation.

One of the first studies linking *CHK2* mutations to cancer was made in a subset of patients with Li-Fraumeni syndrome, a familial cancer predisposition syndrome, which is usually characterized by germline mutation of *TP53* gene (Bell et al., 1999). LFS is characterized by early onset of tumor development in multiple organs with a predominance of breast cancer, brain tumors, adrenal cortical tumors, as well as leukemias and sarcomas (Li and Fraumeni, 1969; Malkin et al., 1990; Srivastava et al., 1990; Varley et al., 1997). Patients with *CHK2* mutations had intact *TP53* gene indicating that mutations in *CHK2* and *TP53* were mutually exclusive. This suggests that CHK2 and p53 might act in the same signaling pathway. Two distinct *CHK2 mutations* were identified in LFS patients. First mutation, CHK2 110delC, encodes a C-terminal truncated protein without any kinase activity (Bartkova et al., 2004). The second mutation, I157T, is a point mutation in the FHA domain, which can potentially disrupt CHK2 dimerization, and thereby, prevent DNA damage induced CHK2 activation (Cai et al., 2009; Schwarz et al., 2003b). Additionally, the *CHK2* I157T mutation also impairs the ability to CHK2 to recognize its substrate, p53 (Falck et al., 2001a; Falck et al., 2001b)

In addition to LFS, these mutations have also been linked to several other familial cancers. Analysis of *CHK2* 110delC mutation in cancer families indicated that this mutation increases the risk of breast and prostate cancer (Meijers-Heijboer et al., 2002; Meijers-Heijboer et al., 2003; Seppala et al., 2003). The I157T mutation increases the risk of developing ovarian, thyroid, kidney, colorectal, bladder carcinomas and leukemia

(Cybulski et al., 2004; Kilpivaara et al., 2006; Matyjasik et al., 2007; Rudd et al., 2006; Szymanska-Pasternak et al., 2006). Subsequent studies have identified three additional *CHK2* mutations. S428F, present in the Ashkenazi-Jewish population increases the risk of breast cancer (Shaag et al., 2005). IvS2 + 1G>A and 5395del has been reported to increase the risk of breast and prostate cancer (Cybulski et al., 2007; Dong et al., 2003; Gorski et al., 2005). Sporadic mutations of *CHK2* have been found in a broad spectrum of tumors, including tumors of the breast, lung, vulva, urinary bladder, colon, and ovary, as well as lymphomas and osteosarcomas (Bartkova et al., 2004; Hangaishi et al., 2002; Haruki et al., 2000; Miller et al., 2002; Reddy et al., 2002; Sullivan et al., 2002; Tavor et al., 2001; Tort et al., 2002).

The *CHK2* gene is also regulated in cancers through epigenetic modification and splice variants. Zhang *et. al. (2004)* reported hypermethylation of *CHK2* locus in non-small cell lung cancer cell lines and primary lung cancer specimen (Zhang et al., 2004b). More than ninety different splice variants of CHK2 were detected in stage III breast cancer. Most of the splice variants disrupt the kinase domain, and therefore, may serve as dominant negative mutants (Craig and Hupp, 2004).

1.7 Targeting CHK2 in cancer

Given the role of CHK2 in the DDR pathway, CHK2 can potentially be a lucrative therapeutic target in various cancers. However, given the role of CHK2 in promoting p53-mediated apoptosis (Hirao et al., 2002; Hirao et al., 2000; Jack et al., 2002; Jallepalli et al., 2003; Lovly et al., 2008; Takai et al., 2002), it would be critical to understand the genetic background of cancers, especially their p53 status, before

considering CHK2 inhibition. Relevance of p53 is further stressed by a study performed

by the laboratory of Michael Yaffe (Jiang et al., 2009). They reported that the stable

knockdown of CHK2 in transformed MEFs with wild-type p53 decreased DNA damage-

induced apoptosis. However, loss of CHK2 in p53 null MEFs resulted in an increase in

apoptosis. In support of this observation, numerous other studies have shown that

inhibition of CHK2 in combination with DNA damaging agents provide therapeutic

advantage by disrupting the DNA repair and cell cycle arrest, which results in prolonged

DNA damage and eventually apoptosis. For example, antisense knockdown of CHK2 in

HEK293 cells increased apoptosis by disrupting S- and G2- checkpoints in response to

IR or VP16 treatment (Yu et al., 2001b). Similarly, inhibition of CHK2 using antisense

oligonucleotides (AsODN) increased IR-induced apoptosis in HeLa cells (Gao et al.,

2009). Moreover, CHK2 has been shown to promote release of survivin, an anti-

apoptotic factor, and promote survival in xenograft models (Lopergolo et al.). CHK2

inhibition also enhanced the level of mitotic catastrophe when used in combination with

either doxorubicin (Castedo et al., 2004) or cisplatin (Vakifahmetoglu et al., 2008).

Various CHK2 specific inhibitors have been developed and tested preclinically

One of theses inhibitors, PV1019, is able to prevent IR-induced apoptosis in mouse

thymocytes (Jobson et al., 2009). This supports the resistance of thymocytes to IR-

induced apoptosis in CHK2 null mice (Hirao et al., 2002; Takai et al., 2002).

Interestingly, PV1019 was able to increase apoptosis in ovarian cells lines that had also

been treated with chemotherapeutic agents. Similarly, another potent CHK2 inhibitor,

NSC109555, synergistically increased gemcitabine-induced apoptosis in various

pancreatic cell lines (Duong et al.). In contrast, the CHK2 inhibitor, CCT241533, failed to

potentiate apoptosis in response to chemotherapy, but increased cell death when cells were treated with PARP-inhibitors (Anderson et al.).

Since most of the tumors have inactivated p53 either through inactivating mutations within p53 or through over-expression of MDM2 or MDMX, inhibition of CHK2 could potentially provide a therapeutic advantage if used in combination with chemotherapeutic agents. Furthermore, CHK2 inhibitors could increase the therapeutic index of chemotherapy by preventing the detrimental side effect in on other organs. However, further studies are needed to identify subsets of cancer patients who might benefit from CHK2 inhibitors and to understand the underlying mechanism by which CHK2 inhibitors sensitize tumor cells to cell death.
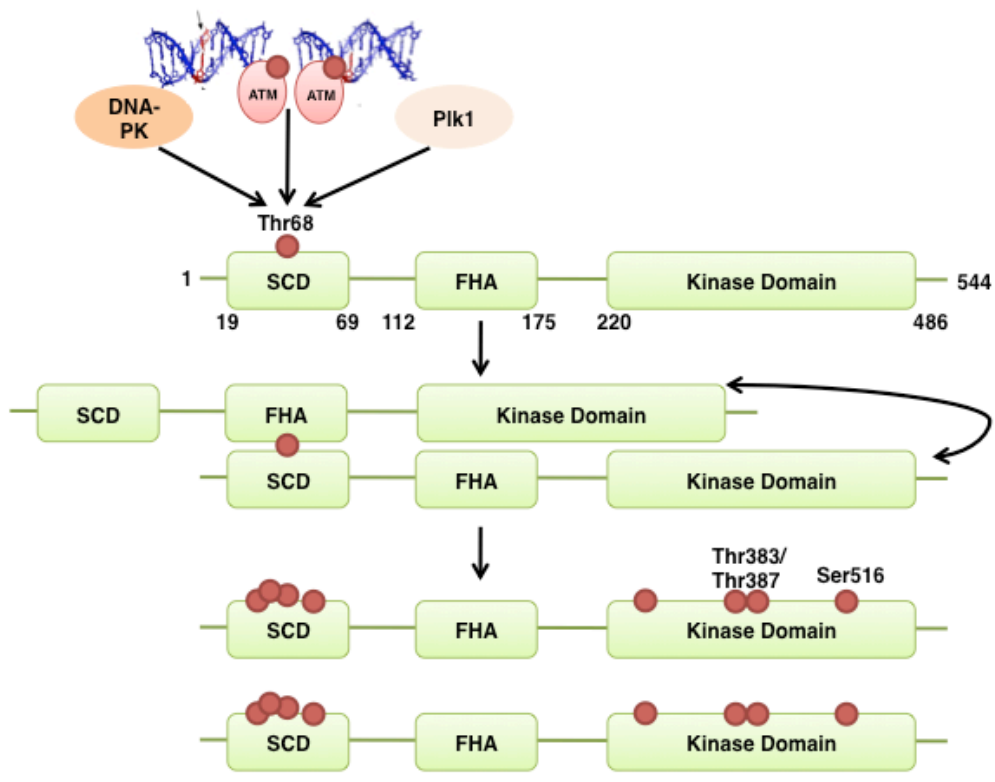
Fig 1.1 Oligomerization-induced CHK2 activation in response to DNA DSBs

In response to DNA DSBs, ATM phosphorylates CHK2 on Thr68. Thr68

phosphorylation induces CHK2 dimerization through its interaction with the FHA domain

of another CHK2 molecule. Dimerization induces a cascade of cis- and trans-

autophosphorylation event that completely activates the CHK2 kinase.
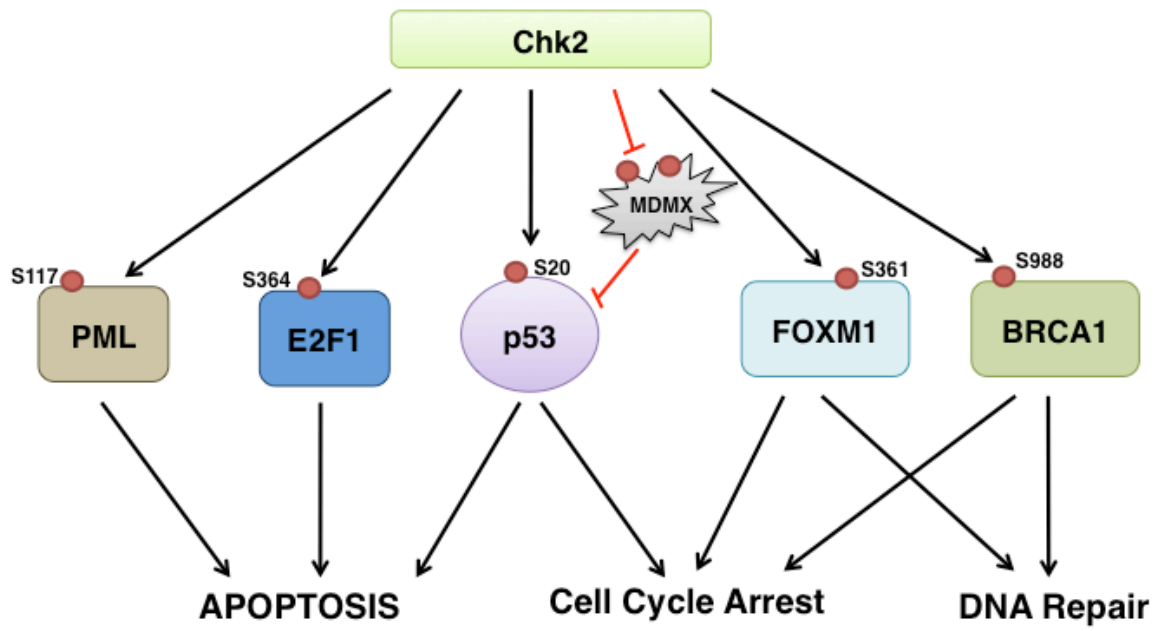
Fig. 1.2 Downstream effectors of CHK2

Upon activation, CHK2 phosphorylates a wide-variety of downstream effectors that mediate cell cycle arrest, DNA repair and/or apoptosis
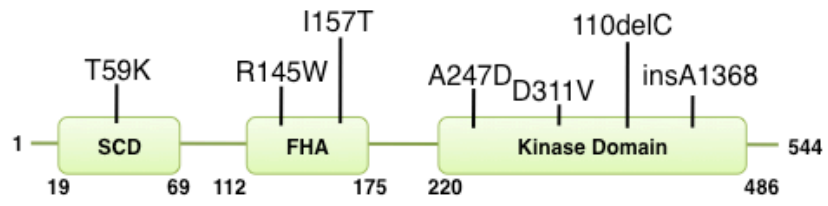
Fig. 1.3 Schematic representing location of *CHK2* mutations characterized in a variety of cancers.

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37

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Chapter 2:

**Inhibitor of apoptosis proteins (IAPs) identified as CHK2 effectors
in the DNA damage response pathway**

(Unpublished Data)

Inhibitor of apoptosis proteins (IAPs) identified as CHK2 effectors in the DNA damage response pathway

Anurag Agarwal[1] and Helen Piwnica-Worms[2]

[1]Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110, [2]Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77230

Running Title: CHK2 regulates apoptosis by inhibiting cIAPs

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*Corresponding author. Mailing address: Helen Piwnica-Worms, Department of Cancer Biology, Unit 1906, PO Box 301429, The University of Texas MD Anderson Cancer Center, Houston, TX 77230-1429 phone: 713-745-1221; fax: 713-745-1812; email: hpiwnica-worms@mdanderson.org

2.1 Abstract

Checkpoints function to maintain genome stability and are essential for normal development and organismal homeostasis. Perturbations in checkpoint control contribute to pathological conditions such as cancer, developmental disorders and neurodegenerative diseases. The checkpoint protein CHK2 is a serine/threonine protein kinase that becomes activated in response to DNA double strand breaks (DSBs) and its activity is critical for inducing cell cycle arrest or apoptosis in cells with DNA damage. CHK2 is an important regulator of p53 as CHK2-deficient mice have defects in p53 stabilization, p53-dependent transcriptional activation, and p53-dependent apoptosis. Here we show that CHK2 deficient HCT116 and MCF7 cells are fully capable of activating the p53-signaling pathway in response to VP16 treatment yet fail to efficiently undergo apoptosis. Inhibition of cIAP1 and cIAP2, two members of the Inhibitor of Apoptosis (IAP) family, restored apoptosis in CHK2 deficient cells. Interestingly, CHK2 interacted with cIAP1 and cIAP2 but not with the third family member, XIAP. In addition, CHK2 phosphorylated cIAP2, inhibited its E3 ubiquitin ligase activity in vitro and extended its half-life in cells. Thus, CHK2 functions as a tumor suppressor protein not only by activating pro-apoptotic regulators (p53) but also by inhibiting anti-apoptotic regulators (cIAPs).

2.2 Introduction

The tumor suppressor protein, Checkpoint Kinase 2 (CHK2), is a serine/threonine protein kinase that functions in the cellular response to DNA double strand breaks (DNA DSBs). Heterozygous germline mutations in *CHK2* are associated with a p53-independent variant form of the Li-Fraumeni syndrome (Bell et al., 1999). Additionally, *CHK2* mutations are also found in sporadic cancers (Dong et al., 2003; Schutte et al., 2003; Sodha et al., 2002) and down regulation of CHK2 protein has been reported in bladder carcinomas, breast carcinomas, and lymphomas (Bartkova et al., 2004; Sullivan et al., 2002; Tort et al., 2002). In response to DNA DSBs, CHK2 promotes cell cycle arrest and/or apoptosis. It has been proposed that CHK2 mediates these effects through the regulation of p53. CHK2 functions immediately upstream of p53 by phosphorylating p53 at serine 20 and promoting its stabilization in response to DNA DSBs (Chehab et al., 2000; Shieh et al., 2000). In turn, p53 transcribes members of the Bcl-2 family of pro-apoptotic genes like *BAX* (Miyashita and Reed, 1995; Reed et al., 1998), *PUMA* (Yu et al., 2001), and *NOXA* (Oda et al., 2000) that drive mitochondrial membrane permeabilization, release of cytochrome c, and subsequent activation of the caspase cascade to induce apoptosis. BAX consists of a homo-dimerization/hetero-dimerization (BH3) domain (Kelekar and Thompson, 1998), a characteristic of all Bcl-2 pro-apoptotic family members and a transmembrane domain. When induced by p53 in response to DNA damage, BAX translocates to the outer-membrane of mitochondria and forms a pore (Eskes et al., 1998; Jurgensmeier et al., 1998; Schendel et al., 1997). This results in the release of cytochrome-c (Bossy-Wetzel et al., 1998; Kroemer et al., 1997) and apoptosis inducing factor (AIF) (Zamzami et al., 1996) from mitochondria. In

55

the cytosol, cytochrome-c forms a complex with Apaf1, a homologue of the

Caenorhabditis elegans protein ced-4, and caspase 9, which triggers caspase activation

and cell death (Adams and Cory, 2002; Aligue et al., 1997)

In response to whole body radiation, CHK2 null mice show defects in several features

of p53 regulation and function including p53 phosphorylation at serine 20, p53

stabilization, activation of p53-target genes and p53-dependent apoptosis (Hirao et al.,

2002; Takai et al., 2002). However, several studies have demonstrated that

phosphorylation of p53 on serine 20 is regulated by kinases in addition to CHK2 and

that CHK2 has substrates in addition to p53. For example, siRNA depletion of CHK2 in

three different cancer cell lines failed to alter p53 stabilization or p53 phosphorylation on

serine 20 (Ahn et al., 2003). In addition, homozygous deletion of *CHK2* in HCT116 cells

had no effect on the ability of ionizing radiation (IR) to induce p53 stabilization or serine

20-phosphorylation, but did impair the ability of IR to induce apoptosis (Jallepalli et al.,

2003). Lovly et. al also identified a novel auto-phosphorylation site (S379) that is

required for CHK2 signaling to the apoptotic machinery. A mutant of CHK2 encoding

alanine in place of serine at position 379 (S379A) retains kinase activity but U2OS cells

expressing this mutant are impaired in their ability to undergo apoptosis following

exposure to VP16. This was also true for U2OS cells stably expressing a kinase-dead

mutant of CHK2 (Lovly et al., 2008b). Thus, both kinase activity and S379

phosphorylation are required for the pro-apoptotic effector function of CHK2.

Paradoxically, p53 accumulation, p53 modification (phosphorylation of S20 and

acetylation of K382) (Fig. 2.1) and p53 transcriptional activity (p21, PUMA) (Fig. 2.2)

were similar between VP16 treated cells expressing WT CHK2 and those expressing

the S379A mutant. These results suggest that cancer cells have evolved additional ways to regulate p53 in the absence of CHK2 and that CHK2 has additional effectors in the DNA damage-induced apoptotic pathway. CHK2 has been shown to regulate BRCA1, E2F-1, MDMX, PML and p73 although the role of these substrates in CHK2-mediated apoptosis has not been well established (Chen et al., 2005; Lee et al., 2000; Pereg et al., 2006; Stevens et al., 2003; Urist et al., 2004; Yang et al., 2002).

This study identifies members of the inhibitor of apoptosis (IAP) family as downstream effectors of CHK2 in the DNA damage apoptotic response pathway. The IAP family is defined by the presence of an N-terminal conserved 70 amino acid motif called Baculovirus IAP Repeat (BIR) (Birnbaum et al., 1994). In this section, I will introduce three IAP family members, cellular IAP1 (cIAP1), cIAP2 and X-linked IAP (XIAP) with a focus on cIAP1 and cIAP2. cIAPs and XIAP have three functional domains: three tandem BIR domains that primarily mediate protein-protein interactions, a ubiquitin binding domain that mediates interactions with other polyubiquitinated proteins (Blankenship et al., 2009; Gyrd-Hansen et al., 2008), and a C-terminal RING domain that confers the E3 ligase activity through which IAPs ubiquitinate their substrates (Mace et al., 2008). Unlike XIAP, cIAPs have an additional domain, Caspase Recruitment Domain (CARD), following the UBA domain (Lopez et al., 2011). The CARD domain regulates the RING activity of the cIAPs through autoinhibition (Fig. 2.3). Deletion of the CARD domain increases the half-life of cIAP1 from greater than 6 h to about 2 h.

IAPs, especially, cIAP1, cIAP2 and XIAP, function as endogenous negative regulators of apoptosis. They inhibit apoptosis through direct regulation of caspases,

and through caspase-independent mechanisms. In this section, I will focus on the regulation of caspases by cIAPs and XIAP and introduce the caspase-independent mechanism in Chapter 3. Among all of the IAP family members, XIAP is the most potent inhibitor of caspases. Caspases are a family of cysteine proteases that execute apoptosis in response to various forms of stress (Abe et al., 2002; Barnett et al., 2009). Normally, caspases exist in an inactive or pro-caspase form. Upon specific stimuli, caspases undergo auto- and trans-activation through proteolytic cleavage and are converted to their active forms denoted cleaved caspase (CC). Caspases can be divided into two categories: a) initiator caspases, like caspase-9 and caspase-8, that sense the apoptotic stimuli, get activated through auto-proteolytic cleavage, and signal downstream; b) executioner caspases, like caspase-3 and caspase-7, that get activated upon proteolytic cleavage by the initiator caspases, and in turn, cleave a variety of proteins, including PARP (PARP cleavage is a classical marker of apoptosis), to initiate apoptosis. XIAP can directly bind and inhibit the initiator caspase, caspase-9, as well as the effector caspases, caspase -3 and caspase -7 (Chai et al., 2001; Deveraux et al., 1998; Deveraux et al., 1997; Huang et al., 2001; Riedl et al., 2001; Silke et al., 2001; Takahashi et al., 1998). XIAP blocks the homo-dimerization of caspase-9 and prevents its activation (Shiozaki et al., 2003). XIAP inhibits the effector caspases through a different mechanism. XIAP binds to the cleaved forms of caspase-3 and -7and inhibits their activity by excluding substrate binding (Chai et al., 2001; Riedl et al., 2001; Scott et al., 2005). Although cIAPs were initially shown to bind and inhibit caspase-3, -7 and -9 *in vitro* (Deveraux et al., 1998; Labbe et al., 2011), it was later shown that cIAPs can bind but are not able to inhibit the protease activity of caspases in cells (Eckelman and

Salvesen, 2006). However, cIAP1 has been shown to ubiquitinate effector caspases and target active caspases for proteosomal degradation (Choi et al., 2009; Damgaard and Gyrd-Hansen, 2011; Mica et al., 2004). On the other hand, mono-ubiqutination of caspase-3 and -7 by cIAP2 might provide novel ways of regulating caspase activity by cIAPs (Huang et al., 2000). In addition to caspases, cIAPs regulate a multitude of cellular processes like cell survival (Biton and Ashkenazi; Gill et al., 2009), cell migration (Oberoi et al., 2012), inflammation (Gyrd-Hansen and Meier, 2010b; Labbe et al., 2011), and immunity (Bertrand et al., 2009; Damgaard and Gyrd-Hansen, 2011; Wong et al., 2010) through the NFκB family of transcription factors.

cIAPs have been shown to play a crucial role in several human malignancies and tumor development. XIAP is upregulated in various cancers (Li et al., 2007; Mizutani et al., 2007; Tamm et al., 2000). The genetic locus of cIAPs, 11q22, is also amplified in numerous cancers like multiple myeloma, lymphomas, non-small cell lung carcinoma, renal carcinoma, and hepatocellular carcinomas (Bashyam et al., 2005; Dai et al., 2003; Gyrd-Hansen and Meier, 2010b; Imoto et al., 2001). Additionally, more than 50% of MALT lymphomas harbor the t(11,18)(q21;q21) translocation that results in fusion of the BIR domains of cIAP2 with the carboxy domain of MALT1 which drives tumor progression and leads to increased chemo-resistance (Akagi et al., 1999; Dierlamm et al., 1999). Knockdown of XIAP increases the sensitivity of chemotherapy in colorectal (Connolly et al., 2009), breast (Lima et al., 2004), lung (Cheng et al.; Hu et al., 2003), and pancreatic (Lopes et al., 2007) cell lines. Similarly, cIAP2 knockdown also increases the efficacy of chemotherapy in various cancer cell lines (Karasawa et al.,

2009; Lopes et al., 2007; Miura et al., 2009; Nagata et al.). Thus IAP inhibition may provide a promising cancer treatment.

IAPs are able to regulate themselves through their RING domain. Both, cIAPs and XIAP, are able to auto-ubiquitinate and target themselves for degradation by the proteosome (Mace et al., 2008). Homo-dimerization of the RING domain is essential for auto-ubiquitination and proteosomal degradation of IAPs (Mace et al., 2008; Nakatani et al., 2013). Moreover, cIAP1 can ubiquitinate other members of the IAP family through hetero-dimerization and promote their destruction (Cheung et al., 2008; Conze et al., 2005). Numerous endogenous regulators of cIAPs have also been identified to date. For example, HtrA2/Omi is a serine protease that inactivates cIAPs and XIAP by an irreversible proteolytic cleavage (Jin et al., 2003; Verhagen et al., 2002; Yang et al., 2003). Mitochondrial protein, SMAC/DIABLO, binds to the BIR3 domain of cIAPs to promote their dimerization-induced auto-ubiquitination and proteosomal degradation (Feltham et al., 2011; Shiozaki and Shi, 2004). On the other hand, SMAC/DIABLO is thought to abrogate XIAP-mediated caspase inhibition (Huang et al., 2003). In turn, IAPs can also ubiquitinated and degrade SMAC to form a negative feedback loop (Hu and Yang, 2003; Morizane et al., 2005). Therefore, small molecules mimicking SMAC binding to IAPs, also known as SMAC mimetics, are being actively developed for cancer treatment (Dynek and Vucic, 2013; Fulda, 2012; Mannhold et al., 2010). In numerous cell lines, SMAC mimetics augment chemotherapy-induced apoptosis in both NFkB – dependent and –independent manners (Berger et al.; Dai et al., 2008; Huerta et al.; Li et al.; Stadel et al.; Yang et al., 2011).

Here we demonstrate that CHK2 deficient cancer cells are impaired in their ability to undergo apoptosis in response to DNA DSBs, despite normal activation of p53 signaling. Restoration of apoptosis in CHK2 deficient cells was achieved by inhibiting cIAP1/2 with BV6 or by knocking down cIAP1/2. cIAP1 and cIAP2 co-precipitated with kinase-active but not kinase-dead CHK2 and CHK2 inhibited the E3 ubiquitin ligase activity of cIAP2. Thus, CHK2 regulates apoptosis by not only activating pro-apoptotic regulators (p53) but also by inhibiting anti-apoptotic regulators (cIAP1/2).

2.3 Material and Methods

Cell Culture and Treatments

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)(Life Technologies) supplemented with 10% BGS (HyClone) and 1% L-glutamine (Sigma-Aldrich). HEK293T cells were maintained in DMEM supplemented with 10% FBS (Atlanta Biologics) and 1% L-glutamine. HCT116 parent, HCT116 CHK2$^{-/-}$ and HCT116 p53$^{-/-}$ cells were obtained from The Core Cell Center at Johns Hopkins University. HCT116 cells were maintained in McCoys 5A medium (Life Technologies) supplemented with 10% FBS. MCF7 cells were maintained in DMEM supplemented with 10% FBS and 1% L-Glutamine. In some cases cells with treated with complete media containing the indicated concentration of VP16 (Sigma-Aldrich). For treatment of cells with BV6 (Genentech), cells were incubated with complete media containing 5 mM BV6 for 4 h. Following that cells were supplemented with complete media containing DMSO or VP16 to a final concentration of 25 μM. For inhibition of the proteosomal degradation of proteins, HEK293T cells were treated with complete media containing 50 μM MG132 (Sigma-Aldrich). For measuring the half-life of proteins, cells were grown in complete media containing 10 μg/mL of cycloheximide (CHX; Sigma-Aldrich).

Constructs, Cloning and Transfections

FLAG-CHK2 WT, FLAG-CHK2 D368N, Myc-CHK2 WT, Myc-CHK2 D368N, His-CHK2 WT, and His-CHK2 D368N have been described (Lovly et al., 2008a). FLAG-cIAP1 was generated by inserting the Hind III/Eco R1 fragment generated by PCR of cIAP1 cDNA from PGEX4T-cIAP1 (Roy et al., 1997) into the corresponding sites of 3X-FLAG-CMV10 vector (Sigma-Aldrich). FLAG-cIAP2 was generated by inserting the Hind III/Eco R1

fragment generated by PCR of cIAP2 cDNA from PGEX4T-cIAP2 (Roy et al., 1997) into the corresponding sites of 3X FLAG CMV10 vector (Sigma-Aldrich). myc-cIAP2 was generated by inserting the Bam H1/Eco R1 fragment generated by PCR of cIAP2 cDNA from pGEX4T-cIAP2 (Roy et al., 1997) into the corresponding sites of pcDNA3.1-myc vector (Lovly et al., 2008a). FLAG-cIAP2 T35A/C557A mutant were generated using QuickChange Site-Directed Mutagenesis kits (Stratagene). pcDNA3-myc-XIAP was purchased from Addgene (Addgene# 11833). CHK2 shRNA (GAACAGATAAATACCGAACAT and GACTCCAGTGGTAATCTACT) was obtained from Washington University Genome Center and Children's Discovery Institute. HEK293 cells were transfected using Superfect (Qiagen). HEK293 cells were plated at a density of 0.6×10^6 cells/6 cm dish. The next day, 3 μg of total plasmid and 20 μl of Superfect reagent was mixed in a tube containing 200 μl of Opti-MEM media (Life Technologies) and incubated at room temperature for 5 min. The cocktail was added to the plates containing 1 ml of complete media. After 6 h, cells were washed with PBS and grown in complete media. Cells were harvested 24 h post transfection. HEK293T and HCT116 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, HEK293T or HCT116 cells were plated at a density of 0.6×10^6 cells/6 cm and 1.5×10^6 cells/6 cm dish respectively. The next day, 2 - 2.5 μg of total plasmid was mixed with 500 μl of Opti-MEM media in tube 1 and incubated at room temperature for 5 min. In tube 2, 6 - 7.5 μL of Lipofectamine 2000 was mixed with 500 μL of Opti-MEM media and incubated at room temp for 5 min. After 5 min, tube 1 and tube 2 were mixed and incubated at room temperature for 20 – 30 min. After the incubation, the transfection cocktail was added to the plates containing 2 mL of

complete media. 24 h post-transfection, the cells were washed with 1X PBS and media was replaced. For RNAi transfections, Luciferase GL3 duplex and SMARTpool siRNAs specific to NEMO, cIAP1, cIAP2 and XIAP (ThermoScientific) were transfected using DharmaFECT siRNA transfection reagent 2 (ThermoScientific). Briefly, HCT116 cells were plated at a density of 0.3×10^6 cells/60mm dish for the next day transfection. For transfection, 7.5 μl of 20 μM siRNA was mixed with 300 μl of Opti-MEM media in tube 1, and 6 μl of Dharmafect 2 was mixed with 300 μl of Opti-MEM media in tube 2. Both the tubes were incubated at the room temperature for 5 min. Tube 1 was mixed with tube 2 and incubated for an additional 30 min. Meanwhile, the media on the cells was replaced by 2.5 mL of complete media. Post-incubation, the transfection cocktail was added to the cells in a dropwise manner. 18 h post-transfection, the media was replaced by 4 mLs of complete media.

Stable cell lines were generated by using lentivirus infection as described previously (Stewart et al., 2003). Briefly, HEK293T cells were plated at 6×10^5 cells/6 cm dish the day before transfection. The next day, cells were transfected with a mixture of 1 μg viral DNA encoding the gene of interest and 1 μg pHR'8.2deltaR packaging plasmid at a ratio of 8:1 with pCMV-VSV-G envelope plasmid using Mirus LTR1 transfection reagent (Mirus). Target cells were plated at 1×10^6 cells/10 cm dish the day before infection with the lentivirus. After HEK293T cells were transfected with viral DNA for 48 h, supernant from the HEK293T cells containing the viral particles were transferred to the target cells with the addition of 10 μg/ml protamine sulfate for 4 h. Media containing virus was then removed and media was replaced. Infected cells were allowed to recover for 24 h and then were re-infected with the lentivirus. Infected cells were

trypsinized and plated in media containing puromycin 48 h after the first infection with lentivirus.

Western blotting, Antibodies and Immunoprecipitation

Cells were lysed in mammalian cell lysis buffer (MCLB (50 mM TrisHCl pH 8.0, 2 mM DTT, 5 mM EDTA, 0.5% NP-40, 100 mM NaCl,)) supplemented with 1 mM microcystin (Sigma-Aldrich), 1 mM sodium orthovanadate (Sigma-Aldrich), 2 mM PMSF (Sigma-Aldrich), protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Calbiochem). Clarified lysates were resolved directly by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. Membranes were blocked in TBST (20 mM Tris HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk for 1h and incubated overnight in primary antibody diluted in TBST/5% nonfat milk. Membranes were washed in TBST, incubated with HRP-conjugated secondary antibody for 1 h in TBST/5% nonfat milk, and washed again in TBST. Membranes were incubated with ECL (Pierce) and analyzed using a GelDoc Imaging System (BioRad). Cleaved caspase -3, XIAP, and cIAP2 antibodies were from Cell Signaling. p53, p21, pS15 p53, pS20 p53, and ubiquitin antibodies were from Santa Cruz Biotechnology. CHK2, K-48 ubiquitin, and K-63 ubiquitin antibodies were from EMD Milipore. Actin and Tubulin were from Sigma-Aldrich. cIAP1 antibody was from R & D system. Endogeous cIAP2 in HCT116 and MCF7 cells was detected using cIAP2 antibody from BD Biosciences and R&D system respectively. β-catenin antibody was from BD Biosciences, and GAPDH antibody was from Advanced ImmunoChemical. The antibody specific for cIAP2 phosphorylated on threonine 35 was generated by immunizing rabbits

with the phosphopeptide CELYRMS-pT-YSTFP coupled to keyhole limpet hemocyanin (KLH).

For co-immunoprecipitation, cells were lysed in MCLB. Cell lysates were pre-cleared with protein A-agarose (Thermo Scientific) and incubated with anti-Flag M2 (Sigma-Aldrich) or anti-myc 9E-10 (SantaCruz Biotechnology) affinity gel. Immunocomplexes were washed 4 times with MCLB, eluted by boiling in the Laemmli buffer at 95°C for 5 min, resolved by SDS-PAGE and analyzed by Western blotting.

Cell Death and Caspase-3 Activity Assays

Cell death as measured by Sub-G1 DNA content was assessed using propidium iodide staining and fluorescent associated cell sorting (FACS) as described previously (Lovly et al., 2008a). Cell death as measured by annexin and PI double positive cells was measured using Annexin V : FITC Apoptosis Detection Kit I (BD Biosciences) as per manufacturers protocol. Caspase-3 activity was measured by utilizing the caspase-3 fluorogenic substrate Ac-DEVD-AFC (SantaCruz Biotechnology). The cells were lysed in mammalian cell lysis buffer (MCLB: 50 mM Tris HCl pH 8.0, 100 mM NaCl, 2 mM DTT, 5 mM EDTA, 0.5% NP-40) and incubated with 5 μg of the peptide at 37°C. The fluorescence was measured over time for a period of 150 min using FLUOstar Optima (BMG Labtech) and the data was analyzed using FLUOstar version 1.30R3 (BMG Labtech).

Ubiquitination assay

His tagged CHK2 was purified from bacteria as described previously (Schwarz et al., 2003a). 300 ng of His-tagged cIAP2 (R&D System) was incubated in the presence or absence of increasing concentrations (30 ng – 300 ng) or 300ng of His-tagged CHK2

KA or CHK2 D368N in the ubiquitination buffer (50mM Tris-HCl pH 7.4, 2 mM ATP, 2.5 mM MgCL2, 0.5 mM DTT and 0.05% NP-40) at 30°C for 30 min. 100 ng of recombinant E1 (Sigma-Aldrich), 300 ng of recombinant UbcH5c (Sigma-Aldrich) and 1 mg of bovine with either wild type, K68R, or K63R ubiquitin (Sigma-Aldrich) were added to the reaction. Reactions were incubated for another 60 min at 30°C. Reactions were stopped by boiling at 95°C for 5 min in the presence of Laemmli buffer.

For inhibiting CHK2 kinase activity, 2 mM of CHK2 inhibitor II hydrate (Sigma-Aldrich) or AZD-7762 (Axon Medcam BV) were incubated with 300 ng of recombinant his tagged CHK2 in the ubiquitination buffer for 10 min. Ubiquitination of cIAP2 was then performed as described above.

Kinase Assays

GFP-Tagged CHK2 was transfected in 293T and immunoprecipitated as described above. Immunoprecipitated CHK2 was incubated with 5 µg of recombinant recombinant cIAP2 (R&D system) in complete kinase buffer (50 mM Tris-HCl pH 7.5, 12.5 mM MgCl$_2$, 1 mM DTT, 440 µM ATP and 7 µCi γ[^{32}P]ATP [>4000 Ci/mmol]) at 37°C for 30 minutes. Samples were boiled, resolved by SDS-PAGE, and transferred onto nitrocellulose.

High-Resolution nano-LC-Mass Spectrometry.

5 µg of recombinant CHK2 and cIAP2 were incubated in presence or absence of in ubiquitination buffer (50 mM Tris-HCl pH 7.4, 2 mM ATP, 2.5 mM MgCL$_2$, 0.5 mM DTT and 0.05% NP-40) at 30°C for 30 min. After the reaction, proteins were first precipitated using a 2D Clean-up Kit (GE Healthcare Life Sciences) according to the manufacturer's protocol. The precipitated proteins were solubilized in 20 ml of 8 M urea, 100 mM Tris,

pH 8.5. Proteins were reduced with 5mM Tris (2-carboxyethyl)phosphine reducing agent (Tcep, Thermo Scientific), alkylated with 20 mM Iodoacetamide (IAM; Sigma Aldrich). The reaction was quenched with 10 mM DTT (Thermo Scientific), followed by digestion with 1mg LysC (Roche) for 4 h at 37 °C with agitation. The solution was diluted to 2 M urea and digested overnight with 4 mg trypsin (Sigma-Aldrich) at 37°C on thermomixer. Digested protein was acidified to 5% formic acid (Fluka) and peptides were desalted using C4 tip (Glygen) followed by C1 tip (Glygen) according to the manufacturers protocol.

The endoprotease digests were analyzed by using nano-LC-MS with a linear ion trap orbitrap mass spectrometer (Orbitrap ELITE) (Thermo Fisher Scientific) (Michalski et al., 2012). Chromatographic separations were performed by using a nano-LC 2D Plus (Eksigent, Dublin, CA) for gradient delivery and a cHiPLC-Nanoflex system (15-cm × 200-μm C_{18} packing (ChromXP C18-CL, 3 μm, 120 Å; Eksigent). The liquid chromatograph was interfaced to the mass spectrometer with a nanospray source (PicoView PV550; New Objective, Woburn, MA). Mobile-phase components were 1% FA in water (solvent A) and 1% FA in 99% ACN (solvent B). After equilibration of the column in 98% solvent A/2% solvent B, the samples were injected (10 μl) by using an AS2 autosampler (Eksigent), at a flow rate of 750 nl/min. The peptides were separated by using an AcN gradient at 400 nl/min, as follows: isocratic elution at 2% solvent B, 0 to 5 min; 2% solvent B to 25% solvent B, 5 to 110 min; 25% solvent B to 80% solvent B, to 170 min; 80% solvent B to 2% solvent B, 170 to 175 min; isocratic elution at 2% solvent B, 175 to 190 min. The total cycle time, including column equilibration, sample loading, and gradient elution of peptides, was 217 min.

The survey scans (m/z 350–1200) (MS1) were acquired at 60,000 resolution at m/z 400) in the Orbitrap in profile mode, and the product-ion mass spectrometry spectra (MS2) were acquired at 7500 resolution in profile mode, after high energy collision-induced dissociation. The maximal injection times for the LTQ and Orbitrap were 50 ms and 200 ms, respectively. The automatic gain-control targets for the LTQ and the Orbitrap were and 3×10^4 and 1×10^6 respectively. The MS1 scans were followed by ten MS2 events in the linear ion trap with collision activation in the ion trap (parent threshold, 15,000; isolation width, 2 Th; normalized collision energy, 30%; activation Q, 0.250; activation time, 30 ms). Dynamic exclusion was activated for 60 s after MS2 acquisition. A repeat count of 3, a repeat duration of 45 s, and a maximal exclusion list size of 500 were used. The following ion source parameters were used: capillary temperature, 275°C; source voltage, 2.7 kV; source current, 100 µA; tube lens, 79 V. The data were acquired by using Xcalibur 2.0.7 (Thermo Fisher).

2.4 Results

2.4.1 Regulation of p53 by DNA damage is intact in HCT116 cells lacking CHK2

CHK2 promotes apoptosis in a p53-dependent manner in normal cells that have been exposed to agents that induce DNA double strand breaks (DSBs) (Takai et al., 2002). We tested whether CHK2 was required for VP16-induced apoptosis in a p53 proficient colon cancer cell line, HCT116 (Bunz et al., 1998). Parental and CHK2$^{-/-}$ HCT116 cells were cultured for 48 h in the presence of increasing concentrations of VP16. CHK2$^{-/-}$ HCT116 cells underwent significantly less apoptosis (Fig. 2.4A), exhibited reduced cleaved caspase 3 activity (Fig. 2.4B) and had lower levels of cleaved caspase 3 (Fig. 2.4C) than did parental HCT116 cells. The observed differences between CHK2 proficient and CHK2 deficient cells were lost at higher doses of VP16 (200 mM). Stable knockdown of CHK2 in HCT116 cells (Fig. 2.5A) and in MCF7 cells, another p53 proficient cancer cell line (Fig. 2.6A), impaired the ability of VP16 to induce cleaved caspase 3 activity (Fig. 2.5B, 2.6B) and apoptosis (Fig. 2.5C). MCF7 cells are resistant to DNA damage-induced apoptosis and so higher concentrations of VP16 were used for these experiments.

p53 was monitored to determine if the reduced apoptotic response of CHK2$^{-/-}$ HCT116 cells to VP16 was due to an inability of these cells to fully activate p53 in response to DNA DSBs. p53 stabilization (Fig. 2.4C) and phosphorylation on both Ser 15 and Ser 20 (Fig. 2.7A, B) were normal in CHK2$^{-/-}$ HCT116 cells following VP16 treatment. Next, p53 transcriptional activity was assessed by monitoring its downstream effectors. p21 and BAX accumulated in both parental and CHK2$^{-/-}$ HCT116 cells following VP16 treatment (Fig. 2.8A) and RNA levels for p21, BAX and PUMA were induced to similar levels in

both cell lines (Fig. 2.8B). Similarly, p53 stabilization and accumulation of p21 and BAX were normal in HCT116 and MCF7 cells knocked down for CHK2 (Fig. 2.5A, 2.6A). Thus, despite normal activation of p53, cells lacking CHK2 were impaired in their ability to undergo apoptosis in response to DNA DSBs.

CHK2 was depleted in p53$^{-/-}$ HCT116 cells to determine if CHK2 regulated p53-independent apoptotic pathways (Fig. 2.9A). Neither p21 or BAX accumulated when p53 null HCT116 cells were treated with VP16 (Fig. 2.9A). CHK2 depletion did not significantly affect levels of cleaved caspase 3 in VP16-treated p53 null HCT116 cells (Fig. 2.9B) but did result in an increase in apoptosis in p53 null cells (Fig. 2.9C). This is agreement with a previous study reporting that loss of CHK2 in p53 null mouse embryonic fibroblasts increased apoptosis in response to DNA damage (Jiang et al., 2009). Taken together, these results suggested that CHK2 has effectors in addition to p53 that contribute to the p53-dependent apoptotic response of HCT116 cells following VP16 treatment.

cIAPs provide anti-apoptotic activity in VP16-treated CHK2 null cells

Given that the ability of DNA damage to activate p53 appeared normal in the absence of CHK2, we next sought to determine why apoptosis was impaired in VP16-treated CHK2$^{-/-}$ cells. The intrinsic apoptotic pathway is regulated downstream of PUMA and BAX by the mitochondrial protein SMAC/DIABLO (second mitochondrial activator of caspases/direct IAP binding protein with low pI). In response to apoptotic stimuli, SMAC is released from the mitochondria into the cytosol where it can bind to members of the inhibitor of apoptosis (IAPs) and reduce their ability to interact with caspases thereby

perpetuating the apoptotic signal (Wang, 2001). To determine if cIAPs were protecting

CHK2 null cells from VP16-induced apoptosis, parental and CHK2 null HCT116 cells

were cultured in the presence of BV6, a cIAP1/2 inhibitor, prior to VP16 treatment.

Consistent with our earlier observations, CHK2 deficiency impaired the ability of VP16

to induce cleaved caspase 3 activity (Fig. 2.10A) and apoptosis in HCT116 cells (Fig.

2.10B, C). Importantly, BV6 rescued the ability of VP16 to generate cleaved caspase 3

activity (Fig. 2.10A) as well as apoptosis (Fig. 2.10B, C) in CHK2$^{-/-}$ HCT116 cells. BV6

was also capable of rescuing cleaved caspase activity in VP16-treated CHK2 deficient

MCF7 cells (Fig. 2.11A). cIAP1 loss was used to verify BV6 activity in HCT116 (Fig.

2.10D) and MCF7 (Fig. 2.11B) cells. These results suggested that cIAPs provide anti-

apoptotic activity in VP16-treated CHK2 null cells and that CHK2 normally functions to

negatively regulate cIAPs in order to drive p53 proficient cells with DNA DSBs into

apoptosis.

cIAP1 and cIAP2 both contribute to reduced apoptosis in CHK2 null cells

Next knockdown experiments were performed to determine if loss of cIAP1 and cIAP2

were both required to rescue cleaved caspase 3 activity in VP16-treated CHK2 null

cells. Given that BV6, which inhibits both cIAP1 and cIAP2, rescued cleaved caspase 3

activity in VP16 treated CHK2 null HCT116 cells and in CHK2 deficient MCF7 cells (Fig.

2.10, 2.11), we depleted both cIAP1 and cIAP2 in parental and CHK2 null HCT116 cells

prior to VP16 treatment (Fig. 2.12A). As seen in Fig. 2.12B and 2.12C, concomitant

depletion of both cIAP1 and cIAP2 was able to rescue VP16 induced apoptosis in CHK2

null HCT116 cells. However individual knockdown of either cIAP2 (Fig. 2.13A) or cIAP1

(Fig. 2.14B) did not restore levels of apoptosis in VP16-treated CHK2 null cells to those measured in VP16-treated parental cells (Fig. 2.13B, 2.14B). In fact, depletion of cIAP2 actually impaired the ability of VP16 to induce apoptosis (as measured by levels of cleaved caspase 3 activity) in both parental and CHK2 null HCT116 cells (Fig. 2.13B). This was confirmed through the use of individual cIAP2 siRNA (C1-4) from the pooled siRNA (Fig. 2.13C, D). Finally, depletion of XIAP did not impact cleaved caspase 3 levels (Fig. 2.15A), cleaved caspase 3 activity (Fig. 2.15B) or apoptosis (Fig. 2.15C) in VP16 treated parental and CHK2 null cells.

Taken together our results suggest that CHK2 promotes apoptosis in response to DNA damage in at least two ways. CHK2 phosphorylates p53 resulting in the activation of pro-apoptotic signaling and CHK2 negatively regulates cIAP1 and cIAP2 to turn-off anti-apoptotic signaling.

CHK2 interacts with and inhibits E3 ligase activity of cIAP2.

To determine how CHK2 might be regulating the IAPs, CHK2 was monitored for its ability to interact with IAP family members. CHK2 oligomerizes and becomes hyperphosphorylated through auto/trans-phosphorylation mechanisms when it is overexpressed in either HEK293 cells or in bacteria (King et al., 2007; King et al., 2006; Schwarz et al., 2003b). This results in its full activation of CHK2 in the absence of any DNA damage. By contrast, CHK2 mutants lacking kinase activity fail to become hyperphosphorylated under these conditions. Thus, HEK293 cells enabled us to monitor interactions between both kinase-active and kinase-inactive forms CHK2 with IAP family members. As seen in Fig. 2.16, kinase-active CHK2 co-precipitated with cIAP2 (Fig.

2.16A lane 2) and cIAP1 (Fig. 2.16B lane 2) but not with XIAP (Fig. 2.16C lane 3).

Interestingly, the ability of CHK2 to interact with cIAP1 and cIAP2 required its kinase

activity as neither cIAP2 (Fig. 2.16A, lane 3) nor cIAP1 (Fig. 2.16B lane 3) co-

precipitated with the kinase-inactive CHK2 mutant (CHK2-KD).

Next, experiments were conducted to identify domains in cIAP2 that were required for

its interactions with CHK2. Different cIAP2 deletion mutants (Fig. 2.17A) were

ectopically expressed in HEK293T cells in the presence or absence of either wild-type

or kinase-dead CHK2. As seen in Fig. 2.17B, deletion of the linker region between the

BIR3 domain and the UBA domain abrogated the interaction between CHK2 and cIAP2

(compare lane 14 and lanes 2, 5, 8, and 11). Similar results were obtained with various

cIAP2 deletion mutant created by deleting its functional domains from the C-terminus

(data not shown). This shows that the linker region (L) of cIAP2 mediates its interaction

with CHK2.

When cIAP2 is overproduced in cells, it undergoes homodimerization, auto-

ubiquitination and in this way regulates its own proteasome-mediated degradation

(Bertrand et al., 2008). Thus, we were able to monitor the ability of CHK2 to regulate

cIAP2 turnover when they were co-produced in HEK293T cells. Interestingly, cIAP2

accumulated in cells when it was co-produced with kinase-active (Fig. 2.18, lane 3) but

not with kinase-dead (lane 5) CHK2. Kinase-active CHK2 had modest effects on cIAP1

levels (lane 9) and no apparent effect on XIAP levels (lane 15). Ectopically produced

cIAP1, cIAP2 and XIAP accumulated in cells cultured in the presence of the proteasome

inhibitor MG132 indicating that all three family members were undergoing ubiquitin-

mediated proteolysis (Fig. 2.18B, lanes 2, 8, and 14). Interestingly, addition of MG132 to

the culture media failed to increase levels of cIAP2 above the already high levels

observed when cIAP2 and kinase-active CHK2 were co-produced (compare lanes 3 and

4). This indicated that CHK2 inhibited the ability of cIAP2 to promote its own turnover.

To confirm whether CHK2 was indeed regulating the E3 ligase activity of cIAP2, cIAP2

deletion mutants that lacked the RING domain (Fig. 2.18B) were co-expressed with

CHK2. As seen previously, ectopically expressed cIAP2 was rapidly degraded (Fig.

2.18C lane 1 and 5) as cIAP2 levels increased in the presence of MG132 (Fig. 2.13C

compare lanes 1 and 2). However, cIAP2 deletion mutants lacking the RING domain

were already present at high levels and did not accumulate in the presence of MG132

indicating that wild type cIAP2 utilizes its own RING domain to mediate its destruction

(Fig. 2.18C compare lanes 7 and 8; lanes 11 and 12). Wild type, but not kinase-dead,

CHK2 was able to stabilize wild type cIAP2 (Fig. 2.18C lane 3) but failed to stabilize

already high levels of cIAP2 ΔR or cIAP2 ΔC mutants. This provided further evidence

that cIAP2 regulates its own turnover through its E3 ubiquitin ligase activity and that

CHK2 potentially impairs the ability of cIAP2 to degrade itself in the cells. On the other

hand, MG132-treatment resulted in higher levels of cIAP1 (Fig 18A compare lanes 9

and 10) and XIAP (Fig 18A compare lanes 15 and 16) in cells where kinase-active

CHK2 was also produced indicating that cIAP1 and XIAP continued to undergo

ubiquitin-mediated proteolysis even in the presence of CHK2. Consistent with the

conclusion that CHK2 stabilizes cIAP2 by inhibiting its ubiquitin-mediated proteolysis *in

vivo* , was the observation that the half-life of c-IAP2 was extended from less than 15

min in the absence of CHK2 to approximately 60 min in the presence of CHK2 (Fig 19

A, B). By contrast, CHK2 did not effect the half-life of cIAP1 (Fig. 2.19 C, D) or XIAP (Fig. 2.19E, F).

Ubiquitination reactions were performed *in vitro* with purified proteins to determine if CHK2 directly inhibited the E3 ubiquitin ligase activity of cIAP2. As seen in Fig. 2.20A, purified cIAP2 migrated as expected for a 70 kDa protein on SDS-gels (lane 1). cIAP2 became stochiometrically ubiquitinated and ran as a heterogenous population of polyubiqutinated species when incubated *in vitro* in the presence of E1, E2 and ubiquitin (Fig. 2.0A lane 2). The ability of cIAP2 to autoubiquitinate was inhibited when assays were carried out in the presence of kinase-active (lanes 3-7) but not kinase-dead (lanes 8-12) CHK2.   This was evident by the presence of the 70 kDa form of cIAP2 (lanes 3-7) in blots probed with cIAP2 antibody; the disappearance of the most polyubiquitinated forms of cIAP2 detected in blots probed with ubiquitin-specific antibodies (Ub blot), as well as well as those probed with antibodies specific for either K63 or K48 linkage specific polyubiquitin. The ability of CHK2 to inhibit the E3 ubiquitin ligase activity of cIAP2 was further tested by including a CHK2 inhibitor in the reaction. As seen in Fig. 2.20B, CHK2 lost the ability to inhibit the autoubiquination of cIAP2 when its kinase activity was blocked by two different CHK2 inhibitors, CHK2 inhibitor II (Arienti et al., 2005) and AZD7762 (Zabludoff et al., 2008). Taken together, these results demonstrate that CHK2 binds to cIAP2 (Fig. 2.16A) and inhibits its E3-ubiquitin ligase activity in a kinase-dependent manner (Fig. 2.0).

As seen in Fig. 2.20, cIAP2 modification by K63-linked ub was much stronger compared to the modification by K48-linked ubiquitin. K48 and K63-linked ubiquitin provide distinct signaling characteristics to a protein. Proteins modified through K48-

linked ubiquitin are primarily targeted for destruction through the ubiquitin-proteosome system (Thrower et al., 2000). On the other hand, K63-linked ubiquitin serves as a signaling scaffold without altering the protein stability (Jacobson et al., 2009; Xu et al., 2009). K63-linked ubiquitination by cIAPs have been shown to be critical in their downstream effectors, especially in the NFκB pathway (Bertrand et al., 2011; Bertrand et al., 2008). Thus, we next questioned if CHK2 could preferentially inhibit the ability of cIAP2 to promote K63-linked ubiquitination. To test this, we performed the *in vitro* ubiquitination assays in the presence of ubiquitin that cannot be linked to the substrates either through lysine 48 (K48R ubiquitin) or lysine 63 (K63R ubiquitin) (Fig. 2.21). In agreement with our previous finding, cIAP2 was modified by K63-linked ubiquitin preferentially over K48-linked ubiquitin modification. In the presence of K63R ubiquitin (Fig. 2.1 lanes 5 – 8), cIAP2 was not modified by K63-linked ubiquitin. cIAP2 modification by K48-linked ubiquitin was increased and significantly higher compared to cIAP2 modification seen in the presence of wild-type ubiquitin (compare lanes 2 and 6). Kinase-active CHK2 was able to inhibit this increased cIAP2 modification through K48-linked ubiquitin (compare lanes 10 and 11). Similarly, in the presence of K48R ubiquitin, cIAP2 failed to be modified by K48-linked ubiquitin and the K63-linked ubiquitin modification of cIAP2 was unaltered when compared to its modification in the presence of the wild-type ubiquitin (compare lanes 9 – 12). As seen previously, CHK2 was able to inhibit cIAP2 modification under these conditions as well.

Taken together these results indicate that *in vitro*, cIAP2 preferentially modifies its substrate through K63-linked ubiquitin compared to K48-linked ubiquitin and that CHK2

is able to inhibit the E3 ubiquitin ligase activity of cIAP2 with no apparent selectivity towards K63 or K48-linked ubiquitin.

cIAP2 is a CHK2 substrate

We next asked if cIAP2 was a direct substrate of CHK2. As seen in Fig. 2.22A, CHK2 phosphorylated cIAP2 *in vitro*. To identify sites of phosphorylation, reactions were scaled up, kinase assays were performed in the presence of unlabeled ATP and reaction products were subjected to mass spectrometry. This analysis identified threonine (Thr) 35 as a CHK2 phosphorylation site *in vitro*. Threonine 35 represented a consensus CHK2 phosphorylation site and was conserved across species (Fig. 2.22B). Sequence alignment indicated that the residues surrounding Thr35 were conserved in cIAP1 but were not conserved in XIAP (Fig. 2.2B). To determine if cIAP2 was phosphorylated on Thr35 in a CHK2-dependent manner in cells, cIAP2 was co-produced with kinase-active CHK2, kinase-dead CHK2 or CDC25C as a negative control. A mutant of cIAP2 lacking E3 ubiquitin ligase activity (Huang et al., 2000) was expressed to eliminate proteasome-mediated degradation of cIAP2. Thr35 phosphorylation was monitored using a phosphospecific Thr35 antibody. As seen in Fig. 2.22C, cIAP2 was phosphorylated on Thr35 when it was co-produced with kinase-active (lane 2) but not kinase-dead (lane 3) CHK2 in HEK293T cells. The antibody was specific for Thr35-phosphorylated cIAP2 as a mutant of cIAP2 encoding alanine in place of Thr35 was not recognized by the phospho-specific antibody (lane 5).

We next determined if Thr35 was essential for CHK2-mediated stabilization of cIAP2. Consistent with our previous observation, CHK2 promoted accumulation of wild

type cIAP2 (Fig. 2.3A compare lanes 1 and 3).

The cIAP2 mutant that cannot be phosphorylated on threonine 35 (cIAP2 T35A) underwent rapid degradation which was inhibited by the proteosome inhibitor MG132. CHK2 was able to stabilize the cIAP2 T35A mutant just like wild type cIAP2 (compare lanes 3 and 9). This suggested that phosphorylation of Thr35 was not sufficient to drive cIAP2 accumulation. cIAP2 contains three additional CHK2 consensus phosphorylation sites (RXXS/T), including S81, T175, and T261. Substitution of all four sites for alanine also did not impair the ability of CHK2 to regulate cIAP2 (Fig. 2.23B lanes 3, 7, 11, and 15). In addition to Thr35, mass spectrometry has also identified Ser155 and Ser566 residues on cIAP2 as additional CHK2 phosphorylation sites. I am currently in the process of mutating these residues to alanine and analyzing the effect of these mutations of CHK2-mediated cIAP2 stabilization.

Taken together our results suggest that CHK2 promotes apoptosis in response to DNA damage in at least two ways. CHK2 phosphorylates p53 resulting in the activation of pro-apoptotic signaling and CHK2 negatively regulates cIAPs to turn-off anti-apoptotic signaling.

Discussion

Initial loss of function studies in mice and in cells derived from these mice established CHK2 as a key regulator of p53 in the DNA damage response pathway (Hirao et al., 2002; Takai et al., 2002). DNA DSBs activate ATM, which in turn phosphorylates CHK2, leading to its full activation. Activated Chk2 phosphorylates p53 on Ser 20 – a key modification to promote p53 stabilization (Chehab et al., 2000; Shieh et al., 2000). Here we demonstrate that CHK2 is dispensable for p53 phosphorylation on Ser 20 in two different p53 proficient cancer cell lines (HCT116 and MCF7) following VP16 treatment. That cancer cells have redundant protein kinases that phosphorylate p53 on serine 20 in response to DNA damage has been observed previously (Ahn et al., 2003; Jallepalli et al., 2003).

Paradoxically, p53 regulation (phosphorylation of Ser 15 and Ser 20) and function (induction of p21, BAX and PUMA) appeared normal in CHK2 deficient HCT116 and MCF7 cells following VP16 treatment, yet these cells were impaired in their ability to undergo apoptosis. This led us to investigate whether CHK2 regulated proteins downstream of p53 in the intrinsic apoptotic pathway that had more proximal roles in regulating the executors of apoptosis. We observed specific interaction between CHK2 and cIAPs, but not with XIAP. Moreover, inhibition of cIAP1 and cIAP2 through the SMAC mimetic, BV6, rescued levels of apoptosis in CHK2 deficient HCT116 and MCF7 cells to levels measured in CHK2 proficient parental cells. Members of the IAP family (XIAP, cIAP1 and cIAP2) are potent inhibitors of cell death in response to various stress signals including DNA DSBs (de Almagro and Vucic, 2012). cIAPs are known to regulate cell death either through regulation of caspases or through regulation of the

NF-κB pathway (Gyrd-Hansen and Meier). cIAPs can bind to active form of caspases and target them for ubiquitin dependent proteosomal degradation (Choi et al., 2009). Inhibition of both cIAP1 and cIAP2 using siRNA rescued apoptosis in CHK2 null cells to levels comparable to that of VP16- treated parental cells. Individual loss of cIAP1 or cIAP2 alone was not sufficient to increase cell death in cells lacking CHK2. Thus, cIAPs provided anti-apoptotic activity in VP16-treated CHK2 deficient cells. This suggested that CHK2 normally functions to inhibit cIAPs and in this way sensitizes cells with DNA DSBs to p53-dependent apoptosis. In support of this, CHK2 inhibited the E3 ubiquitin ligase activity of cIAP2 *in vitro* and extended its half-life in cells. Although CHK2 bound to cIAP1, we were unable to detect alterations in the half-life of cIAP1 in the presence of CHK2. cIAP1 is known to heterodimerize with cIAP2 and target it for degradation (Silke et al., 2005). Thus, CHK2 may regulate cIAP1 indirectly through cIAP2.

The ability of CHK2 to bind to and regulate cIAP2 required the kinase activity of CHK2. This suggested that CHK2 phosphorylated cIAP2 to inhibit its E3 ubiquitin ligase activity. Indeed, we demonstrated that CHK2 phosphorylates cIAP2 on Thr35 *in vitro* and cIAP2 is phosphorylated on Thr35 when co-produced with CHK2 in cells. CHK2 phosphorylates its substrate using the consensus motif: LXRXXpS/T (O'Neill et al., 2002). Interestingly, cIAP2 contains four CHK2 consensus motifs including Thr35 (LXRXXpT$_{35}$), Ser81 (RXXS$_{81}$), Thr145 (RXXT$_{145}$), and Thr261 (RXXpT$_{261}$) and all the sites are evolutionarily conserved. Mass spectrometry of *in vitro* phosphorylated cIAP2 identified Thr35 but not others as a site of CHK2 phosphorylation site.

cIAP1 and cIAP2 contain four distinct functional domains including three consecutive BIR domains that mediate protein-protein interactions, a ubiquitin binding (UBA) domain

that binds to poly-ubiquitinated proteins, a C-terminal RING domain that ubiquitinates its substrates and a caspase recruiting domain (CARD), which has been proposed to regulate the activity of the RING domain (Lopez et al., 2011). The structure of the ring domain of cIAP2 indicates that it exists as a dimer and dimerization of the ring domain is essential for its function (Mace et al., 2008). Thr35 resides within the first BIR1 domain of cIAP2 and this domain may have a role in dimerization (Lin et al., 2007; Zhou et al., 2005). The first BIR domain has been shown to be important for maintaining the E3 ligase activity of the cIAP2-MALT1 fusion protein found in MALT Lymphoma (Zhou et al., 2005). Furthermore, AKT phosphorylates the BIR domain of XIAP, thereby disrupting dimerization and inhibiting its E3 ubiquitin ligase activity (Dan et al., 2004; Mace et al., 2008). cIAP2 is known to homodimerize and to heterodimerize with cIAP1 (Silke et al., 2005). Thus, a logical hypothesis is that phosphorylation of cIAP2 on Thr35 by CHK2 disrupts cIAP2 homo/hetero-dimerization thereby inhibiting its E3 ubiquitin ligase activity. However, we have found that a mutant of cIAP2 encoding alanine for threonine at position 35 can still be regulated by kinase-active CHK2 suggesting there may be additional regulatory mechanisms that remain to be identified. Moreover, mutation of Thr35 alone or in combination with the other CHK2 consensus sites failed to abrogate the effect of CHK2 on cIAP2 stabilization. Mass spectrometry has also identified two additional residues, Ser155 and Ser566, on cIAP2 as CHK2 phosphorylation sites. Ser155 is present in the BIR2 domain of cIAP2 which has been previously shown to be important for caspase binding (Eckelman and Salvesen, 2006). More importantly, Ser566 is present in the RING domain of cIAP2. Analysis of the RING domain indicates that this residue might be present in the hydrophobic core, and

therefore, phosphorylation of Ser566 might disrupt the RING domain and the E3 ligase activity of cIAP2. Ser566 is conserved in cIAP1 but is mutated to an alanine in XIAP. Given that we have observed CHK2 interaction with cIAP1 and cIAP2, but not XIAP, Ser566 has a strong potential for mediating the effects of CHK2 by disrupting cIAP2's RING activity.

In summary, we have demonstrated that in p53 proficient cancer cells, CHK2 is dispensable for p53 activation in response to DNA DSBs. However, apoptosis is impaired in the face of p53 signaling because the cIAPs inhibit apoptosis downstream of p53. We conclude that CHK2 normally functions to inhibit cIAPs and in this way sensitize cells with DNA DSBs to p53-dependent apoptosis (Fig. 2.4). These studies have implications for how CHK2 inhibitors might be used clinically. On the one hand, CHK2 inhibitors would be expected to prevent the activation of p53 by IR or chemotherapy in normal cells, thereby serving as a radio- or chemo-protector. On the other hand, loss of CHK2 in p53 deficient cancer cells has been shown to enhance apoptosis in response to DNA damage (Hirao et al., 2002; Jallepalli et al., 2003; Lovly et al., 2008a; Takai et al., 2002). Thus, one might be able to protect normal cells in the body from the damaging side effects of treatment while at the same time enhance tumor cell killing with CHK2 inhibitors.
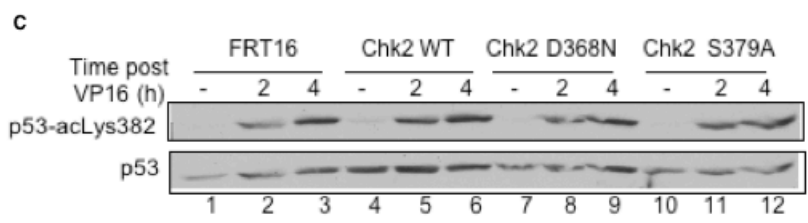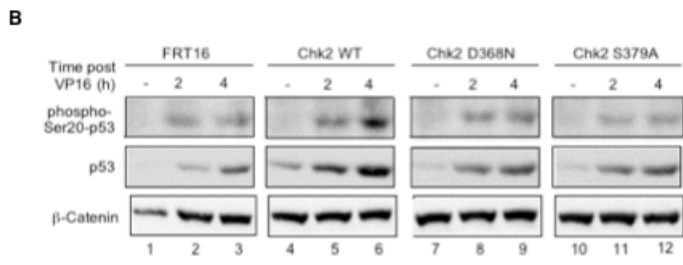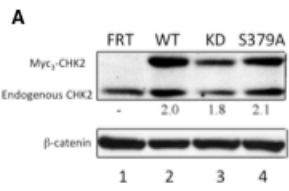
A

	FRT	WT	KD	S379A
Myc₃-CHK2				
Endogenous CHK2	-	2.0	1.8	2.1
β-catenin				
	1	2	3	4

B

	FRT16	Chk2 WT	Chk2 D368N	Chk2 S379A
Time post VP16 (h)	- 2 4	- 2 4	- 2 4	- 2 4
phospho-Ser20-p53				
p53				
β-Catenin				
	1 2 3	4 5 6	7 8 9	10 11 12

C

	FRT16	Chk2 WT	Chk2 D368N	Chk2 S379A
Time post VP16 (h)	- 2 4	- 2 4	- 2 4	- 2 4
p53-acLys382				
p53				
	1 2 3	4 5 6	7 8 9	10 11 12

Fig. 2.1 CHK2 is not required for post-translational modification of p53 in VP16-treated U2OS cells

(A) Cell lysates of parental U2OS (FRT) cells and U2OS cells stably expressing WT and mutant forms of CHK2 were prepared and subjected to western blotting for the indicated proteins

(B, C) Parental U2OS (FRT) cells and U2OS cells stably expressing WT and mutant forms of CHK2 were treated with 10μM VP16 for the indicated periods of time. Cell lysates were prepared and subjected to western blotting for the indicated proteins

These experiments were performed by Dr. Christine Lovly

A

B

86
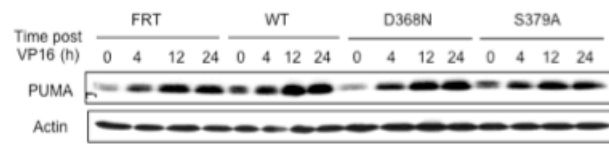
Fig. 2.2 CHK2 activity is dispensable for p53 transcriptional activity in VP16 treated U2OS

(A) Parental U2OS (FRT) cells and U2OS cells stably expressing WT and mutant forms of CHK2 were treated with 10μM VP16 for the indicated periods of time. Cell lysates were prepared and subjected to western blotting for the indicated proteins

(B) Parental U2OS (FRT) cells and U2OS cells stably expressing WT and mutant forms of CHK2 were treated with 10μM VP16 for the indicated periods of time. Total RNA was isolated and subjected to qPCR for p21 and GAPDH

These experiments were performed by Dr. Christine Lovly
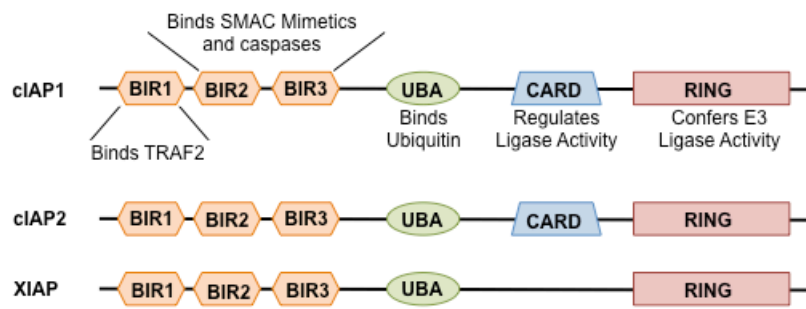
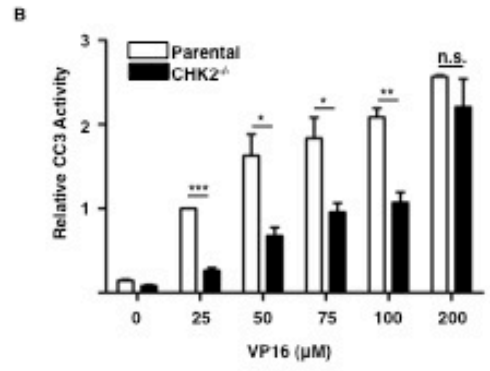**Fig. 2.3 Schematic of domain structure of the IAP family**

A
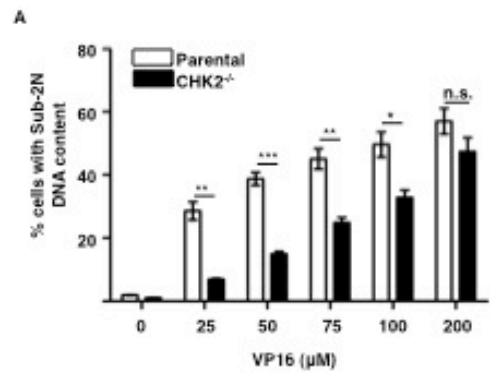

B

C

90
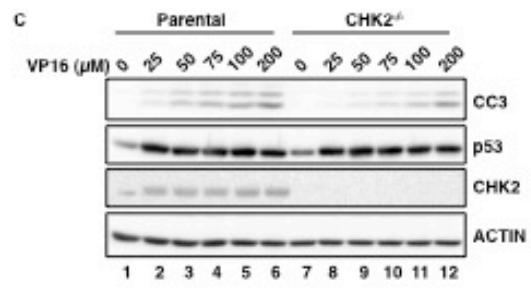
Fig 2.4 CHK2 promotes VP16-induced apoptosis in HCT116 cells

(A, B, C) Parental and CHK2$^{-/-}$ HCT116 cells were treated with VP16 at the indicated concentrations for 48 h. The percentage of cells containing sub-2N DNA content was assessed by propidium iodide (PI) staining followed by flow cytometry (A). Cell lysates were prepared and assayed for cleaved caspase 3 activity as described in Materials and Methods (B) or were subjected to Western blotting with the indicated antibodies (C). The results in panels A, and B are presented as mean (± s.e.m.) of three or more independent experiments. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, n.s. (not significant)

A

HCT116 Parent

shRNA   GFP   CHK2
VP16   −   +   −   +

CHK2
p53
p21
BAX
CC3
GAPDH

1   2   3   4

B

C

HCT116 Parent w/ GFP shRNA
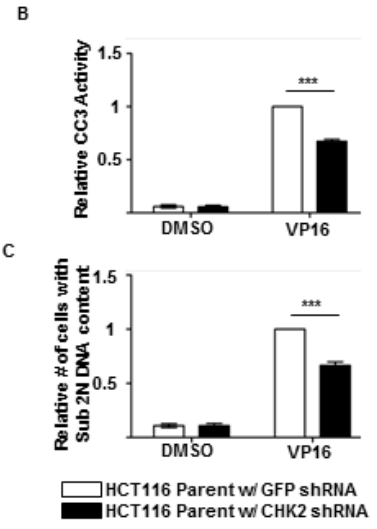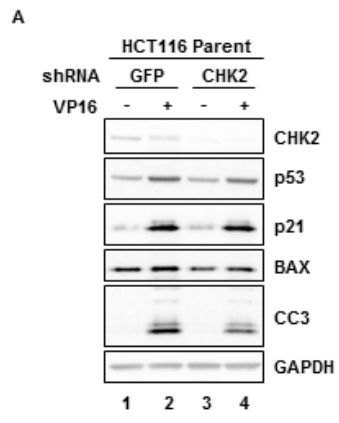HCT116 Parent w/ CHK2 shRNA

Fig 2.5 Stable knockdown of CHK2 abrogates VP16-induced apoptosis in HCT116 cells.

(A, B, C) Parental HCT116 cells stably expressing either GFP shRNA (control) or CHK2 shRNA were treated with 25 mM VP16 for 48 h. Cell lysates were subjected to Western blotting (A), or were assayed for cleaved caspase 3 activity (B). The percentage of cells containing sub-2N DNA content was assessed by PI staining followed by flow cytometry (C).

The results in panels A, B, E and F are presented as mean (± s.e.m.) of three or more independent experiments. ***p<0.0005

A

shRNA			Luc					CHK2		
VP16 (µM)	0	100	200	300	400	0	100	200	300	400

CHK2
p53
p21
BAX
GAPDH

1 2 3 4 5 6 7 8 9 10

B

□ MCF-7 w/ Luc shRNA
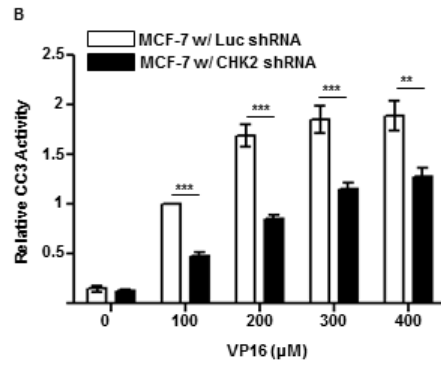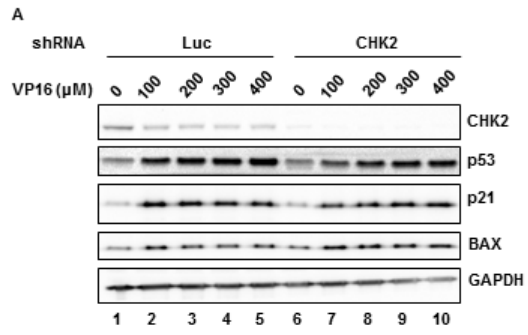■ MCF-7 w/ CHK2 shRNA

Relative CC3 Activity

VP16 (µM)

Fig. 2.6 CHK2 promotes VP16-induced apoptosis in MCF7 cells

(A, B) MCF-7 cells stably expressing control (luciferase) or CHK2 shRNA were cultured

in the presence of VP16 at the indicated concentrations for 72 h. Cell lysates were

prepared and were subjected to Western blotting with the indicated antibodies (A) or

assayed for cleaved caspase 3 activity (B).

The results in panels A, B, E and F are presented as mean (± s.e.m.) of three or more

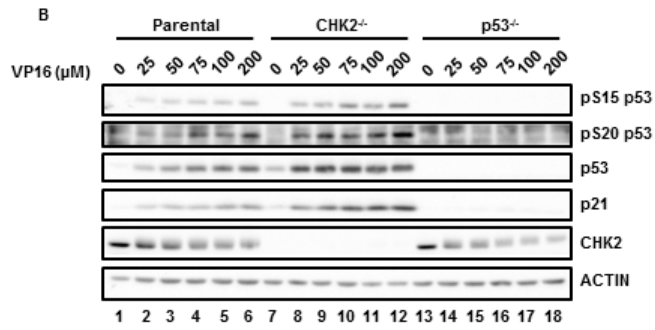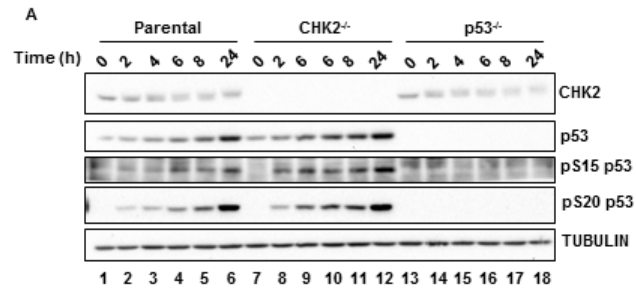independent experiments. **p<0.005, ***p<0.0005

Fig. 2.7 CHK2 is dispensable for p53 stabilization and phosphorylation in response to VP16 treatment

(A) Parental HCT116 cells or HCT116 cells null for either CHK2 (CHK2$^{-/-}$) or p53 (p53$^{-/-}$) were cultured in the presence of 25 μM of VP16 for the indicated times. Cell lysates were subjected to Western blotting with the indicated antibodies.

(B) Parental HCT116 cells or HCT116 cells null for either CHK2 (CHK2$^{-/-}$) or p53 (p53$^{-/-}$) were cultured in the presence of indicated concentrations of VP16 for 8 h. Cell lysates were subjected to Western blotting with the indicated antibodies.
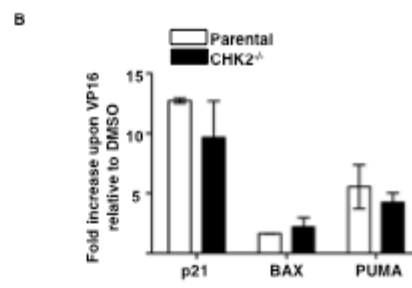
A

Parental CHK2$^{-/-}$

VP16 (μM)   0   25   50   75   100   200   0   25   50   75   100   200

CHK2
p53
BAX
p21
GAPDH

1   2   3   4   5   6   7   8   9   10   11   12

B

Parental
CHK2$^{-/-}$

Fold increase upon VP16 relative to DMSO
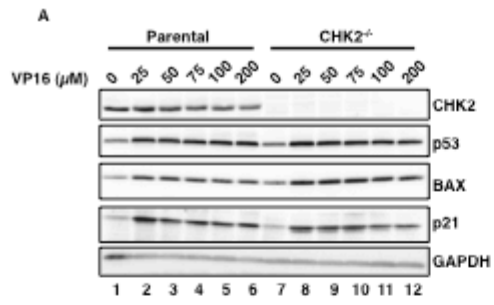
p21   BAX   PUMA

98

Fig. 2.8 Loss of CHK2 does not affect VP16-induced p53 transcriptional activity

(A) Parental and CHK2$^{-/-}$ HCT116 cells were treated with VP16 at the indicated

concentrations for 48 h. Cell lysates were prepared and subjected to Western blotting

with the indicated antibodies.

(B) Parental and CHK2$^{-/-}$ HCT116 cells were cultured in the presence of 25 µM VP16 for

48 h. Total RNA was isolated and was subjected to qPCR for the indicated genes.
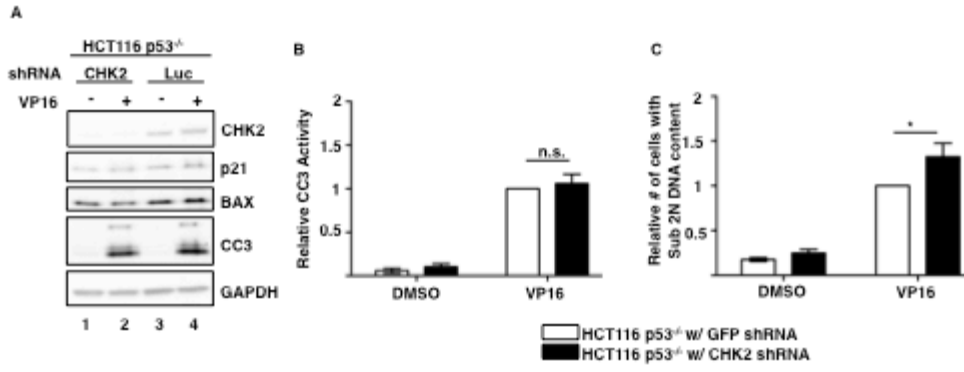
A

HCT116 p53⁻/⁻
shRNA   CHK2   Luc
VP16   –   +   –   +

CHK2
p21
BAX
CC3
GAPDH

1   2   3   4

B

Relative CC3 Activity

n.s.

DMSO   VP16

C

Relative # of cells with Sub 2N DNA content

*

DMSO   VP16

☐ HCT116 p53⁻/⁻ w/ GFP shRNA
■ HCT116 p53⁻/⁻ w/ CHK2 shRNA

**Fig. 2.9 CHK2 is dispensable for VP16-induced cleaved caspase 3 activity in p53$^{-/-}$ HCT116 cells**

(A, B, C) p53$^{-/-}$ HCT116 cells stably expressing either GFP shRNA (control) or CHK2 shRNA were treated with 25 μM VP16 for 48 h. Cell lysates were subjected to Western blotting (A), or were assayed for cleaved caspase 3 activity (B). The percentage of cells containing sub-2N DNA content was assessed by PI staining followed by flow cytometry (C).

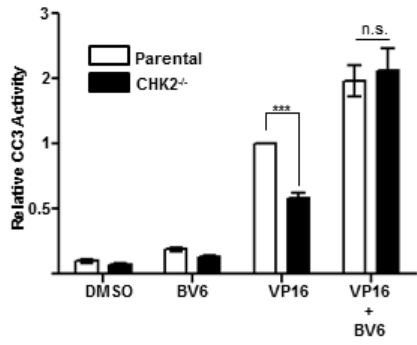The results in panels A, B, E and F are presented as mean (± s.e.m.) of three or more independent experiments. *p<0.05
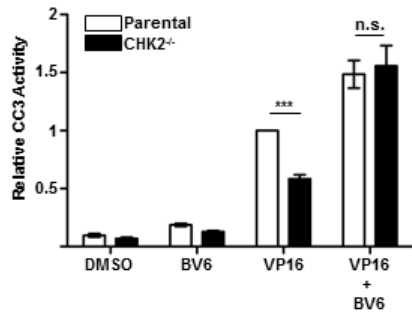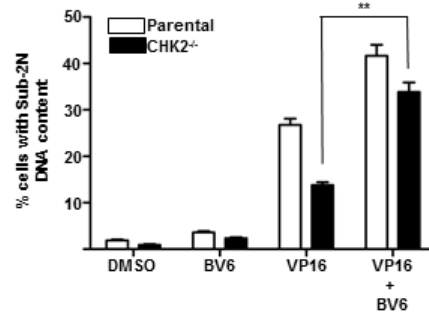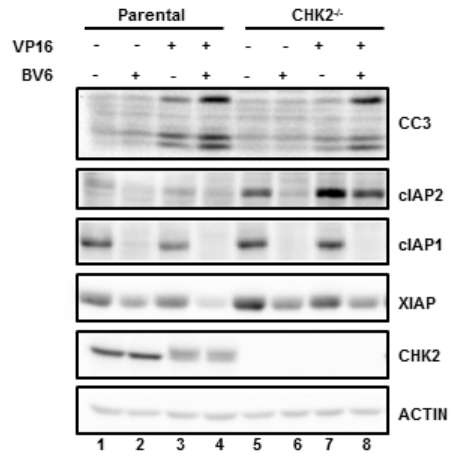
Fig. 2.10 Inhibition of cIAP using SMAC mimetic, BV6, rescues VP16-induced apoptosis in CHK2 null cells

(A, B, C, D) Parental and CHK2$^{-/-}$ HCT116 cells were cultured in the presence of 5 μM BV6 for 4 h followed by 25 μM VP16 for an additional 48 h. Cell lysates were prepared and assayed for cleaved caspase-3 activity (A). The percentage of cells containing sub-2N DNA content was assessed by PI staining followed by flow cytometry (B). Percentage of annexin V and PI double positive cells were assessed by flow cytometry (C). Cell lysates were also subjected to western blotting with the indicated antibodies (D).

The results in panels A are represented as mean ± s.e.m. and represent three or more independent experiments. ***p<0.0005, **p<0.005, n.s. (not significant)

A

B

104
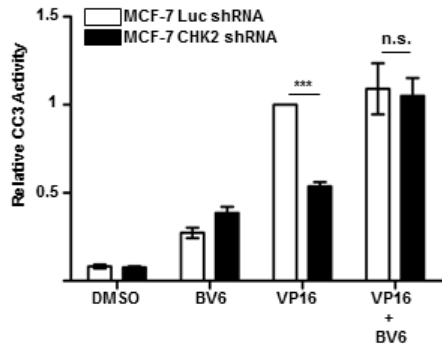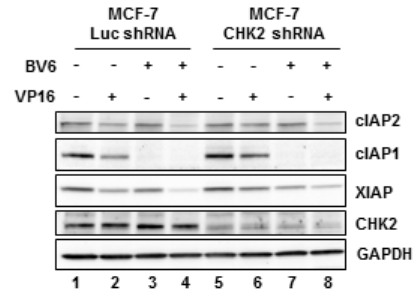
Fig. 2.11 BV6 rescues VP16-induced apoptosis in MCF7 cells lacking CHK2

(A, B) MCF7 cells stably expressing control (luciferase (Luc)) or CHK2 shRNA were cultured in the presence of 5 μM BV6 for 4 h followed by 200 μM VP16 for an additional 72 h. Cell lysates were prepared and assayed for cleaved caspase-3 activity (A) or subjected to Western Blotting for the indicated antibodies (B).

The results in panels A are represented as mean ± s.e.m. and represent three or more independent experiments. ***p<0.0005, n.s. (not significant)

A

|	Parental				CHK2$^{-/-}$			
siRNA	GL3		C1+C2		GL3		C1+C2	
VP16	-	+	-	+	-	+	-	+

cIAP2
cIAP1
XIAP
CC3
TUBULIN

1   2   3   4   5   6   7   8

B

Relative CC3 Activity

siRNA: GL3 / cIAP1+cIAP2 / GL3 / cIAP1+cIAP2
VP16: - - + +

***

C

%AnnexinV(+) and PI(+) cells

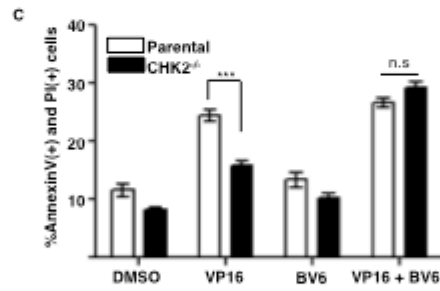DMSO   VP16   BV6   VP16 + BV6

*** n.s

Fig. 2.12 cIAP1 and cIAP2 are both required for inhibition of VP16-induced apoptosis in CHK2 null cells

(A, B, C) Parental and CHK2$^{-/-}$ HCT116 cells transiently expressing GL3 (control) or pooled cIAP1 and cIAP2 (C1 + C2) siRNAs for 18 h were cultured in the presence of vehicle (DMSO, -) or 25 μM VP16 (+) for an additional 48 h. Cell lysates were prepared and subjected to Western blotting with the indicated antibodies (A) or were assayed for cleaved caspase-3 activity (B). Percentage of annexin V and PI double positive cells were assessed by flow cytometry (C).

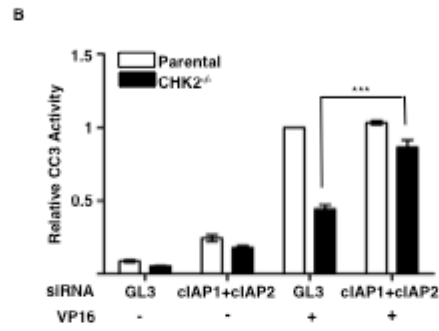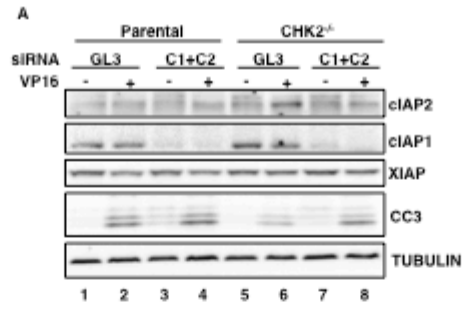The results in panels A are represented as mean ± s.e.m. and represent three or more independent experiments. ***p<0.0005, n.s. (not significant)

A

B

C

D
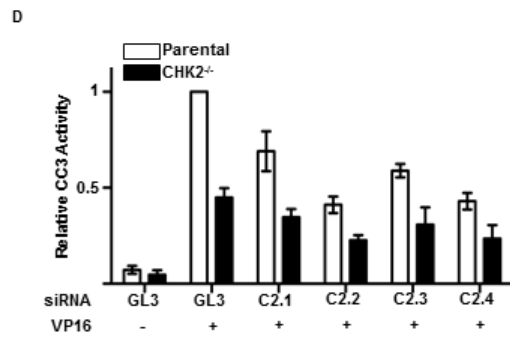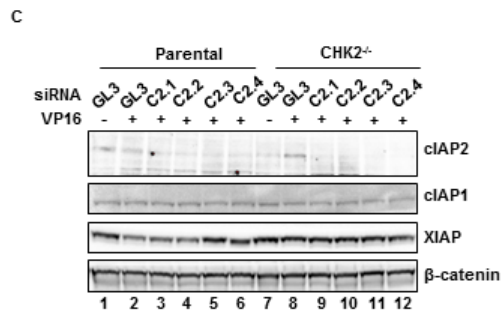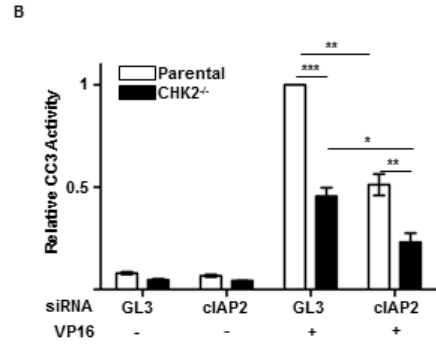
Fig. 2.13 cIAP2 loss reduced VP16-induced apoptosis in HCT116 cells

(A, B) Parental and CHK2$^{-/-}$ HCT116 cells transiently expressing GL3 (control) or pooled

cIAP2 (C2) siRNAs for 18 h were cultured in the presence of vehicle (DMSO, -) or 25

μM VP16 (+) for an additional 48 h. Cell lysates were prepared and subjected to

Western blotting with the indicated antibodies (A) or were assayed for cleaved caspase-

3 activity (B).

 (C, D) Parental and CHK2$^{-/-}$ HCT116 cells transiently expressing GL3 (control) or

individual cIAP2 (C2.1 – C2.4) siRNAs for 18 h were cultured in the presence of vehicle

(DMSO, -) or 25 µM VP16 (+) for an additional 48 h. Cell lysates were prepared and

subjected to Western blotting with the indicated antibodies (C), or were assayed for

cleaved caspase-3 activity (D).

The results in panel B, and D, and F are presented as mean (± s.e.m.) of two or more

independent experiments. *p<0.05, **p<0.005, ***p<0.0005.

A

| siRNA | GL3 | GL3 | C1.1 | C1.2 | C1.3 | GL3 | GL3 | C1.1 | C1.2 | C1.3 |
| VP16 | - | + | + | + | + | - | + | + | + | + |

Parental   CHK2⁻/⁻

cIAP2
cIAP1
XIAP
CHK2
CC3
GAPDH

1 2 3 4 5 6 7 8 9 10

B
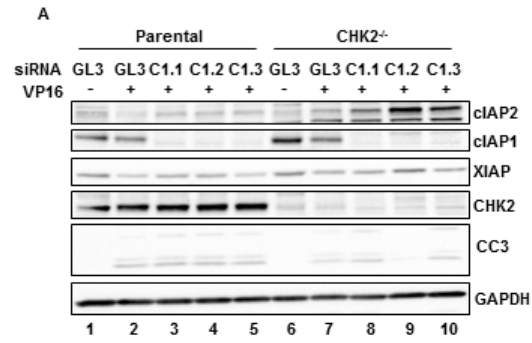
Fig. 2.14 Loss of cIAP1 alone does not affect VP16-induced apoptosis in HCT116 cells

(A, B) Parental and CHK2^{-/-} HCT116 cells transiently expressing GL3 (control) or

individual cIAP1 (C1.1 – C1.3) siRNAs for 18 h were cultured in the presence of vehicle

(DMSO, -) or 25 μM VP16 (+) for an additional 48 h. Cell lysates were prepared and

subjected to Western blotting with the indicated antibodies (A) or were assayed for

cleaved caspase-3 activity (B).

The results in panel B is presented as mean (± s.e.m.) of three or more independent

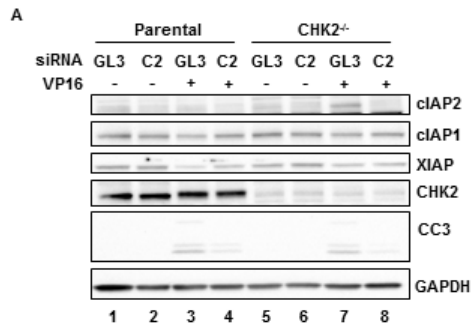experiments. *p<0.05, ***p<0.0005.
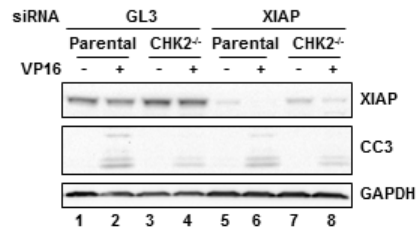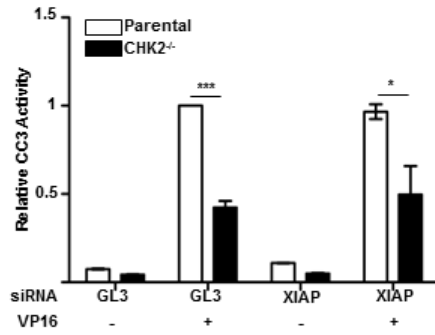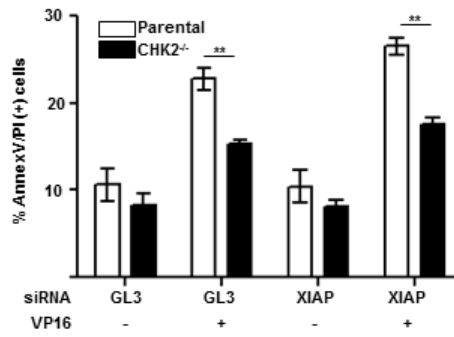
Fig. 2.15 Loss of XIAP does not affect VP16-induced apoptosis in HCT116 cells.

(A, B, C) Parental and CHK2$^{-/-}$ HCT116 cells transiently expressing GL3 (control) or

pooled XIAP siRNAs for 18 h were cultured in the presence of vehicle (DMSO, -) or 25

μM VP16 (+) for 48 h. Cell lysates were prepared and subjected to Western blotting with

the indicated antibodies (A) or were assayed for cleaved caspase-3 activity (B).

Percentage of annexin V and PI double positive cells were assessed by flow cytometry

(C)

The results in panel B, and C are presented as mean (± s.e.m.) of three or more

independent experiments. *p<0.05, **p<0.005, ***p<0.0005.

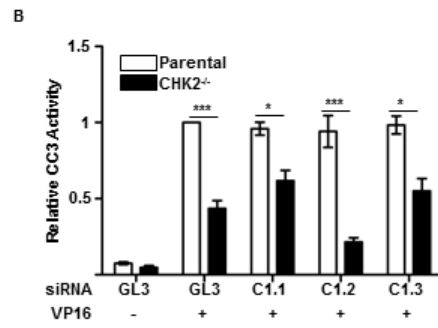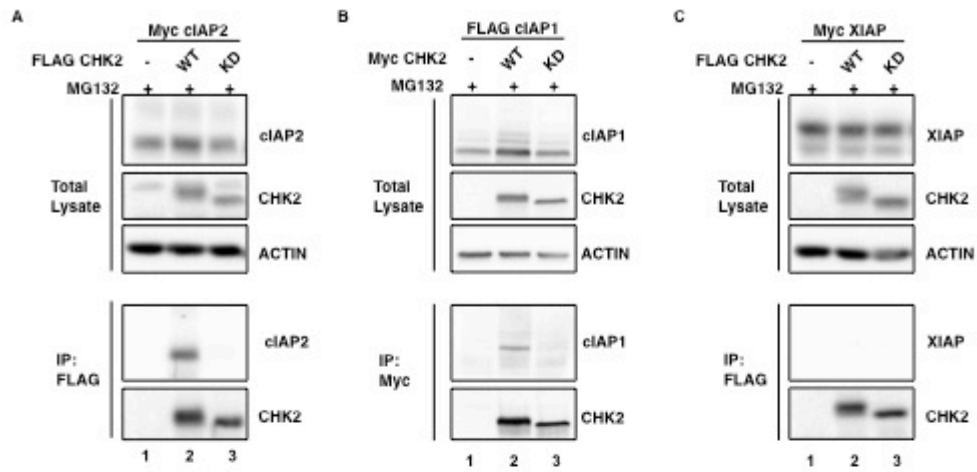**Fig. 2.16 Wild type CHK2 binds to cIAP1 and cIAP2 but not XIAP**

(A, B, C) HEK293T cells were transfected with plasmids encoding tagged forms of the

indicated proteins for 18 h. Cells were then cultured in the presence of 50 μM MG132

for an additional 6 h. Cell lysates were prepared and either resolved directly by SDS-

PAGE (Total lysate) or were incubated with either anti-Flag (A, C) or anti-Myc affinity gel

(B) and precipitates were resolved by SDS-PAGE. Western blotting was performed with

antibodies specific for the indicated proteins.

A

B

Total Lysate

Myc cIAP2: 1R 2R 3R LR UR
FLAG CHK2: W/T KD W/T KD W/T KD W/T KD W/T KD

Myc

CHK2

ACTIN

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

IP:FLAG

Myc cIAP2: 1R 2R 3R LR UR
FLAG CHK2: W/T KD W/T KD W/T KD W/T KD W/T KD

Myc

CHK2

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
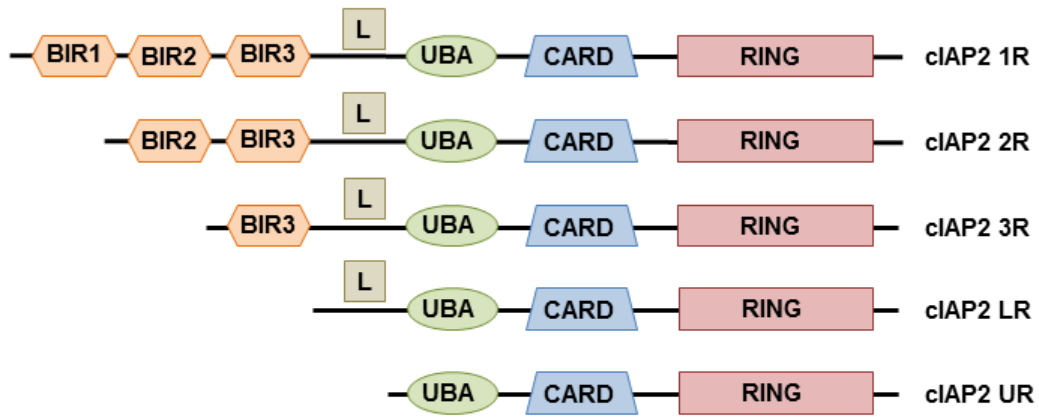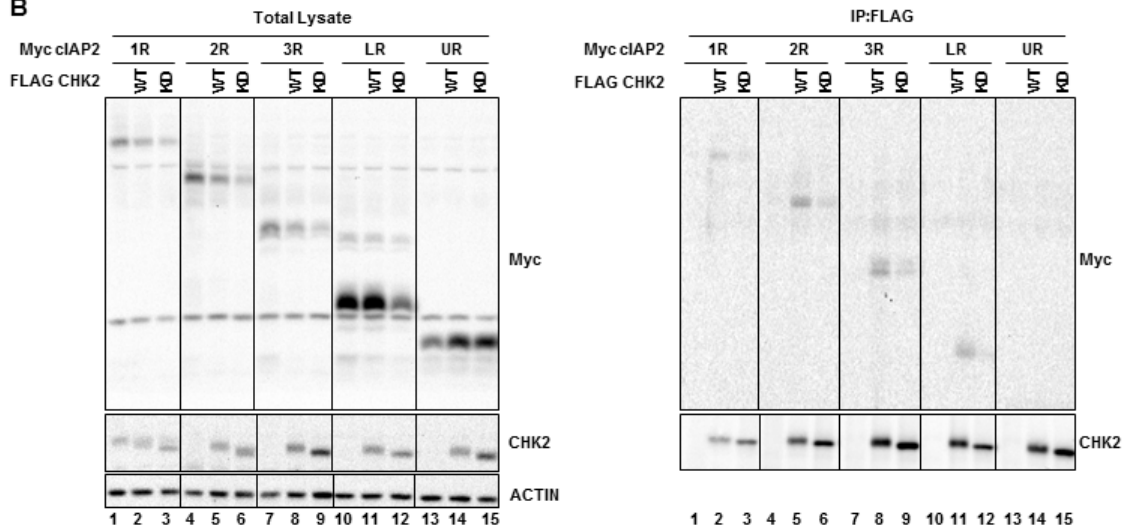
Fig. 2.17 cIAP2 Linker region between BIR3 and UBA domain mediates its interaction with CHK2

(A) Schematic of domain structure of wild type (1R) and deletion mutants of cIAP2 (2R, 3R, LR, and UR).

(B) HEK293T cells were transfected with plasmids encoding tagged forms of the indicated proteins for 18 h. Cells were then cultured in the presence of 50 μM MG132 for an additional 6 h. Cell lysates were prepared and either resolved directly by SDS-PAGE (Total lysate) or were incubated with anti-Flag and precipitates were resolved by SDS-PAGE. Western blotting was performed with antibodies specific for the indicated proteins.

A

FLAG cIAP2

			WT	WT	KD	KD
FLAG CHK2	-	-	WT	WT	KD	KD
MG132	-	+	-	+	-	+

cIAP2
CHK2
ACTIN

1 2 3 4 5 6

FLAG cIAP1

FLAG CHK2	-	-	WT	WT	KD	KD
MG132	-	+	-	+	-	+

cIAP1
CHK2
ACTIN

7 8 9 10 11 12

Myc XIAP

FLAG CHK2	-	-	WT	WT	KD	KD
MG132	-	+	-	+	-	+

XIAP
CHK2
ACTIN

13 14 15 16 17 18

B

BIR1 — BIR2 — BIR3 — L — UBA — CARD — RING   cIAP2 WT

BIR1 — BIR2 — BIR3 — L — UBA — CARD   cIAP2 ΔR

BIR1 — BIR2 — BIR3 — L — UBA   cIAP2 ΔC

C

		WT				ΔR		ΔC	
FLAG cIAP2		WT		WT	KD		ΔR	WT	ΔC	WT
Myc CHK2	-		WT		KD		-	WT	-	WT
MG132	-	+	-	+	-	+	-	+	-	+	-	+	-	+

cIAP2
CHK2
ACTIN

1 2 3 4 5 6 7 8 9 10 11 12 13 14

118
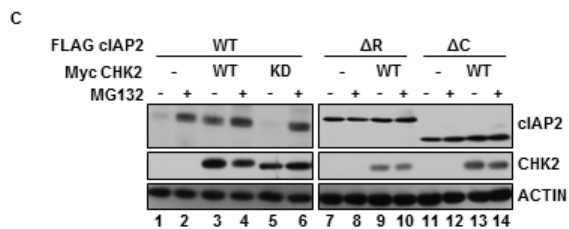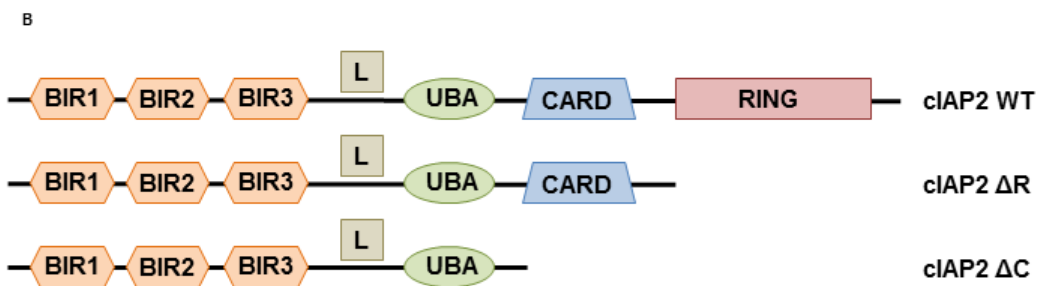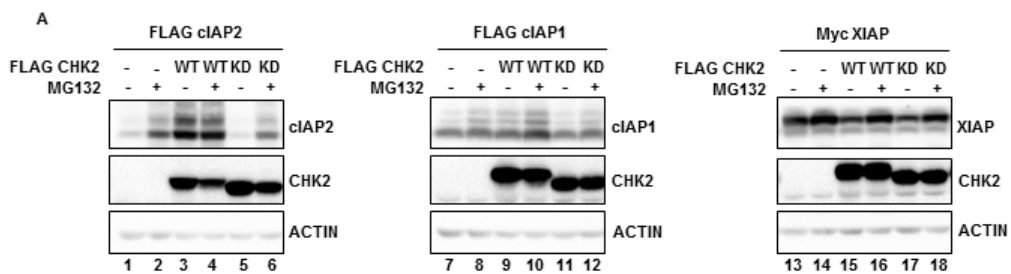
Fig. 2.18 CHK2 promotes cIAP2 accumulation which requires RING domain mediated cIAP2 degradation.

(A) HEK293T cells were transfected with plasmids encoding tagged forms of the indicated proteins for 18 h. Cells were then cultured in the presence of vehicle (-, DMSO) or 50 μM MG132 (+) for an additional 6 h. Cells lysates were subjected to Western blotting with the indicated antibodies.

(B) Schematic of domain structure of wild type and deletion mutant cIAP2

(C) HEK293 cells were transfected with plasmids encoding tagged forms of the indicated proteins for 18 h. Cells were then cultured in the presence of vehicle (-, DMSO) or 50 μM MG132 (+) for an additional 6 h. Cells lysates were subjected to Western blotting with the indicated antibodies.

A

FLAG cIAP2

control | CHK2 WT | CHK2 KD

CHX (min)   0 15 30 45 60   0 15 30 45 60   0 15 30 45 60

IP: FLAG   cIAP2

Total Lysate   CHK2
                                                               ACTIN

1   2   3   4   5   6   7   8   9   10   11 12 13 14 15

B

Relative levels of cIAP2

Control
CHK2 WT
CHK2 KD

min

C

FLAG cIAP1

Myc CHK2 WT | Myc CHK2 KD

CHX (h)   0   4   6   8   0   4   6   8   0   4   6   8

cIAP1
CHK2
ACTIN

1   2   3   4   5   6   7   8   9   10 11 12

D

Relative levels of cIAP1

Control
CHK2 WT
CHK2 KD

Hours

E

Myc XIAP

FLAG CHK2 WT | FLAG CHK2 KD

CHX (h)   0   4   6   8   0   4   6   8   0   4   6   8

**
*   XIAP
                                                               CHK2
                                                               ACTIN

1   2   3   4   5   6   7   8   9   10 11 12

* Endogenous   ** Ectopic

F

Relative levels of cIAP1

Control
CHK2 WT
CHK2 KD

Hours

120
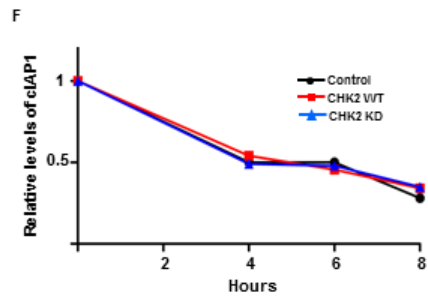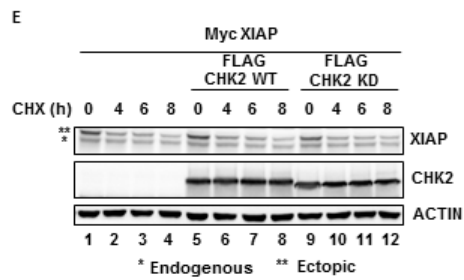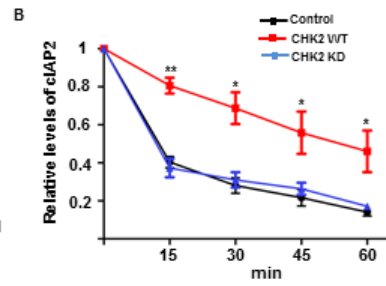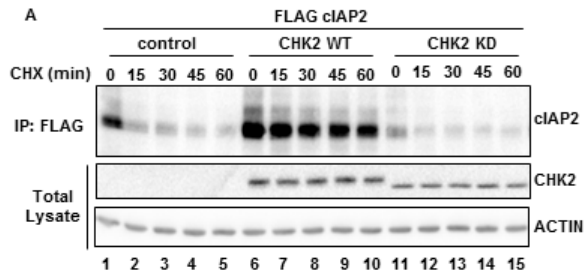
Fig. 2.19 CHK2 increases the half-life of cIAP2 but fails to alter the half-life of cIAP1 and XIAP

(A, B) HEK293 cells were transfected with plasmids encoding the indicated proteins for 24 h. Cells were then cultured in the presence of 10 μg/ml cycloheximide (CHX) for the indicated times. Cell lysates were prepared and either resolved directly by SDS-PAGE (Total Lysate) or were incubated with anti-Flag affinity gel and precipitates were resolved SDS-PAGE and analyzed by Western blotting. (A) cIAP2 levels were quantified using densiometry from 3 independent experiments like the one shown in panel A (B). Results are represented as mean (± s.e.m.). *p<0.05, **p<0.005

(C, D) HEK293T cells were transfected with plasmids encoding the indicated proteins for 24 h. Cells were then cultured in the presence of 10 μg/ml cycloheximide (CHX) for the indicated times. Cell lysates were prepared and subjected to Western blotting for the indicated antibodies (C). cIAP1 levels were quantified using densiometry from 3 independent experiments like the one shown in panel C (D). Results are represented as mean (± s.e.m.). ***p<0.005
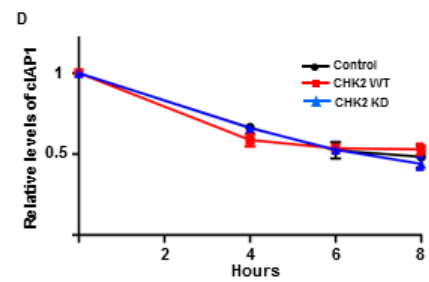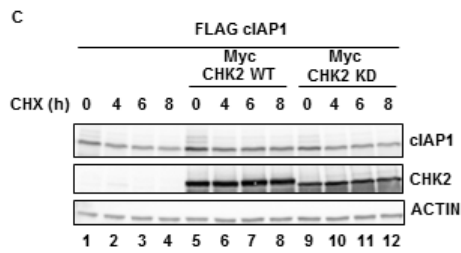
(E, F) HEK293T cells were transfected with plasmids encoding the indicated proteins for 24 h. Cells were then cultured in the presence of 10 μg/ml cycloheximide (CHX) for the indicated times. Cell lysates were prepared and subjected to Western blotting for the indicated antibodies (E). XIAP levels were quantified using densiometry like the one shown in panel C (F).

A

CHK2	–	–	WT		KD	
E1+E2+Ub	–	+	+	+	+	+
cIAP2	+	+	+	+	+	+

K-63 Ub

K-48 ub

Ub

cIAP2

CHK2

1 2 3 4 5 6 7 8 9 10 11 12

B

CHK2 inhibitor II   AZD7762

CHK2	–	–	WT	KD	–	–	WT	KD	–	–	WT	KD
E1+E2+Ub	–	+	+	+	–	+	+	+	–	+	+	+
cIAP2	+	+	+	+	+	+	+	+	+	+	+	+

K-63 Ub

K-48 Ub

Ub

cIAP2

CHK2

1 2 3 4 5 6 7 8 9 10 11 12
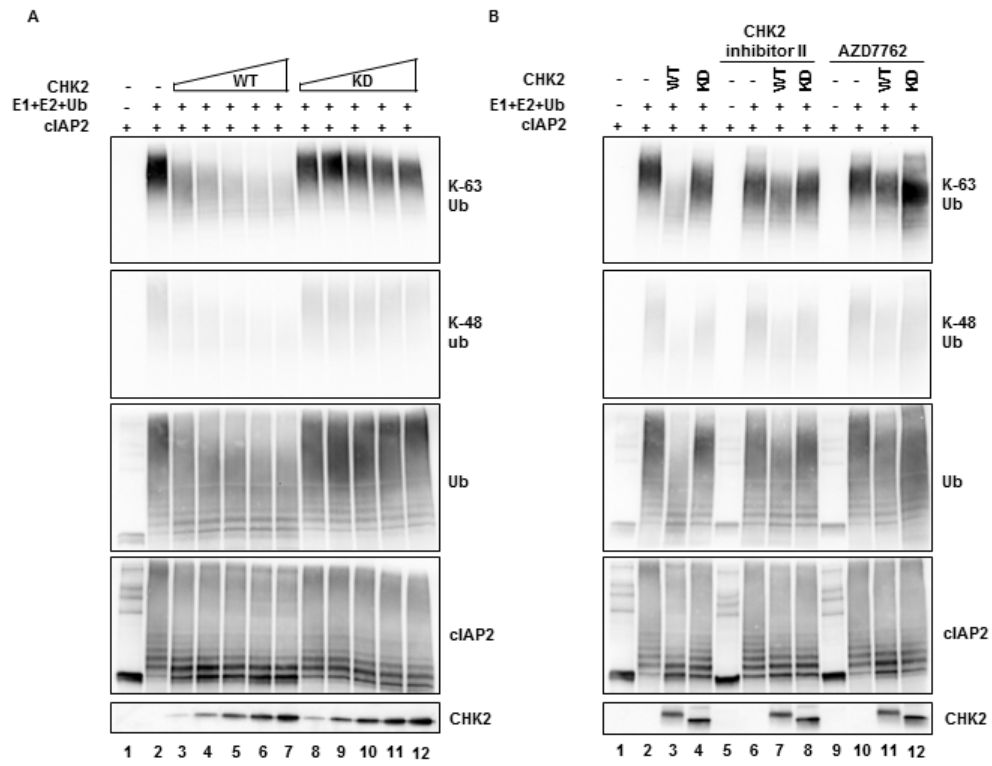
Fig. 2.20 CHK2 inhibits the E3 ubiquitin ligase activity of cIAP2 *in vitro*

(A) cIAP2 was incubated in the absence (-) or presence (+) of increasing concentrations of either CHK2 WT or CHK2 KD for 30 min in ubiquitination buffer at 30°C. Ubiquitination reactions were then carried out *in vitro* as described in Materials and Methods. Reactions were terminated by boiling in Laemmli sample buffer, resolved by SDS-PAGE and analyzed by Western blotting for the indicated proteins or protein modifications.

(B) cIAP2 was incubated in the absence (-) or presence (+) of either CHK2 WT or CHK2 KD with or without 2 mM of CHK2 inhibitor II or AZD7762, at 30°C for 30 min in ubiquitination buffer. Ubiquitination reactions were then carried out *in vitro* as described in the Materials and Methods. Reactions were terminated by boiling in Laemmli sample buffer, resolved by SDS-PAGE and analyzed by Western blotting for the indicated proteins or protein modifications.

A

Ub	WT Ub				K63R Ub				K48R Ub			
CHK2			KA	KD			KA	KD			KA	KD
E1+E2		+	+	+		+	+	+		+	+	+
cIAP2	+	+	+	+	+	+	+	+	+	+	+	+

K63 Ub

K48 Ub

Ub

cIAP2

CHK2

1   2   3   4   5   6   7   8   9   10   11   12

Fig. 2.21 CHK2 inhibits both K63- and K48-linked ubiquitin modification of cIAP2 *in vitro*

(A) cIAP2 was incubated in the absence (-) or presence (+) of either CHK2 WT or CHK2 KD at 30°C for 30 min in ubiquitination buffer. Ubiquitination reactions were then carried out *in vitro* with the indicated mutants of ubiquitin as described in the Materials and Methods. Reactions were terminated by boiling in Laemmli sample buffer, resolved by SDS-PAGE and analyzed by Western blotting for the indicated proteins or protein modifications.
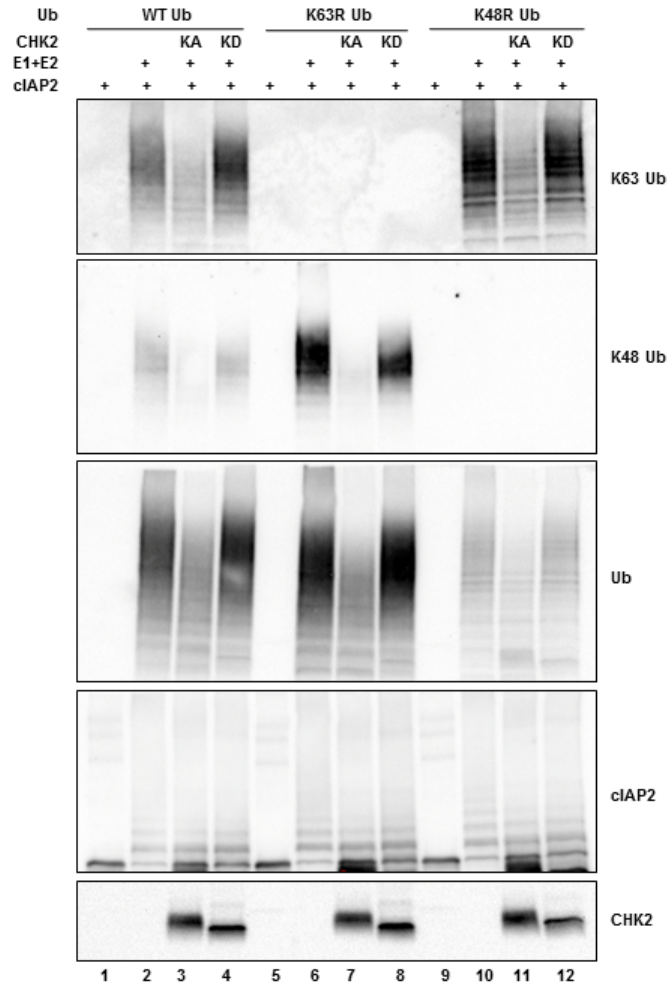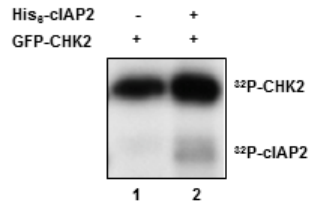
A

His₈-cIAP2   −   +
GFP-CHK2   +   +

^{32}P-CHK2

^{32}P-cIAP2

1   2

B

					*		*				*				
Homo sapiens cIAP2	S	C	E	L	Y	R	M	S	T	Y	S	T	F	P
Homo sapiens cIAP1	S	C	E	L	Y	R	M	S	T	Y	S	T	F	P
Homo sapiens XIAP	V	E	E	F	Y	R	L	K	T	F	A	N	F	P
Mus musculus cIAP2	S	C	E	L	Y	R	L	K	T	Y	S	T	F	P
Rattus norvegicus cIAP2	S	C	E	L	Y	R	L	K	T	Y	S	A	F	P
CHK2 consensus				L	X	R	X	X	S/T					

C

FLAG cIAP2 C557A   FLAG cIAP2 C557A/T35A

Myc-Cdc25C   +   −   −   +   −   −
Myc-CHK2   −   WT   KD   −   WT   KD

Total Lysate — Myc

— ACTIN

IP: FLAG — pT35 cIAP2

— cIAP2

1   2   3   4   5   6

126
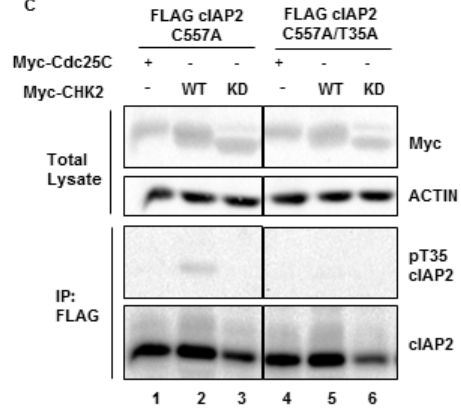
Fig. 2.22 CHK2 phosphorylates cIAP2 on Thr35

(A) GFP-tagged CHK2 was precipitated from 293T cells and incubated with

recombinant human cIAP2 in the presence of complete kinase buffer. Reactions were

resolved by SDS-PAGE and visualized by autoradiography.

(B) Sequence alignment of cIAP2 Thr35 and surrounding residues.

(C) HEK293T cells were transfected with plasmids encoding the indicated proteins for

24 h. Cell lysates were prepared and either resolved directly by SDS-PAGE (Total

Lysate) or were incubated with anti-Flag affinity gel and precipitates were resolved

SDS-PAGE and analyzed by Western blotting.

A

FLAG cIAP2 WT   FLAG cIAP2 T35A

FLAG CHK2   -   WT   KD   -   WT   KD
MG132   -   +   -   +   -   +   -   +   -   +   -   +

cIAP2

CHK2

ACTIN

1   2   3   4   5   6   7   8   9   10 11 12

B

FLAG cIAP2   T35A   T35A/S81A   T35A/S81/   T35A/S81/
                                             T175A   T175A/T261A
FLAG CHK2   -   WT   -   WT   -   WT   -   WT
MG132   -   +   -   +   -   +   -   +   -   +   -   +   -   +   -   +

cIAP2

CHK2

ACTIN

1   2   3   4   5   6   7   8   9   10 11 12 13 14 15 16

128

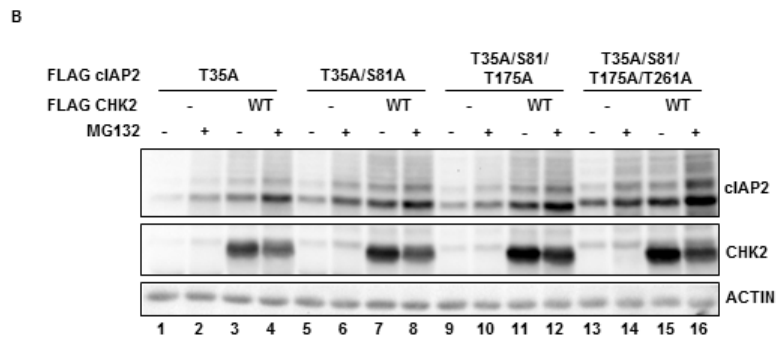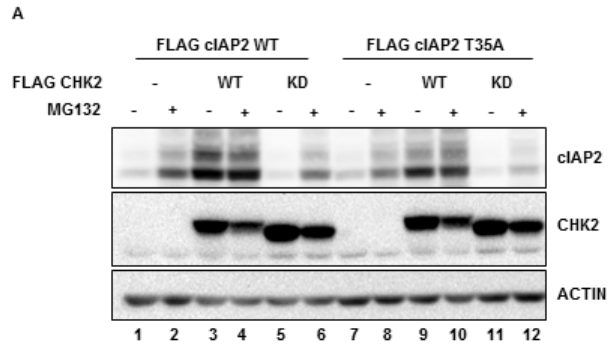**Fig. 2.23 Potential CHK2 target residues of cIAP2 do not play a role in CHK2-mediated cIAP2 stabilization**

(A, B) HEK293T cells were transfected with plasmids encoding tagged forms of the indicated proteins for 18 h. Cells were then cultured in the presence of vehicle (-, DMSO) or 50 μM MG132 (+) for an additional 6 h. Cells lysates were subjected to Western blotting with the indicated antibodies.
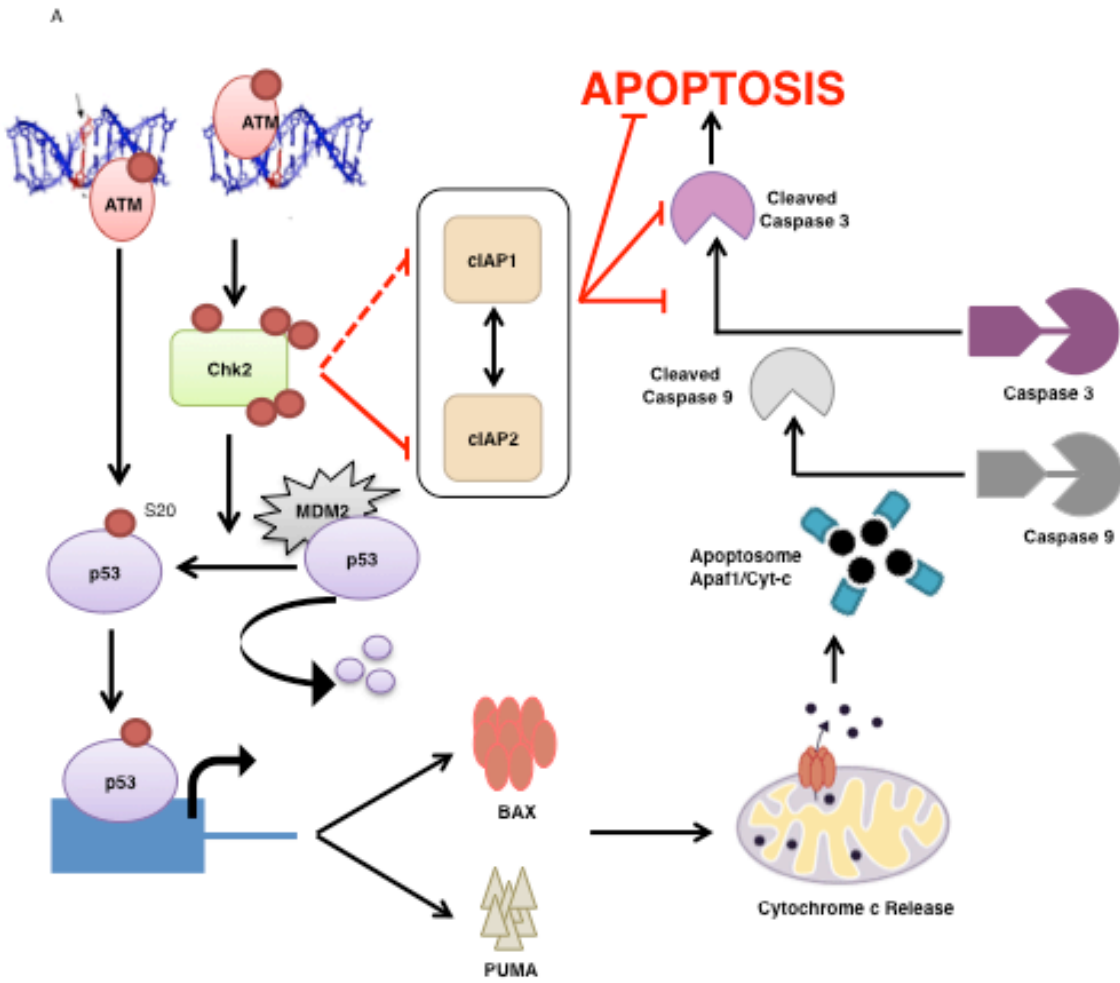
Fig. 2.24 CHK2 promotes VP16-induced apoptosis through phosphorylation of p53 and inhibition of cIAPs

(A) DNA DSBs activate ATM, which in turn phosphorylates CHK2. CHK2 homodimerizes, which leads to additional phosphorylation and activation. Activated CHK2 phosphorylates p53 on Ser20 leading to p53 accumulation. In cancer cells lacking CHK2 other kinases phosphorylate p53 on Ser20. p53 induces expression of the pro-apoptotic proteins BAX and PUMA. This leads to mitochondrial membrane permeabilization and release of cytochrome-c. Cytochrome-c interacts with Apaf-1 and activates the caspase cascade to execute apoptotic cell death. cIAPs oppose the apoptotic program and CHK2 binds to, phosphorylates and inhibits the E3 ubiquitin ligase activity of cIAP2 thereby sensitizing cells to p53 mediated apoptosis. In CHK2 deficient cancer cells the ability of DNA DSBs to induce p53-dependent apoptosis is impaired.

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Chapter 3:

Role of CHK2 in the extrinsic apoptotic pathway through the regulation of NFκB

(Unpublished Data)

3.1 Abstract

Chemotherapeutic agents are widely used in cancer treatment. Most chemotherapeutic drugs cause DNA damage and induce apoptosis either through mitochondrial permeabilization (intrinsic apoptosis) or death-receptor activation (extrinsic apoptosis). The checkpoint kinase CHK2, is activated in response to DNA damage and has traditionally been proposed to promote cell death through the mitochondrial pathway. Here, we show that CHK2 can potentially play a key role in death receptor-induced apoptosis through negative regulation of the NFκB pathway. In response to DNA damage, CHK2 inhibited early activation of NFκB and prevented upregulation of its pro-survival targets. CHK2 was also required for VP16-mediated sensitization of HCT116 cells to the TNFα superfamily member, TRAIL. Thus, CHK2 serves at the crossroad of the intrinsic and extrinsic apoptosis pathways and has a much broader role in cell death than its role in the regulation of the tumor suppressor protein, p53.

3.2 Introduction

Current cancer therapies, for example, chemotherapy and ionizing radiation (IR), exert their anti-tumor effect by triggering apoptosis in cancer cells. The checkpoint kinase, CHK2, plays a key role in DNA damage-induced apoptosis (Hirao et al., 2002; Hirao et al., 2000; Jallepalli et al., 2003; Lovly et al., 2008). CHK2 null mice are protected from whole body radiation, and over-expression of CHK2 can promote apoptosis in response DNA damaging agents like VP16 (Hirao et al., 2000; Lovly et al., 2008; Takai et al., 2002). Traditionally, it was proposed that CHK2 signals to the apoptotic machinery through regulation of the tumor suppressor, p53. Recent studies have challenged the idea that the only role of CHK2 in apoptosis is mediated through p53. I, along with several other groups have previously shown that cells lacking CHK2 are impaired in their ability to induce apoptosis despite normal p53 regulation (Ahn et al., 2000; Chen et al., 2005; Lopergolo et al.; Pereg et al., 2006). Moreover, CHK2 can promote apoptosis in a p53-independent manner through the regulation of downstream proteins like E2F-1, PML, Che1 and survivin (Bruno et al., 2006; Lee et al., 2000; Lopergolo et al.; Stevens et al., 2003; Venere et al., 2002; Yang et al., 2002). However, the role of these proteins as a CHK2 effector in response to DNA damage has not been well established. Previously, I demonstrated that CHK2 can bind to and phosphorylate cellular inhibitor of apoptosis protein, cIAP2, to inhibit its E3 ligase activity. Furthermore, CHK2-mediated inhibition of cIAPs is essential for DNA damage-induced apoptosis in p53 proficient cell lines. cIAPs are members of the Inhibitor of Apoptosis (IAP) family that serve as negative regulators of apoptosis. Although, cIAPs were initially shown to inhibit the

145

caspase activity directly, recent studies have indicated that cIAPs are unable to regulate active caspases under physiological conditions. However, cIAPs can ubiquitinate cleaved forms of effector caspase and target them for proteosomal degradation. cIAPs can also inhibit apoptosis by regulating the activation of the NFκB family of transcription factors. Moreover, the cIAP2 promoter is highly responsive to NFκB activity and helps in setting a "feed-forward" activation loop that actively promotes cell survival (Chu et al., 1997; Mahoney et al., 2008a). Here we identified CHK2 as a novel regulator of NFκB pathway. CHK2 inhibits NFκB activation through inhibition of the E3 ubiquitin ligase activity of cIAPs.

The NFκB family contains five family members: p50, p52, RelA, RelB and c-Rel. While all the members of the NFκB family share a Rel homology domain in their N-terminus, a subfamily including RelA, RelB and c-Rel also contain a trans-activation domain in their C-terminal protein domain. NFκB dimers bind to κB sites within the promoters/enhancers of target genes and regulate transcription through the recruitment of co-activators and co-repressors. The NFκB pathway is divided into two distinct pathways: a) the canonical pathway, and b) the non-canonical pathway. In this chapter I will focus on the canonical pathway and the role that cIAPs play in this pathway.

The canonical NFκB pathway is activated by various stimuli including DNA damage, Tumor Necrosis Factor α (TNFα), TNF Related Apoptosis inducing Ligand (TRAIL), Fas ligand (FasL) and others. All these stimuli eventually converge on the activation of the inhibitors of κB (IκB) kinase (IKK) complex. The hetero-trimeric IKK complex consists of two catalytic subunits, IKKα and IKKβ, and a regulator unit, IKKγ, which is also known as NFκB Essential Modifier (NEMO). Upon activation, IKKα or

146

IKKβ, phosphorylate the IκB inhibitor protein, IκBα (DiDonato et al., 1996; Mercurio et al., 1997; Zandi et al., 1997). Normally, IκBα masks the nuclear localization sequence (NLS) of NFκB transcription factors and retains them in the cytosol (Beg et al., 1995; Henkel et al., 1992). Upon phosphorylation by the IKK on Ser32, IκBα is recognized by the SCPβ^{TrCP} E3 ubiquitin ligase and targeted for proteosomal destruction (Brown et al., 1995; Chen et al., 1995; DiDonato et al., 1996). As a result the NFκB transcription factors like p50/p65 move into the nucleus where they promote and inhibit transcription of a wide variety of genes.

Different stimuli activate IKK through distinct mechanisms. One of the most characterized activators of NFκB is TNFα. TNFα is a pleiotropic cytokine that signals through the TNFα receptor 1 (TNFR1). Upon TNFα binding, TNFR1 rapidly recruits TNFR-associated death domain (TRADD). In turn, TRADD recruits Receptor Interacting Protein 1(RIP1) (Hsu et al., 1996a) and TNFR associated factor 2 (TRAF2) (Hsu et al., 1996b; Hsu et al., 1995) to the membrane. TRAF2 binds to the BIR1 domain of cIAPs (Rothe et al., 1995; Samuel et al., 2006; Vince et al., 2009) and brings them into the complex where cIAPs promote K-63 linked ubiquitination of RIP1 (Bertrand et al., 2011; Bertrand et al., 2008). This complex of TRAF2/cIAPs/RIP1 is often referred to as complex I. Ubiquitinated RIP1 interacts with both TAK1 and NEMO and serves as the scaffold to bring NEMO and TAK1 in close proximity (Bertrand et al., 2008). TAK1 phosphorylates IKKα/β in their activation loops to activate their kinase activities and drive nuclear translocation of NFκB transcription factors. In the absence of cIAPs, RIP1 associates with Fas associated death domain protein (FADD) and caspase-8, also known as complex II, that leads to caspase-8 activation and apoptosis (Feoktistova et

al.; Ikner and Ashkenazi; Park et al.). Caspase-8 can directly cleave the effector caspase-3. Caspase-8 can also amplify the apoptotic signal through Bid cleavage and Bax-mediated mitochondrial permeabilization (Li et al., 1998). NFκB can inhibit the complex II and caspase-8 activation by increasing the transcription of cFLIP. cFLIP competes with caspase-8 for FADD binding and prevents oligomerization-induced activation of caspase-8 (Irmler et al., 1997; Krueger et al., 2001). In the absence of cIAPs, RIP1 can also bind to RIP3 and induce caspase-independent cell death known as necroptosis (Cho et al., 2009; Feoktistova et al.; Holler et al., 2000). While TNFα primarily activates pro-survival signal through NFκB, TRAIL promotes complex II formation and caspase-8 activation.

Cells also activate the pro-survival signals of NFκB in response to DNA damage (Criswell et al., 2003; Huang et al., 2003; Li et al., 2001). Inhibition of NFκB promotes apoptosis in cells with damage. However, DNA damage-induced NFκB activation does not require assembly of a protein complex at the cell membrane but rather gets activated in the nucleus, and is then transported to the cytoplasm. DNA DSBs activate IKK through the formation of RIP1/NEMO/TAK1 complex without the requirement of membrane receptors (Wu et al.; Yang et al.). However, a universal requirement of RIP1 for DNA DSB-induced NFκB activation has recently been debated (Hinz et al.; Wong et al.). cIAP1 and cIAP2 play a critical and non-redundant role in the activation of NFκB in response to DNA DSBs (Jin et al., 2009). While cIAP1 is required for mono-ubiquitination of NEMO, an essential step in NFκB activation, cIAP2 regulates a downstream process (Feoktistova et al.; Jin et al., 2009).

In this study, I demonstrated that loss of CHK2 activates NFκB in the early stages of DNA damage and alters the ability of NFκB to promote the transcription of pro-survival genes (*cIAP2*) rather than the transcription of pro-apoptotic genes (*TNFα and IL-8*). I also demonstrated that CHK2 plays an essential in synergistic activation of cell death in response to VP16 and TRAIL treatment through it ability to regulate cIAP2.

3.3 Materials and Methods

Cell Culture and Treatments

HCT116 parent, HCT116 CHK2$^{-/-}$ and HCT116 p53$^{-/-}$ cells were obtained from The Core Cell Center at Johns Hopkins University. HCT116 cells were maintained in McCoys 5A medium (Life Technologies) supplemented with 10% FBS. MCF7 cells were maintained in DMEM supplemented with 10% FBS and 1% L-Glutamine. In some cases cells with treated with complete media containing the indicated concentration of VP16 (Sigma-Aldrich). For inhibition of NFκB, IKK inhibitors, BAY 11072 (Sigma-Aldrich) and BMS 35541 (Sigma-Aldrich), cells were treated with supplemented media to a final concentration of 7.5 μM or 10 μM. For treatment with caspase 3 inhibitor, zDEVD-FMK (BD Bioscience), caspase 8 inhibitor, zIETD-FMK (BD Bioscience), and RIP1 kinase inhibitor, necrostatin 1 (Nec-1)(Sigma-Aldrich), cells were treated with complete media containing respective amount of the inhibitor for four hours. Cells were then supplemented with complete media containing DMSO or VP16 to a final concentration of 25 μM. For blocking TNFα signaling, cells were incubated with complete media containing VP16 alone or VP16 and TNF receptor soluble factor 1A (TNFRSF1A-Fc) (R&D systems) for 48 h. In some cases, 24 h after VP16 treatment, cells were supplemented with complete media containing TNFRSF1A-Fc to a final concentration of 10 μg/mL. For TRAIL-induced apoptosis, cells were grown in complete media supplemented with 10μM VP16 for 48 h. Cells were supplemented with 100ng/mL TRAIL (Milipore) for final 24 h.

Western blotting, Antibodies and Immunoprecipitation

Cells were lysed in mammalian cell lysis buffer (MCLB (50 mM Tris HCl pH 8.0, 2 mM DTT, 5 mM EDTA, 0.5% NP-40, 100 mM NaCl,) supplemented with 1 mM microcystin (Sigma-Aldrich), 1 mM sodium orthovanadate (Sigma-Aldrich), 2 mM PMSF (Sigma-Aldrich), protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Calbiochem). Clarified lysates were resolved directly by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. Membranes were blocked in TBST (20 mM Tris HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk for 1 h and incubated overnight in primary antibody diluted in TBST/5% nonfat milk. Membranes were washed in TBST, incubated with HRP-conjugated secondary antibody for 1 h in TBST/5% nonfat milk, and washed again in TBST. Membranes were incubated with ECL (Pierce) and analyzed using a GelDoc Imaging System (BioRad). Cleaved caspase -3, XIAP, and cIAP2 antibodies were from Cell Signaling. CHK2 antibody was from EMD Milipore. Actin and Tubulin were from Sigma-Aldrich. cIAP1 antibody was from R & D system. Endogeous cIAP2 in HCT116 and MCF7 cells was detected using cIAP2 antibody from BD Biosciences and R&D system respectively. β-catenin antibody was from BD Biosciences, and GAPDH antibody was from Advanced ImmunoChemical.

Transfections

HCT116 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, HCT116 cells were plated at a density of 1.5 x 10^6 cells/6 cm dish. The next day, 2 - 2.5 μg of total plasmid was mixed with 500 μl of Opti-MEM media in tube 1 and incubated at room temperature for 5 min. In tube 2, 6 - 7.5 μL of Lipofectamine 2000 was mixed with 500 μL of Opti-MEM media and incubated at

room temp for 5 min. After 5 min, tube 1 and tube 2 were mixed and incubated at room temperature for 20 – 30 min. After the incubation, the transfection cocktail was added to the plates containing 2 mL of complete media. 24 h post-transfection, the cells were washed with 1X PBS and media was replaced. For RNAi transfections, Luciferase GL3 duplex and SMARTpool siRNAs specific to NEMO and RIP (ThermoScientific) were transfected using DharmaFECT siRNA transfection reagent 2 (ThermoScientific). Briefly, HCT116 cells were plated at a density of 0.3×10^6 cells/60mm dish for The next day transfection. For transfection, 7.5 μl of 20 μM siRNA was mixed with 300 μl of Opti-MEM media in tube 1, and 6 μl of Dharmafect 2 was mixed with 300 μl of Opti-MEM media in tube 2. Both the tubes were incubated at the room temperature for 5 min. Tube 1 was mixed with tube 2 and incubated for an additional 30 min. Meanwhile, the media on the cells was replaced by 2.5 mL of complete media. Post-incubation, the transfection cocktail was added to the cells in a dropwise manner. 24 h post-transfection, the media was replaced by 4 mLs of complete media.

Stable cell lines were generated by using lentivirus infection as described previously (Stewart et al., 2003). Briefly, HEK293T cells were plated at 6×10^5 cells/6 cm dish the day before transfection. The next day, cells were transfected with a mixture of 1 μg viral DNA encoding the gene of interest and 1 μg pHR'8.2deltaR packaging plasmid at a ratio of 8:1 with pCMV-VSV-G envelope plasmid using Mirus LTR1 transfection reagent (Mirus). Target cells were plated at 1×10^6 cells/10 cm dish the day before infection with the lentivirus. After HEK293T cells were transfected with viral DNA for 48 h, target cells were infected in the presence of 10 μg/ml protamine sulfate for 4 h. Virus was then removed and media was replaced. Infected cells were allowed

to recover for 24 h and then were re-infected with lentivirus. Infected cells were trypsinized and plated in media containing puromycin 48 h after the first infection with lentivirus.

Cell Death and Caspase-3 Activity Assays

Cell Death was assessed using propidium iodide staining and fluorescent associated cell sorting (FACS) as described previously (Lovly et al., 2008). For identifying Annexin V and PI double positive cells, Annexin V : FITC Apoptosis Detection Kit I (BD Biosciences) was used as per manufacturers protocol. Caspase-3 activity was measured by utilizing the caspase-3 fluorogenic substrate Ac-DEVD-AFC (SantaCruz Biotechnology). The cells were lysed in mammalian cell lysis buffer (MCLB: 50 mM Tris HCl pH 8.0, 100 mM NaCl, 2 mM DTT, 5 mM EDTA, 0.5% NP-40) and incubated with 5 μg of the peptide at 37°C. The fluorescence was measured over time for a period of 150 min using FLUOstar Optima (BMG Labtech) and the data was analyzed using FLUOstar version 1.30R3 (BMG Labtech).

NFκB activity luciferase assay

HCT116 cells were transiently transfected using Lipofectamine 2000 with 0.5 μg of $\kappa B_5 \rightarrow$FLuc (ref) and 2μg of pRL-TK. Cells were allowed to recover overnight and then plated in black-walled 96-well plates (Fischer Scientific). The next day, cells were exposed to VP16 or vehicle (DMSO) for the specified durations and indicated concentrations. Bioluminescence measurements were acquired in an IVIS 100 imaging system using a final concentration of 150 μg/ml of D-Luciferin for firefly luciferase and 400 nM of coelenterazine for renilla luciferase.

Quantitative PCR

Total RNA was isolated from the cells using RNeasy mini (Qiagen). 2 μg of total RNA was reverse transcribed to synthesize cDNA using the SuperScript III First Strand Synthesis kit (Invitrogen). Real time PCR was performed with TaqMan Gene Expression Probe (Life Technologies) for cIAP1 (Hs01112284_m1), cIAP2 (Hs00985031_g1), TNFα (Hs01113624_g1), and IL-8 (Hs00174103_m1) using Bio-Rad CFX96 PCR instrument (95°C for 10 min, then 40 cycles of 95°C for 30 s, 61°C for 1 min, and 74°C for 1 min).

3.4 Results

CHK2 inhibits NFκB-induced pro-survival signals

NFκB is known to promote cell survival in response to DNA damage and IAPs are critical regulators of the NFκB pathway in response to a wide variety of stimuli. Previously, we demonstrated that cIAPs provide anti-apoptotic activity in p53 proficient cancer cells that are deficient in CHK2 (see Chapter 2). In this study we asked, if cIAPs provided anti-apoptotic activity in CHK2 deficient cells through the regulation of the NFκB pathway.

Proteosomal degradation of IκBα allows the NFκB dimers to translocate into the nucleus and activate the transcription of their target genes (Beg et al., 1995; Henkel et al., 1992). Thus, we monitored IκBα levels in VP16-treated HCT116 cells as a read out for the activation of the NFκB pathway. CHK2$^{-/-}$ HCT116 contained lower levels of IκBα protein at baseline compared with parental HCT116 cells (Fig. 3.1A, compare lanes 1 and 7) and IκBα was more quickly lost in CHK2 null cells following VP16 treatment (Fig. 3.1A). Interestingly, after VP16 treatment, the anti-apoptotic protein, cIAP2, accumulated in CHK2$^{-/-}$ HCT116 cells (lanes 8-12) but not in parental cells (lanes 2-6). On the other hand, in both parental and CHK2 null HCT116 cells, VP16 induced a reduction in cIAP1 and XIAP levels in a dose-dependent manner (Fig. 3.1A).

Although IκBα levels fell in both parental and CHK2$^{-/-}$ HCT116 cells after 48 h of VP16 treatment (Fig. 1A), quantitative RT-PCR revealed differential upregulation of NFκB-target genes like TNFα, and cIAP2 in the two cell lines (Fig. 3.2B). Following VP16 treatment, activation of pro-apoptotic genes like TNFα and IL-8 was much more robust in parental compared with CHK2 null HCT116 cells (Fig. 3.2B). By contrast,

activation of the anti-apoptotic cIAP2 gene was more robust in CHK2 null cells compared to parental HCT116 cells (Fig. 3.2B). As noted earlier, a corresponding increase in cIAP2 protein was also observed in VP16-treated CHK2$^{-/-}$ HCT116 cells (Fig. 3.2A, lanes 7 – 12). Since it has been previously shown that early activation of NFκB pathway has an anti-apoptotic role, while late activation of NFκB plays a pro-apoptotic role (Biton and Ashkenazi), we evaluated the NFκB pathway at earlier time points after VP16 treatment. Interestingly, after VP16 treatment for 24 h, CHK2$^{-/-}$ HCT116 cells had significantly lower levels of IκBα protein when compared to VP16 treated parental HCT116 cells (Fig. 3.2C). This indicated that NFκB pathway is activated in CHK2$^{-/-}$ HCT116 cells earlier than in parental cells. To confirm this we utilized a bioluminescence based NFκB reporter system described previously. The reporter plasmid was transiently expressed in the cells followed by VP16 treatment. Similar to IκBα levels, CHK2$^{-/-}$ HCT116 cells showed higher bioluminescence at basal level when compared to parental HCT116 cells (Fig. 3.2D). Upon VP16 treatment, bioluminescence in CHK2$^{-/-}$ HCT116 cells increased significantly more when compared to the increase observed in parental cells. However, this difference was not observed when cells were treated with higher concentration of VP16 (100 and 200μM). Similar results were observed when bioluminescence was assessed after 12 h of VP16 treatment (Fig. 3.2E).

NFκB pathway does not contribute to VP16-induced apoptosis in HCT116 cells

Due to differential activation of NFκB target genes in VP16 treated parental and CHK2$^{-/-}$ HCT116 cells, we assessed the role of NFκB in these cells. We pre-treated cells with the IKK inhibitor, BAY 11072, to inhibit the NFκB pathway and then followed with VP16

treatment (Fig. 3.2). 48 h of treatment with BAY 11072 induced significant death at baseline in both parental and CHK2 null cells (Fig. 3.2A). Therefore, we incubated the cells with two different IKK inhibitors, BAY 11072 (Fig. 3.2B, C and D) and BMS345541 (Fig. 3.3) for the final 24 h of the VP16 treatment. VP16 caused a reduction in IκBα levels (Fig. 3.2B and Fig. 3.3A compare lane 1, 4, 7, and 10) and an increase in Ser532 phosphorylation of p65 subunit of NFκB dimer (marker for NFκB transcriptional activation)(Fig. 3.3A) in both parental and CHK2$^{-/-}$ HCT116 cells. Pre-treatment with BAY 11072 (Fig. 3.2B lanes 4 – 6 and 10 - 12) and BMS 345541 (Fig. 3.3B lanes 4-6 and 10-12) rescued IκBα levels in HCT116 cells in a dose dependent manner.

Consistent with our previous observation, VP16 induced cIAP2 accumulation in CHK2$^{-/-}$ HCT116 (Fig. 3.2B, 3.3A compare lane 7 and 10). However, CHK2 null cells failed to increase cIAP2 levels in the presence of either IKK inhibitors (Fig. 3.2B, Fig. 3.3A). This suggested that following VP16 treatment, NFκB activation is required for cIAP2 upregulation in CHK2$^{-/-}$ HCT116 cells. Although inhibition of IKK reduced cIAP2 accumulation in CHK2 null HCT116 cells, it failed to alter the cleaved caspase 3 activity (Fig. 3.2C, 3.3B) or apoptosis (Fig. 3.2D). However, BMS 354451 treatment marginally increased apoptosis in both parental and CHK2$^{-/-}$ HCT116 cells. However, cell death was still lower in CHK2 null cells compared to parental cells (Fig. 3.3C).

To inhibit the NFκB pathway throughout VP16 treatment, we transiently knocked down key components of the pathway, including RIP1 (R1) or NEMO (N1), using a pool of 4 different siRNAs targeting their respective proteins. RIP1 (Fig. 3.4A, lanes 2, 5, 8, and 11) and NEMO (Fig. 3.4A. lanes 3, 6, 9, and 12) were both efficiently knocked down in HCT116 cells. Knockdown of NEMO, but not RIP1, abolished the VP16-induced

reduction in the IκBα levels (Fig. 3.4A). This observation is consistent with a previous

report that RIP1 is dispensable for DNA-damage induced NFκB activation (Hinz et al.).

Loss of NEMO prevented the accumulation of cIAP2 in VP16 treated CHK2 nulls cells

(Fig. 3.4A, compare lanes 10 and 12). RIP1 loss had no impact on cIAP2 levels (Fig.

3.4A, lanes 9 and 10). This supports our earlier observation that the NFκB pathway is

required for VP16-induced upregulation of cIAP2 in CHK2$^{-/-}$ HCT116 cells. Knockdown

of RIP1 failed to alter apoptosis or cleaved caspase 3 activity in either parental or

CHK2$^{-/-}$ HCT116 cells (Fig. 3.4B, C). On the other hand, knockdown of NEMO reduced

the cleaved caspase 3 activity in both parental and CHK2$^{-/-}$ HCT116 cells with only a

small reduction in apoptosis of parental cells (Fig. 3.4B, C). However, apoptosis and

cleaved caspase 3 activity in CHK2 null cells remained lower compared to parental cells

even after NEMO loss. The results obtained with NEMO and RIP1 knockdown were

confirmed with individual siRNAs present in the pooled siRNA cocktail. All NEMO

specific siRNAs, with the exception of N2, were able to deplete NEMO resulting in

stabilization of IκBα (Fig. 3.5A lanes 3 – 6 and 9 – 12). Like the pooled siRNAs for

NEMO, individual siRNAs had almost no effect on apoptosis (Fig. 3.5B) but did result in

a reduction in cleaved caspase -3 activity in both parental and HCT116 cells (Fig. 3.5C).

On the other hand, individual siRNAs targeting RIP1 (R1-4) were each capable of

depleting RIP1 although to varying degrees. siRNA-R1 was the most efficient at

depleting RIP1 levels *in vivo* (Fig. 3.5D lanes 3 and 9). RIP1 knockdown had no effect

on apoptosis (Fig. 3.5E) or cleaved caspase 3 activity (Fig. 3.5F).

TNFα does not contribute to VP16-induced apoptosis in HCT116 cells

Next we asked if upregulation of pro-apoptotic genes (*TNFα* and *IL-8)* played a role in promoting apoptosis in VP16 treated parental HCT116 cells. Upon binding to its cell surface receptors, TNFα promotes the formation of FADD, RIP1 and caspase-8 complex, also known as complex II. Additionally, in response to TNFα stimulation, RIP1 can associate with RIP3 to induce caspase-independent necrosis (Cho et al., 2009; Feoktistova et al.; Holler et al., 2000). Thus, we treated HCT116 cells with a caspase-8 inhibitor (Casp8 in) and a RIP1 kinase inhibitor (Nec-1) alone or in combination and monitored cleaved caspase 3 activity and cell death. As seen in Fig. 3.6, VP16 induced cleaved caspase 3 activity, as well as apoptosis, in the HCT116 cells. CHK2 null cells had lower caspase 3 activity and underwent less cell death when compared to parental cells. Pre-treatment with caspase-3 inhibitor completely inhibited caspase-3 activity (Fig. 3.6A), but did not alter cell death in either parental or CHK2 null cells (Fig. 3.6B). Nec-1 decreased caspase-3 activity in both the parental and CHK2 null cells. Interestingly, inhibition of caspase-8, both in the presence or absence of Nec-1, completely abolished caspase 3 activity in parental and CHK2 null cells. This suggests that caspase-8 is required for the activation of the effector caspase 3. Cell death, as measured by annexin and PI double positive cells, decreased in parental cells in the presence of caspase-8 inhibitor and Nec-1 alone and levels of cell death were not further decreased when the two inhibitors were added together. Cell death in CHK2 null cells was unaltered under identical experimental conditions.

Since, caspase-8 inhibitor was successful in inhibiting caspase-3 activation, we wanted to test whether TNFα was responsible for cell death in the VP16 treated parental cells. We were unable to detect any TNFα in the supernatant of cultures of

parental cells before or after VP16 treatment. This could be due to the fact that TNFα

levels were below the level of detection in the ELISA. To test this, we incubated cells

with soluble TNF receptor (TNFRS1) to prevent any TNFα that may have been in the

culture supernatant from signaling back into the cells. As seen in Fig. 3.7, treatment of

cells with TNFRS1 for 48 h (Fig. 3.7) or during the final 24 h prior to harvest (Fig. 3.7)

had no effect on IκBα or cIAP2 levels (panel A). Caspase-3 activity (panel B) and cell

death (panel C) were also unaltered under both conditions.

Taken together these results suggest that HCT116 cells do not secrete TNFα in

response to VP16 treatment. However, caspase-8 was required for caspase-3

activation.

CHK2 is required for synergistic effect of VP16 and TRAIL cell death

Our data demonstrated that CHK2 inhibits NFκB activity but this regulation is

dispensable for VP16-induced apoptosis. Therefore, we wanted to explore CHK2-

mediated regulation of NFκB in a different system. TRAIL is a member of the TNF

superfamily and induces apoptosis in various cancer cell lines. However, some cancer

cell lines are resistant to TRIAL but can be sensitized using chemotherapeutic agents

(Keane et al., 1999). It has previously been reported that HCT116 BAX$^{-/-}$ cells are

resistant to TRAIL but pre-treatment of these cells with VP16 can sensitize them to

TRAIL-mediated cell death (Wang and El-Deiry, 2003). Thus, we next tested the role of

CHK2 in VP16-induced sensitization of BAX null cells to TRAIL. We stably knocked

down CHK2 in BAX null HCT116 cells and treated the cells with 10 μM VP16 for 24 h followed by treatment with 100 ng/mL TRAIL for another 24 h (Fig. 3.8A). As seen in Fig. 3.8, VP16 and TRAIL treatment alone failed to induce cleaved caspase-3 activity or apoptosis (measured by sub-G1 DNA content) in BAX null HCT116 cells. However, pre-treatment of BAX-null cells with VP16 significantly enhanced TRAIL-induced cleaved caspase-3 activity (Fig. 3.8B) and apoptosis (Fig. 3.8C). Since, cIAP2 can provide anti-apoptotic signals in response to TRAIL treatment, we monitored cIAP2 protein in BAX null HCT116 cells after VP16 and TRAIL treatment. Interestingly, we observed differential regulation of cIAP2 in the BAX null cells expressing control shRNA compared with those expressing CHK2 shRNA. cIAP2 accumulated in both control and CHK2 shRNA expressing cells that had been treated with TRAIL (Fig. 3.8A lanes 2 and 6). VP16 failed to significantly effect cIAP2 levels (Fig. 3.8A lanes 3 and 7). However, pre-treatment with VP16, prevented TRAIL-induced accumulation of cIAP2 in BAX null cells expressing GFP shRNA (Fig. 3.8A compared lanes 2 and 4) but not in cells expressing CHK2 shRNA (Fig. 8A compared lanes 6 and 8).

Taken together these results suggest that CHK2 is essential for VP16-mediated sensitization of cells to TRAIL-mediated cell death. In response to VP16 treatment, CHK2 prevents TRAIL-induced cIAP2 upregulation and thereby promotes apoptotic signals within the cells.

3.5 Discussion

In response to various stimuli like, DNA damage, TNFα and TRAIL, cIAPs exert their anti-apoptotic effects through the regulation of the NFκB pathway (Dai et al., 2009; Hinz et al.; Jin et al., 2009; Mahoney et al., 2008b). cIAPs ubiquitinate several key proteins in the NFκB pathway to promote its activation. In turn, cIAPs are themselves transcriptional targets of NFκB dimers. This forms a positive feed-forward loop to establish a strong pro-survival signal. Previously, we have shown that CHK2 inhibits the anti-apoptotic signals of cIAP2 to promote chemotherapy-induced cell death in p53 proficient cell lines (refer chapter 2). In this study we demonstrated that CHK2 can regulate the NFκB pathway and inhibit its early activation in response to VP16 treatment. However, CHK2 mediated regulation of NFκB was dispensable for VP16-induced apoptosis. Furthermore, we have uncovered a potential role of CHK2 in the extrinsic apoptotic pathway. We demonstrated that CHK2 is essential for VP16-mediated sensitization of cells to TRAIL-mediated cell death.

Although NFκB is thought to primarily promote cell survival, some studies have uncovered pro-apoptotic functions of NFκB as well. Recently, Biton *et. al.* (2011) showed that DNA damage activates NFκB in two waves. In the initial stages of DNA damage, NFκB provides anti-apoptotic signal and promotes cell survival. However, upon prolonged exposure to DNA damage, cells secrete TNFα in a NFκB-dependent manner to promote apoptosis. Similar to the observation made by Biton*et. al (2011),* we observed that in CHK2 null HCT116 cells, NFκB was activated at early stages of DNA damage (12 and 24h) (Fig. 3.1C, D, E) and activated the anti-apoptotic gene, *cIAP2*

(Fig. 3.1A, B). On the other hand, in parental cells, NFκB was activated at later stages (48 h) and induced the transcription of the pro-apoptotic genes, *TNFα and IL-6*. Thus we hypothesized that CHK2 prevents early activation of NFκB and drives the cells towards cell death.

Although, loss of CHK2 altered the ability of NFκB to activate its pro-apoptotic genes, inhibition of NFκB through two distinct IKK inhibitors, BAY-11072 and BMS-345541, failed to rescue cell death or cleaved caspase activity in CHK2 null cells (Fig. 3.2, 3.3). Moreover, depletion of NEMO (Fig. 3.4, 3.5) was also not sufficient to increase cell death in cells lacking CHK2. Surprisingly, NEMO knockdown reduced cleaved caspase activity (Fig. 4B, 5C) in both the parental and CHK2 null cells. Similar observations were made upon depletion of cIAP2 in HCT116 cells (Fig. 2.13). Knockdown of NEMO reduced basal levels of cIAP2 protein in HCT116 cells, as well as in VP16-induced levels in CHK2 null cells. It should be noted that loss of NEMO did not completely remove cIAP2 protein from HCT116 cells. Inhibition of caspase-3 activity was more prominent upon cIAP2 depletion compared with NEMO depletion. Thus, decreases in caspase-3 activity in response to NEMO depletion might be driven, in part, through reduction in cIAP2 levels. To our knowledge, a reduction in apoptosis in response to cIAP2 loss has not been previously reported. We predict that this observation may be a result of close association between cIAP1 and cIAP2. Under normal conditions, cIAP1 targets cIAP2 for proteosomal degradation and loss of cIAP1 increases cellular levels of cIAP2 (Fig. 2.13). Thus cIAP2 might keep cIAP1 away from its other substrates. Upon cIAP2 loss, a pool of cIAP1 that was earlier bound to cIAP2 might become available to inhibit apoptosis. In support of this hypothesis, concomitant

163

loss of cIAP1 and cIAP2 does not impact cleaved caspase 3 activity in parental HCT116 cells (Chapter 2 Fig. 10). However, further experiments would be required to test this hypothesis.

We observed that inhibition of caspase 8 suppressed caspase 3 activity in HCT116 cells (Fig. 3.6). VP16 induced transcription of the NFκB target gene, *TNFα* (Fig. 3.1). Since TNFα induces cell death through activation of caspase-8, inhibition of NFκB through loss of NEMO might reduce TNFα release, and thereby reduce cell death. However, we were unable to detect any TNFα in the supernatant of cultured HCT116 cells and blocking of TNFα signaling did not affect cell death (Fig. 3.7). It has been previously reported that although DNA damaging agents upregulate members of TNFα superfamily, they do not contribute to caspase-8 activation or cell death (Milner et al., 2002).

Although CHK2 mediated regulation of NFκB was dispensable for VP16-induced apoptosis, we found that CHK2 was required for VP16-mediated sensitization of BAX null cells to TRAIL. In resistant cells, TRAIL activates NFκB and upregulates cIAP2 expression (Kim et al.; Lin et al., 2000). Inhibition of NFκB or cIAP2 can sensitize resistant cells to TRAIL treatment (Bockbrader et al., 2005; Franco et al., 2001; Jonsson et al., 2003; Kim et al., 2002; Li et al., 2004; Seo et al.). VP16-treatment inhibited TRAIL-induced cIAP2 levels and induced cell death. However, loss of CHK2 impaired VP16-mediated inhibition of cIAP2 accumulation and reduced TRAIL-mediated cell death. Thus, we hypothesized that VP16 activates CHK2, which in turn, can inhibit NFκB and promote TRAIL-induced cell death.

Overall, this study expands the role of CHK2 from the intrinsic apoptotic pathway to the extrinsic apoptotic pathway by establishing its role in NFκB regulation. Since, NFκB inhibition can increase the efficacy of various DNA damaging agents, CHK2 inhibitors might provide survival benefits for both cancer and normal cells. Therefore, careful evaluation of the effects of CHK2 on the NFκB pathway would be key before developing CHK2 inhibitors for clinical applications.
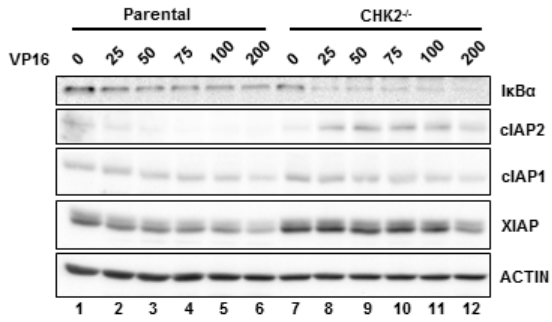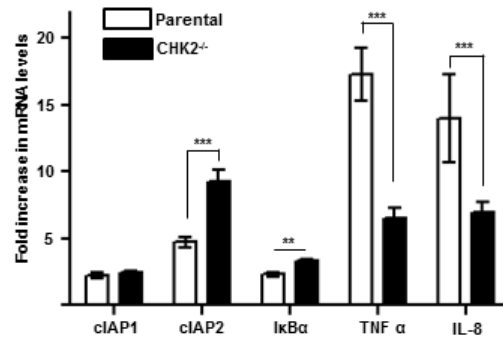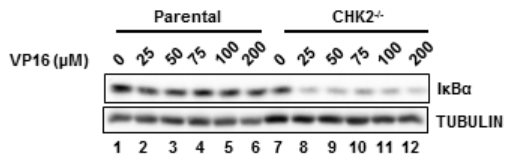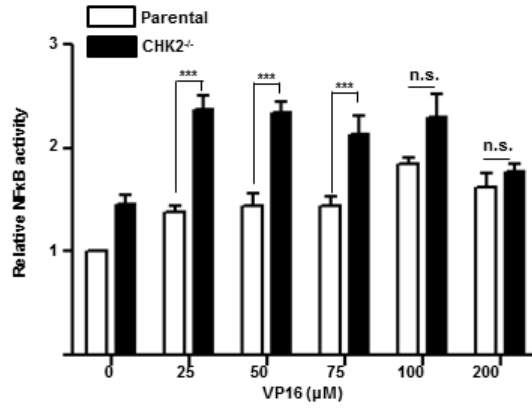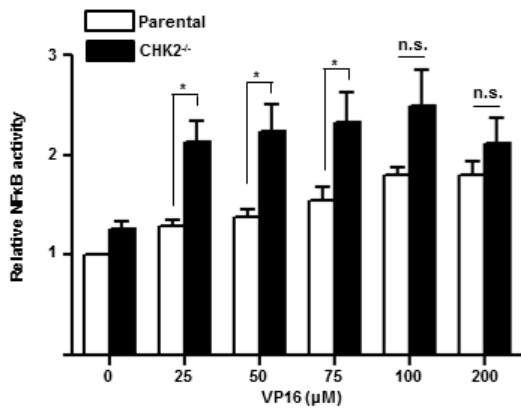
Fig. 3.1 CHK2 inhibits VP16-induced early activation of NFκB

(A) Parental and CHK2$^{-/-}$ HCT116 cells were treated with VP16 at the indicated concentrations for 48 h. Cell lysates were prepared and were subjected to Western blotting with the indicated antibodies.

(B) Parental and CHK2$^{-/-}$ HCT116 cells were cultured in the presence of 25 μM VP16 for 48 h. Total RNA was isolated and was subjected to qPCR for the indicated genes.

(C) Parental and CHK2$^{-/-}$ HCT116 cells were treated with VP16 at the indicated concentrations for 24 h. Cell lysates were prepared and were subjected to Western blotting with the indicated antibodies.

(D, E) Parental and CHK2$^{-/-}$ HCT116 were transiently transfected with κB$_5$-FLUC and TK-RLUC reporter plasmids and treated with VP16 at the indicated concentrations for 24 h. Bioluminescence was then measured as described in Experimental Procedures. The results in panels B, D and E are presented as mean ($\pm$ s.e.m.) of three or more independent experiments. *p<0.05, **p<0.005, ***p<0.0005, n.s. (not significant)
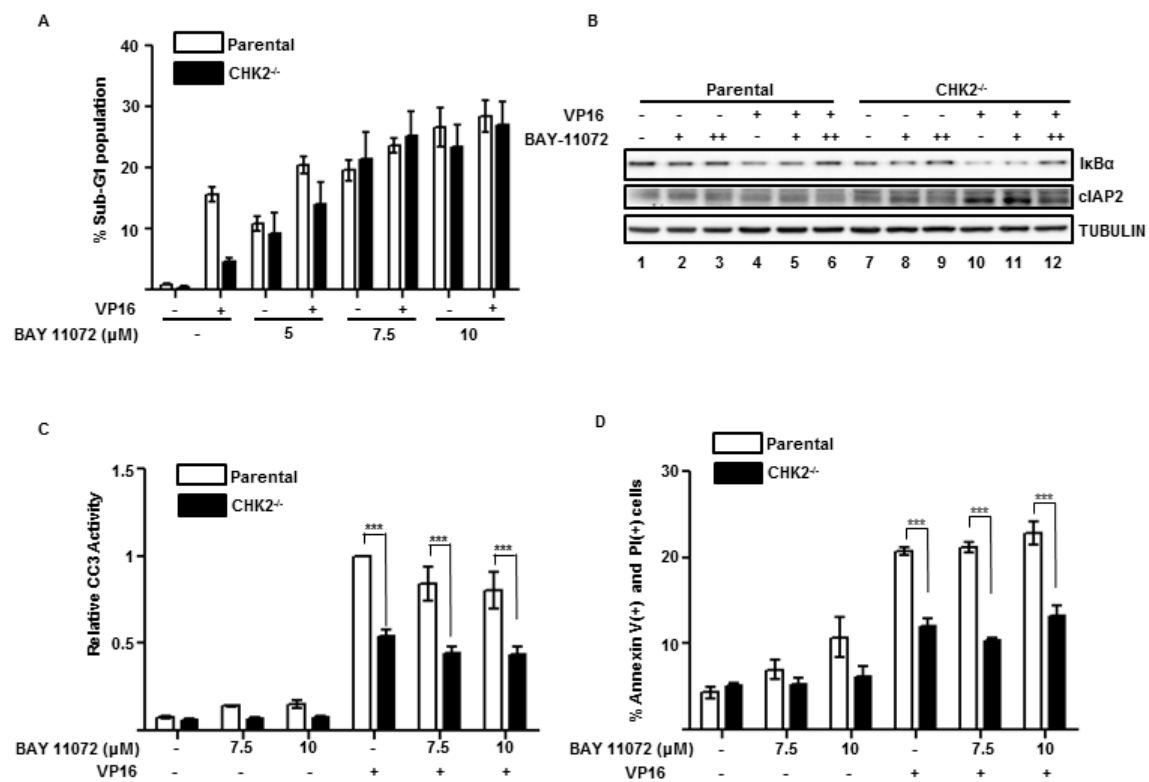
Fig. 3.2. IKK inhibitor, BAY-11072, does not rescue cell death in CHK2 null cells

(A) Parental and CHK2$^{-/-}$ HCT116 cells were cultured in the presence of 5, 7.5 or 10 μM

Bay-11072 for 4 h followed by 25 μM VP16 for an additional 48 h. The Percentage of

cells containing sub-2N DNA content was assessed by PI staining followed by flow

cytometry.

(B, C, D) Parental and CHK2$^{-/-}$ HCT116 cells were cultured in the presence of 25 μM

VP16 for an additional 48 h. Cells were supplemented with 7.5 or 10 μM BAY-11072 for

the final 24 h. After 48h, cell lysates were prepared and were subjected to western

blotting (B) or assayed for cleaved caspase-3 activity (C). The percentage of cells

staining positive for annexin V and PI was assessed by flow cytometry (D).

The results in panels A, C and D are presented as mean ($\pm$ s.e.m.) of three or more

independent experiments. ***p<0.0005, n.s. (not significant)

A

	Parental						CHK2−/−					
VP16	−	−	−	+	+	+	−	−	−	+	+	+
BMS 345541	−	+	++	−	+	++	−	+	++	−	+	++

pS532 p65
Total p65
IκBα
cIAP2
cIAP1
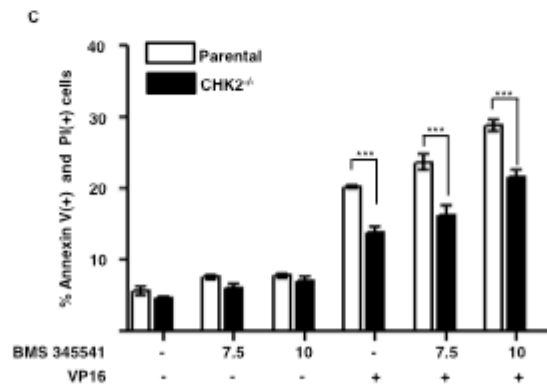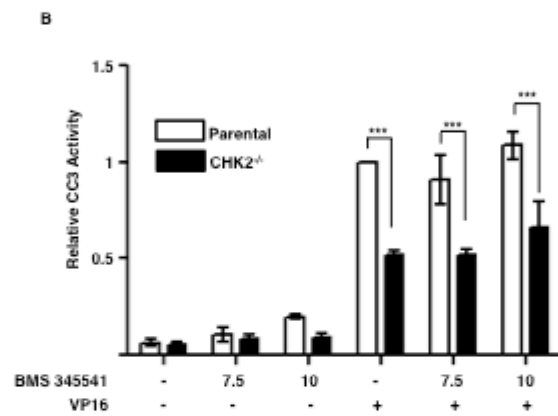XIAP
TUBULIN

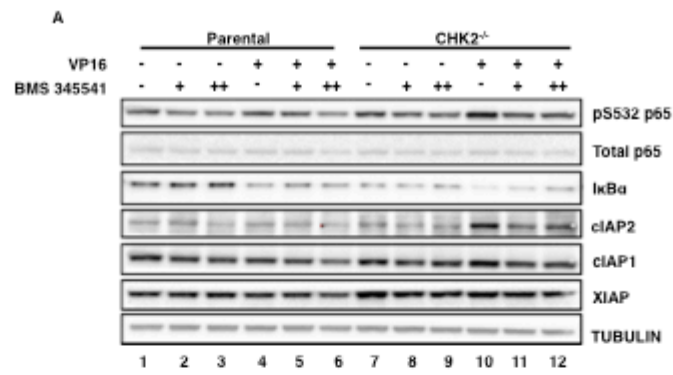1 2 3 4 5 6 7 8 9 10 11 12

B

C

Fig. 3.3 IKK inhibitor, BMS-345541, does not rescue cell death in CHK2 null cells

(A, B, C) Parental and CHK2$^{-/-}$ HCT116 cells were cultured in the presence of 25 μM VP16 for an additional 48 h. Cells were supplemented with 7.5 or 10 μM BMS-345541 for the final 24 h. After 48h, cell lysates were prepared and were subjected to western blotting (A) or assayed for cleaved caspase-3 activity (B). The percentage of cells staining positive for annexin V and PI was assessed by flow cytometry (C). The results in panels B and C are presented as mean ($\pm$ s.e.m.) of three or more independent experiments. ***$p<0.0005$, n.s. (not significant)

A

Parental | CHK2⁻/⁻

| siRNA | GL3 | R1 | N1 | GL3 | R1 | N1 | GL3 | R1 | N1 | GL3 | R1 | N1 |
| VP16 | − | − | − | + | + | + | − | − | − | + | + | + |

RIP1
NEMO
IκBα
cIAP2
TUBULIN

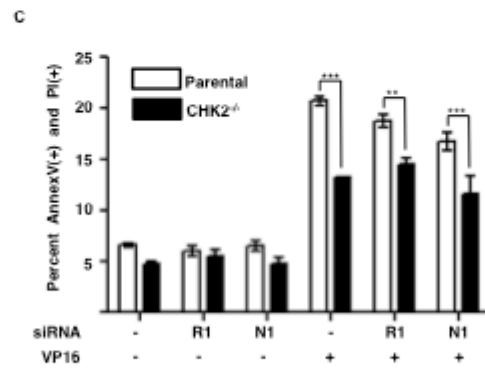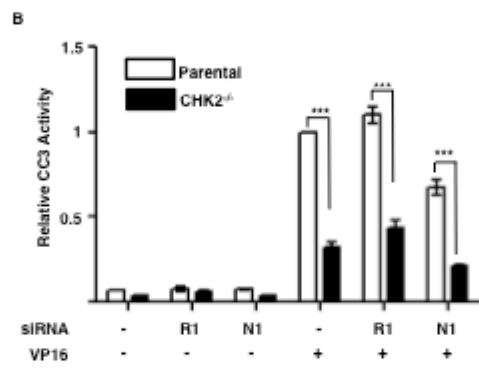1 2 3 4 5 6 7 8 9 10 11 12

B

C

Fig. 3.4 Knockdown of RIP1 and NEMO using pooled siRNA does not rescue cell death in CHK2 null cells

(A, B, C) Parental and CHK2$^{-/-}$ HCT116 cells transiently expressing GL3 (control), RIP siRNA (R1) or NEMO siRNA (N1) for 18 h were cultured in the presence of 25 µM VP16 for an additional 48 h. Cell lysates were prepared and subjected to Western blotting for the indicated antibodies (A) or assayed for cleaved caspase-3 activity (B). The percentage of cells staining positive for annexin V and PI was assessed by flow cytometry (C).

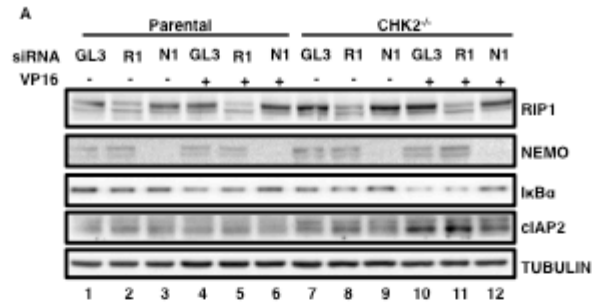The results in panels B and C are presented as mean (± s.e.m.) of three or more independent experiments. ***$p < 0.0005$, n.s. (not significant)

A

Parental | CHK2⁻/⁻

siRNA	GL3	GL3	N1	N2	N3	N4	GL3	GL3	N1	N2	N3	N4	
VP16	−	+	+	+	+	+	−	+	+	+	+	+	
													NEMO
													IκBα
													TUBULIN
	1	2	3	4	5	6	7	8	9	10	11	12	

D

Parental | CHK2⁻/⁻

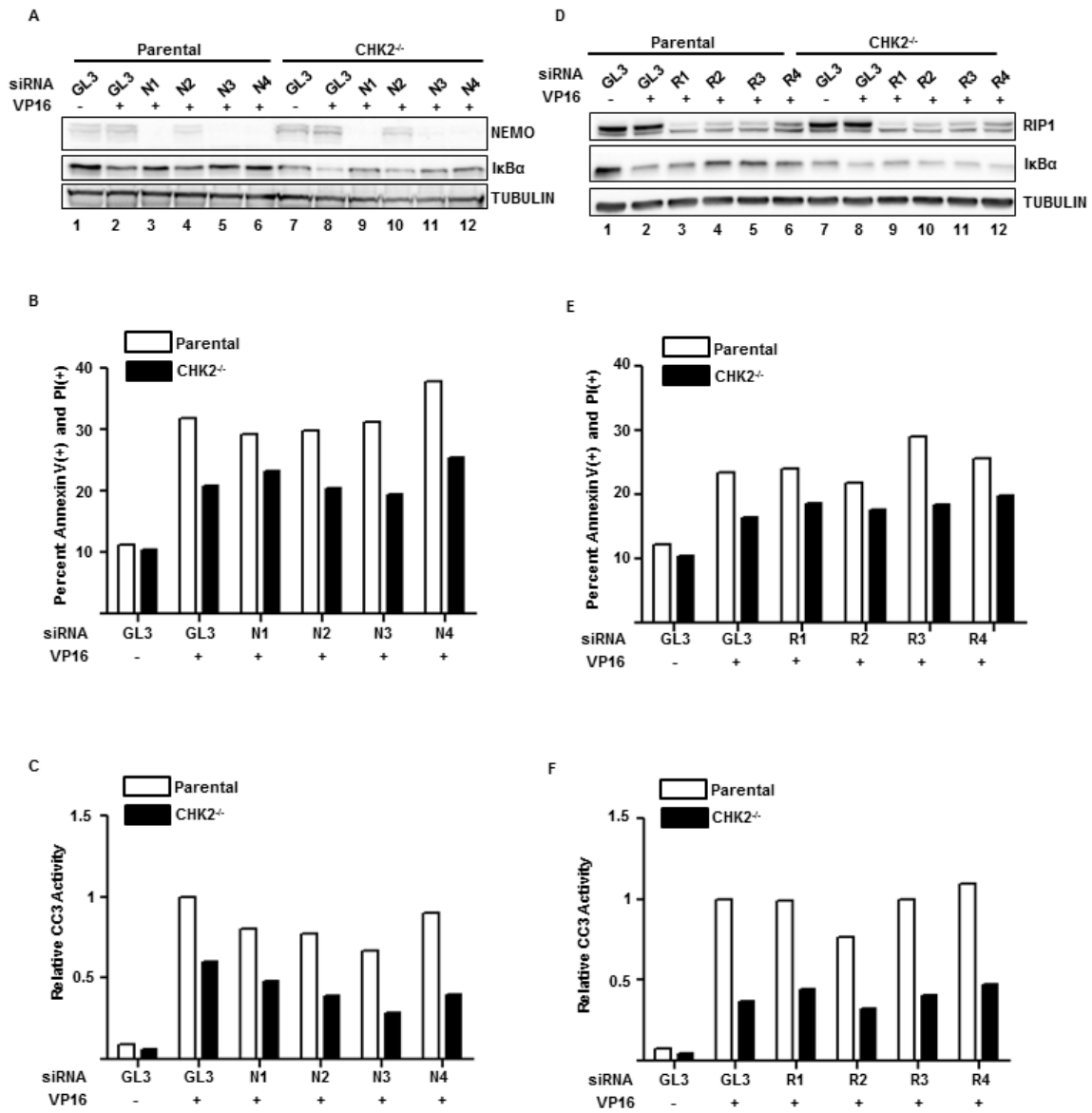| siRNA | GL3 | GL3 | R1 | R2 | R3 | R4 | GL3 | GL3 | R1 | R2 | R3 | R4 | |
VP16	−	+	+	+	+	+	−	+	+	+	+	+	
													RIP1
													IκBα
													TUBULIN
	1	2	3	4	5	6	7	8	9	10	11	12	

B

C

E

F

Fig. 3.5 Knockdown of RIP1 and NEMO using individual siRNA does not rescue cell death in CHK2 null cells

(A, B, C) Parental and CHK2$^{-/-}$ HCT116 cells transiently expressing GL3 (control) or individual NEMO (N1-4) siRNAs for 18 h were cultured in the presence of vehicle (DMSO, -) or 25 μM VP16 (+) for an additional 48 h. Cell lysates were prepared and subjected to Western blotting with the indicated antibodies (A). The percentage of cells staining positive for annexin V and PI was assessed by flow cytometry (B). Cell lysates were also assayed for cleaved-caspase 3 activity (C)

(D, E, F) Parental and CHK2$^{-/-}$ HCT116 cells transiently expressing GL3 (control) or individual RIP1 (R1-4) siRNAs for 18 h were cultured in the presence of vehicle (DMSO, -) or 25 μM VP16 (+) for an additional 48 h. Cell lysates were prepared and subjected to Western blotting with the indicated antibodies (A). The percentage of cells staining positive for annexin V and PI was assessed by flow cytometry (B). Cell lysates were also assayed for cleaved-caspase 3 activity (C)
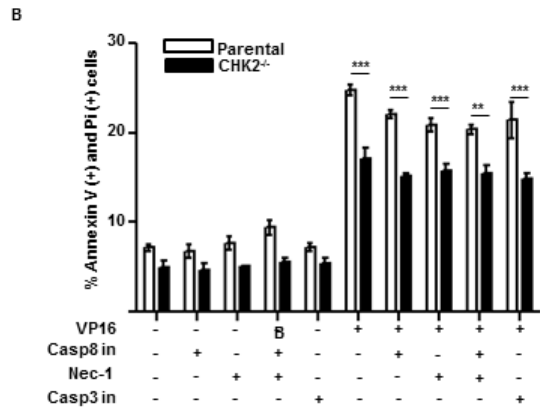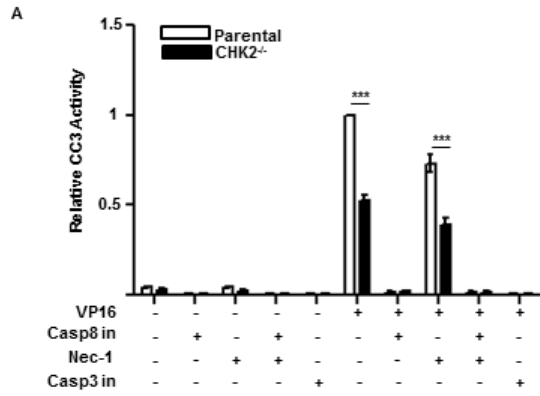
A

B

Fig. 3.6 Caspase-8 is required for caspase-3 activation in VP16-treated HCT116 cells

(A, B) Parental and CHK2$^{-/-}$ HCT116 cells were cultured in the presence of 20μM zIETD-FMK (Casp8 in), 30μM necostatin-1 (Nec-1), or 20μM zDEVD-FMK (Casp3 in) for 4 h followed by 25 μM VP16 for an additional 48 h. Cell lysates were prepared and assayed for cleaved caspase-3 activity (A). The percentage of cells staining positive for annexin V and PI was assessed by flow cytometry (B).

The results in panels B and C are presented as mean (± s.e.m.) of three or more independent experiments. *p<0.05, **p<0.005, ***p<0.0005, n.s. (not significant)

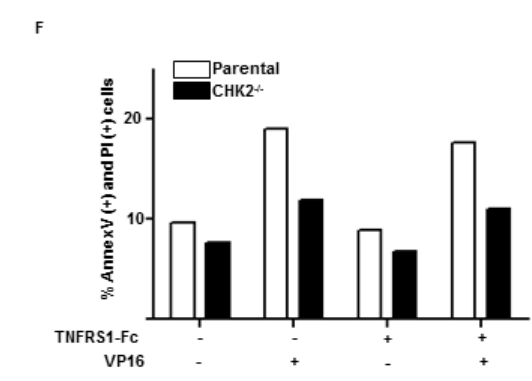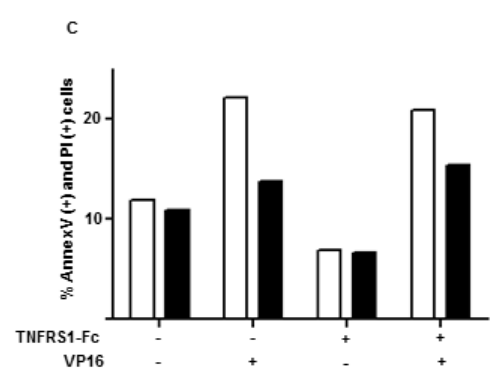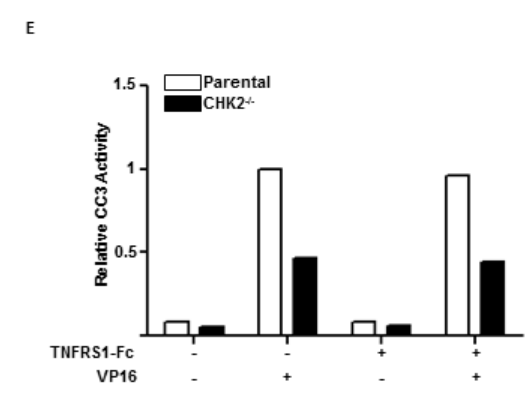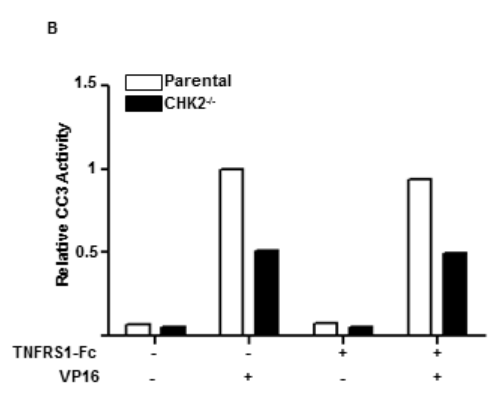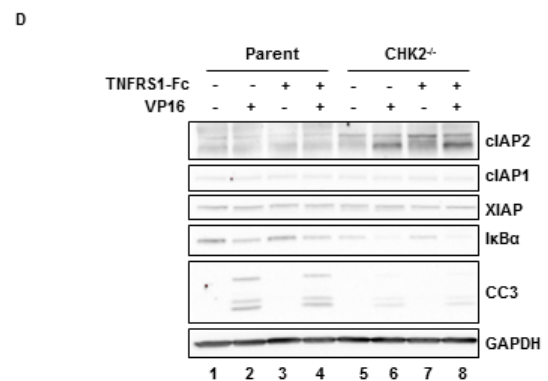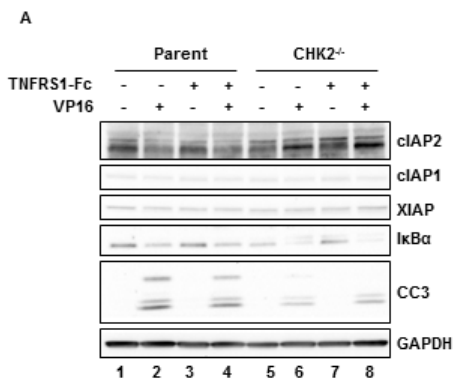**Fig. 3.7 TNFα does not play a role in VP16-treated HCT116 cells**

(A, B, C) Parental and CHK2$^{-/-}$ HCT116 cells were cultured in the presence of 10μg/mL of TNFRSF1-Fc and 25 μM VP16 for. Cell lysates were prepared subjected to western blotting for the indicated antibodies (A) and assayed for cleaved caspase-3 activity (B). The percentage of cells staining positive for annexin V and PI was assessed by flow cytometry (C).

(D, E, F) Parental and CHK2$^{-/-}$ HCT116 cells were cultured in the presence of 25 μM VP16 for. Cells were supplemented with 10μg/mL of TNFRSF1-Fc for the final 24 h. After 48 h, cell lysates were prepared subjected to western blotting for the indicated antibodies (D) and assayed for cleaved caspase-3 activity (E). The percentage of cells staining positive for annexin V and PI was assessed by flow cytometry (F).

A

GFP shRNA   CHK2 shRNA

VP16   -   -   +   +   -   -   +   +
TRAIL   -   +   -   +   -   +   -   +

CHK2

ACTIN

cIAP2

ACTIN

1   2   3   4   5   6   7   8
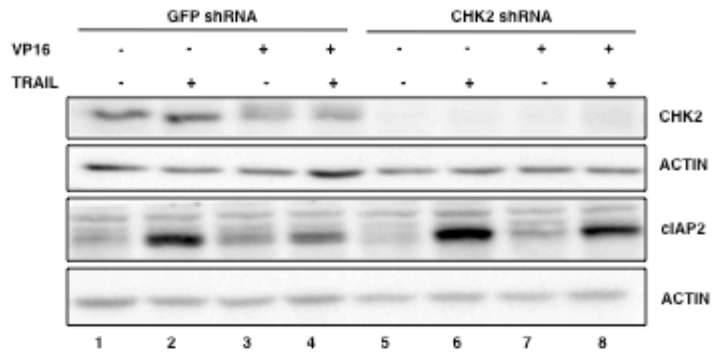
B
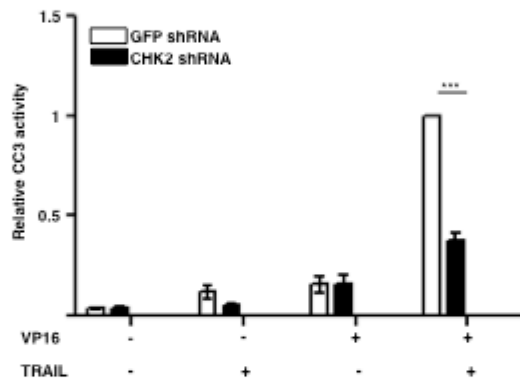
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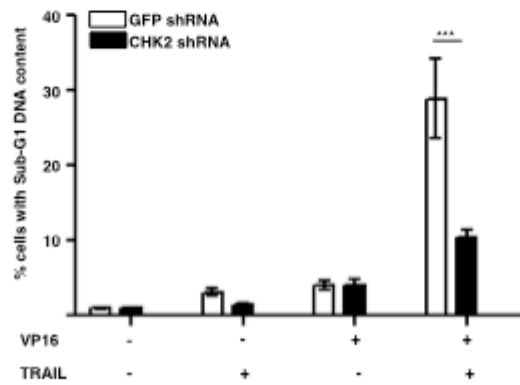
Fig. 3.8 CHK2 is required for VP16-induced sensitization to TRAIL

(A, B, C) HCT116 BAX$^{-/-}$ cells stably expressing control (GFP) shRNA or CHK2 shRNA were treated with 10 μM VP16 for 48 h. Cells were supplemented with 100ng/mL TRAIL for the final 24 h. Cell lysates were prepared and subjected to western blotting for the indicated antibodies (A) or assayed for cleaved caspase 3 activity (B). The percentage of cells containing sub-2N DNA content was assessed by PI staining followed by flow cytometry (C)

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Chapter 4:

Summary, Discussion and Future Directions

4.1 Summary

The data presented in this dissertation provides further insight into the role of CHK2 in DNA damage-induced apoptosis. Traditionally, it has been proposed that CHK2 promotes apoptosis through phosphorylation-mediated stabilization of p53. I have provided evidence that CHK2 is dispensable for p53 regulation and transcriptional activation of its target genes (*p21, BAX,* and *PUMA*). However, despite normal p53 activity, cells lacking CHK2 are impaired in their ability to undergo cell death. We have identified cIAPs as novel CHK2 effectors that provide anti-apoptotic signals in the absence of CHK2. CHK2 binds to Inhibitor of Apoptosis (IAP) family members, cIAP1 and cIAP2, but not XIAP, and potentially inhibits their activity to promote VP16-induced cell death. I have further shown that CHK2 can inhibit the NFκB pathway potentially through the regulation of cIAPs and plays an important role in the cellular response to TNFα superfamily member, TRAIL. Overall, this work provides preliminary evidence that CHK2 plays an important role in both extrinsic and intrinsic apoptosis pathway through the inhibition of cIAPs.

4.2 Discussion

4.2.1 Role of CHK2 in VP16-induced p53 stabilization and cell death

CHK2 plays a central role in the cellular response to DNA damage. DNA double strand breaks activate CHK2 in an ATM-dependent manner (Ahn et al., 2002; Matsuoka et al., 2000; Schwarz et al., 2003). In turn, CHK2 promotes cell cycle arrest and/or apoptosis through regulation of numerous downstream effectors. One of the most widely studied CHK2 effectors is the tumor suppressor protein, p53. CHK2 can phosphorylate p53 on Ser20 and disrupt the interaction of p53 with its E3 ubiquitin ligase, MDM2 (Chehab et al., 2000; Chehab et al., 1999). As a result, p53 is stabilized within the cell and induces transcription of various genes involved in cell cycle arrest (*p21*) and apoptosis (*BAX, PUMA* and *NOXA*). CHK2 null mice are protected from ionizing radiation-induced cell death and fail to stabilize p53 and upregulate its transcriptional targets (Hirao et al., 2000; Takai et al., 2002). This provided the first evidence that CHK2 is essential for p53 regulation and that it induces apoptosis through p53. However, subsequent studies in various cancer cell lines have challenged this notion. Cancer cells are able to phosphorylate p53 on Ser20, stabilize p53, and induce transcription of p53-targets even in the absence of CHK2 (Ahn et al., 2003; Jallepalli et al., 2003). In support of these studies, I have also shown that p53 regulation was intact in HCT116 and MCF7 cells deficient in CHK2. In response to VP16, parental and CHK2 null HCT116 cells had similar levels of total p53, pSer20 p53, and pSer15 p53. Despite normal regulation of p53, both CHK2 deficient HCT116 and MCF7 cells underwent significantly less cell death when compared with their CHK2 proficient counterparts. Interestingly, the reduction in cell death decreased with increasing amount of damage. This suggests that

at lower levels of damage, CHK2 is essential for cellular apoptosis, but is dispensable at higher levels.

Taken together, our data, along with studies from different groups, have demonstrated that CHK2 signals to the apoptotic machinery independent of its role in p53 regulation. The goal of my thesis work was to identify additional CHK2 effectors that are required for DNA damage induced cell death.

4.2.2 cIAPs inhibit VP16-induced apoptosis in the absence of CHK2

In order to identify the downstream effectors of CHK2 that play a key role in the apoptotic pathway, we turned our attention towards the family of Inhibitor of Apoptosis proteins (IAPs). IAP family members, especially cIAP1, cIAP2 and XIAP, are endogenous inhibitors of cell death. We found that simultaneous inhibition of both cIAP1 and cIAP2 rescued VP16-induced apoptosis in cells lacking CHK2. On the other hand, XIAP had no effect on cell death.

cIAPs are well-known regulators of cell death. They can bind and ubiquitinate the effector caspase, caspase 3, and target it for proteosomal degradation (Choi et al., 2009; Mica et al., 2004). On the other hand, cIAPs can also activate the NFκB pathway in response to DNA damage and promote cell survival signals (Jin et al., 2009). Inhibition of cIAPs can sensitize cells to various forms of DNA damaging agents (Gill et al., 2009; Karasawa et al., 2009). This has prompted the development of several IAP inhibitors, known as SMAC mimetics, for cancer treatment. One such SMAC mimetic, BV6, is specific to cIAPs and can induce their rapid degradation (Blankenship et al.,

2009; Varfolomeev et al., 2007). I demonsrated that BV6 increased apoptosis in CHK2 deficient cells to levels comparable to that measured in CHK2 proficient cells.

This result provided the first indication that cIAPs might provide anti-apoptotic signals to prevent DNA damage-induced apoptosis in CHK2 deficient cells. Thus, we conclude that CHK2 can promote DNA damage induced apoptosis through two distinct pathways: a) by regulating p53 and promoting transcription of its pro-apoptotic targets including *BAX* and *PUMA*, and b) by negatively regulating both cIAP1 and cIAP2 to relieve their anti-apoptotic activities. However, it remains unclear how cIAPs provide anti-apoptotic signals in the absence of CHK2. cIAPs can target cleaved caspases for proteosomal degradation. Therefore, monitoring the ubiquitination status of effector caspases in the presence and absence of CHK2 is expected to provide further insight into the mechanism of cIAP-mediated inhibition of cell death.

4.2.3 CHK2 mediated regulation of cIAPs

To undersand mechanistically how CHK2 might regulate the cIAPs, we monitored for interactions between CHK2 and cIAP1, cIAP2 and XIAP. We observed that both cIAP1 and cIAP2, but not XIAP, interacted with CHK2 when co-produced in HEK293T cells. cIAPs are known E3 ubiquitin ligases that modify their substrates by attaching single or multiple ubiquitin molecules. In addition to ubiquitination of other proteins, cIAPs can also regulate their own cellular levels through auto-ubiqutiantion resulting in proteosomal degradation (Dueber et al., 2011; Feltham et al., 2011; Mace et al., 2008). CHK2 inhibited cIAP2 auto-ubiquitination *in vitro* in a phosphorylation dependent manner. cIAPs are known to conjugate various forms of ubiquitin, K63- and K48-linked

ubiquitin, and CHK2 was able to inhibit both forms auto-ubiquitination. Consistent with this, CHK2 promoted cIAP2 accumulation by inhibiting cIAP2 turnover in the cells. As a result, the cIAP2 half-life was extended from less than 15 min in the absence of CHK2 to more than an hour in the presence of CHK2. CHK2 mediated regulation of cIAP2 required its kinase activity as kinase-dead mutant of CHK2 had no effect on cIAP2 function. Although, cIAP1 and cIAP2 both interacted with CHK2, CHK2 promoted cIAP2 accumulation but not cIAP1 or XIAP accumulation.. cIAP1 is known to interact with cIAP2 and induce its destruction (Silke et al., 2005). Thus, it is plausible that CHK2 inhibits cIAP1 indirectly through its interaction with cIAP2. Therefore, understanding the effect of CHK2 on cIAP1 in the presence and absence of cIAP2 is key to understanding how CHK2 regulates cIAP1.

Mass spectrometry of phosphorylated cIAP2 identified Thr35 as a CHK2 phosphorylation site *in vitro*. Thr35 of cIAP2 fall within the CHK2 consensus phosphorylation motif (L X R X X S/T). Phosphorylation of cIAP2 on Thr35 increased when it was co-produced with CHK2 in vivo. However, we found that CHK2 was capable of regulating a mutant of cIAP2 that could not be phosphorylated on Thr35. In addition, a mutant of cIAP2 lacking all four potential CHK2 phosphorylation sites (Ser81, Thr145, and Thr261) was still stabilized in the presence of CHK2. Mass spectrometry also identified Ser155 and Ser566 as CHK2 phosphorylation sites. Ser155 is present within the BIR2 domain of cIAP2, and this domain is known to play a role in caspase binding. Interestingly, Ser566 is present within the hydrophobic pocket of the RING domain of cIAP1 and is conserved between cIAP1 and cIAP2. XIAP encodes alanine at

the equivalent position. Current studies are aimed at determining if phosphorylation of cIAP2 on Ser155 and Ser566 by CHK2 inhibits cIAP2 E3 ubiquitin ligase activity.

4.2.4 Role of CHK2 in regulation of the NFκB pathway

The NFκB pathway promotes cell survival in response to a wide variety of stimuli, including DNA damage (Hinz et al., 2010; Huang et al., 2003b; Miyamoto, 2010; Wu et al., 2006; Wu et al., 2010). cIAP1 and cIAP2 are required for NFκB activation and can set a "feed-forward" activation loop that actively promotes cell survival (Bertrand et al., 2011; Bertrand et al., 2008; Chu et al., 1997; Mahoney et al., 2008). Loss of CHK2 in HCT116 cells results in the early activation of NFκB and upregulation of its pro-survival target, *cIAP2*. On the other hand, in the presence of CHK2, NFκB was only activated at later stages and induced the transcription of pro-apoptotic factors (*TNFα* and *IL-8*). TNFα can activate cell death through the activation of caspase-8. Indeed, caspase 8 is required for VP16-induced caspase-3 activity in HCT116 cells. Surprisingly, inhibition of NFκB did not increase apoptosis in CHK2 null cells. Similarly, inhibition of TNFα signaling did not inhibit cell death in the parental cells. However, loss of cIAP2 or NEMO did reduce cell death in both parental and CHK2 null cells. Thus, NFκB might play a more complex role in the VP16-treated parental and CHK2 null cells (Biton and Ashkenazi, 2011; Huang et al., 2003a; Kasibhatla et al., 1998).

We demonstrated that CHK2 is required for VP16-induced sensitization of cells to the apoptotic ligand, TRAIL. Activation of NFκB and its pro-survival target, *cIAP2*, has been shown to promote TRAIL resistance (Franco et al., 2001; Keane et al., 2000; Kim et al., 2002; Seo et al.). Thus, we hypothesize that CHK2 inhibits NFκB activation to

194

promote TRAIL-induced apoptosis. Consistent with this hypothesis, VP16 is unable to prevent TRAIL-induced cIAP2 accumulation in the absence of CHK2. Therefore, in addition to its role in the intrinsic apoptosis pathway, CHK2 might also play an important role in the death receptor-induced extrinsic apoptotic pathway.

4.3 Future Directions

CHK2 inhibitors are currently being considered for cancer treatments. Numerous studies have shown that CHK2 inhibitors can increase cell death in cell lines with non-functional p53 (Jiang et al., 2009; Jobson et al., 2009). This has been attributed to the effects of CHK2 on cell cycle arrest. My thesis work has provided insight into a novel p53-independent role of CHK2 in both the intrinsic and extrinsic apoptotic pathways. My data suggests that inhibition of CHK2, even in p53 mutant cancers, might provide survival benefit through the ability of cIAPs to inhibit apoptosis. Thus, understanding the effects of CHK2 inhibitors on cIAPs and the NFκB pathway would be crucial before considering their clinical application.

On the other hand, SMAC mimetics, like BV6, are currently being developed for clinical application. It is believed that SMAC mimetics can enhance the efficacy of chemotherapeutic agents. However, if CHK2 is able to efficiently inhibit cIAPs in response to DNA damage, SMAC mimetics might not provide any therapeutic benefit. As seen in my work, concomitant loss of cIAP1 and cIAP2 did not increase DNA-damage induced cell death in HCT116 and MCF7 cells. However, cIAP upregulation (Bashyam et al., 2005; Dai et al., 2003; Gyrd-Hansen and Meier, 2010; Imoto et al., 2001) and CHK2 downregulation (Bartkova et al., 2004; Sullivan et al., 2002; Tort et al., 2002) is observed in various cancer subtypes. In such cancer subtypes, CHk2-mediated cIAP inhibition might be suboptimal. Combination of SMAC mimetics with chemotherapeutic agents that induce DNA double strand break might provide therapeutic advantage in these cancer subtypes. Moreover, *CHK2* is mutated in a subset of patients with Li-Fraumeni syndrome, a familial cancer predisposition

syndrome (Bell et al., 1999). Li-Fraumeni patients with *CHK2* mutations have intact

TP53 gene and thus, can potentially benefit from combination of chemotherapy and

SMAC mimetic to relieve the cytoprotective effects of cIAPs in the absence of CHK2

function.

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