cIAP2 negatively regulates proliferation and tumourigenesis by repressing IKK activity and maintaining p53 function

By

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A thesis submitted to the Faculty of Graduate and Post-doctoral Studies, University of Ottawa, in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

The cellular inhibitor of apoptosis protein (cIAP)-2 plays an important role in the protection against apoptosis by inhibiting the endogenous IAP inhibitor Smac, thus allowing other members of the IAP family, such as XIAP to block caspases. Additionally, cIAP2 functions as a ubiquitin ligase and mediates survival/proliferative signaling through NF-κB. cIAP2 is overexpressed in many human cancers and is believed to play an oncogenic role. This led to the development of small molecule IAP antagonists aimed at eliciting apoptosis in cancer cells. However, the loss of cIAP2 is also associated with multiple myeloma, in which constitutively active NF-κB signaling contributes to pathogenesis of the disease and suggests that cIAP2 may also perform a tumour suppressive function.

We demonstrate a novel role for cIAP2 in maintaining p53 levels in mammary epithelial cells that express wildtype p53. Downregulation of cIAP2 resulted in activation of IKKs, which led to increased Mdm2-mediated degradation of p53. cIAP2 depletion also led to increased phosphorylation of ERK1/2. Reduction of p53 levels, in combination with survival signaling provided by NF-κB and MEK-ERK pathways were associated with increased colony formation *in vitro* and increased DMBA-induced adenocarcinomas in cIAP2-null mice.

Treatment of cells with IAP antagonists resulted in significant cytotoxicity only in p53-mutant MDA-MB-231 cells, which was associated with autocrine production of TNF-α. We propose that the transcription of TNF-α is potentiated by gain-of-function mutation in p53 since downregulation of mutant p53 in MDA-MB-231 cells decreased TNF-α mRNA. Downregulation of cIAPs in p53-mutant cells resulted in a decrease in nuclear IKK-α, which may result in decreased IKK-α-mediated survival signaling. In

contrast, cIAP downregulation in p53-wildtype cells resulted in no change in nuclear IKK-α, degradation of the corepressor SMRT and cell survival. We show that the effects of cIAP2 downregulation are context-dependent. Downregulation of cIAP2 in p53-wildtype cells results in a decrease in p53 and an increase in survival and proliferative signaling. These results suggest a tumour suppressor function for cIAPs that may account for cIAP mutation-associated cancers such as multiple myeloma. Moreover, our data also defines gain-of-function p53 mutation as a possible contributor to IAP antagonist sensitivity.

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LIST OF ABBREVIATIONS

AA	amino acid
ARF	alternate reading frame
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad-3-related
BID	BH3-interacting domain death agonist
BIR	baculovirus IAP repeat
BSA	bovine serum albumin
CBP	CREB binding protein
CDK	cyclin-dependent kinase
c-FLIP	caspase-8/(FLICE) inhibitory protein
cIAP1	cellular inhibitor of apoptosis protein 1
cIAP2	cellular inhibitor of apoptosis protein 2
CREB	cAMP-response element-binding protein
DIABLO	direct IAP binding protein with low PI
DMBA	7,12-Dimethylbenz(a)anthracene
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAPK	DNA-dependent protein kinase
dNTP	deoxyribonucleotide triphosphate
DR5	death receptor 5
EGFR	epidermal growth factor receptor
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
ERK	extracellular signal-regulated kinase

FADD	FAS-associated death domain
FASL	Fas ligand
FBS	fetal bovine serum
FGF-2	fibroblast growth factor 2
FLICE	FADD-like IL-1β-converting enzyme
GADD45	growth arrest and DNA damage
HDAC	histone deacetylase
hEGF	human epithelial growth factor
HRP	horseradish peroxidase
Hsp	heat shock protein
IAP	inhibitor of apoptosis protein
ID4	inhibitor of DNA binding 4
IGF2	insulin growth factor 2
IκB	inhibitor of kappa B
IKK	inhibitor of kappa B kinase
JNK	c-Jun amino-terminal kinases
kDa	kilodalton
kDIF	keratinocyte differentiation-inducing factor
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
Mdm2	murine double minute 2
MDR1	multi drug resistance 1
MEF	mouse embryonic fibroblast
MEK	MAPK/ERK kinase
MIP-1α	macrophage inflammatory protein-1α
MK2	MAPK-activated protein kinase 2
MOI	multiplicity of infection

mTOR	mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEAA	non-essential amino acids
NEMO	NF-κB essential modifier
NES	nuclear export sequence
NF-κB	nuclear factor-kappa B
NIK	NF-κB inducing kinase
NLL	nuclear localization sequence
OD	optical density
OIS	oncogene-induced senescence
PBS	phosphate-buffered saline
PCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PI3K	phosphatidylinositol 3-kinase
PIAS	protein inhibitor of STAT
PML	promyelocytic leukemia
PNK	polynucleotide kinase
PP1	protein phosphatase 1
PTM	post-translational modification
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
RE	response element
RHD	Rel homology domain
RING	really interesting new gene
RIP1	receptor interacting protein 1
RIPA	radioimmunoprecipitation assay
SCC	squamous cell carcinoma

SCF	Skp1-Culin-Roc1/Rbx1-Hrt-1-F-box
Ser	serine
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
Smac	second mitochondria-derived activator of caspases
SMRT	silencing mediator of retinoid and thyroid receptors
SRC	steroid receptor coactivator
SUMO	small ubiquitin-like modifier
SUSP	SUMO-specific protease
TAF	TATA-binding protein-associated factor
TBS-T	tris-buffered saline and tween
TNF-α	tumour necrosis factor-α
TNFR	tumour necrosis factor receptor
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor-associated factor 2
TRAF3	TNF receptor-associated factor 3
TRAIL	TNF-related apoptosis inducing ligand
Wip	Wild-type p53-induced phosphatase 1
XIAP	X-chromosome-linked inhibitor of apoptosis protein

ACKNOWLEDGEMENTS

First and foremost I must thank my supervisor, Dr. Christine Pratt, whose limitless enthusiasm and passion for science has been a tremendous source of inspiration. From you I have truly learned to believe in myself, and more importantly, to believe in my data. I feel very fortunate to have you as a mentor.

I thank my Thesis Advisory Committee: Dr. Doug Gray, Dr. David Lohnes, Dr. Bruce McKay and Dr. Laura Trinkle-Mulcahy for their guidance and scientific discussions.

To my labmates, past and present - Nathalie Gionet, Minying Niu, Deidre McVeigh, Lisa Hubers, Sam Ruddy, Andrea Sau - thank you for all of your support, friendships and for making this an unforgettable journey. To Beta and Massih, thank you for being the keepers of my sanity.

Last but most definitely not least, I would like to thank my Mom, Andy and 婆婆. My accomplishments have only been possible because of your endless love and support.

CHAPTER I - INTRODUCTION

The transformation of a normal cell into a cancer cell depends on the acquisition of key characteristics that enable it to establish malignancy. These attributes, as described by Hanahan and Weinberg in their seminal publication "The hallmarks of cancer" (Hanahan and Weinberg, 2000), include self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, and evasion of apoptosis. These characteristics reflect alterations in cell signaling pathways that control motility, proliferation and survival in a normal cell (Irish et al., 2006). Understanding these changes and their impacts is challenging, given the extraordinary complexity and intricacy of the different signaling pathways that contribute to each process.

From a therapeutic standpoint, the understanding of the mechanisms by which cancer cells evade apoptosis is particularly relevant, since it may lead to identification of targets that can be exploited in cancer cells, and also provide insight into mechanisms of therapeutic resistance. As such, a substantial effort is being invested in developing strategies to target the apoptotic pathway for cancer therapy.

Apoptosis

Apoptosis, or programmed cell death, is the process by which damaged or unwanted cells are dismantled through proteolysis, then engulfed by neighbouring cells. The cell undergoes distinct morphological changes, including shrinkage, membrane blebbing, degradation of DNA by endonucleases and finally the breakage of the cells into small vesicles (Schulze-Bergkamen and Krammer, 2004). This process is essential to the proper development and homeostasis of an organism and deregulation of this process is associated with a variety of human pathologies including autoimmune diseases,

neurodegenerative disorders, and cancer (Thompson, 1995; Vaux and Flavell, 2000; Green and Evan, 2002).

Caspases

The key effector proteins of apoptosis are a family of cysteine proteases called caspases, of which 11 have been identified in humans. These proteins, once activated, cleave their substrates after specific aspartic acid residues. There are over 200 substrates known in mammals, some of which are required for the cell to maintain viability (Earnshaw et al., 1999; Nicholson, 1999). Those caspases that have a role in apoptosis are divided into two classes: the initiator caspases, which include caspase-2, -8, -9 and -10; and the effector caspases, which include caspases-3, -6 and -7 (Fuentes-Prior and Salvesen, 2004). The caspases are tightly regulated and are initially expressed in cells as inactive procaspase precursors. Upon induction of apoptosis, the initiator caspases are activated first by oligomerization, which cleave and activate effector caspases. Proteolysis by the effector caspases leads to cell death through the activation of nucleases, diminished DNA repair, inhibition of protein synthesis, cleavage of major structural proteins in the cytoplasm and nucleus, and disruption of signal transduction required for cellular homeostasis (Earnshaw et al., 1999; Salvesen and Dixit, 1997). The effector caspases can also cleave and activate initiator caspases to initiate an amplification loop (Slee et al., 1999).

Intrinsic and Extrinsic apoptotic pathways

The apoptotic response in mammals is mediated through two pathways: the intrinsic and the extrinsic pathways (Figure 1.1). The intrinsic pathway is initiated by stress stimuli detected from within the cell, such as hypoxia, oncogene activation and

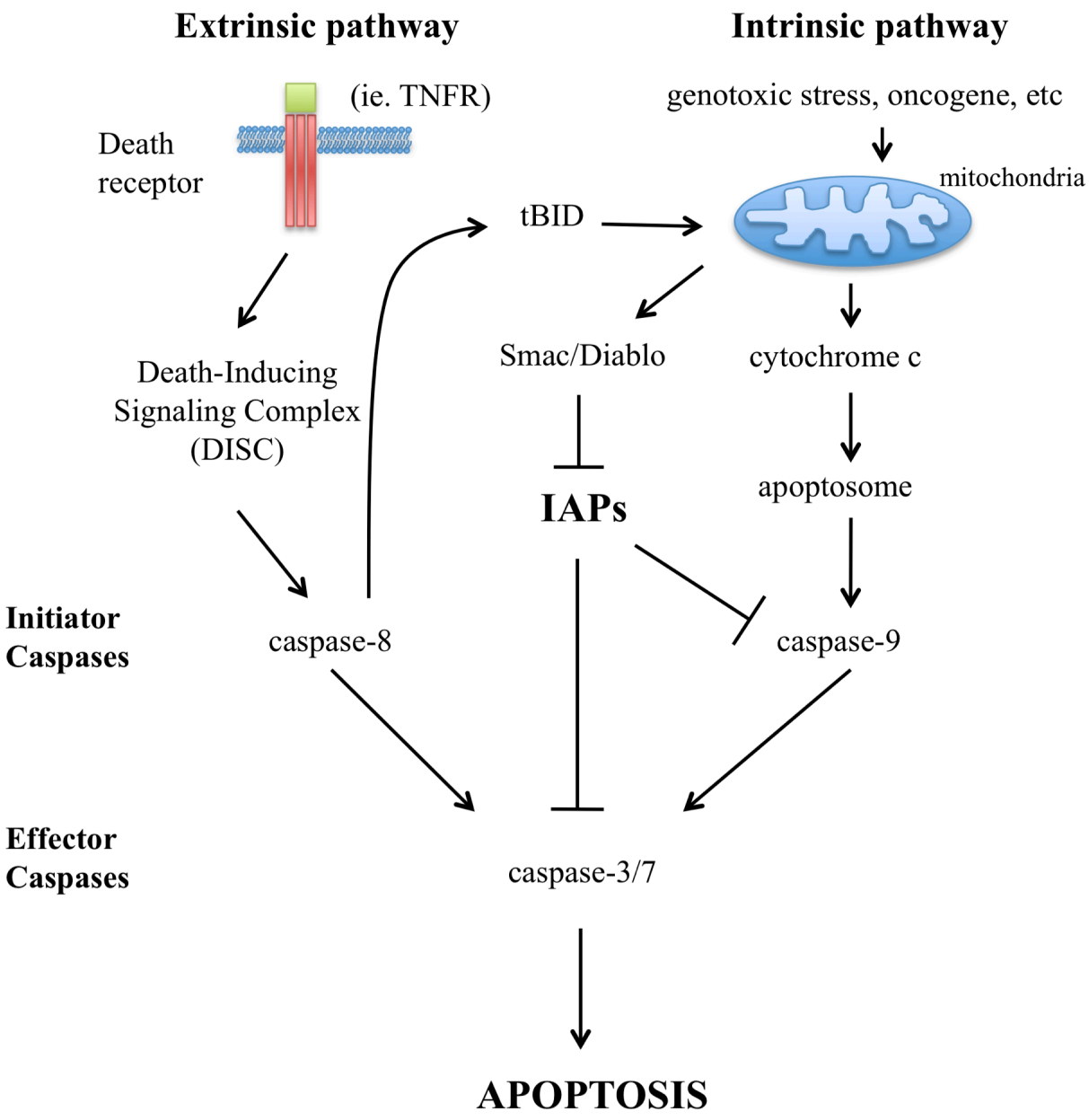


Figure 1.1. The intrinsic and extrinsic apoptosis pathways.
See text for details. BID, BH3-interacting domain death agonist; IAPs, inhibitor of apoptosis proteins; Smac/Diablo, second mitochondria-derived activator of caspases/direct IAP binding protein with low PI; TNFR, tumour necrosis factor receptor.

DNA damage. These stresses transduce signals that result in permeabalization of the outer mitochondrial membrane, leading to the release of cytochrome c and other pro-apoptotic molecules that form the apoptosome. Caspase-9 is then activated and, in turn, activates the cascade of downstream caspases (Jiang and Wang, 2004). In contrast, the extrinsic pathway is triggered by the binding of an extracellular death ligand, such as tumour necrosis factor-α (TNF-α) to its cell-surface death receptor, the TNF receptor (TNFR). A death signaling complex is formed and results in the activation of caspase-8 and subsequent activation of caspase 3 (Debatin and Krammer, 2004). Although the intrinsic and extrinsic pathways are initiated by different stimuli and involve different effectors, crosstalk exists between the two pathways. The extrinsic pathway can signal to the intrinsic pathway through caspase-8-mediated proteolysis of BID (BH3-interacting domain death agonist) to generate tBID, which can promote mitochondrial cytochrome c release and assembly of the apoptosome (Fuentes-Prior and Salvesen, 2004).

Numerous signaling pathways regulate apoptosis and there is significant crosstalk between the different pathways, some of which are pro-apoptotic and others anti-apoptotic. The outcome is determined by the integration of these signals. Given that multiple pathways can impact apoptosis, it is not surprising that cancer cells have developed many different mechanisms to evade apoptosis. The following sections will briefly describe a few pathways governing apoptosis that are commonly deregulated in cancer.

Inhibitor of apoptosis proteins

Once apoptosis has been initiated and the cascade of caspase cleavage/activation has begun, there remains a final defense against cell death: the inhibitor of apoptosis

proteins (Hengartner, 2000). The inhibitor of apoptosis proteins (IAPs) are potent suppressors of apoptosis and the human family is comprised of eight members: cellular IAP 1 (cIAP1)/hIAP2/MIHB/BIRC2, cellular IAP 2 (cIAP2)/hIAP1/MIHC/BIRC3, X-linked IAP (XIAP)/hILP/MIHA/BIRC4, neurona apoptosis inhibitory protein (NAIP)/BIRC1, melanoma IAP (ML-IAP)/KIAP/livin/BIRC7, survivin/TIAP/BIRC5, Apollon/BRUCE/BIRC6, and IAP like protein 2 (ILP2)/Ts-IAP/BIRC8 (Salvesen and Duckett, 2002) (Figure 1.2). All IAP proteins are characterized by the presence of one to three baculovirus IAP repeat (BIR) domains, which are zinc-binding regions of approximately 70 amino acids that mediate protein-protein interactions (Hinds et al., 1999). A number of IAPs also contain a RING (really interesting new gene) domain that confers ubiquitin protein ligase (E3) activity, and are capable of auto-ubiquitination, as well as ubiquitination of proteins involved in apoptosis and signaling (Srinivasula and Ashwell, 2008). The specific function of IAPs as ubiquitin ligases will be discussed in further detail in a later section.

The most thoroughly characterized IAP member is XIAP, which directly binds to and inhibits caspases 3, 7 and 9 (Deveraux et al., 1997). To date, XIAP is the only member of the IAP family to exhibit direct binding and potent inhibition of the caspases under physiological conditions, while other members have been shown to only do so weakly (Eckelman and Salvesen, 2006; Eckelman et al., 2006). Upon induction of apoptosis, XIAP is inhibited by the endogenous IAP antagonist Smac (second mitochondria-derived activator of caspase), also known as DIABLO (direct IAP binding protein with low PI). Smac is localized to the mitochondria under normal conditions. Under apoptotic stress, it is released in a similar manner as cytochrome c (Du et al., 2000; Verhagen et al., 2000).

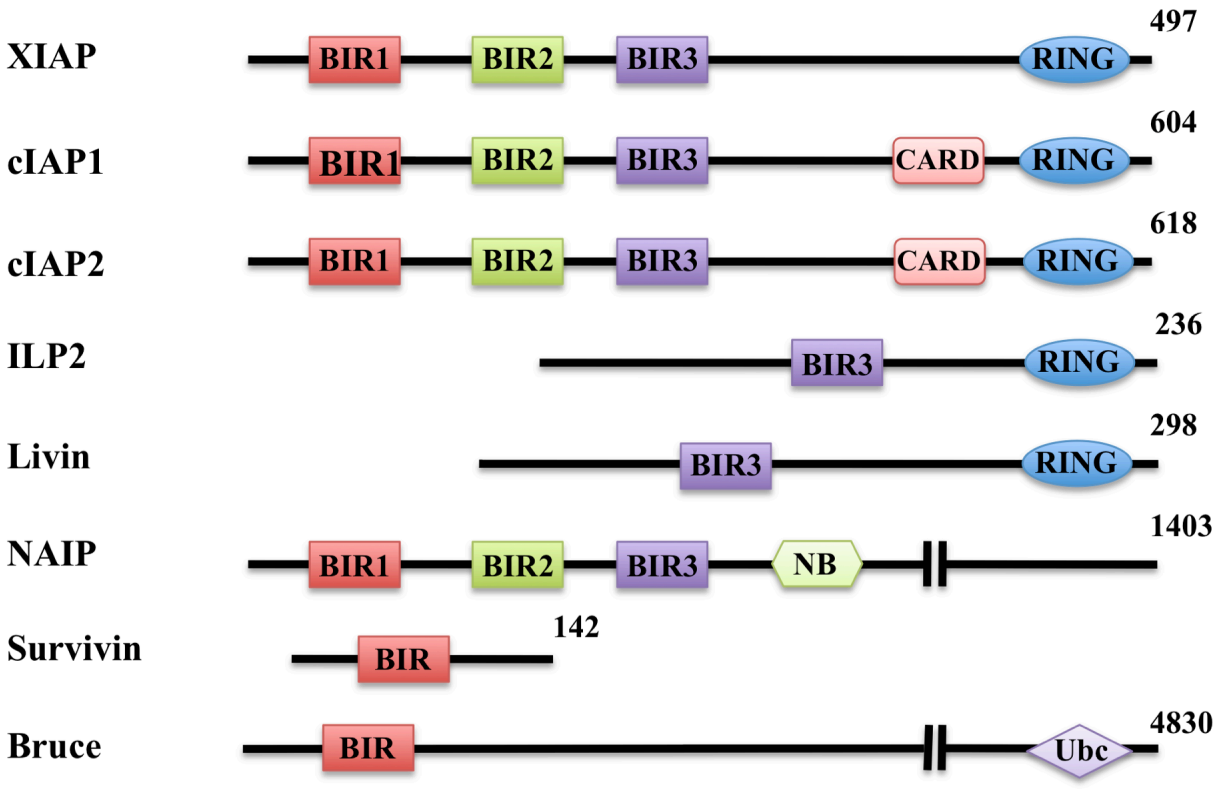


Figure 1.2. Inhibitor of apoptosis protein (IAP) family
Human inhibitor of apoptosis proteins (IAP). To date, eight IAP family members have been identified: X-linked IAP (XIAP, BIRC4, hILP, MIHA), cellular IAP 1 (cIAP1, MIHB, hIAP2, BIRC2) and cellular IAP 2 (cIAP2, MIHC, hIAP1, BIRC3), IAP-like protein 2 (ILP2, TsIAP, BIRC8), livin (ML-IAP, kIAP, BIRC7), NLR family apoptosis inhibitory protein (NAIP, BIRC1), survivin (BIRC5), and BRUCE (apollon, BIRC6). The number of amino acids in each protein is indicated on the right. BIR: baculovirus IAP repeat; RING: really interesting new gene; CARD: caspase activation recruitment; NB: nucleotide binding domain; Ubc: ubiquitination enzyme motif.

During the release from the mitochondria, Smac is processed to reveal an IAP-binding motif (IBM), which binds to the BIR domains of XIAP and prevents its interaction with caspases (Liu et al., 2000; Wu et al., 2000).

Cellular Inhibitor of Apoptosis proteins

cIAP1 and cIAP2 can bind caspases, but do not directly inhibit them (Eckelman and Salvesen, 2006). Instead, they exert their anti-apoptotic effects through protein-protein interactions and by modulating the levels of other proteins through their function as ubiquitin ligases. Firstly, cIAP1/2 can bind to Smac and sequester it away from XIAP, allowing XIAP to inhibit caspases and suppress apoptosis (Hu and Yang, 2003). Furthermore, the cIAPs can target caspases and Smac for degradation by mediating their ubiquitination (Choi et al., 2009; Hu and Yang, 2003).

Ubiquitination is an important post-translational modification that can regulate the stability and activity of a protein, depending on the type of ubiquitin linkage that is applied. Ubiquitin, a 8.5 kDa polypeptide, is conjugated to its targets through a series of sequential reactions involving E1, E2 and E3 enzymes. The E1 enzyme activates ubiquitin and transfers it to the E2 enzyme, which forms an E2-ubiquitin thioester. This complex next interacts with an E3 ubiquitin ligase, which recognizes and specifies the substrate to be ubiquitinated, resulting in the transfer of the ubiquitin to the substrate (Kerscher et al., 2006). Attachment of at least four ubiquitins linked in a chain via their lysine 48 residue marks a protein for degradation by the proteasome (Chau et al., 1989). In contrast, the use of lysine 63 linkages in either monoubiquitination or polyubiquitination leads to modification of protein function but does not result in degradation (Hochstrasser, 2006). The ubiquitin ligase activity of the cIAPs is conferred

by the presence of the RING domain in their carboxy terminus and their substrates include themselves and proteins involved in signaling (Vaux and Silke, 2005). The targets of ubiquitination by the cIAPs include multiple substrates in the tumour necrosis factor receptor (TNFR) complex. Following ligand binding, cIAP1/2 activates receptor interacting protein (RIP)-1 through ubiquitination, which results in the activation of NF-κB signaling. Additionally, cIAP1 ubiquitinates TNFR-associated factor (TRAF)-2 (Li et al., 2002) while cIAP2 ubiquitinates TNFR-associated factor (TRAF)-1 (Lee et al., 2004), resulting in degradation of those proteins in both cases.

True to its name, cIAP2 plays an important role in the inhibition of apoptosis. As such, it is induced to promote survival during cellular stresses such as detachment from extracellular matrix (Liu et al., 2006) and ER stress (Hamanaka et al., 2009). It is also induced by pro-survival signaling such as nuclear factor (NF)-κB (Chu et al., 1997; Hong et al., 2000). Not surprisingly, its anti-apoptotic activity is exploited for tumour cell survival, and its expression is induced by potent oncogenes such as Ras (Liu et al., 2005) and E6 (Wu et al., 2010). As such, many members of the IAP family, including the cIAPs are overexpressed in human cancers (Wright and Duckett, 2005). cIAP1 is closely related to cIAP2, sharing 73% amino acid identity (Uren et al., 1996). It is an established oncogene seen in DNA amplicons along with cIAP2 in mice and human tumours, and can cooperate in transformation with oncogenes such as Myc (Xu et al., 2007).

p53

The human gene *TP53* encodes p53, a transcription factor that plays a critical role in tumour suppression and is activated in response to a variety of malignancy-associated stresses, such as DNA damage, oncogenic activation and hypoxia (Balint and Vousden,

2001). Activation of p53 initiates signaling cascades that can result in cell-cycle arrest, senescence, differentiation and apoptosis, with the response being dependent on factors such as the type and intensity of stress and the cell type (Murray-Zmijewski et al., 2008). A simplified illustration of p53 activation and regulation of its downstream targets is shown in Figure 1.3. In general, the function of p53 activation is to inhibit aberrant cell growth, thereby halting tumour development. The importance of the tumour suppressor role played by p53 is underscored by its frequent mutation or inactivation in cancer (Hainaut and Hollstein, 2000). Additionally, germline mutations in p53 define Li-Fraumeni syndrome, which predisposes to a wide spectrum of early-onset cancers (Varley, 2003).

Regulation of p53 by Mdm2

In normal unstressed cells, p53 protein is rapidly turned over, with a half-life varying between 6 and 20 minutes depending on the cell type. Its degradation is mediated predominantly through interaction with its negative regulator, murine double minute 2 (Mdm2) (Kubbutat et al., 1997). Mdm2 functions as an E3 ubiquitin ligase that targets p53 for ubiquitination, leading to subsequent poly-ubiquitination by p300 and resulting in its degradation (Haupt et al., 1997; Kubbutat et al., 1997). Ubiquitination by Mdm2 also promotes nuclear export of p53, possibly by revealing a nuclear export sequence (NES) and inducing p53 into a monomeric form, allowing it access to the nuclear export machinery (Stommel et al., 1999). Thus, modification by Mdm2 serves to both target p53 for degradation and to sequester p53 in the cytoplasm, where it is unable to activate transcription. The cellular level of Mdm2 can determine the modification of p53. Low levels of Mdm2 induce mono-ubiquitination of p53 to signal p53 for nuclear export

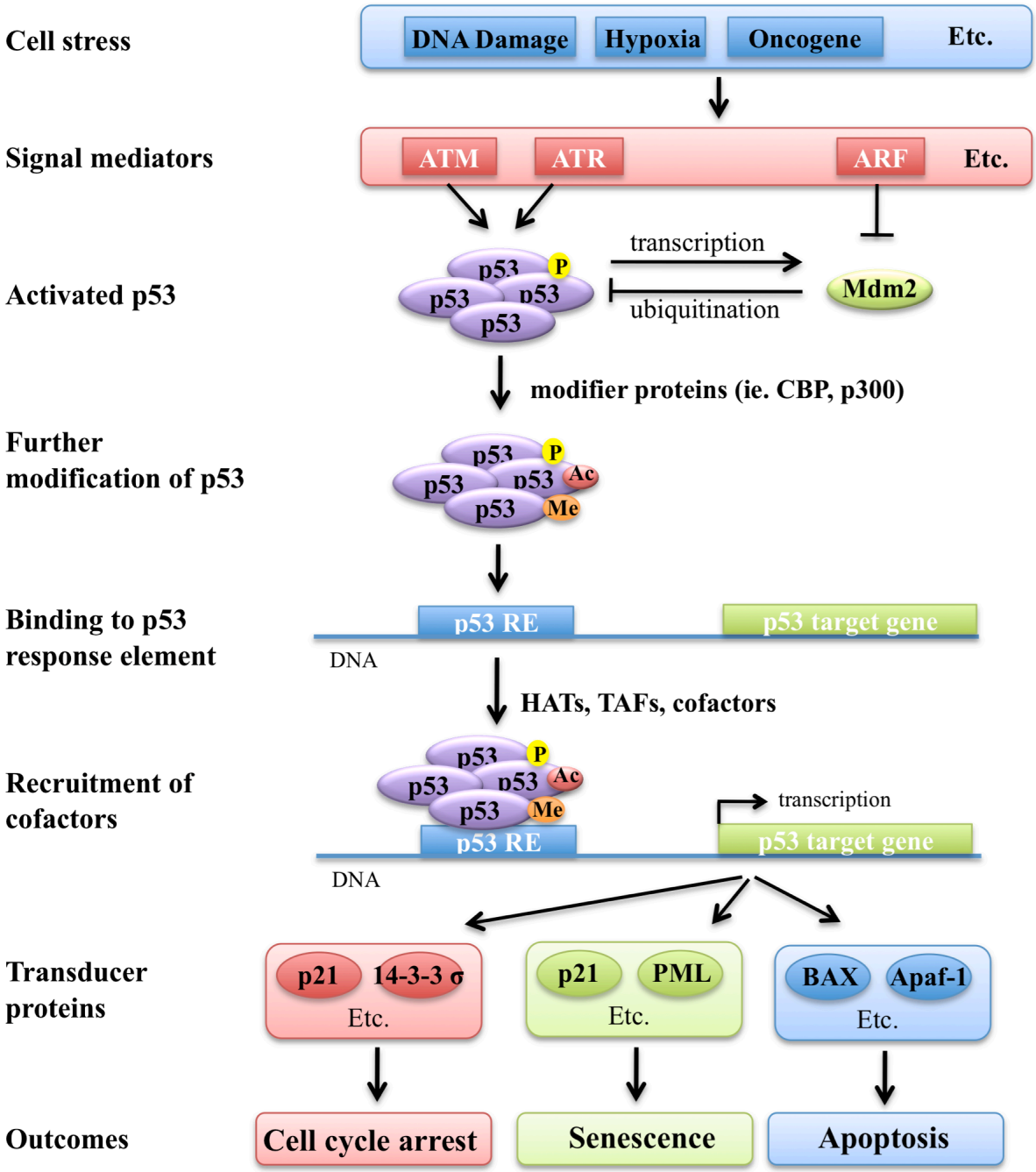


Figure 1.3. p53 regulates cycle arrest, senescence and apoptosis
See text for details. Ac, acetyl group; ARF, alternate reading frame; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad-3-related; CBP, Creb binding protein; Mdm2, murine double minute 2; Me, methyl group; PML, promyelocytic leukemia; RE, response element; TAFs, TATA-binding protein-associated factors. Modified from Riley et al., 2008.

whereas high levels results in poly-ubiquitination and degradation (Li et al., 2003). In addition to ubiquitination, Mdm2 can bind p53 at its amino terminus to directly block the interaction of p53 with transcriptional coactivators, thereby inhibiting the ability of p53 to activate its target genes (Momand et al., 1992). When activation of p53 does occur, one of its target genes is Mdm2, thereby establishing a negative feedback loop that attenuates the p53 response (Harris and Levine, 2005).

Regulation of Mdm2 occurs through its interaction with the tumour suppressor ARF ($p19^{ARF}$ in mice, $p14^{ARF}$ in humans). ARF sequesters Mdm2 in the nucleolus, where it is unable to interact with p53. Additionally, ARF prevents export of Mdm2-p53 complexes to the cytoplasm, which occurs through the nucleolus, thereby allowing accumulation of p53 (Tao and Levine, 1999).

Modulation of Mdm2 activity by post-translational modification

Mdm2 is subject to regulation by post-translational modifications (PTMs) that modulate its activity. In addition to ubiquitinating p53, Mdm2 can auto-ubiquitinate to result in its own proteasomal degradation (Fang et al., 2000). Mdm2 can also be phosphorylated at multiple sites and these sites can be categorized into three clusters. First, a group of residues, Tyr294, Ser395, Ser407, Tyr276 and Ser17 are phosphorylated in response to DNA damage and result in inhibition of Mdm2 function (Meek and Hupp, 2010). A second group, serines 240, 242, 246, 253, 256, 260, 262 and 269 are phosphorylated under normal unstressed conditions. These sites are hypo-phosphorylated following DNA strand breaks and result in stabilization of p53 (Blattner et al., 2002). Lastly, a group of four sites, serines 157, **166**, 186 and 188, lie in close proximity to the nuclear export and import sequences. Phosphorylation of these sites is stimulated by

signaling pathways activated by growth factors and mitogens (Meek and Hupp, 2010). Phosphorylation of Ser166 and Ser186, mediated principally by Akt (Ashcroft et al., 2002), have been identified as key targets in response to proliferative and survival signaling (Meek and Hupp, 2010). Akt participates in a number of survival pathways that, when deregulated, contribute to tumourigenesis. It phosphorylates and inactivates the proapoptotic proteins BAD and procaspase 9, can activate NF-κB, and can downregulate p53 through its phosphorylation of Mdm2 (reviewed in (Altomare and Testa, 2005). The phosphorylations at Ser166 and Ser186 stimulate nuclear entry of Mdm2 where it can interact with p53 (Mayo and Donner, 2001), increase its association with p300 (Zhou et al., 2001) and block the inhibition of Mdm2 by its negative regulator, ARF (Zhang et al., 1998). Ultimately, Ser166 and Ser186 phosphorylation of Mdm2 leads to increased turnover of p53, inhibition of p53-mediated transactivation and protection against p53-induced cell death (Mayo and Donner, 2001; Ogawara et al., 2002).

Mdm2 activity towards p53 can also be induced by modification with SUMO (small ubiquitin-like modifier). Mammalian cells express three SUMO proteins - SUMO1, SUMO2 and SUMO3. Similar to the ubiquitination pathway, sumoylation involves an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase (Johnson, 2004). Most organisms contain a single SUMO E1 enzyme, a heterodimer consisting of SAE1 and SAE2 (Desterro et al., 1999; Okuma et al., 1999). Additionally, in contrast with the ubiquitination pathway in which dozens of E2s participate in the ubiquitination of distinct sets of substrates, the SUMO pathway involves only one E2 enzyme, Ubc9 (Johnson, 2004). The E3 ligases that sumoylate Mdm2 include members of the PIAS (protein inhibitor of activated STAT) family, PIAS1 and PIASxβ, as well as RanBP2

(Miyauchi et al., 2002). The factors leading to the sumoylation of Mdm2 are still unclear, but it appears that Mdm2 becomes sumoylated in transit to the nucleus by RanBP2 in the nuclear pore, then further sumoylated by PIAS proteins in the nucleus (Miyauchi et al., 2002). Sumoylation redirects the ubiquitin ligase activity of Mdm2 from itself to p53, thus increasing the efficiency of p53 ubiquitination and degradation. Consistent with this, SUMO-specific protease (SUSP) 4 has been shown to de-sumoylate Mdm2, resulting in its auto-ubiquitination. Upon induction of SUSP4 by UV stress, Mdm2 is degraded, leading to stabilization of p53 (Lee et al., 2006).

Activation of p53

In response to cellular stresses, inhibition of p53 is relieved by various mechanisms that signal either to p53 directly, or to Mdm2. The half-life of p53 increases from minutes to hours and it accumulates in the nucleus where it binds as a tetramer in a sequence-specific manner to its response element (RE) (McLure and Lee, 1998). p53 then recruits transcription proteins, such as TATA-binding protein-associated factors (TAFs) and histone acetyltransferases (HATs), to the promoter-enhancer regions of target genes to facilitate transcription (Farmer et al., 1996; Thut et al., 1995). Binding of p53 to an RE can also directly repress transcription of some genes although it is not yet clear what distinguishes a transcriptional-activator site from a transcriptional-repressor site. p53-mediated repression can be accomplished through three mechanisms: steric hindrance to inhibit binding of a transactivating protein (Budhram-Mahadeo et al., 1999; Lee et al., 1999); squelching/inactivation of other DNA-bound and -unbound transcriptional activators through protein-protein interactions (Farmer et al., 1996; Seto et al., 1992; Truant et al., 1993); and recruitment of corepressors, such as histone deacetylases

(HDACs) to the promoter-enhancer region of a gene, where they deacetylate the lysine residues of histones in chromatin, thereby repressing transcription (Murphy et al., 1999). The genes regulated by p53 include those involved in cell cycle arrest, senescence and apoptosis (Murray-Zmijewski et al., 2008).

In cases where DNA damage is not severe, activation of p53 results in cell cycle arrest to allow for repair. A key target of transcriptional activation by p53 that mediates this arrest is $p21^{WAF1}$, which inhibits cell cycle progression at the G1/S boundary by inhibiting the activity of cyclin-dependent kinase (CDK)-cyclin complexes and by inhibiting DNA replication (Chen et al., 1995; Luo et al., 1995). Additionally, DNA-damaging agents strongly induce 14-3-3 σ in a p53-dependent manner to result in a G2 arrest (Hermeking et al., 1997).

When cellular damage is too severe for repair, p53 activates transcriptional programs that result in apoptosis or senescence. p53-mediated apoptosis can occur through both the intrinsic and extrinsic pathways, where it targets multiple points in the apoptotic program to ensure that the process is carried out (Fridman and Lowe, 2003). A number of pro-apoptotic proteins are induced by p53, including members of the Bcl-2 family, such as BAX, which is a critical mediator of cell death induced by chemotherapeutic agents (Miyashita and Reed, 1995). Other targets include components of the apoptotic machinery, including caspase 6, an effector caspase (MacLachlan and El-Deiry, 2002), and Apaf-1 (Moroni et al., 2001; Robles et al., 2001), which activates caspase 9. In addition to transcriptional activation, p53 can facilitate apoptosis by suppressing the expression of pro-survival genes. Genes that are repressed by p53 include

members of the anti-apoptotic family, such as *Bcl-2*, *Bcl-xL*, and *survivin* (Vousden and Lu, 2002).

In order to prevent damaged cells from replicating, p53 activation can induce a permanent cell cycle arrest, known as senescence. Senescence can occur as a result of telomere dysfunction, known as replicative senescence, and also from aberrant oncogene activation, known as oncogene-induced senescence (OIS). In contrast to cell cycle arrest and apoptosis, the targets involved in p53-dependent senescence are not well understood. A number of potential mediators have been identified, such as $p21^{WAF1}$, promyelocytic leukemia (PML) and plasminogen activator inhibitor-1 (PAI-1), although the specific roles of each in senescence remains to be elucidated (Qian and Chen, 2010).

Post-translational modifications (PTMs) play a key role in the activation of p53, both by uncoupling p53 from Mdm2 to enable its stabilization (Li et al., 2002; Sakaguchi et al., 2000; Shieh et al., 1997; Unger et al., 1999), and by increasing its transcriptional activity (Lambert et al., 1998; Sakaguchi et al., 1998; Dumaz and Meek, 1999). Different stress signals appear to activate varying enzyme activities that modify p53 at different residues, thus relaying the nature of the stress signal to the protein and promoting a transcriptional program that responds to that particular stress signal (Colman et al., 2000). The most common PTM of p53 is phosphorylation. Twenty-one phospho-serine and -threonine residues have been identified thus far (Meek and Anderson, 2009). One of the most common sites of phosphorylation of p53 occurs at serine 15, mediated principally by ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad-3-related) in response to genotoxic stress. Phosphorylation at this residue induces nucleation and also primes p53 for subsequent modification of other residues (Bode and

Dong, 2004; Saito et al., 2003; Sakaguchi et al., 1998). Importantly, although serine 15 phosphorylation of p53 does not directly disrupt the interaction between p53 and Mdm2 (Dumaz and Meek, 1999), it is required for subsequent phosphorylation at threonine 18, which directly disrupts the interaction (Dumaz et al., 1999). Thus, phosphorylation of serine 15 is associated with p53 protein stability. Multiple kinases can phosphorylate the same residue on p53 - for example, serine 15 can also be phosphorylated by ERK (extracellular signal-regulated kinase) (Persons et al., 2000), DNA protein kinase (DNAPK) (Lees-Miller et al., 1992), and p38 (She et al., 2000), and the same kinase may phosphorylate multiple residues. This redundancy allows for specific patterning of p53 PTMs and may also provide a compensatory mechanism (Bode and Dong, 2004). Further modifications by acetyltransferases, such as CREB-binding protein (CBP), p300 and p300/CBP-associated factor (PCAF), and methyltransferases, such as SET9, which acetylate and methylate p53, respectively, further stabilizes the protein and increases site-specific DNA-binding (Riley et al., 2008).

Inactivation of p53

The protective function of p53 is compromised in approximately half of all human cancers and typically occurs as a result of somatic mutations (Hollstein et al., 1991). A summary of the mechanisms of p53 inactivation is shown in Figure 1.4. Inactivation may occur as a result of a direct mutation in *TP53*, or an alteration that affects the regulation of p53 protein. For example, expression of the E6 oncoprotein in cervical cancer can bind p53 and result in its rapid degradation (Scheffner et al., 1990). Amplification of Mdm2 in soft-tissue sarcomas results in a similar loss of p53 through binding and subsequent degradation (Momand et al., 1992; Oliner et al., 1992). Frequently, the loss of functional

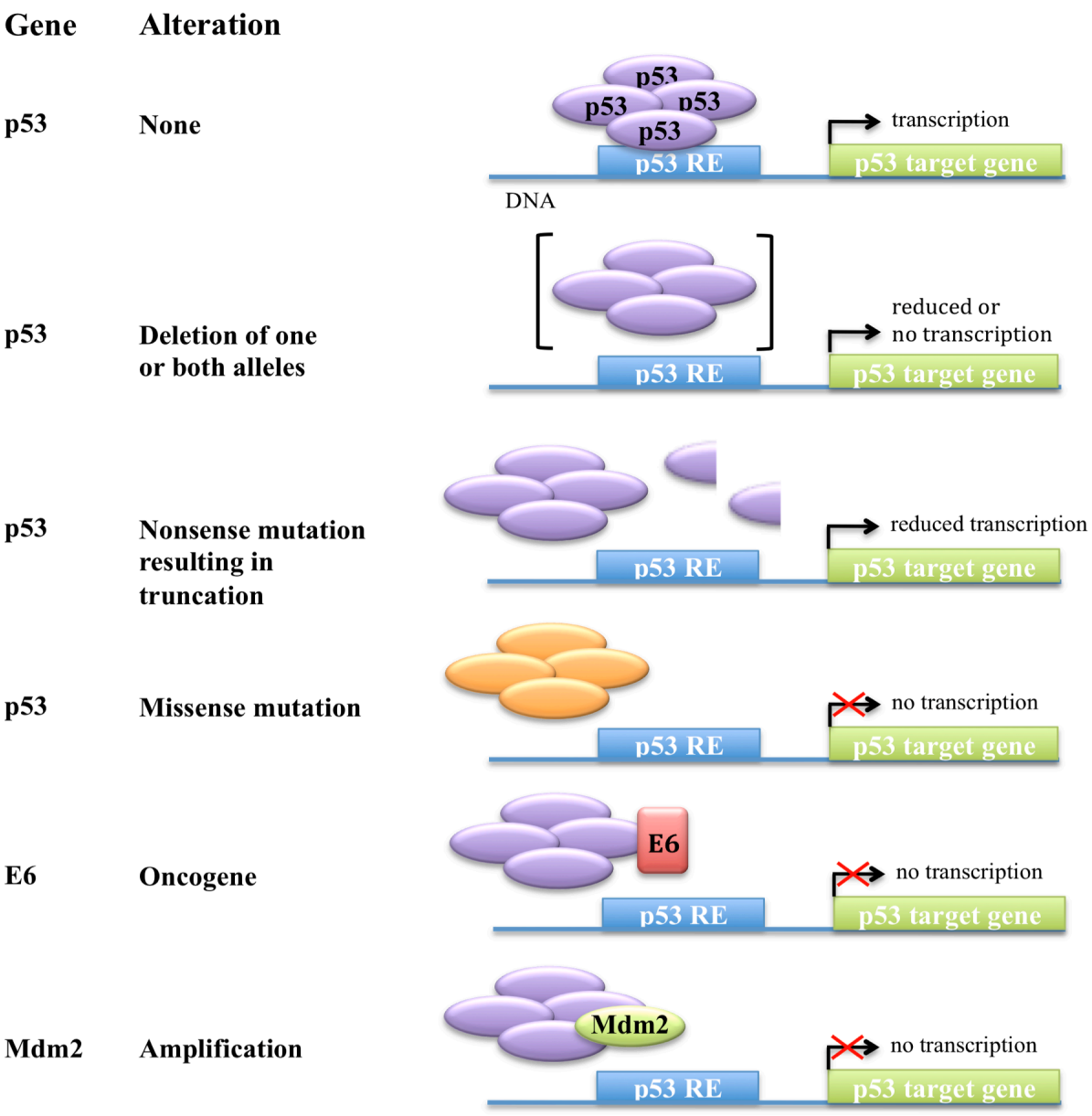


Figure 1.4. Mechanisms of p53 inactivation
Common mechanisms by which p53 is inactivated in cancer cells. See text for detailed description of each alteration. Mdm2, murine double minute 2; RE, response element. Modified from Vogelstein and Kinzler, 2002.

p53 is a consequence of a somatic mutation in *TP53*. p53 mutations often attenuate the sequence-specific DNA-binding of the mutant protein to the wild-type p53 responsive element, resulting in a loss of their tumour suppressive function (Kato et al., 2003). Additionally, these mutant proteins can act as dominant-negative inhibitors of wild-type p53, due to the formation of hetero-tetramers consisting of the mutant protein with the wild-type protein that are defective in sequence-specific DNA binding (Milner and Medcalf, 1991; Milner et al., 1991). Mutant p53 protein frequently accumulates to high levels in tumours and tumour cell lines, which has been used to identify patients with p53 mutations. This is due to the inability of mutant p53 to transcriptionally activate Mdm2 (Midgley and Lane, 1997).

Gain of function (GOF) mutations in p53

Unlike most tumour-suppressor genes, which are frequently inactivated by deletions or truncating mutations, *TP53* is often altered by a single mono-allelic missense mutation and overexpressed as a stable full-length protein, which suggests the existence of a selection pressure for maintaining expression of the mutant protein (Brosh and Rotter, 2009). Consistent with this, many mutant forms of p53 acquire new oncogenic properties that are independent of wild-type p53, termed the gain-of-function hypothesis. This was demonstrated in *TP53*-null cells, which when transfected with mutant p53, displayed an enhanced ability to form tumours in mice (Shaulsky et al., 1991; Wolf et al., 1984). In fact, when p53 was first characterized more than 30 years ago, p53 was initially mistakenly identified as an oncogene due to observations that p53 had tumour-promoting abilities and was overexpressed in a large number of cancers (Levine and Oren, 2009).

Gain-of-function mutations of p53 may enhance the oncogenic potential of a cell through a variety of mechanisms. Common p53 mutants gain the ability to interact with and inactivate p63 and p73 (Di Como et al., 1999; Gaiddon et al., 2001), members of the p53 family that have key tumour suppressing roles (Deyoung and Ellisen, 2007). Despite the fact that about two thirds of mutations of p53 occur in its DNA-binding domain (Kato et al., 2003), mutant p53 has been well documented to modulate gene transcription (Kim and Deppert, 2004; Strano et al., 2007). Therefore it appears that the mutations do not necessarily alter the transcriptional potential of p53, but rather the target specificity. Mutant p53 can interact with various transcription factors and become tethered to the response element of the transcription factor. Through its transactivation domain, mutant p53 recruits transcriptional activators and augment the transcription of that transcription factor's targets (Di Agostino et al., 2006). Variability exists in the identity of genes affected by different p53 mutants, but many of the genes regulated by mutant p53 in human cancers promote survival and proliferation, inhibit apoptosis, increase invasiveness and angiogenesis, among other effects (Brosh and Rotter, 2009). Accordingly, various human tumours with mutant p53 are often more aggressive and patients with tumours harbouring mutant p53 are predicated to have poorer prognoses than patients with tumours lacking the p53 protein (Borresen-Dale et al., 1998; Falette et al., 1998; van Slooten et al., 1999).

NF-κB Signaling pathway

Activation of the nuclear factor (NF)-κB signaling pathway can be viewed as being antagonistic to signaling by the p53 pathway, in that it largely promotes survival with some exceptions (Ak and Levine, 2010). The NF-κB family of transcription factors

bind to κB elements in the promoters/enhancers of target genes and can modulate gene expression through the recruitment of coactivators and corepressors (Hayden and Ghosh, 2004). The genes regulated by NF-κB are involved in a large variety of processes, including apoptosis, cell adhesion, proliferation, immune responses, inflammation, cellular stress response and tissue remodeling (Hayden and Ghosh, 2004; Pahl, 1999). In mammalian cells, there are five NF-κB members, RelA (p65), RelB, c-Rel, p105/p50 (NF-κ1) and p100/p52 (NF-κB2) (Figure 1.5), which share an N-terminal Rel homology domain (RHD) that is responsible for DNA binding, dimerization, and interaction with inhibitor of κB (IκB) proteins (Hayden and Ghosh, 2004). Homo- and heterodimers of these subunits constitute the different NF-κB complexes, which are usually kept inactive in the cytoplasm by a family of IκB proteins. This family includes IκBα, IκBβ, IκBε, IκBγ and BCL-3, and is characterized by the presence of five to seven ankyrin repeats that bind the RHD of NF-κB dimers. The established model of IκB function is that IκB-bound NF-κB dimers are retained in the cytoplasm by masking a nuclear localization sequence (NLS), thereby preventing nuclear translocation and DNA-binding. However, recent evidence has shown that IκBα only masks the NLS of p65, but not that of p50. Therefore, the exposed NLS of p50 combined with the nuclear export sequence (NES) of IκBα results in the constant shuttling of IκBα/NF-κB complexes between the nucleus and cytoplasm (Ghosh and Karin, 2002).

Activation of NF-κB signaling

Canonical NF-κB pathway

Activation of NF-κB can occur through multiple mechanisms, the most frequently observed being the canonical pathway (Figure 1.6). In this pathway, IκB is

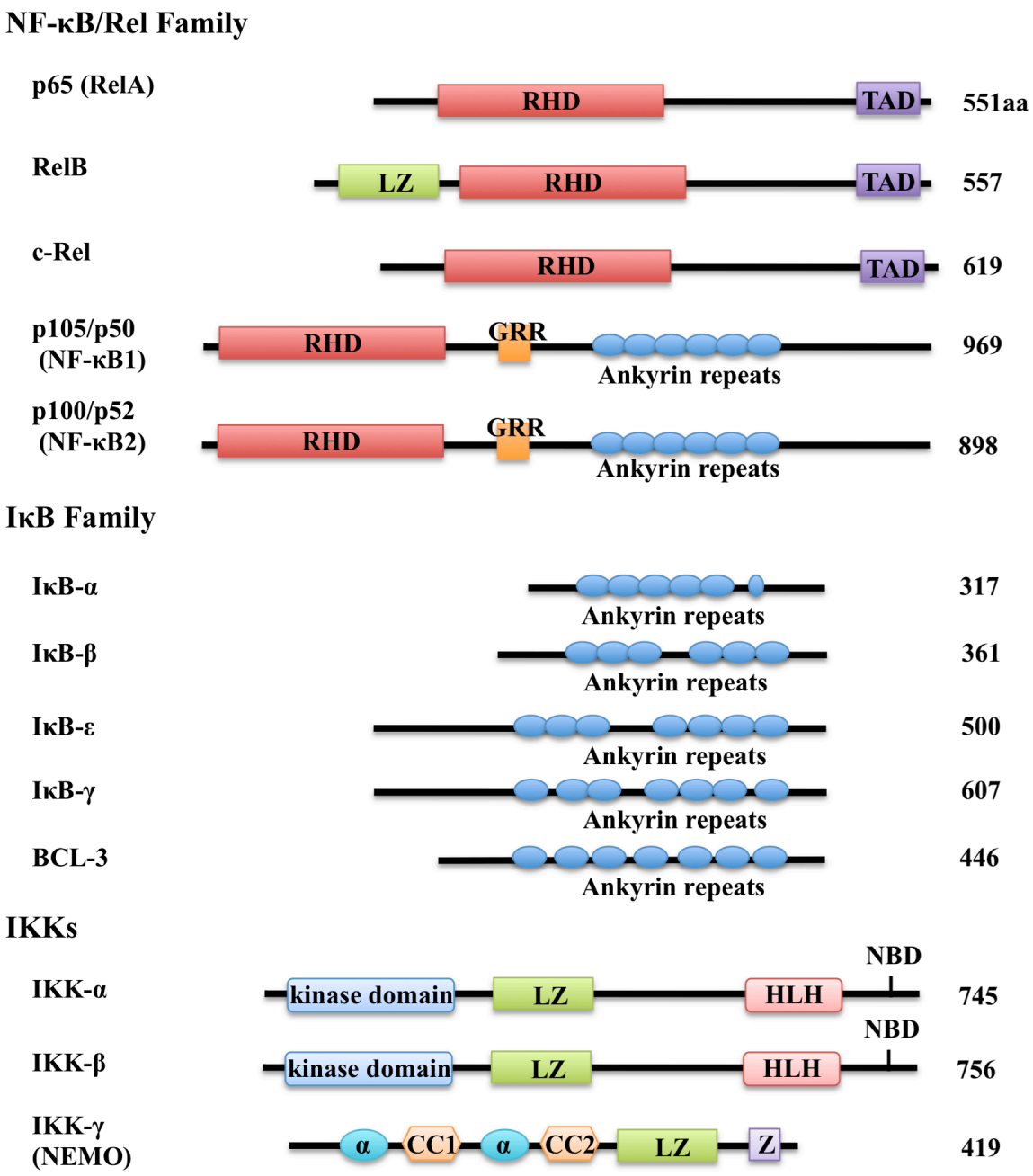


Figure 1.5. Schematic representation of NF-κB, IκB and IKK proteins family of proteins
Members of the NF-κB, IκB and IKK protein families. The number of amino acids in each protein is shown on the right. RHD, Rel homology domain; TAD, transactivation domain; LZ, leucine zipper domain; GRR, glycine-rich region; HLH, helix-loop-helix domain; Z, zinc finger domain; CC1/2, coiled-coil domains, NBD, NEMO-binding domain; α, α-helical domain.

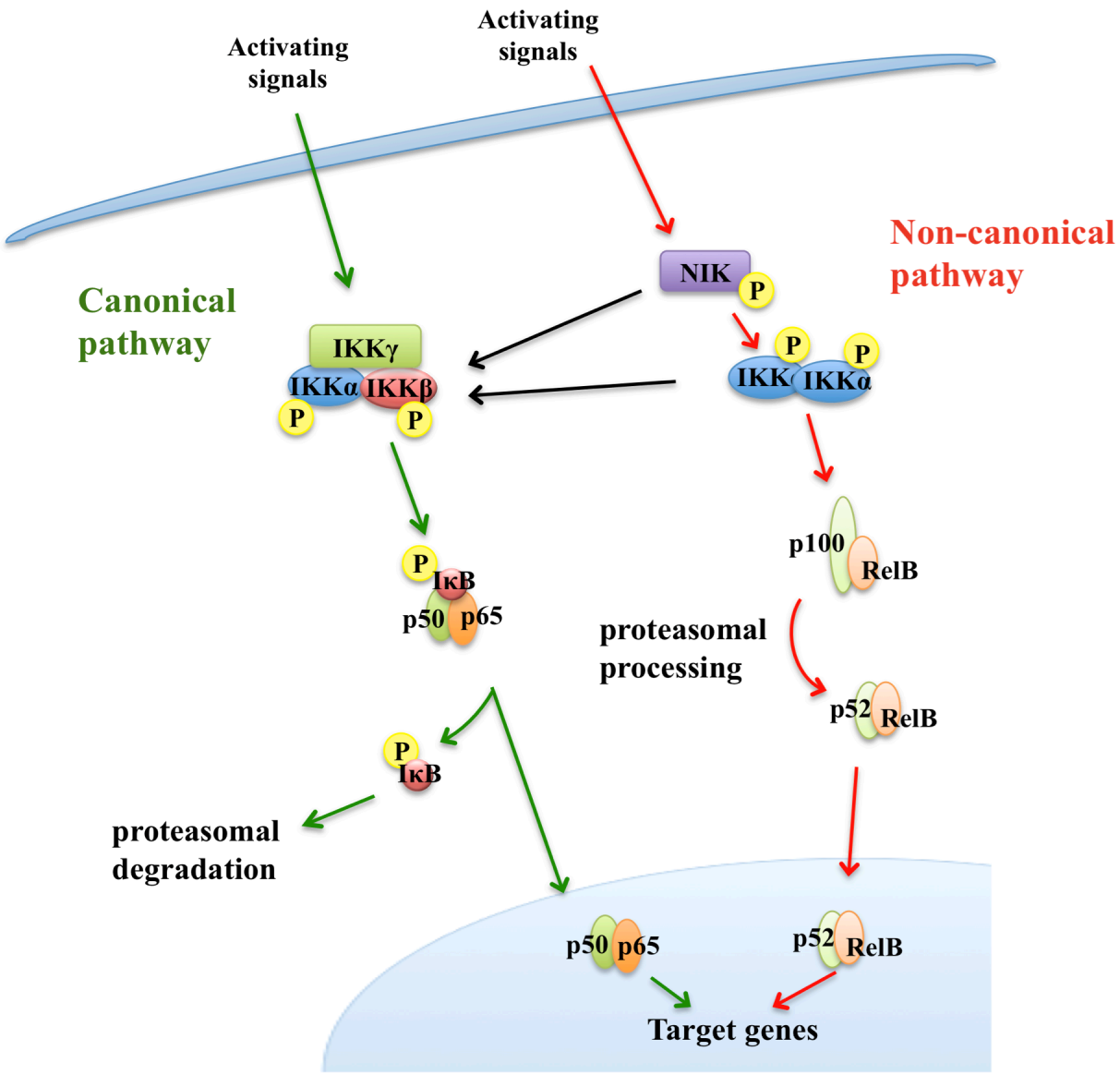


Figure 1.6. Activation of the NF-κB signaling pathway
Activation of the canonical and non-canonical NF-κB pathways. See text for details. IκB, inhibitor of κB; IKK, IκB kinase; NIK, NF-κB inducing kinase; P, phosphorylation.

phosphorylated and targeted for degradation by an activated IκB kinase (IKK) complex (DiDonato et al., 1997; Mercurio et al., 1997). This complex consists of two catalytic subunits IKK-α and IKK-β (also known as IKK1 and IKK2, respectively), as well as a regulatory subunit called IKK-γ, also known as NF-κB essential modifier (NEMO) (Hayden and Ghosh, 2004). Genetic experiments have shown that in the canonical pathway, the predominant IκB kinase is IKK-β (Pasparakis et al., 2006). Phosphorylation of IκB proteins result in their polyubiquitination by β-TrCP-containing Skp1-Culin-Roc1/Rbx1-Hrt-1-F-box (SCF) E3 ubiquitin ligase complexes ($SCF^{\beta\text{-}TrCP}$), leading to their degradation by the proteasome (Alkalay et al., 1995). Following the degradation of IκB, the released NF-κB complex (primarily p65/p50) binds to promoter and enhancer regions containing the κB consensus sequence, where it can either activate or repress transcription (Hayden and Ghosh, 2004).

Non-canonical NF-κB pathway

The non-canonical NF-κB pathway activates a RelB/p52 complex that involves the proteasomal processing of p100 to p52 (Figure 1.6). p100 functions as an IκB-like molecule and inhibits RelB nuclear translocation (Solan et al., 2002), thus its processing serves both to generate p52 and to allow entry of the complex into the nucleus. In contrast to the canonical pathway, activation of the non-canonical pathway does not require IKK-β or NEMO (Claudio et al., 2002). Instead, signaling to NF-κB-inducing kinase (NIK) results in the phosphorylation and activation of IKK-α, which phosphorylates p100 and induces its ubiquitin-dependent processing by the proteasome to p52 in a manner similar to the degradation of IκB. The resulting p52/RelB complex then translocates to its target genes in the nucleus (Xiao et al., 2001). The activity of the non-

canonical NF-κB pathway is largely dependent on the steady state level of NIK. Typically, levels of NIK protein are extremely low, owing to its targeting for constant degradation by a multi-subunit ubiquitin ligase complex composed of TRAF3 (TNF receptor-associated factor 3), TRAF2 (TNF receptor-associated factor 2), and cIAP1/2 (cellular inhibitor of apoptosis protein 1/2) (Liao et al., 2004; Vallabhapurapu et al., 2008; Zarnegar et al., 2008). Deficiencies in TRAF2, TRAF3, or cIAP1/2 lead to accumulation of NIK and results in aberrant p100 processing (Grech et al., 2004; He et al., 2006; Varfolomeev et al., 2007). The importance of NIK negative regulation is highlighted by evidence that shows genetic aberrations in TRAF2, TRAF3 and the cIAPs are associated with activation of both canonical and non-canonical NF-κB signaling in multiple myeloma, where they can contribute to pathogenesis of the disease (Annunziata et al., 2007; Demchenko et al., 2010; Keats et al., 2007). Despite the fact that NIK and IKK-α activation are typically associated with non-canonical signaling, both of these kinases are capable of phosphorylating and activating IKK-β, thereby activating canonical NF-κB signaling (Hacker and Karin, 2006; Nakano et al., 1998; O'Mahony et al., 2000).

Targets of NF-κB activation

Activation of NF-κB signaling regulates a large number of genes involved in a wide range of biological functions including cytokines, adhesion molecules, chemokines, and a number of genes that contribute to survival by promoting proliferation and inhibiting apoptosis (Karin et al., 2002). NF-κB promotes proliferation by inducing the transcription of genes such as cyclin D1 that stimulate transition from G1 to S phase (Guttridge et al., 1999; Hinz et al., 1999), and the oncoprotein c-myc that also induces

pro-proliferation genes (La Rosa et al., 1994). Inhibition of apoptosis by NF-κB is achieved by the transcription of factors including members of the Bcl2 family, such as BFL1 and Bcl-xL (Khoshnan et al., 2000; Wang et al., 1999), caspase-8/FADD (FAS-associated death domain)-like IL-1β-converting enzyme (FLICE) inhibitory protein (c-FLIP) (Kreuz et al., 2001), and cellular inhibitor of apoptosis 2 (cIAP2) (Wang et al., 2003). Another target of NF-κB is its own inhibitor, IκB-α, which establishes an autoregulatory loop that terminates the activation of NF-κB (Hay et al., 1999).

There is also growing evidence to support a pro-apoptotic role for NF-κB signaling. For example, it has been shown that TNF-related apoptosis inducing ligand (TRAIL) induces apoptosis in a number of cell lines by activating NF-κB, resulting in the transcriptional activation of death receptor 5 (DR5) (Shetty et al., 2002). Additionally, NF-κB can upregulate another death receptor, Fas, and its ligand, Fas ligand, in response to a number of pro-apoptotic stimuli (Radhakrishnan and Kamalakaran, 2006). NF-κB-mediated cell death is also facilitated by repression of survival genes such as cIAP and c-FLIP (Poppelmann et al., 2005), which is interesting to note given they are also targets for the pro-survival function of NF-κB. The pro-apoptotic role of NF-κB is not as well characterized as its pro-survival role. However, the cell type, stimulus, context of NF-κB activation, and likely the nature of the NF-κB proteins appear to be important in determining a pro- or anti-apoptotic response.

Nuclear functions of IKK-α

The roles of the IKK complexes discussed so far have focused on the cytoplasmic functions, which stimulate the nuclear translocation of active NF-κB complexes. While the cytoplasmic roles of the IKK complex have been well established, emerging reports

indicate that the IKKs, particularly IKK-α, perform additional roles in the nucleus (Espinosa et al., 2011). Depending on the cell type, activation of IKK-α in the nucleus can play both tumour suppressing and tumour promoting roles. In keratinocytes, nuclear IKK-α is required for production of a group of keratinocyte differentiation-inducing factors (kDIF), which induce growth arrest and terminal differentiation (Sil et al., 2004). The loss of nuclear IKK-α is associated with squamous cell carcinoma (SCC), likely attributable to its control of keratinocyte proliferation. Reduced IKK-α, either through mutations or epigenetic silencing, has been reported in SCC. In murine models, overexpression of IKK-α inhibits chemically induced SCC formation and progression in mice (Liu et al., 2006), and IKK-$\alpha^{+/-}$ display higher incidence of SCC (Park et al., 2007). In addition to tumour suppression, IKK-α mediates cisplatin-induced apoptosis. Nuclear IKK-α is phosphorylated by ATM following cisplatin treatment, resulting in a stabilization of p73 and initiation of apoptosis (Yoshida et al., 2008).

In contrast, one of the consequences of nuclear IKK-α activation is enhanced proliferation. There is evidence to support a critical role for IKK-α in the nucleus to activate NF-κB-responsive genes in an IκB-independent manner. In response to cytokine stimulation, it is required for the direct phosphorylation of histone H3 and expression of NF-κB targets (Anest et al., 2003; Park et al., 2006; Yamamoto et al., 2003). In addition to histone H3, IKK-α phosphorylates SMRT (silencing mediator of retinoid and thyroid receptors), an HDAC-associated corepressor protein that regulates NF-κB. Under basal conditions, SMRT and HDAC3 associate with p50 homodimers on chromatin to promote repression. Following phosphorylation by IKK-α, SMRT and HDAC3 are dissociated from chromatin. SMRT is then degraded by the proteasome and allows recruitment of

active NF-κB complexes to the promoters of target genes to enhance survival (Hoberg et al., 2004). Activation of NF-κB target gene expression by IKK-α can occur at the expense of p53 targets, which augments the survival signal. Nuclear IKK-α phosphorylates CBP, which increases its HAT and transcriptional activity. Additionally, this phosphorylation switches the binding preference of CBP from p53 to NF-κB, thus increasing NF-κB target gene expression while decreasing p53-mediated transcription (Huang et al., 2007). Nuclear IKK-α can also impact NF-κB-independent gene expression. In colorectal cells, IKK-α is recruited to the promoter of Notch-responsive genes where it derepresses SMRT-mediated repression, resulting in expression of genes that promote tumour growth (Fernandez-Majada et al., 2007). Additionally, IKK-α is recruited to estrogen-responsive promoters, where it phosphorylates estrogen receptor (ER)-α, steroid receptor coactivator (SRC)-3 and histone H3 to increase expression of cyclin D1 and c-myc (Park et al., 2005), which contribute to increased proliferation.

Importantly, inflammatory stimuli-mediated activation of nuclear IKK-α has been shown to phosphorylate PIAS1, a SUMO E3 ligase that belongs to a family of transcriptional regulators (Shuai and Liu, 2005). Upon phosphorylation at serine 90 by IKK-α, PIAS1 rapidly associates with promoters of specific NF-κB target genes to repress activation of inflammatory genes, including TNF-α (Liu et al., 2007). Thus IKK-α is a critical regulator of NF-κB signaling in both positive and negative manners.

Crosstalk between NF-κB and p53

Given that NF-κB signaling can regulate a large variety of cellular processes, it is not surprising that intricate crosstalk exists between it and other fundamental cell signaling pathways. An example of this is the complex relationship between NF-κB and

p53. Anti-apoptotic effects exerted by activation of NF-κB can, in part, be achieved by its negative regulation of p53. Tergaonkar et al. (2002) demonstrated that in mouse embryonic fibroblasts (MEFs), activation of IKK-β, but not IKK-α, can increase expression of Mdm2. It was unclear if the increase was a direct consequence of transcriptional activation by NF-κB, although it appeared unlikely since p53 was found to be required for the induction. Direct binding to the Mdm2 promoter has been established for BCL-3, a member of the IκB family, which is required for Mdm2 expression in MCF-7 breast cancer cells. Its overexpression induces an increase in Mdm2 and suppresses p53 transcriptional activation (Kashatus et al., 2006). A recent study in Jurkat cells showed that an activated canonical NF-κB complex involving p50 also binds directly to the promoter of Mdm2 and induces its expression (Busuttil et al., 2010). The expression of Mdm2 is controlled by two promoters, P1 and P2. p53-mediated induction of Mdm2 occurs through binding to the P2 promoter (Barak et al., 1994) while p50 was found to bind the P1 promoter, thus establishing a p53-independent induction of Mdm2 expression by NF-κB (Busuttil et al., 2010). Independent of Mdm2, activated IKK-β phosphorylates p53 at serines 362 and 366, which signals it for ubiquitination by β-TrCP1 followed by degradation. Through this mechanism, activation of the IKK-β lowers p53 levels while increasing NF-κB activity (Xia et al., 2009). The activity of p53 can be impinged on by NF-κB through the upregulation of Mortalin (Li et al., 2010; Li et al., 2010). Mortalin, or Mot-2, is a member of the heat shock protein (Hsp)-70 family and is overexpressed in cancer (Wadhwa et al., 2006). Direct binding of mortalin to p53 has been shown to inactivate p53 by sequestering it in the cytoplasm, resulting in downregulation of its

downstream targets (Wadhwa et al., 1998). Thus, activation of NF-κB signaling can negatively impact p53 levels and activity through multiple mechanisms.

Reciprocally, p53 can negatively regulate NF-κB activity. Overexpression of wildtype p53 can increase the expression of IκB-α, resulting in a reduction of active NF-κB complexes translocating to the nucleus (Shao et al., 2000). Additionally, p53-null mice display constitutively high levels of IKK and NF-κB activity, indicating a role for p53 in limiting NF-κB activity under basal conditions (Komarova et al., 2005). A number of groups have reported on the mutual repression between p53 and NF-κB. Transcriptional activation mediated by either p53 or NF-κB is dependent on the relative level of the other protein, in part due to competition for a limited pool of coactivators such as CREB-binding protein (CBP) and p300. It is interesting to note that mutant p53 is unable to inhibit NF-κB activity, despite the fact that it is able to bind to coactivators, indicating additional mechanisms exist for inhibition by wildtype p53 (Ikeda et al., 2000; Wadgaonkar et al., 1999; Webster and Perkins, 1999).

In contrast to the mutual repression, there is also cooperative crosstalk between NF-κB and p53. For example, apoptosis induced by p53 has been shown to require the activation of NF-κB. This activation of NF-κB occurs by a mechanism that does not require an active IKK complex. Instead, p53 activates the MAPK pathway, resulting in activation of the kinase $pp90^{rsk}$ that phosphorylates IκB and activates NF-κB. Inhibition of NF-κB abrogates p53-mediated apoptosis (Ryan et al., 2000). Mutations in p53, as previously mentioned, can promote survival by activating genes that are not typically targets of wildtype p53. One such target is p100/p52, resulting in activation of the non-canonical NF-κB pathway (Scian et al., 2005). Additionally, mutant p53 has been

reported to enhance the activation of NF-κB in response to TNF-α, although the mechanism whereby this occurs is still unclear (Weisz et al., 2007). Crosstalk between p53 and NF-κB is complex, and the consequence of their interaction, whether it is pro-survival or pro-death, is context-dependent.

Function of cIAPs in NF-κB signaling

The cIAPs can regulate the canonical and non-canonical NF-κB pathways in contrasting ways. cIAP1/2 plays a critical role in TNF receptor (TNFR) signaling to canonical NF-κB (Rothe et al., 1995) (Figure 1.7). Binding of TNF-α to TNFR induces the formation of complex I, consisting of TRADD (TNF receptor-associated death domain), TRAF2 (TNF receptor-associated factor 2), and RIP1 (receptor interacting protein 1) (Hsu et al., 1995). TRAF2 recruits cIAP1/2 to the complex, where they are required for TNFR-induced activation of NF-κB signaling. cIAP1/2 mediates the activating K63-linked polyubiquination of RIP1, which results in the phosphorylation of IKK and subsequent degradation of IκB to activate canonical NF-κB (Varfolomeev et al., 2008). The K63-linked polyubiquitination of RIP1 also suppresses the activation of caspase-8 and formation of the pro-apoptotic complex II (Wang et al., 1998), thereby preventing apoptosis (Figure 1.8). The expression of pro-survival genes stimulated by TNF-α activation of NF-κB signaling is believed to play a major role in the protection against TNF-α-induced cell death (Karin and Lin, 2002).

In contrast, cIAP1/2 can also repress NF-κB activity. As previously mentioned, cIAP1/2 participate in a multi-subunit ubiquitin ligase complex that includes TRAF2 and TRAF3. This complex targets NIK and tonically represses it to limit the activation of both the canonical and non-canonical NF-κB signaling (Liao et al., 2004; Vallabhapurapu et al.,

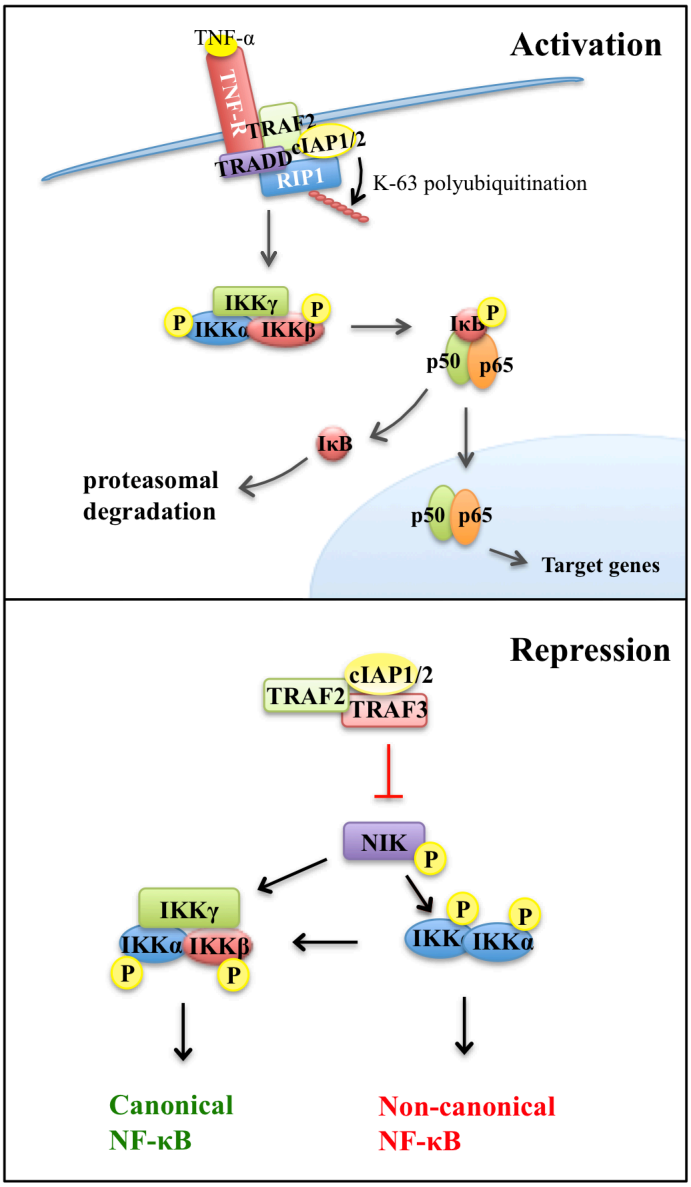


Figure 1.7. cIAP1/2 participates in positive and negative regulation of NF-κB
cIAP1/2 are recruited to an activated TNF receptor where they mediate K-63 polyubiquitination of RIP1. RIP1 subsequently activates the IKK complex, resulting in activation of canonical NF-κB complexes. In contrast, cIAP1/2 represses activation of canonical and non-canonical NF-κB signaling by ubiquitinating NIK, leading to its degradation. See text for details. cIAP, cellular inhibitor of apoptosis; IKK, IκB kinase; NIK, NF-κB inducing kinase; RIP, receptor interacting protein; TNF, tumour necrosis factor; TNFR, TNF receptor; TRAF, TNFR associated factor; TRADD, TNFR associated death domain.

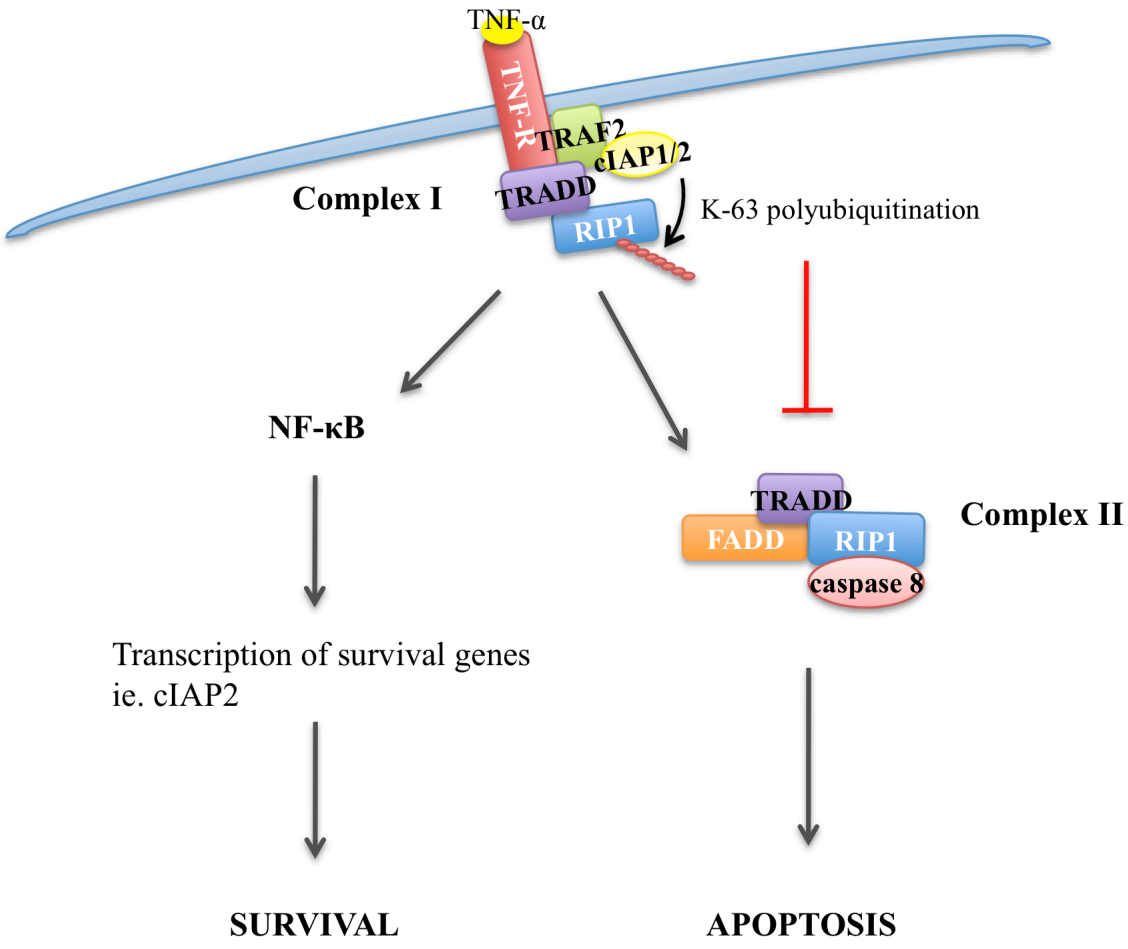


Figure 1.8. cIAP1/2 ubiquitination of RIP1 mediates survival signaling by NF-κB
Upon binding of TNF-α to its receptor TNFR, complex I, containing TRAF2, TRADD, RIP1 and cIAP1/2, is formed. cIAP1/2 mediates K-63 polyubiquitination of RIP1, thus resulting in the activation of NF-κB and downstream transcription of pro-survival genes. In the absence of cIAP1/2, unubiquitinated RIP1 forms complex II with TRADD, FADD and caspase 8, resulting in initiation of apoptosis. cIAP2, cellular inhibitor of apoptosis 2; FADD, Fas-Associated protein with Death Domain; RIP, receptor interacting protein; TNF-R, tumour necrosis factor receptor; TRADD, Tumor necrosis factor receptor type 1-associated death domain; TRAF, TNF-receptor associated factor.

2008; Zarnegar et al., 2008). Mutations in the constituents of this complex, including cIAP1/2, lead to constitutive activation of NF-κB and contribute to the pathogenesis of multiple myeloma (Annunziata et al., 2007; Keats et al., 2007). This is in contrast to their proposed roles as oncogenes, where their anti-apoptotic functions may contribute to aberrant proliferation (Dynek and Vucic, 2010).

Thus, the cIAPs can both positively and negatively regulate the NF-κB pathway. While cIAPs participate in the activating ubiquitination of RIP to result in activation of the canonical pathway in response to TNF/death ligands, they conversely ubiquitinate NIK in an inhibitory manner to suppress the both the canonical and non-canonical pathways.

IAP antagonists

The anti-apoptotic function of IAPs and their overexpression in a wide variety of cancers make them attractive therapeutic targets. As such, a number of strategies to target IAP proteins in cancer are currently under investigation. One focus has been on the generation of molecules that mimic the amino-terminus of mature Smac. These Smac mimetics disrupt IAP:caspase and IAP:SMAC interactions and can stimulate cell death (Vucic and Fairbrother, 2007). Originally designed to target XIAP, these antagonists exhibit higher affinities for the cIAPs, triggering auto-ubiquitination and proteasomal degradation. IAP antagonist-induced autoubiquitination and subsequent degradation of cIAP1 requires TRAF2 while degradation of cIAP2 requires both TRAF2 and cIAP1. Despite the fact that cIAP2 can ubiquitinate RIP1 and NIK in the absence of cIAP1, it does not appear to be able to autoubiquitinate. It is suggested that its own RING domain interacts with that of cIAP1, which promotes the ubiquitination of cIAP2 (Darding et al.,

2011). Following treatment with an IAP antagonist, it is speculated that the downregulation of cIAPs result in the accumulation of NIK, which activates non-canonical NF-κB signaling and leads to autocrine TNF-α production (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). In the absence of cIAPs, activation of survival genes by p65/RelA is greatly reduced (Mahoney et al., 2008; Varfolomeev et al., 2008). TNF-α instead triggers the formation of the pro-apoptotic complex II, consisting of FADD, caspase-8 and de-ubiquitinated RIP1, resulting in apoptosis (Wang et al., 2008). Induction of cell death by IAP antagonists has shown some success in a few cell lines, but its effectiveness as a single agent is limited (Cheung et al., 2009). It appears that the cytotoxicity of IAP antagonists is dependent on the production of TNF-α. However, the factors that determine whether IAP antagonism will induce TNF-α in a particular cancer cell remain unclear.

MAPK signaling

The mitogen-activated protein kinase (MAPK) signaling cascades are comprised of a series of kinases that participate in a signaling relay. The cascade begins with a MAPK kinase kinase (MAP3K), which activates a MAPK kinase (MAP2K) and finally signals to the terminal MAPK. The terminal MAPKs consist of extracellular signal-regulated kinase (ERK)-1/2, c-Jun amino-terminal kinase (JNK), p38 and ERK5. In general, ERKs are activated by growth factor-stimulated cell surface receptors while JNK, p38 and ERK5 are activated by stress and growth factors. The MAP3Ks upstream of ERK1/2 are the Raf kinases, which phosphorylate MEK1/2 (MAP2Ks). MEK1/2 subsequently phosphorylates ERK1/2, resulting in their activation. ERKs can phosphorylate and regulate a large number of substrates, many of which are transcription

factors in the nucleus, leading to changes in gene expression (reviewed in (Yoon and Seger, 2006). The targets of ERK activation often include those that promote survival and proliferation. Therefore, it is not surprising that constitutive ERK activation is frequently observed in cancer (Mebratu and Tesfaigzi, 2009).

Previous observations

7,12-Dimethylbenz(a)anthracene (DMBA)-induced mammary tumour formation in the cIAP2-null mouse was characterized in our laboratory to determine the effects of cIAP2 loss on tumourigenesis. cIAP2$^{-/-}$ mice developed mammary adenocarcinomas following a longer latency compared to cIAP2$^{+/+}$ control littermates (127 days versus 93 days, respectively) (Figure A.1). However, by the end of the study, the cIAP2$^{-/-}$ mice had developed significantly more adenocarcinomas than the control mice (Table 1). Fourteen mammary adenocarcinomas developed in 12 of 21 control animals while the remaining 9 mice developed only benign hyperplasias. In contrast, 19 of 24 cIAP2$^{-/-}$ mice (80%) produced a total of 23 mammary carcinomas, the majority of which were poorly differentiated adenocarcinomas with 5 being completely undifferentiated anaplastic tumours. TUNEL assays performed on tumour sections from cIAP2$^{-/-}$ mice exhibited 6-fold higher numbers of apoptotic bodies compared with control mice (Figure A.2A). However, cIAP2$^{-/-}$ tumours also displayed a nearly 4-fold increase in the percentage of Ki-67-positive mitotic cells compared to control tumours (Figures A.2B). These results indicated that while the loss of cIAP2 led to an increase in apoptosis, this alteration concurrently resulted in an increase in proliferative signaling. The growth rate of tumours was estimated by dividing the wet weight of tumours on resection by the time from initial palpation to sacrifice. Overall, cIAP2$^{-/-}$ tumours displayed a higher mean tumour growth

rate relative to the control tumours (Figure A.2C). When the tumours were examined by western blotting, activation of NF-κB was found in all adenocarcinomas compared to hyperplasias as indicated by an increase in phospho-IKK-β. However, increased phosphorylated IKK-α was only observed in cIAP2-null adenocarcinomas. Together, these *in vivo* observations implicated a role for cIAP2 in limiting cell growth during carcinogen-induced mammary tumourigenesis.

In vitro stable transfection of MCF-10A cells with H-ras and shRNA targeted against cIAP2 resulted in an increase in colonies in a clonogenic assay compared to transfection with H-ras and a control shRNA (Figure A.4). This result supported the idea that cIAP2 plays a role in inhibiting proliferation and survival during transformation.

Statement of the problem

(1) cIAP2 plays an important role in the inhibition of apoptosis in response to survival signaling. In contrast, it also limits activation of NF-κB by maintaining low cellular levels of NIK. Mutation or loss of cIAP2 is associated with the development of multiple myeloma. Characterization of carcinogen-induced tumours in cIAP2-null mice presented an interesting paradox wherein cIAP2 protected against apoptosis, but also appeared to play a role in suppressing both proliferation and development of adenocarcinomas. This data suggests that cIAP2, in addition to preventing apoptosis, also functions to limit survival and proliferative signaling. The precise role of cIAP2 in the context of mammary epithelial tumourigenesis remains unclear.

(2) Current literature reports that use of IAP antagonists *in vitro* has shown limited success, with only a few cell lines being highly susceptible to IAP antagonists as single agents. Sensitivity to IAP antagonists appears to be dependent on autocrine TNF-α

production. However, the factors that determine if a cell line transcribes TNF-α in response to IAP antagonism have not been elucidated.

Hypothesis

In accordance with earlier reports, we suspected that downregulation or loss of cIAP2 in mammary epithelial cells would result in the activation of NF-κB. We hypothesized that depletion of cIAP2 likely regulates additional signaling pathways that promote survival and proliferation, thereby contributing to mammary tumourigenesis. Additionally, differential activity of a factor that modifies NF-κB activity likely determines the susceptibility of a given cell to IAP antagonists by altering the expression of TNF-α.

Objectives

(1) To determine the effects of cIAP2 downregulation on signaling pathways involved in proliferation and tumour progression.

(2) To identify factors that determine sensitivity to IAP antagonists.

CHAPTER II - MATERIALS AND METHODS

Cell Culture

MCF-10A and MCF-10AT1 mammary epithelial cells were maintained in Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F-12) media (Invitrogen) supplemented with 5% (v/v) horse serum (Invitrogen), 1% non-essential amino acids (NEAA) (Invitrogen), 0.5 mg/mL hydrocortisone (Sigma), 10 μg/mL insulin (Sigma), 100 ng/mL cholera toxin (Sigma), 20 ng/mL human epithelial growth factor (hEGF). MCF-7 and ZR-75 cells were maintained in high glucose DMEM (Hyclone) supplemented with 5% fetal bovine serum (FBS) (Hyclone) and 1% NEAA. MDA-MB-231 cells were maintained in low glucose DMEM (Invitrogen) supplemented as with MCF-7 and ZR-75 cells. T-47D cells were maintained in high glucose DMEM supplemented with 10% FBS and 1% NEAA. MDA-MB-231 cells were maintained in low gluclose DMEM supplemented with 5% FBS and 1% NEAA. HEK293 cells were maintained in high glucose DMEM supplemented with 10% FBS and 1% NEAA. Cells were incubated at 37^{o}C under 5% CO_2. For passaging, cells were washed with phosphate-buffered saline (PBS) (137 mM sodium chloride [NaCl], 2.7 mM potassium chloride [KCl], 10 mM disodium hydrogen orthophosphate [Na_2HPO_4], 1.76 mM potassium dihydrogen orthophosphate [KH_2PO_4], pH 7.0-7.4) prior to isolation. PBS was removed by aspiration and trypsin solution (0.25% trypsin, 1 mM ethylenediaminetetraacetic acid [EDTA]) (Invitrogen) was added. Cells were incubated until cells detached, then resuspended in the trypsinizing solution, which was inactivated by addition of serum-containing media. Cells were then plated in complete medium at the desired density.

Reagents

AEG40730 and SM164 were generous gifts from Dr. Robert Korneluk. BMS-345541, MG132, and Nutlin-3 were purchased from Sigma. Doxorubicin was obtained from the Ottawa General Hospital Regional Cancer Centre. UO126 was purchased from Cell Signaling.

Reverse transfection of siRNA

In 60 mm dishes, 1 mL of serum-free OptiMEM (Invitrogen) was mixed with 5 nM siRNA targeted at cIAP1, cIAP2, IKK-α, p53, or non-targeting siRNA (Dharmacon) and 7.5 μL of Dharmafect I (Dharmacon) then incubated at room temperature for 20 minutes. MCF-10AT1 cells (1.5 x 10^5 per dish) were seeded on top of the transfection mixture. 6 hours and 24 hours following transfection, fresh media was changed. Cells were grown for 48 hours prior to harvesting.

siRNA sequences

cIAP1 [AAAGAGAGCCAUUCUGUUCUU], cIAP2 [AUUCGUACAGUUCAUGUU], IKK-α #4 [CCAGAUACUUUCUUUACUA] (ON-TARGETplus), IKK-α #5 [GAAGUUCGGUUUAGUAGCC] (ON-TARGETplus), p53 (siGENOME SMARTpool #M-003329-01), XIAP (siGENOME SMARTpool #M-004098-01), and non-targeting siRNA (Accell SMARTpool) were purchased from Dharmacon.

DNA plasmids and transfection

Constitutively active IKK-α and IKK-β mutants (IKK-α-S176/180E and IKK-β-S177/181EE) were obtained from Dr. M. Karin (University of California, San Diego, USA). The p53 mutant 173L construct (Cys to Lys at amino acid 173) was provided by

Dr. Karen Vousden (University of Glasgow, UK). Two other mutant p53 constructs, p53-V143A (Val to Ala at amino acid 143) and p53-R175H (Arg to His at amino acid 175) were kind gifts from Dr. S. Benchimol (York University, Toronto). The inserts were excised using HindIII and BamHI and subsequently cloned into a pcDNA3 vector.

Transient transfection of DNA was performed using polyethylenimine (PEI) (Polysciences). HEK293 cells were seeded in 6-well plates in complete medium. Transfection mixture was prepared in serum-free medium containing 5 μg DNA and 15 μL PEI, incubated at room temperature for 15 minutes then added to each well. RNA was extracted from cells 48 hours following transfection as described below.

Stable transfection of DNA was performed using Fugene6 (Roche). MCF-7 cells were plated 24 hours prior to transfection in complete medium on 60 mm dishes. Transfection mixture was prepared in serum-free medium containing 2 μg DNA and 6 μL Fugene6, incubated at room temperature for 15 minutes then added to each plate. Forty-eight hours later, cells were replated in selection media containing 1 μg/mL G418.

Adenovirus infection

Cells were plated in complete medium in 60 mm dishes. 24 hours later, cells were infected with adenovirus expressing a dominant negative mutant form (K44A) of IKK-β (Sanlioglu et al., 2001) at 25 multiplicity of infection (MOI). Adenovirus expressing GFP was used as an adenoviral control. Protein was extracted 24 hours later in RIPA buffer and analyzed by western blotting.

Antibodies

The following primary antibodies were used for western blot analysis and EMSA: anti-actin #A-2066 (Sigma); anti-Akt #9272 (Cell Signaling); anti-phospho-Akt (Ser473)

#9271 (Cell Signaling); anti-cIAP1 #AF818 (R&D Systems); anti-cIAP2 #S2700 (Epitomics); anti-ERK1/2 #9102 (Cell Signaling); anti-phospho-ERK1/2(Thr202/Tyr204) #9101 (Cell Signaling); anti-GADD45-α (C-4) #sc-6850 (Santa Cruz); anti-IKKα #2682 (Cell Signaling); anti-IKKβ #2684 (Cell Signaling); anti-phospho-IKKα/β (Ser176/180) #2697 (Cell Signaling); anti-Mdm2 #M4308 (Sigma); anti-phospho-Mdm2(Ser166) #3521 (Cell Signaling); anti-MEK1/2 #9126 (Cell Signaling); anti-phospho-MEK1/2(Ser217/221) #9154 (Cell signaling); anti-NIK #4994 (Cell Signaling); anti-phospho-NIK(Thr559) #sc-12957 (Santa Cruz); anti-p21 (C-19) #sc-397 (Santa Cruz); anti-p50 #06-886 (Millipore); anti-p53 (BP-53) #sc-263 for cell lines (Santa Cruz); anti-p53 (FL-393) #sc-6243 for mouse tissue (Santa Cruz); anti-phospho-p53(Ser15) #9284 (Cell Signaling); anti-p100/p52 #06-413 (Millipore); anti-PIAS1 #ab32219 (Abcam); anti-H-Ras #ab32417 (Abcam); anti-rIAP1 (Aegera) to detect cIAP1 and cIAP2; anti-rIAP3 to (Aegera) to detect XIAP; anti-SMRTe #MA1843 (ABR); anti-TRAF1 #4710 (Cell Signaling); anti-TRAF2 (C-20) #sc-876 (Santa Cruz);

The following secondary antibodies were used for western blot analysis: peroxidase-conjugated goat anti-rabbit IgG (H+L) (Jackson Research Laboratories Inc.); peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson Research Laboratories Inc.);

SDS-polyacrylamide gel-electrophoresis and immunoblotting

Whole cell lysates were prepared from cell cultures washed twice with PBS and scraped in radioimmunoprecipitation assay (RIPA) buffer (1% Nonidet P-40 [NP-40], 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS] in PBS plus Complete Protease Inhibitor Cocktail tablet [Roche] and PhosSTOP Phosphatase Inhibitor Cocktail tablet [Roche]). The cell solution was incubated on ice for a minimum of 10 minutes, vortexed

at maximum speed to rupture cells and centrifuged for 15 minutes, 16 000 x g, 4°C to remove insoluble material. Lysate protein concentrations were determined using the Biorad D_c Protein Assay kit (Biorad). Typically, 10-25 µg of protein were denatured in sample buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreiotol [DTT], 2% SDS, 0.1% bromophenol blue, 10% glycerol) for 5 minutes at 100°C and resolved on a 6-12% SDS polyacrylamide gel by electrophoresis (SDS-PAGE) (Laemmli, 1970). Gels were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) at 100 V for 2 hours or at 20 V overnight at 4°C. The membrane blots were blocked for one hour in 5% non-fat skim milk powder dissolved in Tris-buffered saline (20 mM Tris-HCl pH 7.6, 137 mM NaCl) containing 0.1% Tween-20 (TBS-T), then incubated with the appropriate primary antibody dissolved in blocking solution plus 0.02% sodium azide for 1 hour at room temperature or overnight at 4°C. The blots were washed for 10 minutes twice in TBS-T then incubated for one hour at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit secondary antibody, diluted 1:10 000 in blocking solution. Finally, the blots were washed 4 times, 10 minutes each in TBS-T and the bands were detected using the Immobilon Western Chemiluminescent HRP Substrate (Millipore) by exposing the membrane to radiography film.

Quantitation of western blots and statistical analysis

To quantify the bands obtained via western blot analysis, ImageJ software based analysis (http://rsb.info.nih.gov/ij/) was used. The area under curve (AUC) of the specific signal was corrected for the AUC of the loading control. Statistical analysis was performed using Student's *t*-test. All data were shown as mean with standard error of

mean deviation (mean ± SD). Probabilities of $p < 0.05$ were considered significant and denoted with an asterisk.

Co-immunoprecipitation (co-IP)

MCF-10AT1 cells transfected with siRNA were harvested in co-IP buffer (25 mM Tris-Cl pH7.5, 150 mM NaCl, 50 mM NaF, 0.5 mM EDTA pH8, 0.5% Triton-X, 5 mM β-glycerophosphate, 5% glycerol, 1 mM DTT, 1 mM PMSF and 1 mM $NaVO_3$). Lysates were sonicated for 30 seconds on ice then protein concentration was determined using a Bradford assay (Biorad) according to manufacturer's instructions. 300 μg of protein was incubated with 2 μg of anti-PIAS1 antibody, anti-IKK-α antibody, or rabbit IgG as a control for 2 hours at 4°C with rotation. Protein A/G agarose beads (Roche) were added and incubated overnight at 4°C with rotation. Agarose beads bound with immunoreactive complexes were washed four times with co-IP buffer, each time rotating for 5 minutes at 4°C followed by centrifugation at 200 x g for 2 minutes. Following the final wash, immunoprecipitated complexes were eluted with 60 μL of 2X sample buffer and boiled for 5 minutes. 25 μL of each sample was analyzed using SDS-PAGE as described above with 15 μg of input protein.

Electrophoretic Mobility Shift Assays (EMSA)

Electrophoretic mobility shift assays (EMSA) were performed using nuclear extracts from cultured cells (Fried, 1989). NF-κB oligonucleotides were obtained from Promega (E3291) and end-labeled with T4 polynucleotide kinase (PNK) using [γ-^{32}P]-ATP (Perkin Elmer). Intact nuclei were isolated from cells by washing cells in buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] pH 7.9, 10mM KCl, 1.5 mM magnesium chloride [$MgCl_2$], 500 μM DTT, 500 μM

phenylmethanesulfonylfluoride [PMSF]), centrifuged at 200 x g for 5 minutes at 4°C, then incubated in buffer A + 0.1% NP-40 on ice for 20 minutes. The cell mixture was centrifuged at 20 000 x g for 10 minutes at 4°C and the supernatant (cytoplasmic fraction) was discarded. The nuclear pellet was washed once with buffer A then resuspended in buffer B (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 200 μM EDTA, 25% glycerol, 500 μM DTT, 500 μM PMSF, 500 μM spermidine, 150 μM spermine, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin) and incubated on ice for 45 minutes. Insoluble material was removed by centrifugation at 20 000 x g for 10 minutes at 4°C and the nuclear extract was stored at -80°C until ready for analysis. Protein concentration was determined using a Bradford Assay (Biorad). The EMSA was carried out by incubating 5 μg of protein in a reaction buffer containing 20 mM HEPES pH 7.9, 200 μM EDTA, 200 μM ethylene glycol tetraacetic acid (EGTA), 100 mM KCl, 5% glycerol, 2 mM DTT and 5 μg poly d[I-C] (Roche) at room temperature for 10 minutes. [γ-^{32}P]-ATP-labeled NFκB oligonucleotide was added and incubated at room temperature for 20 minutes. For supershift experiments, 2 μg of anti-p50 antibody was added to the reaction and incubated at room temperature for 20 minutes prior to addition of the labeled probe. Loading buffer was added to the reaction mix then resolved on a 5% native polyacrylamide gel in non-denaturing Tris-glycine buffer (14.4 g/L glycine, 3.03 g/L Tris pH 8.3). Equivalence of extract loading was demonstrated by EMSA with a DNA fragment corresponding to the consensus Sp1 binding site (Promega).

Fractionation

Separation of the nuclear and cytoplasmic extracts was carried out using the same method as in the EMSA protocol. The cytoplasmic fraction obtained was mixed with an

equal volume of buffer C (20 mM HEPES, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT and 0.5 mM PMSF). Protein concentration of the extracts was determined using a Bradford Assay (Biorad). Equal amounts of protein were loaded on an SDS-PAGE gel and western blotting was performed as previously described.

Immunofluorescence

Cells were seeded on glass coverslips and allowed to adhere for 24 hours before being treated as described. Fixation was performed at room temperature with 4% paraformaldehyde (PFA) in PBS for 15 minutes. Cells were permeabalized with 0.5% Triton-X in PBS at room temperature for 5 minutes. Primary antibodies were diluted in 1% bovine serum albumin (BSA) in PBS and incubated at room temperature for 1-3 hours. Alexa488- or CY3-conjugated secondary antibodies were diluted 1:200 in 1% BSA in PBS and incubated at room temperature for 1 hour, protected from light. Washes following antibody incubations were performed once with 0.1% Triton-X in PBS then twice more with PBS. Coverslips were mounted onto glass slides with VectaShield with DAPI (Vector labs) and sealed with nail polish. Images were captured with a 63x oil-immersion objective on a Zeiss Axioimager.Z1 fluorescence microscope using AxioVision software.

MTT cell viability assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assays were performed using the CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega) according to manufacturer's instructions. Briefly, 4 x 10^3 cells were seeded per well in a 96-well plate. 24 hours after seeding, cells were treated as indicated and grown for an additional 30 hours. To each well, 15 μL of dye

solution was added and incubated at 37°C for 4 hours. Following conversion of yellow MTT to purple formazan, cells were lysed using 100 μL of solubilization solution. Samples were incubated at room temperature overnight and absorbance at 570 nm was measured using a Molecular Devices Spectramax M2 platereader. Values are expressed as a percentage compared to vehicle-treated cells. All treatments were performed in triplicate wells.

RNA Extraction, reverse transcription and Quantitative-PCR

Cells were seeded in triplicate on 60 mm dishes and treated 24 hours later with 5 nM SM164 for 4 hours. Cells were trypsinized and collected by centrifugation at 200 x g for 5 minutes at room temperature. Total RNA extraction was performed using an RNeasy mini kit (Qiagen) according to manufacturer's instructions and RNA was eluted in 30 μL nuclease-free water. Optical density (OD) was read on a spectrophotometer at 260 nm to determine quality and concentration of the RNA. Reverse transcription-quantitative PCR (RT-qPCR) reactions were performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen) with 100 ng of RNA and 0.5uM of the respective primers, according to manufacturer's protocol. Forward and reverse SYBR green primers were purchased from Qiagen for GAPDH (QT01192646) and for TNF-α (QT01079561). Reverse transcription was performed at 50°C for 30 minutes followed by initial PCR activation at 95°C for 15 minutes. Forty cycles consisting of a denaturing step at 94°C for 15 seconds, an annealing step at 55°C for 30 seconds then an extension step at 72°C for 30 seconds were performed.

Whole mounts of normal mammary glands

Fourth inguinal mammary glands were extracted from mice and spread onto glass microscope slides. Glands were fixed overnight in Carnoy's fixative (10% glacial acetic acid, 30% chloroform, 60% ethanol). Fixed glands were washed in 70%, 50%, 25% ethanol and finally water for 15 minutes each, then defatted in acetone for 20 minutes three times. Glands were rehydrated in 100% then 95% ethanol for 20 minutes each then stained with hematoxylin for one hour at room temperature. Glands were rinsed clear in tap water then detained with acid alcohol (50% ethanol, 0.2% HCl) for 30 minutes twice. Lastly, glands were dehydrated in 70%, 95%, and 100% ethanol for 20 minutes each then stored in xylene.

cIAP2-null mice

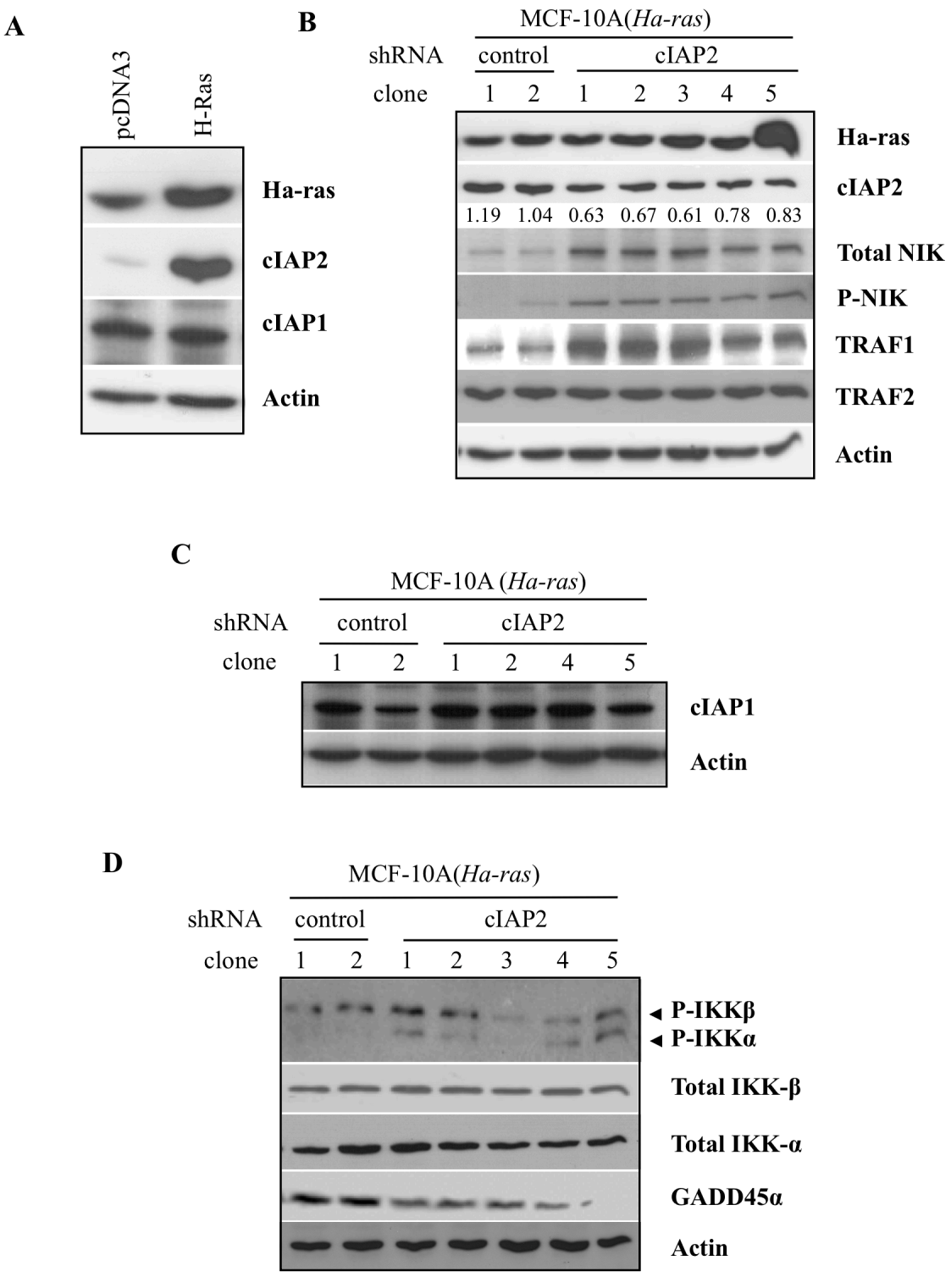
cIAP2$^{-/-}$ mice were generated as described by Conte *et al.* 2006. Mice were housed in a specific-pathogen-free environment, and all experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and protocols approved by the University of Ottawa Animal Care Committee.

CHAPTER III - RESULTS

Objective I: To determine the effects of cIAP2 downregulation on signaling pathways involved in proliferation and tumour progression

Downregulation of cIAP2 activates canonical NF-κB signaling

Preliminary experiments in the laboratory indicated that the downregulation of cIAP2 in H-ras expressing MCF-10A cells resulted in an increase in colony formation in a clonogenic assay (Figure A4). Additionally, tumour initiation by DMBA resulted in increased numbers of highly proliferative adenocarcinomas in a cIAP2-null mouse compared to control wildtype mice. To determine how the loss of cIAP2 can increase mammary tumourigenesis, *in vitro* studies were undertaken to investigate the signaling pathways that cIAP2 may participate in to promote survival and proliferation. MCF-10A cells, a spontaneously immortalized, non-tumourigenic mammary epithelial cell line, express relatively low endogenous levels of cIAP2. Transfection with constitutively active Ha-*ras* increased cIAP2 expression dramatically (Figure 3.1A), which has been reported in literature (Liu et al., 2005). To investigate how cIAP2 downregulation may contribute to a growth advantage in these cells, individual stable clones of MCF-10A cells transfected with either control pcDNA3 alone, Ha-*ras* with a control shRNA or Ha-*ras* with cIAP2 shRNA (hereafter referred to as MCF-10A(pcDNA3), MCF-10A(Ha-*ras*) and MCF-10A(Ha-*ras*/cIAP2 shRNA) respectively) were analyzed by western blotting. Figure 3.1B shows that despite a modest decrease (about 35%) in cIAP2, MCF-10A(Ha-*ras*/cIAP2 shRNA) cells displayed increased levels of TRAF1 and NIK. Since both TRAF1 and NIK are targets of ubiquitination by cIAP2, the clear increase of these two proteins indicates that even moderate disruption in the expression levels of cIAP2 is sufficient to exert downstream effects. Additionally, the levels of TRAF2, a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pcDNA3
H-Ras
Ha-ras
cIAP2
cIAP1
Actin
B
MCF-10A(Ha-ras)
shRNA
control
cIAP2
clone
1 2 1 2 3 4 5
Ha-ras
cIAP2
1.19 1.04 0.63 0.67 0.61 0.78 0.83
Total NIK
P-NIK
TRAF1
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MCF-10A (Ha-ras)
shRNA
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1 2 1 2 4 5
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Actin

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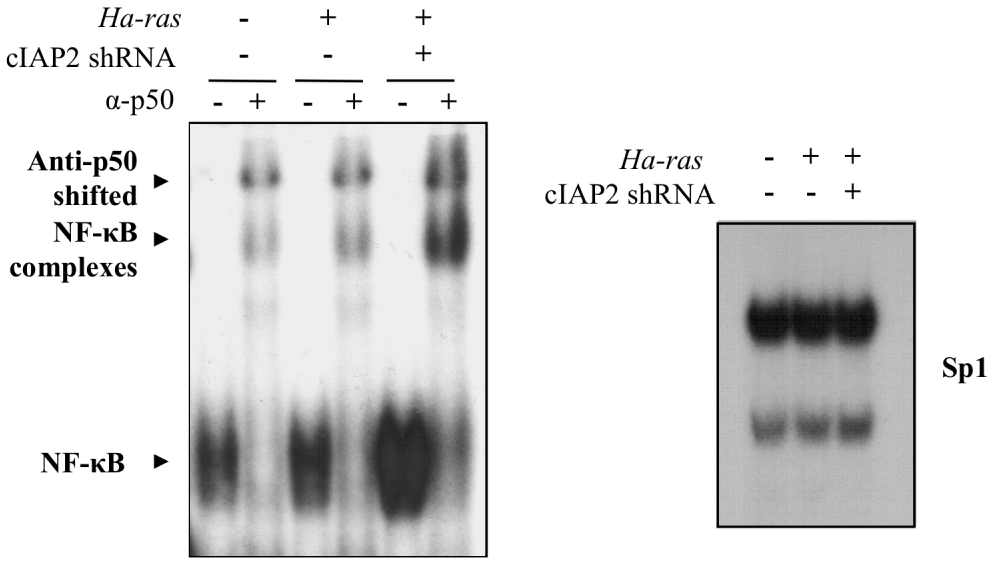


Figure 3.1. Downregulation of cIAP2 activates canonical NF-κB signaling.
A, Protein extracts from MCF-10A cells stably transfected with pcDNA3 or Ha-*ras* were immunoblotted for Ha-ras, cIAP2 and cIAP1. **B,** Protein extracts from stable clones of MCF-10A cells transfected with Ha-*ras* and either control shRNA or cIAP2 shRNA were immunoblotted for Ha-ras, cIAP2, total and phosphorylated NIK. cIAP2 levels were quantified, normalized to actin and indicated below the blot. **C,** Protein extracts from stable clones were immunoblotted for cIAP1. **D,** Protein extracts from clones were immunoblotted with an antibody that recognizes phosphorylated IKK-α and -β, total IKK-α, totally IKK-β and GADD45α. Actin was used as a loading control in all western blotting experiments. **E,** Nuclear extracts from MCF-10A cells stably transfected with control pcDNA3, Ha-*ras* with control shRNA or cIAP2 shRNA were subjected to EMSA analysis. Supershift was performed using an anti-p50 antibody. Active NF-κB complexes and anti-p50 antibody-shifted NF-κB complexes are indicated. SP1 was used as a loading control on the right panel. Western blots and EMSA shown are representative of three independent experiments.

ubiquitination target of cIAP1, were not affected. Immunoblotting using an anti-cIAP1 antibody confirmed the specificity of the shRNA to target cIAP2 and that cIAP1 levels were unchanged (Figure 3.1C).

The increase of NIK in MCF-10A(Ha-*ras*/cIAP2 shRNA) cells was accompanied by an increase in its phosphorylation (Figure 3.1B). Activated NIK can phosphorylate IKK-α and lead to the activation of the non-canonical NF-κB pathway (Hayden and Ghosh, 2004). To determine if NF-κB signaling is activated in MCF-10A(Ha-*ras*/cIAP2shRNA) cells, western blot analysis was performed on whole cell lysates (Figure 3.1D). Levels of phosphorylated IKK-β were increased in MCF-10A(Ha-*ras*) cells compared to MCF-10A(pcDNA3) control cells, with no change in total IKK-β levels. When cIAP2 shRNA was co-transfected with Ha-*ras*, phosphorylated IKK-β levels were similarly increased, but notably, levels of phosphorylated IKK-α were also increased without a change in total levels. Additionally, growth arrest and DNA damage (GADD)-45α, which is subject to negative regulation by NF-κB (Zerbini et al., 2004), was decreased in MCF-10A(Ha-*ras*/cIAP2 shRNA) cells. These results suggest that the increase in IKK-α phosphorylation corresponded to a differential activation of NF-κB signaling in MCF-10A(Ha-*ras*/cIAP2 shRNA) cells.

In addition to their roles in non-canonical NF-κB signaling, both NIK and IKK-α have been shown to activate the canonical signaling pathway by phosphorylating IKK-β (Nakano et al., 1998; Yamamoto et al., 2000). To determine if the increase in phosphorylated IKK-α led to activation of canonical NF-κB complexes, electrophoretic mobility shift assay (EMSA) analysis was performed. As shown in Figure 3.1E, transfection of MCF-10A cells with Ha-*ras* marginally increased NF-κB activity above

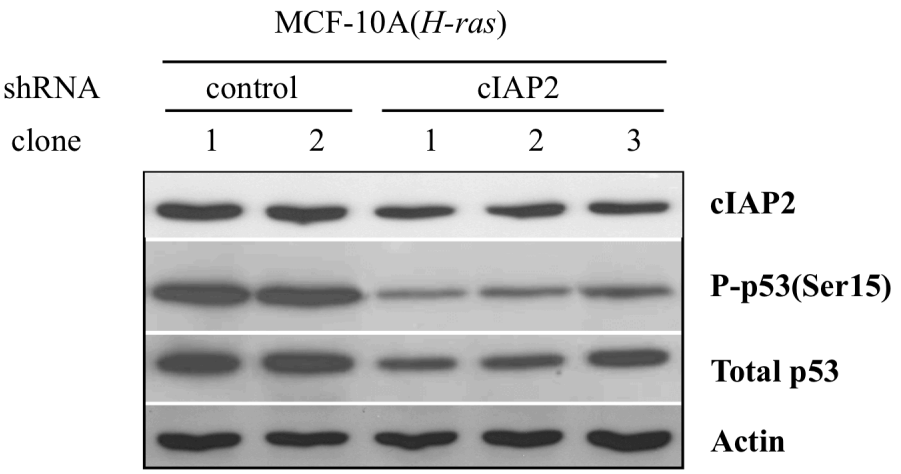
control but co-transfection of cIAP2 shRNA dramatically increased the levels of active NF-κB complexes, including p50, as indicated by a shift using a specific antibody against p50. Importantly, the entire NF-κB complex stimulated by cIAP2 downregulation was shifted by the p50 antibody. Since p50 is a constituent of the canonical NF-κB pathway, and does not participate in non-canonical signaling, cIAP2 downregulation specifically activates the canonical pathway in MCF-10A(H-*ras*) cells. Together, these results show that downregulation of cIAP2 in H-*ras*-transfected MCF-10A cells leads to canonical NF-κB activation, likely as a result of the increase in phosphorylated NIK and strong activation of IKK-α.

Downregulation of cIAP2 decreases wildtype p53

Many of the gene targets regulated by NF-κB signaling are involved in the control of cellular processes such as cell proliferation, apoptosis, and the cellular stress response (Perkins, 2007). An example of this is the tumour suppressor p53 (Tergaonkar et al., 2002). Since MCF-10A cells express wildtype p53, we sought to determine if p53 stabilization or activity was affected by NF-κB activation in MCF-10A(Ha-*ras*/cIAP2 shRNA) cells, which could contribute to the increased colony formation that was observed. Figure 3.2A shows that in the stable clones where cIAP2 was downregulated, both total and phosphorylated p53 (Ser15) levels were decreased.

Under our experimental conditions, MCF-10A(Ha-*ras*) and MCF10-A(Ha-*ras*/cIAP2 shRNA) cells senesced following multiple passages, therefore, further experiments were performed in MCF-10AT1 cells. This cell line was established from a lesion originating from T-24 *ras*-transfected MCF-10A cells xenografted in a mouse (Dawson et al., 1996). These cells do not form invasive carcinomas when xenografted

A


MCF-10A(*H-ras*)
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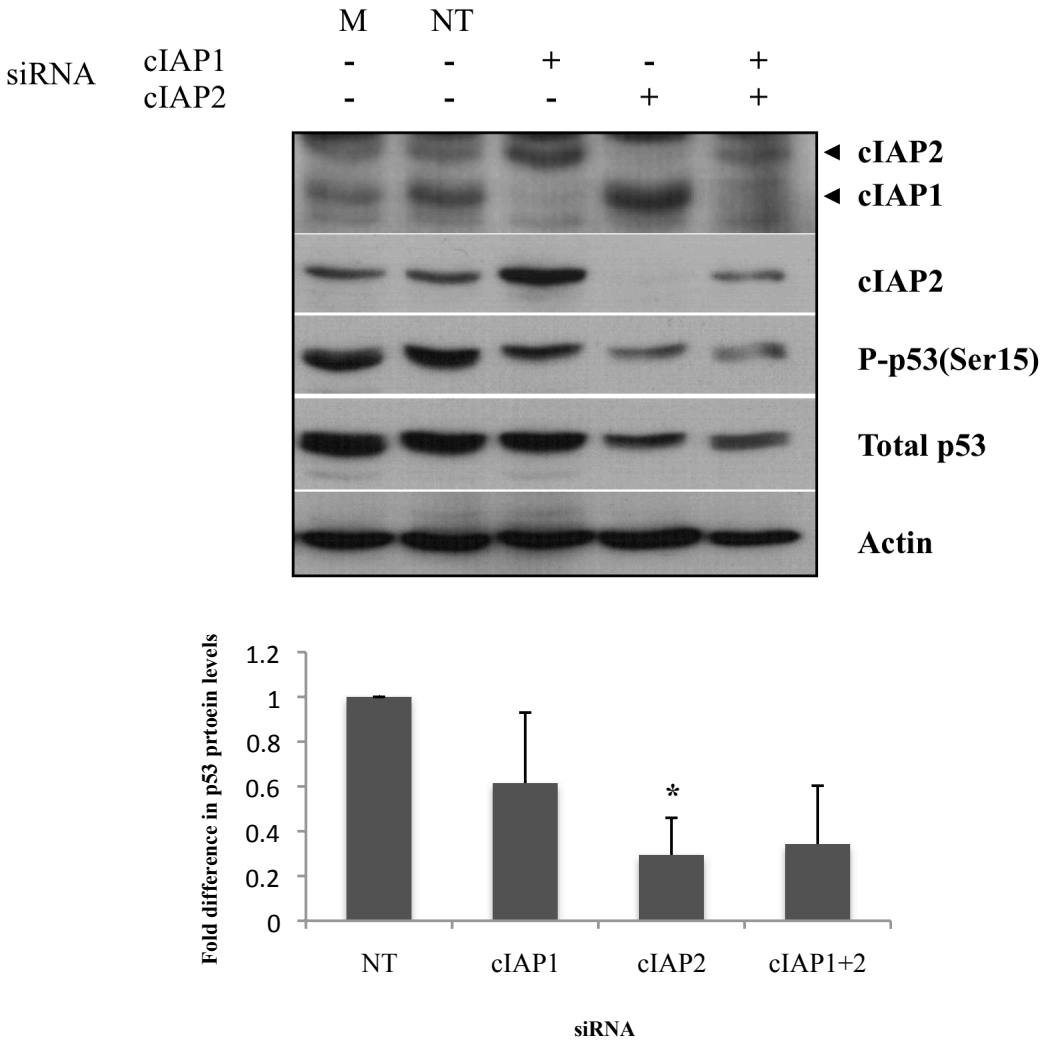
P-p53(Ser15)
Total p53
Actin

B


M
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cIAP2
cIAP1
cIAP2
P-p53(Ser15)
Total p53
Actin
Fold difference in p53 prtoein levels
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cIAP1+2
siRNA

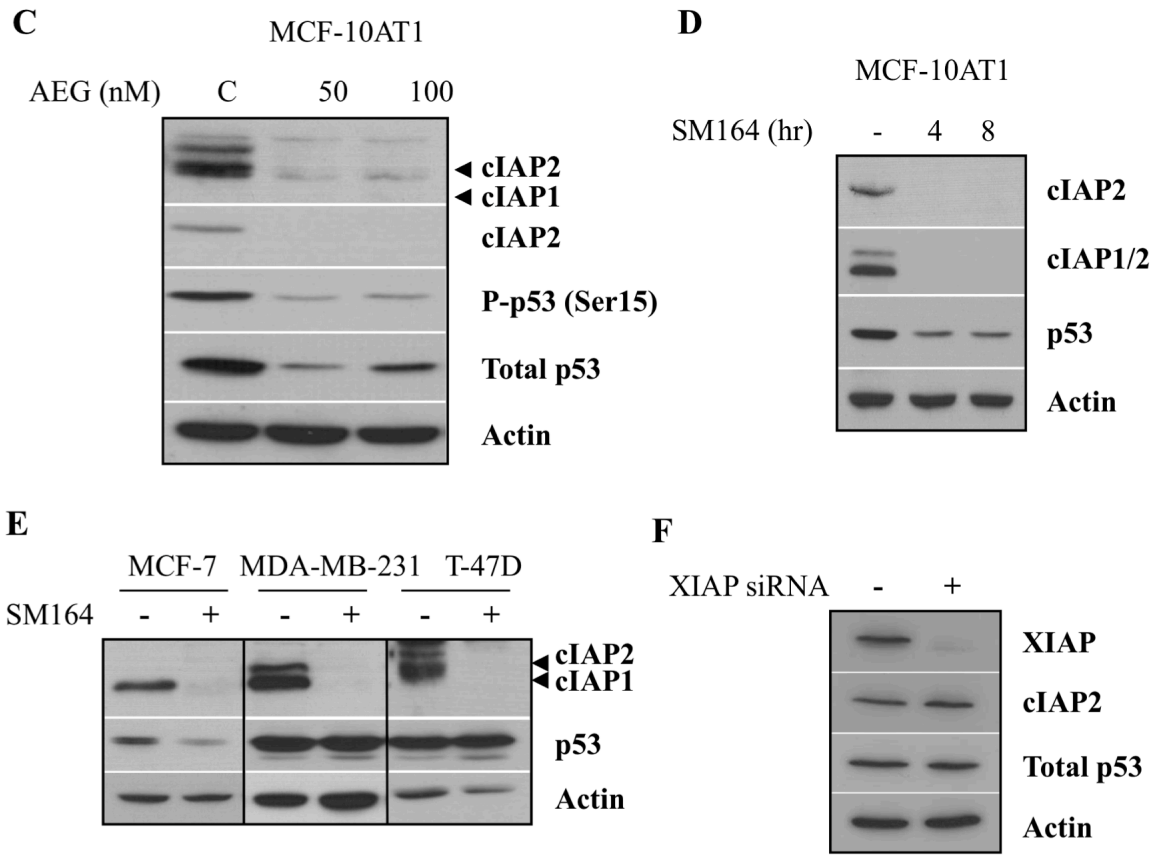


Figure 3.2. cIAP2 downregulation reduces p53.
A, Whole cell lysates from MCF10A cells stably transfected with pcDNA3 or *H-ras* in combination with control or cIAP2 shRNA were immunoblotted for cIAP2, phospho-p53 (Ser15) and total p53. Note that the immunoblot for cIAP2 shown was also shown in figure 3.1B. **B,** MCF-10AT1 cells were mock-transfected (M), transfected with non-targeting siRNA (NT) or siRNA targeting cIAP1 or cIAP2. Whole cell lysate was immunoblotted for cIAP1/2 (using anti-rIAP1 antibody that recognizes both proteins), cIAP2, phospho-p53 (Ser15) and total p53. Quantification of p53 is below. **C,** MCF-10AT1 cells were treated with AEG40730, or vehicle control (C) for 18 hours at the indicated concentrations. Whole cell lysates were immunoblotted for cIAP1/2, phospho-p53 (Ser15) and total p53. **D,** MCF-10AT1 cells were treated with SM164 at 10 nM for the indicated times. Whole cell lysates were immunoblotted for cIAP2, cIAP1/2 (using rIAP1 antibody) and p53. **E,** MCF-7, MDA-MB-231 and T-47D cells were treated with the IAP antagonist SM164 at 10 nM, or DMSO for 18 hours. Whole cell lysates were immunoblotted for cIAP2 and total p53. **F,** MCF-10AT1 cells were transfected with NT or XIAP siRNA . Whole cell lysates were immunoblotted for XIAP, cIAP2 and p53. Actin was used as a loading control in all western blots. Western blots shown are representative of experiments performed in triplicate at minimum. Quantification of protein levels were obtained from three independent experiments. Data are expressed as fold difference compared to non-targeting siRNA and control adenovirus (mean ± SD). * denotes $p < 0.01$ as determined by Student's *t*-test.

and similar to the parental MCF-10A cells, express wildtype p53 (Miller, 2000). MCF-10AT1 cells were transfected with siRNA targeted at cIAP1 and cIAP2. Compared to cells that were either mock-transfected or transfected with non-targeting siRNA, downregulation of cIAP1 resulted in stabilization of cIAP2, consistent with cIAP1 being a ubiquitin ligase for cIAP2. Transfection with the cIAP1 siRNA only slightly decreased phospho-p53 (Ser15), and did not affect total p53 levels. However, cells that were transfected with cIAP2 siRNA, either alone or in combination with cIAP1 siRNA, displayed dramatically decreased levels of both total and phosphorylated p53 (Ser15) (Figure 3.2B).

A number of small molecule IAP antagonists have recently emerged, many of which exert their effects by inducing proteasomal degradation of cIAP1 and 2 (Varfolomeev et al., 2007; Vince et al., 2007). These antagonists function by inducing \ of cIAPs, which exposes the RING domains and induces auto-ubiquitination (Dueber et al., 2011). To determine if pharmacological downregulation of cIAP2 was consistent with RNAi experiments in decreasing p53 levels, MCF-10AT1 cells were treated with the IAP antagonist AEG40730 for 18 hours. At 50 and 100 nM treatments, both cIAP1 and cIAP2 levels were abolished, along with a decrease in both total and phosphorylated p53 (Figure 3.2C). Similar results were observed using a different, more potent IAP antagonist, SM164 (10 nM), where downregulation of cIAP2 and p53 occurred as early as 4 hours following treatment (Figure 3.2D). To test the effects of IAP antagonism in different breast cancer cell lines, including those that harbour p53 mutations, MCF-7, MDA-MB-231 and T-47D cells were treated with SM164 (10 nM) for 18 hours. Figure 3.2E shows that in p53-wildtype MCF-7 cells, treatment with SM164 resulted in loss of cIAP1

expression and a decrease in p53 whereas in p53-mutant MDA-MB-231 and T-47D cells, the loss of cIAPs had no effect on p53 levels. It is important to note that MCF-7 cells do not express detectable levels of cIAP2, although it expresses cIAP1. Therefore, in these cells, downregulation of cIAP1 alone is sufficient to decrease p53 levels.

IAP antagonists have been shown to inhibit XIAP at high concentrations (Bertrand et al., 2008). To determine if XIAP downregulation can regulate p53 levels as demonstrated for the cIAPs, XIAP protein levels were reduced in MCF-10AT1 cells by transfection with a specific siRNA. Figure 3.2F shows that XIAP downregulation did not affect p53 protein levels levels. This result, combined with previous experiments where the concentrations of IAP antagonist, in particular AEG40730, were lower than those reported to inhibit XIAP but still downregulated p53 levels, indicates that XIAP is unlikely to be involved in the decrease in p53 following cIAP depletion by IAP antagonist treatment.

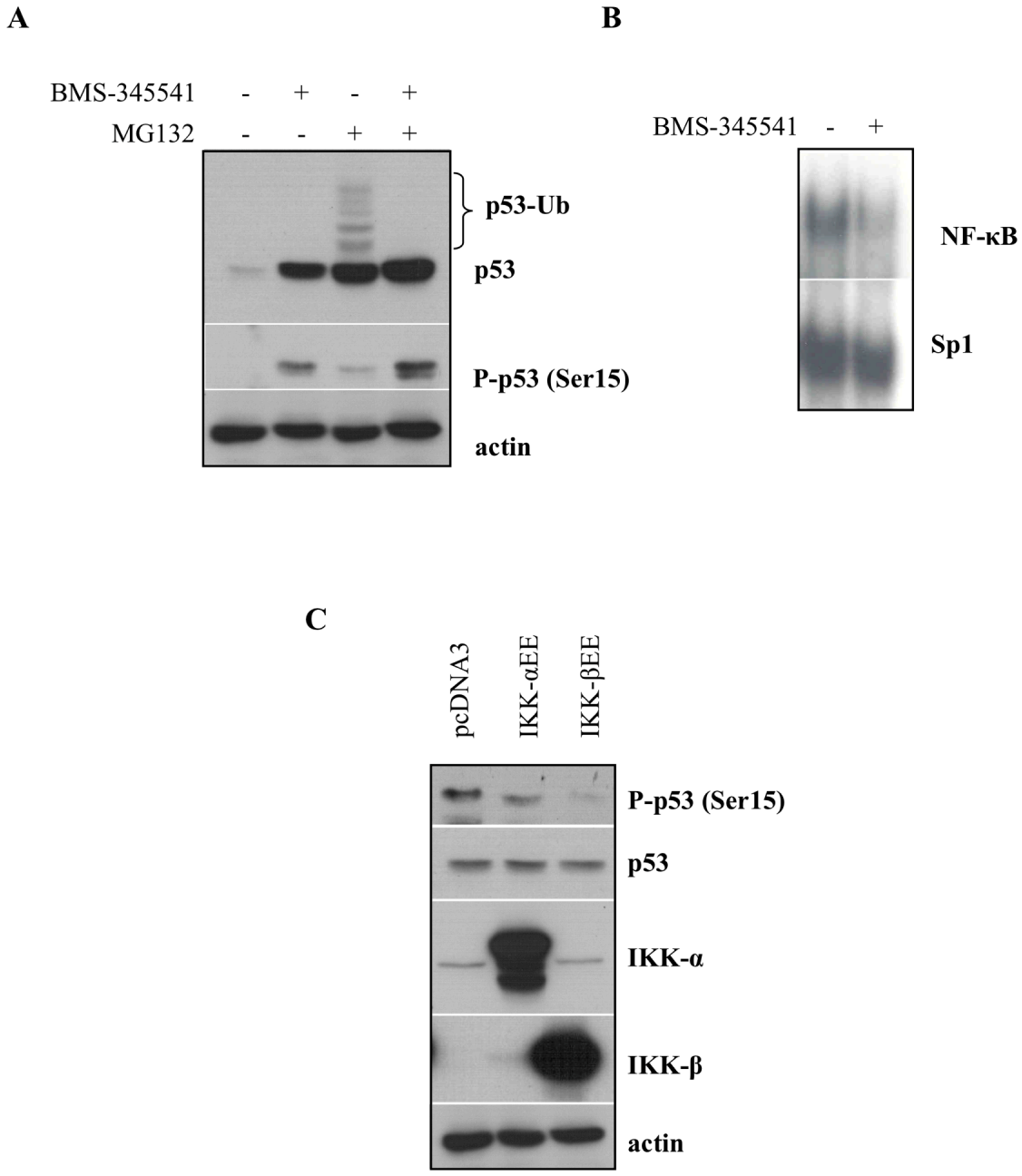
Overall, these results indicate that the downregulation of cIAP2 by multiple means (RNAi, IAP antagonists) consistently decreased the levels of both phosphorylated p53 at serine 15, as well as total levels of p53 protein. While this regulation can also be achieved by cIAP1 downregulation in some contexts, it is XIAP independent.

Regulation of p53 by cIAP2 is mediated by NF-κB

cIAP2 downregulation activated NF-κB signaling, which has been shown to regulate p53 by multiple means (Ak and Levine, 2010). We sought to confirm the role of NF-κB on the regulation of p53 in MCF-7 cells, which express wildtype p53. When the activities of both IKKs were inhibited using the pharmacological inhibitor BMS-345541, both phosphorylated p53 (Ser15) and total p53 levels were increased, indicating that NF-

κB regulates p53 in a negative manner (Figure 3.3A). Treatment with the proteasomal inhibitor MG132 resulted in stabilization of p53 and its ubiquitinated forms, which appear as a number of laddered bands. Co-treatment with BMS-345541 and MG132 resulted in a strong increase of p53 levels, but the laddered bands were absent. This suggests that NF-κB may play a role in promoting the ubiquitination of p53. To confirm that treatment of MCF-7 cells with BMS-345541 resulted in an inhibition of NF-κB signaling, nuclear extracts from treated cells were subjected to EMSA analysis. Figure 3.3B shows that overnight treatment with 5 μM BMS-345541 resulted in a decrease in active NF-κB complexes. To further confirm that activation of NF-κB negatively regulates p53, MCF-7 cells were stably transfected with constitutively active mutants of IKK-α and -β (IKK-α-S176/180EE and IKK-β-S177/181EE). Pooled clones were subject to western blotting analysis and revealed that active IKK-α decreased phospho-p53 (Ser15) levels slightly but expression of active IKK-β resulted in very strong decrease of phospho-p53 (Ser15) (Figure 3.3C). Levels of total p53 were not affected by expression of either mutant. Together, these results confirm that modulation of IKK activity can regulate p53 levels.

To determine if the negative effect of cIAP2 downregulation on p53 was mediated by NF-κB activation in MCF-10AT1 cells, IKK-α expression and IKK-β activity were modulated using siRNA against IKK-α and an adenovirus encoding a dominant-negative kinase-dead mutant of IKK-β (Ad-IKK-βKA). Interference of individual IKKs, or in combination, had no effect on the expression level of p53 (Figure 3.3D lanes 1-4). In the presence of cIAP2 siRNA, interference with the IKKs individually only partially restored p53 levels (lanes 5-7). However, when the activities of both IKK-α and IKK-β were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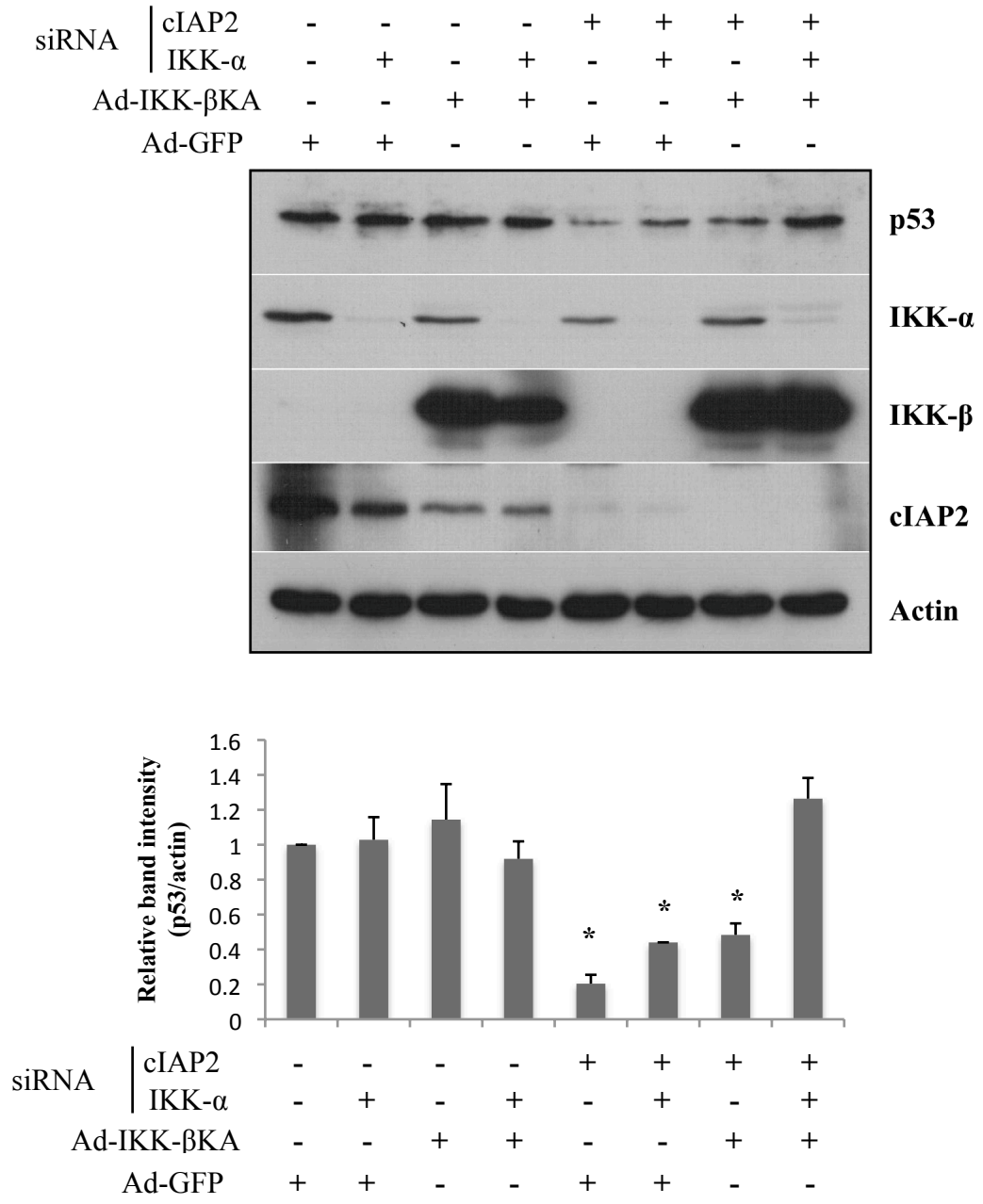
BMS-345541 - + - +
MG132 - - + +
p53-Ub
p53
P-p53 (Ser15)
actin
B
BMS-345541 - +
NF-κB
Sp1
C
pcDNA3
IKK-αEE
IKK-βEE
P-p53 (Ser15)
p53
IKK-α
IKK-β
actin

D


siRNA
cIAP2 - - - - + + + +
IKK-α - + - + - + - +
Ad-IKK-βKA - - + + - - + +
Ad-GFP + + - - + + - -
p53
IKK-α
IKK-β
cIAP2
Actin
Relative band intensity (p53/actin)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
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siRNA
cIAP2 - - - - + + + +
IKK-α - + - + - + - +
Ad-IKK-βKA - - + + - - + +
Ad-GFP + + - - + + - -

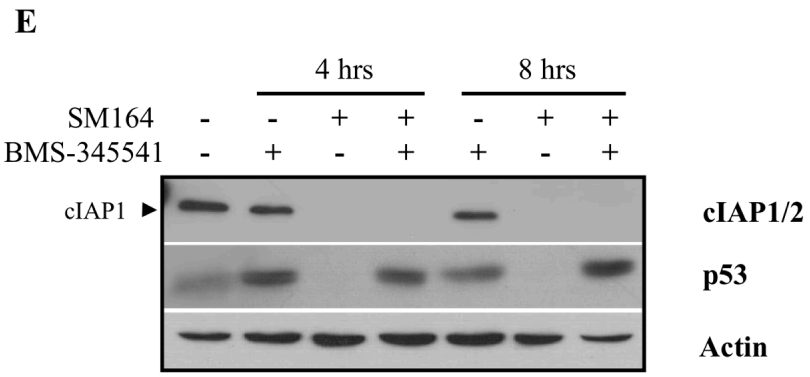
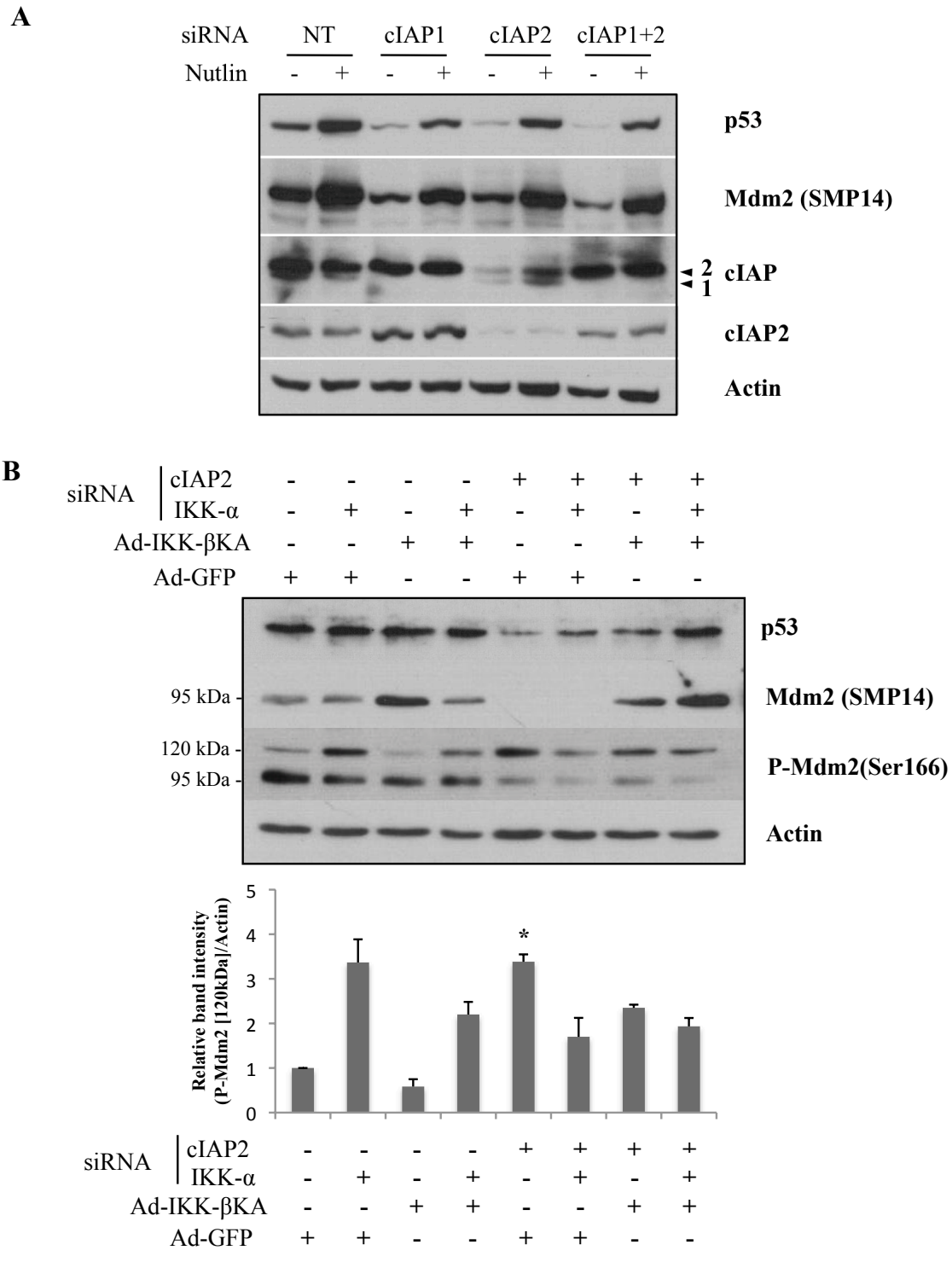


Figure 3.3. Regulation of p53 by cIAP2 is mediated by IKK-α and IKK-β
A, MCF-7 cells were treated with BMS-345541 (5 μM) or vehicle for 24 hrs in the presence or absence of MG132 (10 μM) for the final 6 hrs of culture. Protein lysates were subjected to immunoblotting for p53, and phospho-p53 (Ser15). **B,** Nuclear extracts from MCF-7 cells treated with vehicle or 5 μM BMS-345541 or vehicle for 24 hours were subjected to EMSA analysis using a canonical NF-κB oligonucleotide as a probe. SP1 was used as a loading control. **C,** MCF-7 cells were stably transfected with IKK-αEE or IKK-βEE expression constructs. Pooled colonies were immunoblotted for IKK-α, IKK-β, p53 and phospho-p53(Ser15). **D**,MCF-10AT1 cells were transfected with cIAP2 or IKK-α siRNA then infected with an adenovirus expressing a dominant-negative mutant of IKK-β. Non-targeting siRNA and adenovirus expressing GFP were used as transfection and infection controls. Protein extracts were immunoblotted for p53, IKK-α, IKK-β and cIAP2. Quantification of p53 is below. **E,** MCF-7 cells were treated with vehicle (DMSO), SM164 (100 nM), and/or BMS-345541 (5 μM) for the indicated times. Whole cell lysates were immunoblotted using p53 and rIAP1 (which detects both cIAP1 and cIAP2) antibodies. Actin was used as a loading control in all western blots. Western blots shown are representative of experiments performed in triplicate at minimum. Quantification of protein levels were obtained from three independent experiments. Data are expressed as fold difference compared to non-targeting siRNA and control adenovirus (mean ± SD). * denotes $p < 0.01$ as determined by Student's *t*-test.

impeded, the level of p53 was restored to control levels (lane 8), strongly implicating a role for each IKK in the downregulation of p53 following depletion of cIAP2. To confirm this effect in another p53-wildtype mammary cell line, MCF-7 cells were co-treated with SM164 and the pan IKK inhibitor, BMS-345541. Downregulation of cIAP1 by SM164 treatment consistently resulted in a decrease in total p53 levels (Figure 3.3E). This effect was abrogated when cells were co-treated with BMS-345541, thus supporting the role of IKKs in the regulation of p53 by the cIAPs.

NF-κB regulation of p53 is mediated through Mdm2

NF-κB signaling can impinge on p53 regulation through multiple mechanisms, one of which is mediated by the ubiquitin ligase Mdm2 (Tergaonkar et al., 2002), which modifies and inhibits p53. To determine if the regulation of p53 by cIAP2 is mediated by Mdm2, MCF-10AT1 cells were transfected with cIAP1, cIAP2 or both siRNAs then treated with nutlin-3, a small molecule inhibitor that disrupts the interaction between p53 and Mdm2 (Vassilev et al., 2004). Transfection with either cIAP1 or cIAP2 siRNA resulted in a decrease in p53 levels (Figure 3.4A). Treatment with 10 μM nutlin-3 for 4 hours following cIAP1/2 depletion restored p53 levels, suggesting that Mdm2 is involved in the regulation of p53. Unexpectedly, along with p53, the levels of Mdm2 were also decreased following cIAP siRNA transfection. The antibody used to detect Mdm2 in these experiments, SMP14, was generated against amino acids 154 to 167. An activating phosphorylation of Mdm2 occurs on serine 166, which results in the loss of the epitope and reactivity of the SMP14 antibody (de Toledo et al., 2000). To determine if cIAP2 downregulation results in increased phosphorylation of Mdm2 at Ser166, the same samples from Figure 3.3D were immunoblotted for phospho-Mdm2(Ser166). Figure 3.4B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siRNA
NT
cIAP1
cIAP2
cIAP1+2
Nutlin
- + - + - + - +
p53
Mdm2 (SMP14)
2
1
cIAP
cIAP2
Actin
B
siRNA
cIAP2
IKK-α
Ad-IKK-βKA
Ad-GFP
- - - - + + + +
- + - + - + - +
- - + + - - + +
+ + - - + + - -
p53
95 kDa
Mdm2 (SMP14)
120 kDa
95 kDa
P-Mdm2(Ser166)
Actin
Relative band intensity
(P-Mdm2 [120kDa]/Actin)
0
1
2
3
4
5
*
siRNA
cIAP2
IKK-α
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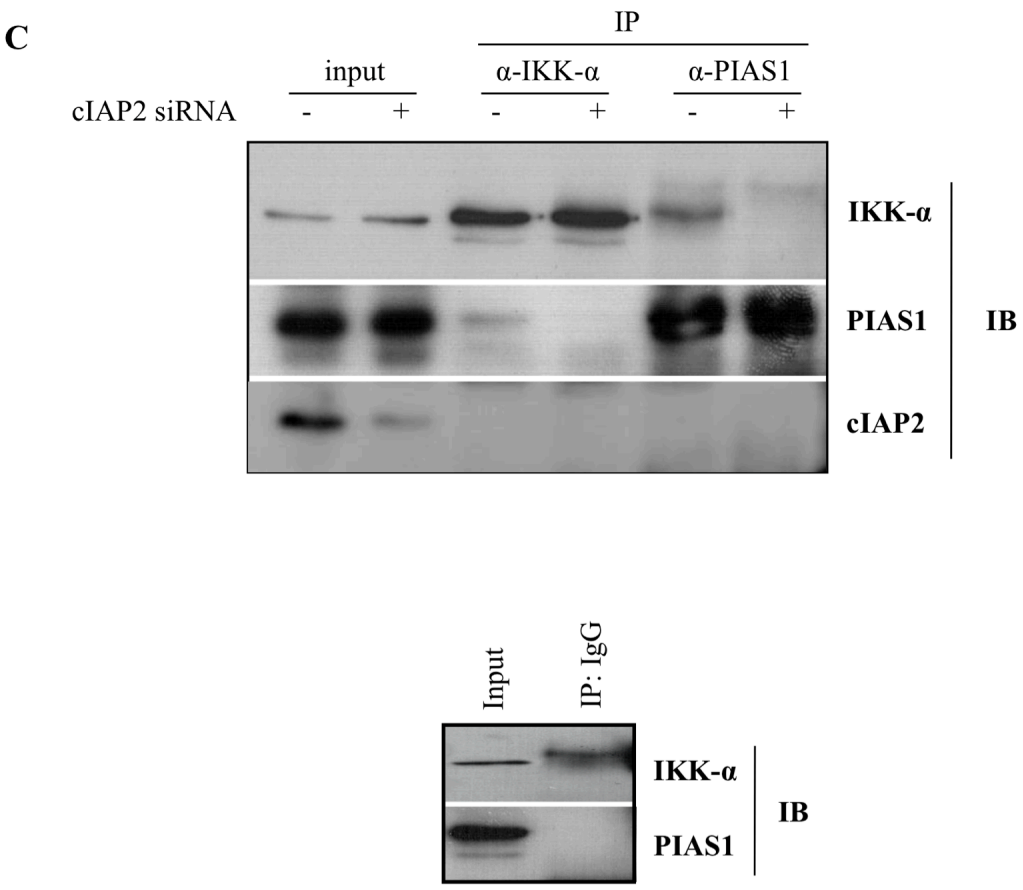


Figure 3.4. NF-κB regulation of p53 is mediated by Mdm2.
A, MCF-10AT1 cells were transfected with either non-targeting (NT), cIAP1 or cIAP2 siRNA for 48 hours then treated with Nutlin-3 (10 μM) for 4 hours prior to harvesting. Whole cell lysates were immunoblotted for p53, Mdm2 (SMP14), rIAP (which detects both cIAP1 and cIAP2), and cIAP2. **B,** MCF-10AT1 cells were transfected with siRNA targeted against cIAP2 or IKK-α then infected with an adenovirus expressing a dominant-negative mutant of IKK-β. Non-targeting siRNA and adenovirus expressing GFP were used as transfection and infection controls. Protein extracts were immunoblotted for p53 (the same blot was presented in Figure 2F), Mdm2 (SMP14) and phospho-Mdm2(Ser166). Quantification of P-Mdm2 (120kDa) is below, as obtained from three independent experiments. Data are expressed as fold difference compared to non-targeting siRNA (mean ± SD). * denotes $p < 0.01$ as determined by Student's t-test. **C,** MCF-10AT1 cells were transfected with either non-targeting (NT) or cIAP2 siRNA for 48 hours. Coimmunoprecipitation assays were performed with whole-cell extracts using anti-IKK-α, anti-PIAS1 or control IgG, followed by immunoblotting for IKK-α, PIAS1 and cIAP2. Western blots shown are representative of experiments performed in triplicate at minimum.

shows that following cIAP2 depletion, there was an increase in an immunoreactive band that migrates at 120 kDa. Mdm2 protein typically migrates at 95 kDa; however, numerous groups have reported that following translocation to the nucleus, which is stimulated by Ser166 phosphorylation (Mayo and Donner, 2001), Mdm2 becomes sumoylated, resulting in an increase in its apparent molecular weight to 120 kDa (Chen and Chen, 2003; Xirodimas et al., 2002). Sumoylation of Mdm2 prevents autoubiquitination and promotes ubiquitination of p53 (Lee et al., 2006; Xirodimas et al., 2002). The increase in the 120 kDa band was associated with a decrease in the 95 kDa Mdm2(Ser166) band, indicating that in addition to the increase in phosphorylation at serine 166, the downregulation of cIAP2 induces a shift to the 120 kDa form of Mdm2. Interference of each IKK expression individually and in combination decreased both the 95 kDa and 120 kDa bands, but depletion of IKK-α resulted in more pronounced decrease of the 95 kDa band than interference with IKK-β. Together, the results implicate a role for both IKK-α and IKK-β in the activation of Mdm2 following downregulation of cIAP2.

The sumoylation of Mdm2 is mediated in part by the SUMO E3 ligase PIAS1. PIAS1 has been shown to interact with IKK-α in an inactive state. Following phosphorylation by IKK-α, PIAS1 is released and functions to repress transcription, a process that requires its sumoylation (Liu et al., 2007). It is possible that the activation of IKK-α following cIAP2 depletion may also release PIAS1, which would allow it to sumoylate its substrates. To determine if cIAP2 downregulation affects the IKK-α:PIAS1 interaction, co-immunoprecipitation was performed to examine IKK-α-PIAS1 complexes. Figure 3.4C shows that under control conditions, endogenous IKK-α interacted with

PIAS1 in MCF-10AT1 cells. However, this interaction was reduced upon cIAP2 downregulation, consistent with activation of IKK-α and release of PIAS1.

cIAP2 is required for p53 upregulation by doxorubicin

IAP antagonism has been reported to augment current chemotherapeutics such as doxorubicin (Awasthi et al., 2011; Weisberg et al., 2007). Doxorubicin is one of the most widely used chemotherapeutic drugs in the treatment of cancer where it causes DNA damage by inhibiting topoisomerase II, which results in the formation of double strand breaks, and by forming DNA adducts (Tewey et al., 1984). Failure to repair this DNA damage leads to p53-mediated cell death (Lowe et al., 1993). To determine if SM164 can affect the induction of p53 by doxorubicin, MCF-10AT1 cells were treated with the chemotherapeutic then analyzed by western blotting. Figure 3.5 shows that treatment with 100 nM doxorubicin for 18 hours dramatically increased levels of total and phosphorylated p53, as well as its downstream target p21. Co-treatment of these cells with SM164 (10 nM) prevented the upregulation of total p53 by doxorubicin, although there remained a low level of active phospho-p53 (Ser15), and only a slight decrease in p21 levels. The kinetics of doxorubicin-induced activation of p53 may occur quicker than the downregulation of p53 by SM164, hence resulting in residual phospho-p53 (Ser15) activity. Treatment with nutlin-3 restored the upregulation of total and phosphorylated p53 by doxorubicin, even in the presence of SM164. These results show that downregulation of cIAPs can interfere with the induction and to a lesser extent, activation of p53 by doxorubicin, through a mechanism involving Mdm2.

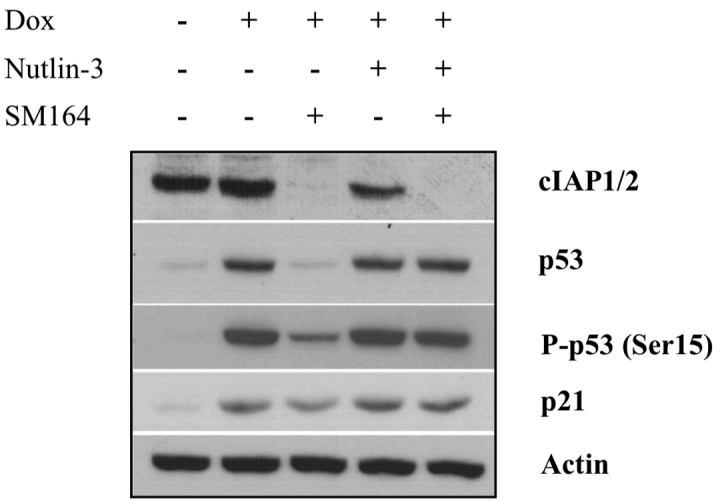


Figure 3.5. cIAP downregulation prevents p53 induction by doxorubicin
MCF-10AT-1 cells were treated with 100 mM doxorubicin for 18 hours in the presence or absence of 10 nM SM164. Whole cell lysates were immunoblotted for cIAP1/2 (using the rIAP antibody that recognizes both proteins), p53, phospho-p53 (Ser15) and p21. Actin was used as a loading control.

cIAP2 downregulation decreases p53 *in vivo*

Our lab had previously characterized DMBA-induced tumour formation in cIAP2-null mice (Figures A.1-2, Table 1). Highly apoptotic tumours formed following a longer latency compared to control mice. However, at the end of the study, more adenocarcinomas had formed in the cIAP2-null mice and the tumours displayed higher mitotic indices than the control mice. Since DMBA-induced tumours typically retain expression of wildtype p53 (Jerry et al., 1994), western blotting was performed to determine if p53 levels were affected by the absence of cIAP2, as in *in vitro* experiments. Immunoblotting of tumour protein extracts revealed that total p53, phospho-p53 (Ser15) and its downstream effector, p21 were all downregulated in the cIAP2-null tumours compared to control tumours (Figure 3.6). Additionally, phospho-IKK-α was differentially increased in cIAP2-null tumours, consistent with in vitro results (Figure A.3A-C). These results demonstrate that the oncogenic signaling resulting from cIAP2 downregulation observed in vitro is similarly applicable in an *in vivo* model.

cIAP2 downregulation activates MAPKs

Given that cIAP2 downregulation led to increased colony formation in a clonogenic assay and resulted in highly proliferative tumours in a null mouse, it is likely that the decrease in cIAP2 impacts multiple pathways that promote survival and proliferation. Although Akt can be activated downstream of NF-κB signaling, activation of Akt was not increased following cIAP2 downregulation (Figure A.6). However, examination of ERK1/2 revealed that transfection of MCF-10AT1 cells with cIAP2 siRNA strongly increased the phosphorylation of ERK1/2, without corresponding changes in total ERK1/2 levels (Figure 3.7A). Similar results were obtained in MCF-7

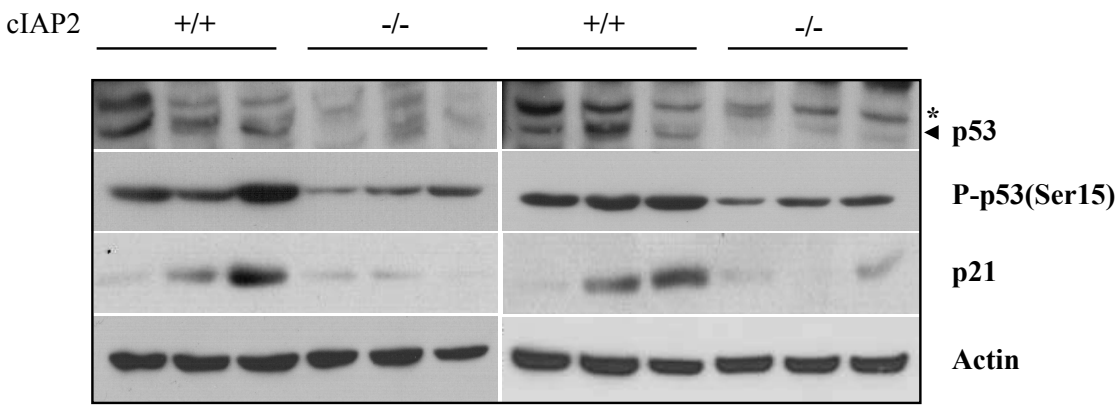


Figure 3.6. p53 and p21 are downregulated in DMBA-induced tumours in cIAP2$^{-/-}$ mice. Protein extracts from DMBA-induced tumors generated in cIAP2$^{+/+}$ or cIAP2$^{-/-}$ mice were immunoblotted with total p53, phospho-p53(Ser15) and p21. The asterisk indicates a non-specific reactive band. Actin was used as a loading control.

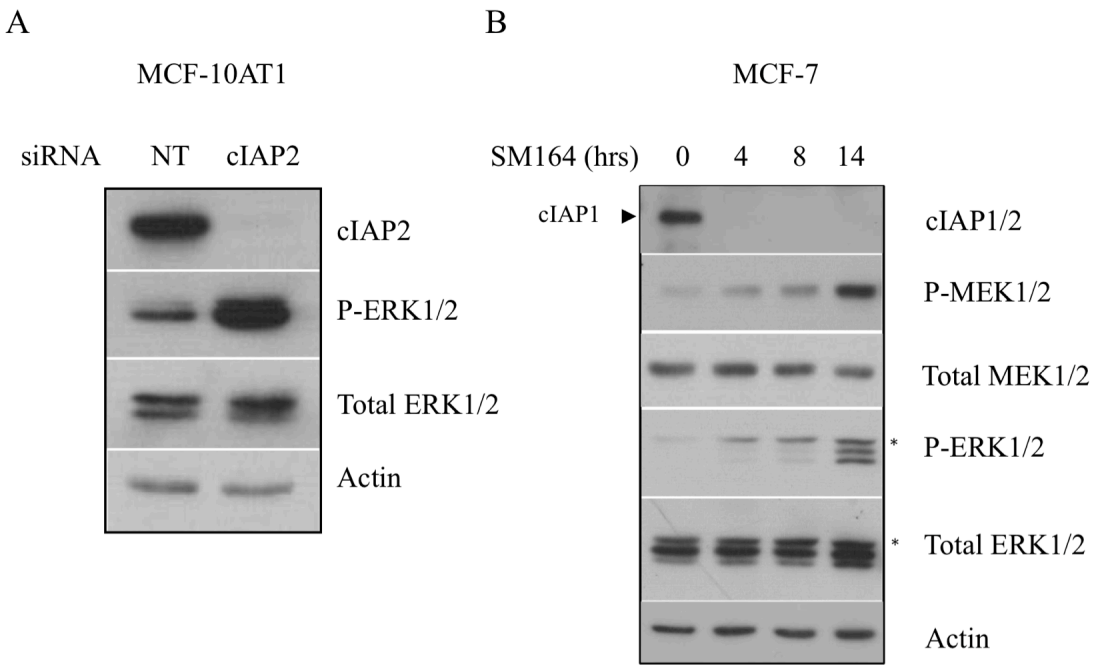


Figure 3.7. cIAP2 downregulation activates ERK1/2
A, MCF-10AT1 cells were transfected with either non-targeting (NT) or cIAP2 siRNA for 48 hours. Cell lysates were immunoblotted for cIAP2, phospho-ERK1/2 and total ERK1/2. **B,** MCF-7 cells were treated with SM164 (10 nM) for the indicated times. Whole cell lysate was immunoblotted for cIAP1/2 (using anti-rIAP1 that recognizes both proteins), phospho-MEK, total MEK, phospho-ERK1/2 and total ERK1/2. Asterisk indicates a non-specific band. Actin was used as a loading control in all experiments. Western blots shown are representative of experiments performed at least three times.

cells, where treatment with the IAP antagonist SM164 (10 nM) resulted in a time-dependent increase in phospho-MEK1/2, upstream of ERK1/2, and phospho-ERK1/2 (Figure 3.7B). Interestingly, treatment with the MEK inhibitor, UO126, resulted in an increase in cIAP2 levels (Figure A.7), suggesting that a reciprocal regulatory relationship exists. Overall, the results indicate that in addition to activating NF-κB and negatively regulating p53, downregulation of cIAP2 could contribute to survival and proliferation by activating the MAPK pathway.

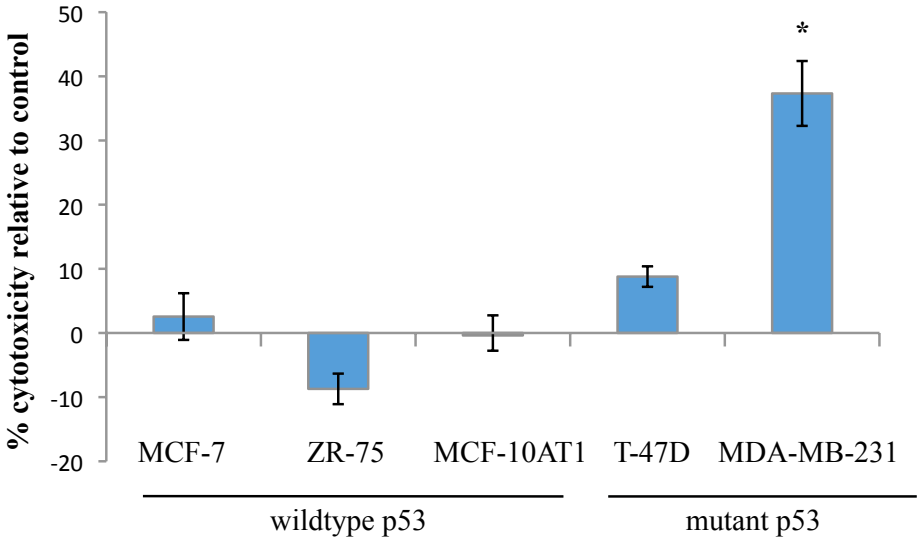
The findings presented thus far indicate that the downregulation of cIAP2 can impact multiple signaling pathways. The downregulation of cIAP2 resulted in activation of the IKKs, increased activity of Mdm2 and decreased p53 levels, and activation of the MAPKs. Together, these alterations contributed to increased proliferation both *in vitro* and *in vivo*.

Objective II: To identify factors that determine sensitivity to IAP antagonists

Cytotoxic response to IAP antagonism is associated with p53 status

IAP antagonists have been found to be effectively cytotoxic in selective cell lines by inducing TNF-α, which results in cell death in the absence of the cIAPs (Varfolomeev et al., 2007; Vince et al., 2007). However, the majority of these cell lines do not express wildtype p53. To determine if p53 status can affect the response to IAP antagonism, a number of p53-wildtype and -mutant breast cancer cell lines were subjected to an MTT cell viability assay following treatment to the IAP antagonist AEG40730. Figure 3.8A shows that p53-wildtype cell lines MCF-7, ZR-75 and MCF-10AT1 showed no change in cell viability following a 30-hour treatment with 100 nM AEG40730 alone. IAP-antagonist-treated p53-mutant T-47D cells showed slight cytotoxicity (~9% cell death)

A


% cytotoxicity relative to control
50
40
30
20
10
0
-10
-20
*
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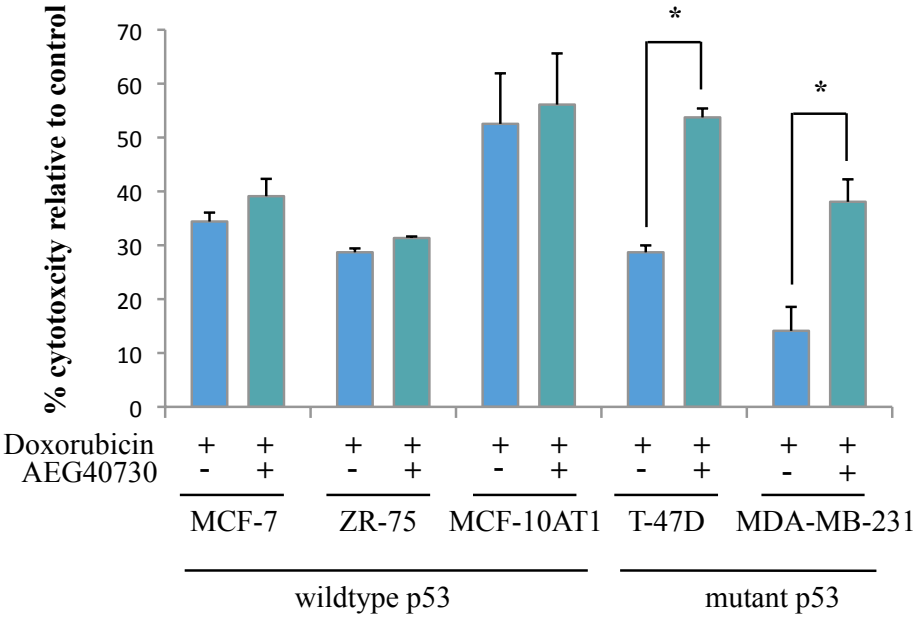
ZR-75
MCF-10AT1
T-47D
MDA-MB-231
wildtype p53
mutant p53

B


% cytotoxcity relative to control
70
60
50
40
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Doxorubicin
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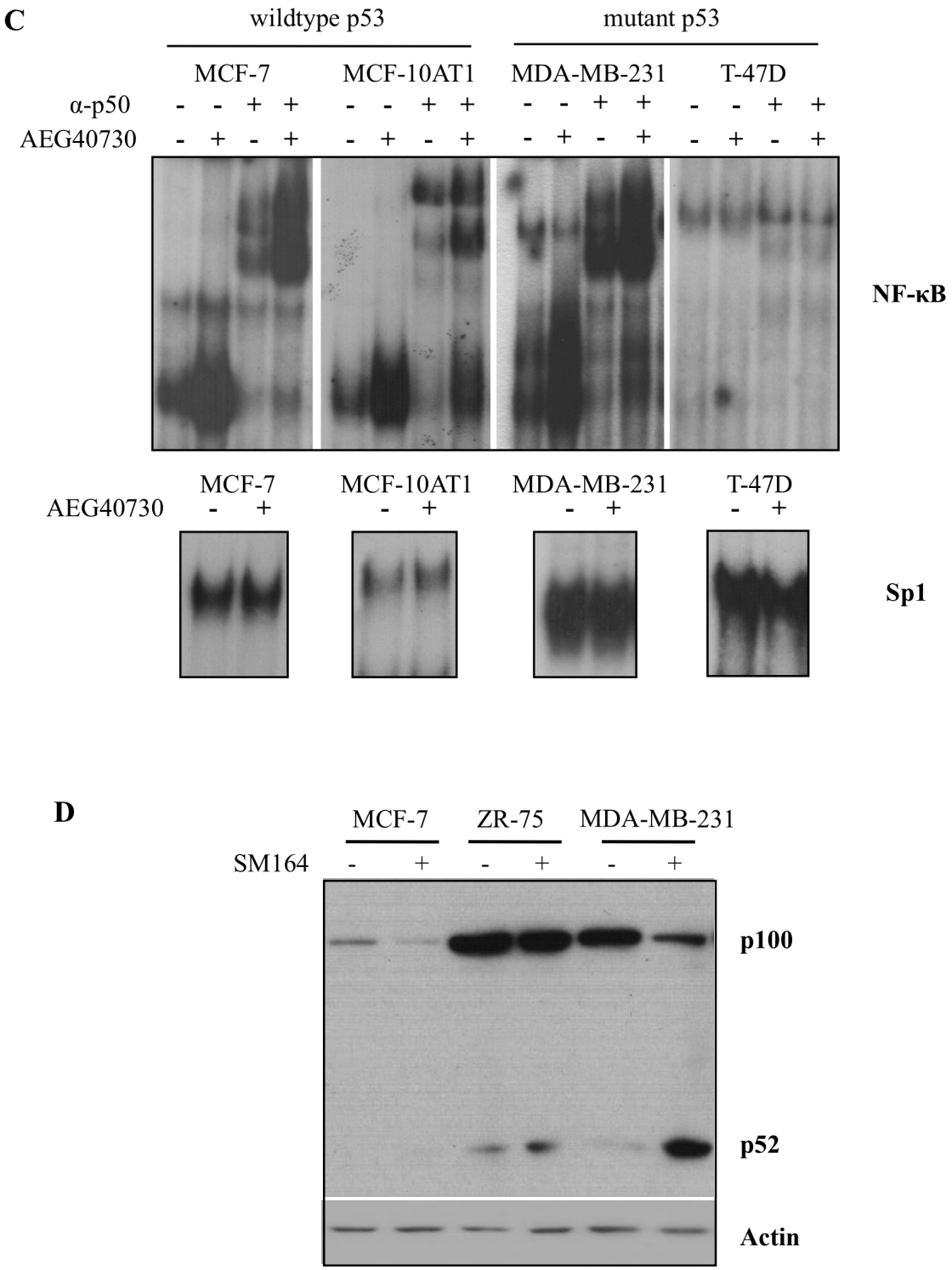
MDA-MB-231
wildtype p53
mutant p53


C
wildtype p53
mutant p53
MCF-7
MCF-10AT1
MDA-MB-231
T-47D
α-p50
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NF-κB
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MCF-10AT1
MDA-MB-231
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SM164
p100
p52
Actin

E

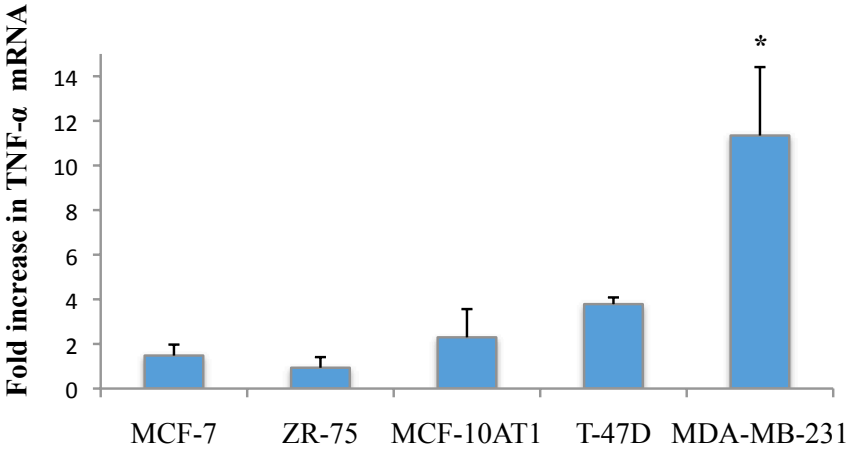


Figure 3.8. p53 status is associated with response to IAP antagonism
A, The indicated cell lines were treated with vehicle (DMSO) or AEG40730 (100nM) for 30 hours. Cell viability was then assessed by an MTT assay and % cytotoxicity is expressed relative to vehicle-treated cells. **B,** MTT assay of cells co-treated with doxorubicin (MCF-7, ZR75 and T47D at 500nM; MCF10A-AT1 at 100nM; and MDA-MB-231 at 200nM) and either vehicle or AEG40730 for 30 hours. Cell viability and % cytotoxicity is expressed relative to vehicle-treated cells. **C,** Nuclear extracts from the indicated cell lines treated with vehicle (DMSO) or AEG40730 (100 nM) for 18 hours were subjected to EMSA analysis. Supershift was performed using an anti-p50 antibody. SP1 was used as a loading control on the bottom panels. **D,** The indicated cell lines were treated with vehicle or SM164 (10 nM) for 6 hours then immunoblotted with an antibody that recognizes p100 and its processed active form p52. Actin was used as a loading control. **E,** Total RNA was extracted from the indicated cell lines treated with SM164 (10 nM) for 4 hours. qPCR was performed to determine TNF-α transcript levels and expressed as fold difference over cells treated with vehicle control (DMSO). GAPDH was used for normalization. Data are means ± sd. (n=3 per group). Asterisk denotes $p < 0.01$ as determined by Student's *t*-test.

while MDA-MB-231 cells, which also express mutant-p53, underwent a dramatic 37% induction of cell death. IAP antagonists have also been reported to augment the cytotoxicity of other chemotherapeutic agents, such as doxorubicin (Awasthi et al., 2011; Weisberg et al., 2007). To determine if AEG40730 is more effective when used in conjunction with doxorubicin, the same cell viability assay was performed with the addition of doxorubicin (MCF-7, ZR75 and T-47D at 500 nM; MCF10A-AT1 at 100 nM; and MDA-MB-231 at 200 nM). The concentrations of Doxorubicin were selected following an initial dose response experiment to determine treatment concentrations for each cell line that would induce a moderate cytotoxic response. Doxorubicin treatment alone resulted in varying levels of cell death in all cell lines (14% - 52%) (Figure 3.8B). Co-treatment with AEG40730 augmented cell killing only in p53 mutant T-47D and MDA-MB-231 cells, but not in MCF-7, ZR-75 or MCF10-AT1 cells. These results suggest that p53 mutation may play a role in the differential sensitivity of the various cell lines to IAP antagonism.

Since cell death induced by IAP antagonists is reported to result from TNF-α generated in response to non-canonical NF-κB signaling (Varfolomeev et al., 2007; Vince et al., 2007), NF-κB activation and subsequent production of TNF-α were examined in a number of breast cancer cell lines treated with IAP antagonist. EMSA analysis showed that with the exception of T-47D, all cell lines tested (MCF-7, MCF-10AT1 and MDA-MB-231) displayed strong increases in NF-κB activity following treatment with AEG40730 (Figure 3.8C). However, western blotting to detect activation of non-canonical NF-κB signaling showed that MCF-7 and ZR75 cells displayed much less processing of p100 to p52 than did MDA-MB-231 cells in response to IAP

antagonism (Figure 3.8D). Subsequently, when q-PCR was performed on RNA extracted from SM164-treated cells, only MDA-MB-231 cells transcribed increased TNF-α mRNA following antagonist treatment, averaging 11-fold higher than vehicle-treated cells (Figure 3.8E). These results indicate that canonical NF-κB activation alone is insufficient for TNF-α induction, and that additional factors may be involved.

SM-induced transcriptional activation of TNF-α is mediated by mutant p53

Gain of function mutations in p53 have been shown to induce expression of p100 (Scian et al., 2005), which is involved in the non-canonical NF-κB pathway and mediates the induction of TNF-α by IAP antagonists (Varfolomeev et al., 2007). Mutant p53 has also been shown to enhance NF-κB activation in response to TNF-α (Weisz et al., 2007). Since our experiments showed that MDA-MB-231 cells, which express mutant p53 (R280K), were the only cell line tested to increase transcription of TNF-α in response to IAP antagonism, we investigated the requirement of mutant p53 in this process. MDA-MB-231 cells were transiently transfected for 48 hours with an siRNA targeting p53 then analyzed by western blotting. Figure 3.9A confirms that the siRNA was effective in downregulating p53(R280K). Similarly-transfected cells were then treated with 10 nM SM164 for the final 5 hours of transfection to perform qPCR to detect TNF-α transcript levels (Figure 3.9B). Control cells transfected with non-targeting siRNA exhibited 18.5-fold increase in transcription of TNF-α mRNA in response to SM164 treatment, consistent with previous results. Notably, TNF-α mRNA induction in cells transfected with p53 siRNA was significantly reduced by 60% following IAP antagonism. To determine if these results were a consequence of an inactivation of wildtype p53 or a gain-of-function mutation, MCF-7 cells were first stably transfected with a

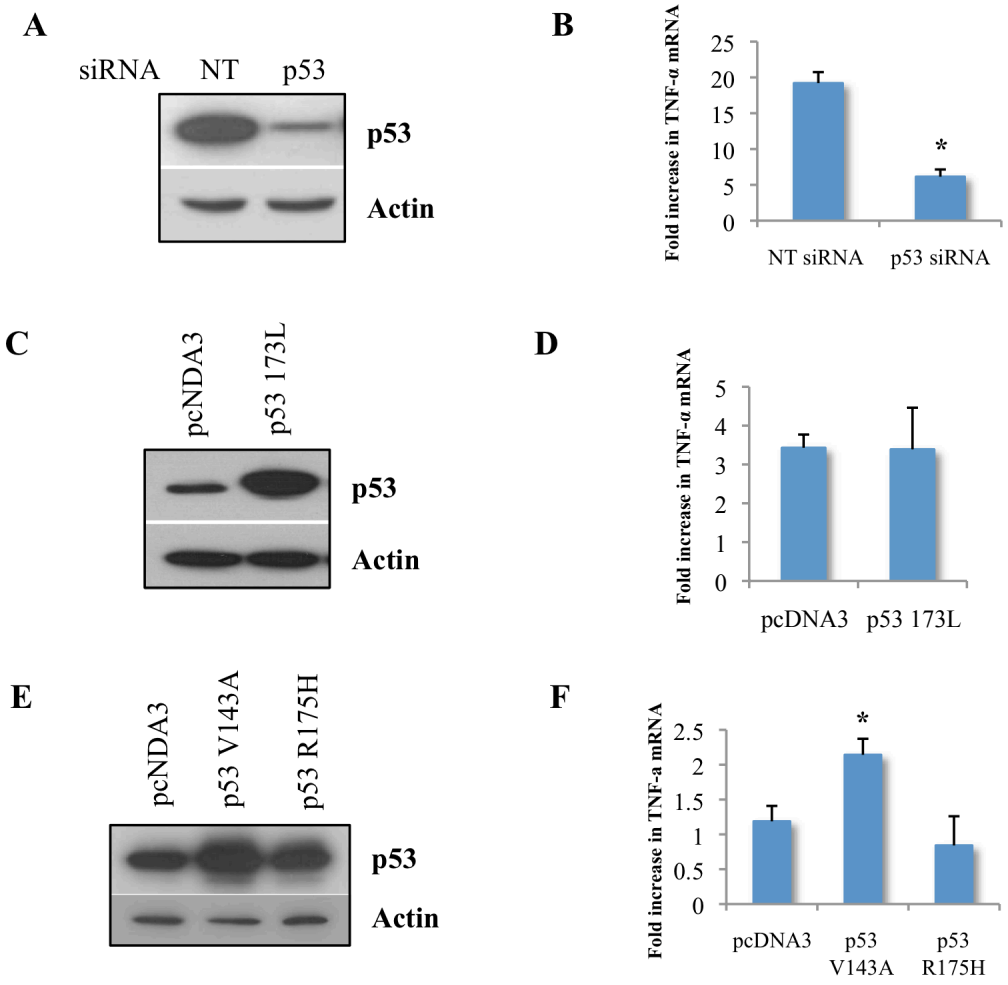


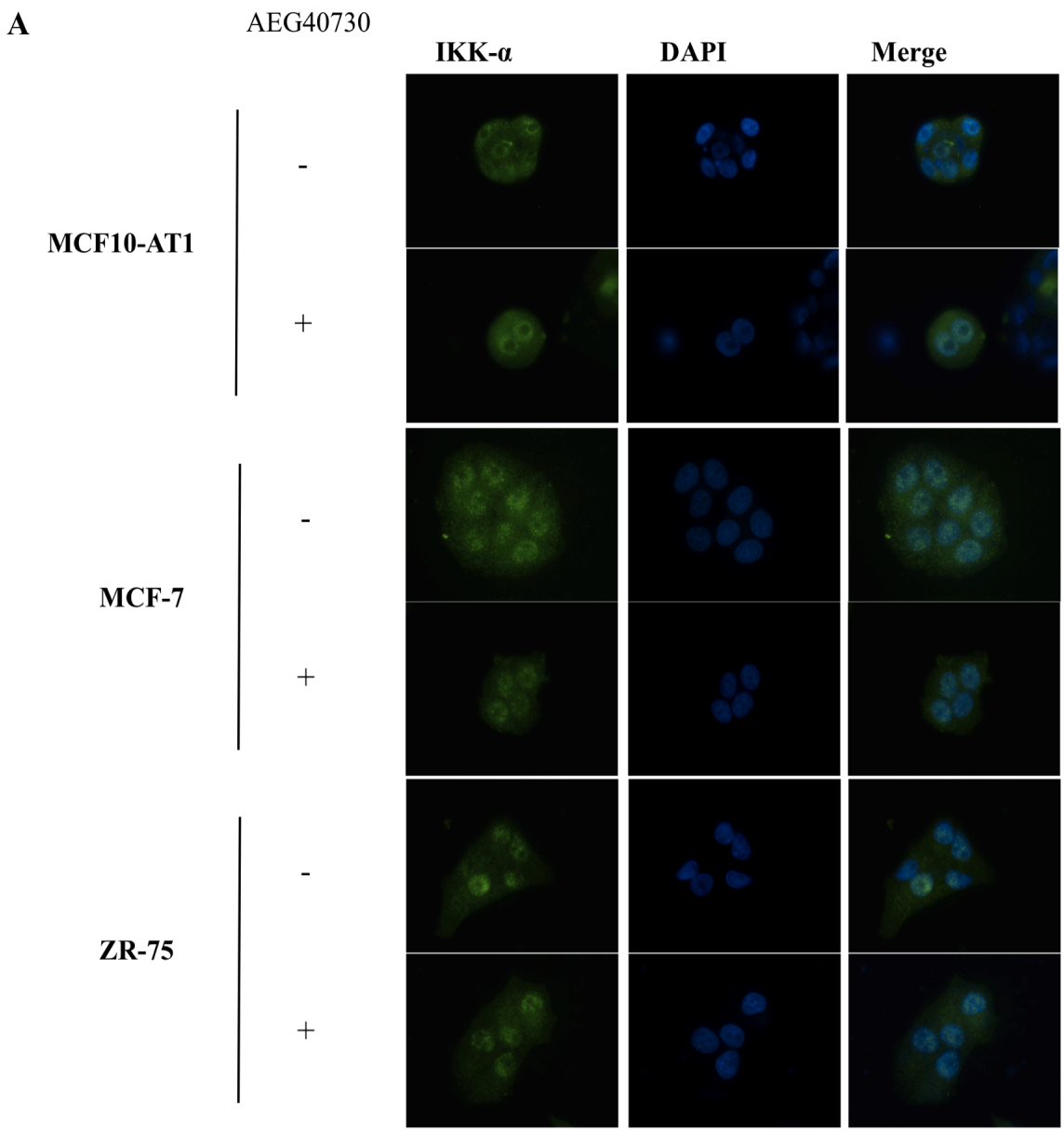
Figure 3.9. IAP-antagonist induced-TNF-α production is mediated by gain of function p53 mutation

A, MDA-MB-231 cells were transfected with either non-targeting (NT) or p53 siRNA. Whole cell lysates were immunoblotted for p53. **B,** Total RNA was extracted from MDA-MB-231 cells transfected with non-targeting (NT) or p53 siRNA for 48 hours then treated with 10 nM SM164 for the final 5 hours. Total RNA was extracted and qRT-PCR was performed to determine TNF-α transcript levels. **C,** MCF-7 cells were stably transfected with control (pcDNA3) plasmid or a plasmid encoding mutant p53 173L. Whole cell lysates were immunoblotted for p53. **D,** MCF-7 cells from **C** were treated with SM164 and qRT-PCR was performed as in **B**. **E,** HEK293 cells were transiently transfected with control (pcDNA3) or the DNA mutants p53 V143A and R175H. Whole cell lysates were immunoblotted for p53. **F,** HEK293 cells from **E** were treated with SM164 and qRT-PCR was performed as in **B.** Actin was used as a loading control in all western blots. For qRT-PCR experiments, data are expressed as fold difference over cells treated with vehicle (DMSO). GAPDH was used for normalization. Data are means ± s.d. (n=3 per group), Asterisk, $p < 0.01$ (Student's *t*-test).

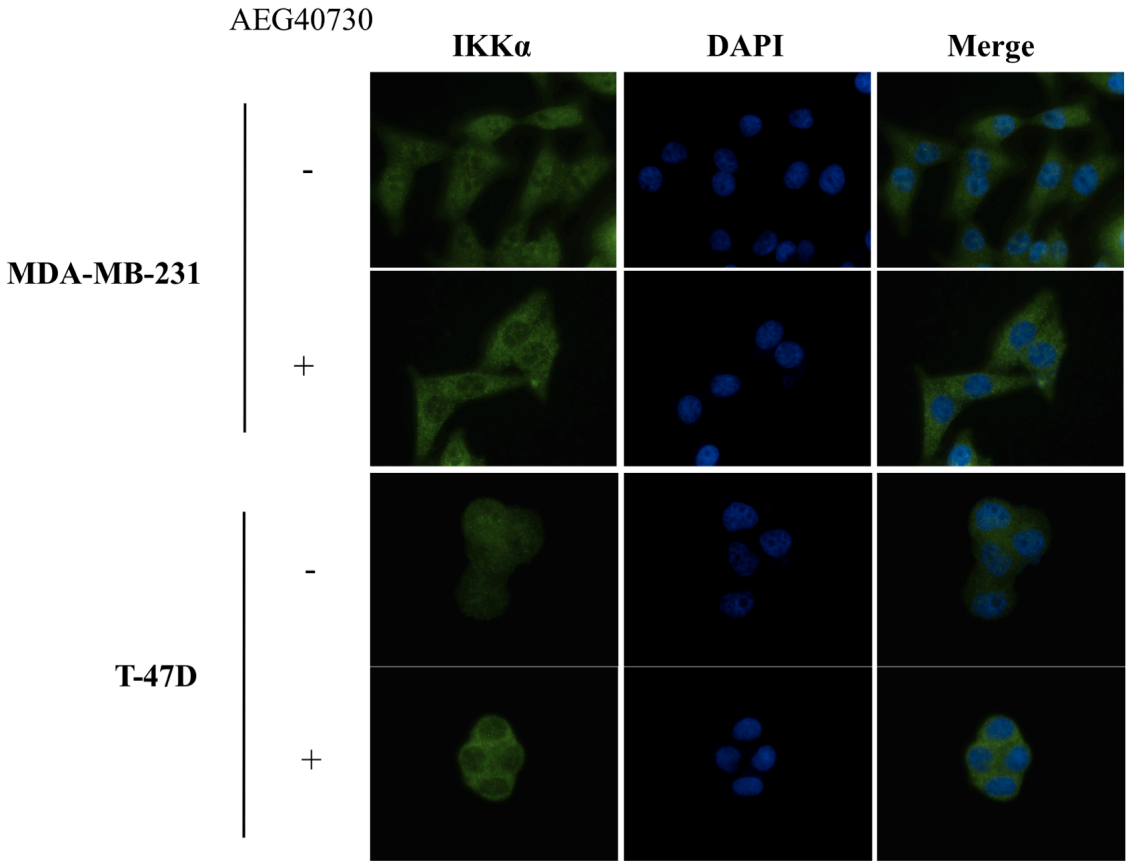
transcriptionally inactive DNA-binding mutant, p53-173L (Ludwig et al., 1996), then extracts were immunoblotted to confirm expression of the mutant (Figure 3.9C). The cells were treated with 10 mM SM164 for 5 hours and qRT-PCR for TNF-α showed that the expression of p53-173L did not alter transcription of TNF-α. Two additional p53 mutants were then tested to see whether they affected TNF-α transcription. HEK293 cells were transfected with two tumour-derived p53 mutants - p53-V143A and p53-R175H (Figure 3.9E). These cells were then treated with SM164 (10 nM) for 5 hours. Figure 3.9F shows that the expression of p53-R175H did not induce the transcription of TNF-α. However, expression of p53-V143A increased TNF-α transcript levels significantly by approximately two-fold. These results suggest that the ability to augment the transcription of TNF-α is a gain-of-function that is not conferred by the p53-173L or p53-175H mutants.

Nuclear IKK-α decreases in response to IAP antagonism in p53-mutant, but not - wildtype cells

cIAP2 downregulation resulted in a differential activation of IKK-α (Figure 3.1D). Since a nuclear function attributed to IKK-α is to promote survival by facilitating the transcription of NF-κB-responsive genes (Hoberg et al., 2004; Yamamoto et al., 2003), AEG40730-treated cells were immunostained for IKK-α to determine its localization. In untreated cells, IKK-α localization was mostly nuclear in all cell lines tested, with some cytoplasmic staining (Figure 3.10A and B). Following treatment with 100 nM AEG40730 for 6 hours, IKKα remained in the nucleus in p53-wildtype MCF-10AT1, MCF-7 and ZR-75 cells (Figure 3.10A). However, when p53-mutant cells MDA-MB-231 and T-47D were treated with AEG40730, IKK-α appeared to be excluded from


A
AEG40730
IKK-α
DAPI
Merge
MCF10-AT1
-
+
MCF-7
-
+
ZR-75
-
+

B



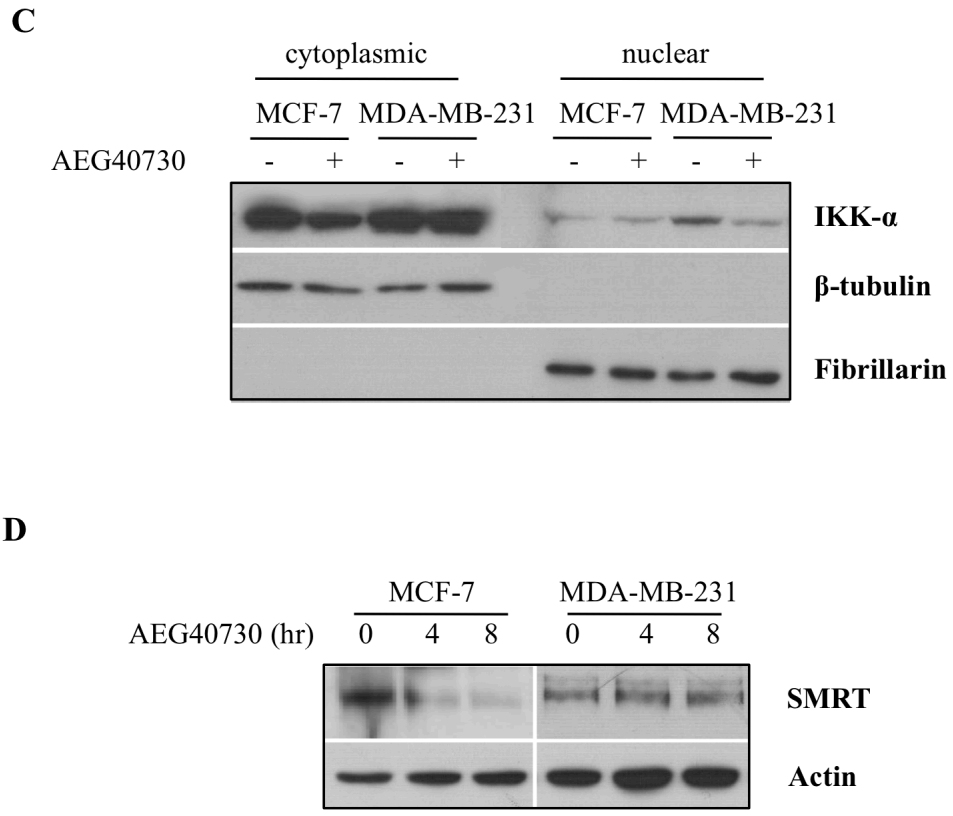


Figure 3.10. Downregulation of cIAP2 in p53-mutant cells decreases nuclear IKK-α
A, p53-wildtype cells MCF-10AT1, MCF-7, ZR-75, and **B,** p53-mutant cells MDA-MB-231 and T-47D were grown on coverslips then treated with AEG40730 (100 nM) for 6 hours. Cells were fixed and immunostained for IKK-α. Coverslips were counterstained with DAPI to indicate nuclei. **C,** MCF-7 and MDA-MB-231 cells were treated with AEG40730 (100 nM) for 6 hours. Nuclear and cytoplasmic fractions, prepared as described in Methods, were immunoblotted for IKK-α. Immunoblotting for β-tubulin and fibrillarin confirmed the purity of the cytoplasmic and nuclear fractions, respectively, as well as to demonstrate equal loading of protein. **D,** MCF-7 and MDA-MB-231 cells were treated with AEG40730 (100 nM) for the indicated times then whole cell lysate was immunoblotted for SMRT. Actin was used as a loading control. Western blots shown are representative of experiments performed at a minimum of two times.

the nucleus (Figure 3.10B). To confirm the decrease in nuclear IKK-α in AEG40730-treated p53-mutant cells, we performed a nuclear/cytoplasmic fractionation of MCF-7 and MDA-MB-231 cells, treated with either vehicle (DMSO) or AEG40730 for 6 hours. As shown in Figure 3.10C, there was no change in the level of nuclear IKK-α following AEG40730 in MCF-7 cells. Notably, consistent with the immunofluorescence data, nuclear IKK-α was decreased in MDA-MB-231 cells treated with AEG40730. One of the nuclear functions of IKK-α is to phosphorylate the corepressor SMRT to facilitate the derepression and subsequent transcription of survival genes regulated by NF-κB (Hoberg et al., 2004). To determine the effects of cIAP2 downregulation on SMRT, MCF-7 and MDA-MB-231 cells were treated with 100 nM AEG40730. After 4 hours of treatment, SMRT levels in MCF-7 cells began to decrease and following 8 hours of treatment, SMRT levels were dramatically diminished (Figure 3.10D). In contrast, SMRT levels did not change following AEG40730 treatment in MDA-MB-231 cells. Together, these results indicate that in p53-mutant MDA-MB-231 cells, IAP antagonist treatment does not result in IKK-α-mediated derepression of SMRT as it does in p53-wildtype MCF-7 cells, which may contribute to the induction of cell death in those cells.

CHAPTER IV - DISCUSSION

Summary

The results in this study present a tumour suppressor role for cIAP2 whereby, as a function of its ubiquitin ligase activity, cIAP2 suppresses NIK protein and prevents activation of NF-κB. Downregulation of cIAP2 through RNAi and using small molecule inhibitors consistently resulted in activation of the canonical NF-κB pathway. Moreover, we demonstrate that the cIAPs regulate p53 protein levels through controlling IKK activity. Both IKK-α and IKK-β were activated, which decreased phospho-p53 (Ser15) and mediated an increase in Mdm2 phosphorylation/activation, resulting in decreased wildtype p53 levels. Activation of IKK-α/β also activated the MEK-ERK pathway, which is typically associated with proliferation (McCubrey et al., 2007). These are likely the mechanisms by which cIAP2 downregulation can increase colony formation *in vitro* and adenocarcinoma development in cIAP2-null mice.

Cytotoxicity of IAP antagonists was found to be most pronounced in a cell line that expressed mutant p53 and we propose a role for gain of function p53 mutations in the transcriptional activation of TNF-α, and therefore sensitivity to IAP antagonists. Lastly, the treatment of p53-mutant cells with the IAP antagonist resulted in a decrease in nuclear IKK-α and unchanged SMRT levels. In contrast, IAP antagonist induced SMRT degradation in p53-wildtype cells without altering nuclear IKK-α. This result suggests that the global transcriptional response to NF-κB activation is not engaged in cells such as MDA-MB-231 cells, thus limiting survival signaling following cIAP depletion.

Overall, in cells that express wildtype p53, cIAP2 depletion results in destabilization of p53 which, together with survival and proliferative signaling provided

by NF-κB and ERK1/2, results in an increased potential for cancer development and progression.

cIAP2 downregulation in mammary epithelial cells activates canonical NF-κB

MCF-10A cells are a non-tumourigenic mammary epithelial cell line (Dawson et al., 1996) that expresses low endogenous levels of cIAP2. Stable transfection of these cells resulted in an induction of cIAP2 (Figure 3.1A), consistent with its role as a facilitator of oncogenic signaling (Liu et al., 2005). Stable cotransfection with the cIAP2 shRNA only resulted in a moderate decrease in cIAP2 levels (Figure 3.1B). This is likely due to the shRNA being opposed by induction of cIAP2 by Ha-*ras* (Liu et al., 2005) and by NF-κB (Chu et al., 1997; Hong et al., 2000) that was activated in response to cIAP2 downregulation. Despite the modest downregulation of cIAP2, both TRAF1 and NIK levels were increased (Figure 3.1B), which is consistent with the participation of cIAP2 in the ubiquitination of NIK as part of a complex with cIAP1, TRAF2 and TRAF3 (Liao et al., 2004; Vallabhapurapu et al., 2008; Zarnegar et al., 2008). These results demonstrate that even a small perturbation in the levels of cIAP2 resulted in a stoichiometric inbalance and led to stabilization of its targets. This is in agreement with the characterization of multiple myeloma, where mutations in any of a number of genes that disrupt the function of TRAF2, TRAF3 or cIAP1/2 result in stabilization of NIK, constitutive activation of NF-κB, and are associated with development of the disease (Keats et al., 2007).

The increases in both total and phosphorylated levels of NIK were associated with activation of NF-κB signaling (Figure 3.1F). Activation of both canonical and non-canonical NF-κB signaling resulting from the stabilization of NIK protein have been

shown in multiple cancers (Neely et al., 2011; Saitoh et al., 2010). NIK is stabilized as a result of different aberrations in different cells, but appears to consistently result in activation of NF-κB signaling and mediate oncogenicity. Specifically, stabilization of NIK resulting from cIAP depletion has been demonstrated through the use of IAP antagonists, and led to subsequent activation of non-canonical NF-κB signaling (Varfolomeev et al., 2007; Vince et al., 2007). The activation of NF-κB was concurrent with an increase in the phosphorylated form of IKK-α, which was not observed in control cells in which cIAP2 expression was intact. Despite the typical association of NIK and IKK-α with activation of the non-canonical NF-κB pathway, recent evidence shows that both of these kinases can activate canonical NF-κB through activation of IKK-β (Hacker and Karin, 2006; Nakano et al., 1998; O'Mahony et al., 2000). It is unclear what factors determine whether the canonical or non-canonical NF-κB pathway is activated when NIK is stabilized. In a panel of cell lines treated with IAP antagonist, MDA-MB-231 cells displayed processing of p100 to p52 while MCF-7 and T47D cells only processed minor amounts of p52 (Figure 3.8D), despite the observation that all cell lines activated canonical NF-κB signaling as shown by EMSA analysis (Figure 3.8C).

The activation of canonical NF-κB signaling in response to cIAP2 downregulation is a key event, given that NF-κB is intricately linked to proliferation and inhibition of apoptosis (Hayden and Ghosh, 2008). The association between aberrant activation of NF-κB signaling and cancer has been well established. Activation of NF-κB in malignancies is a common occurrence and different components of the pathway can contribute to mammary tumourigenesis. As discussed in the following sections, the activation of NF-

κB can impact the survival and proliferation of mammary epithelial cells by multiple mechanisms.

Downregulation of cIAP2 results in a reduction of p53

A critical finding in this study is that cIAP2 downregulation results in a strong decrease in the cellular levels of p53 in an NF-κB-dependent manner. This result provides an important mechanism for the increase in proliferation and survival that was observed both *in vitro* and *in vivo*. p53 is a potent tumour suppressor that is critical for preventing aberrant proliferation (reviewed in (Balint and Vousden, 2001). In response to cIAP2 downregulation, transcriptional activation of survival genes by NF-κB combined with a decrease in functional p53 creates an environment conducive to tumourigenesis. Less consistently, cIAP1 reduction could also reduce p53. Why this occurs is not clear but may be dependent on the ability of cIAP2, which is stabilized in the absence of cIAP1, to fully compensate for cIAP1 functions.

The reduction of p53 resulting from cIAP2 downregulation was consistent across different cell lines that express wildtype p53 and using different methods of cIAP2 downregulation (RNAi and two different IAP antagonists) (Figure 3.2A-E). The development of IAP antagonists was initially targeted at inhibiting XIAP. However, it was found that these compounds displayed higher affinity for the cIAPs and induced their degradation. The concentrations of IAP antagonists used in the present experiments were sufficient for causing degradation of the cIAPs, but below the concentrations reported to inhibit XIAP activity (Bertrand et al., 2008). As demonstrated in Figure 3.2F, downregulation of XIAP in MCF-10AT1 cells had no impact on p53 levels, confirming that this regulation of p53 is XIAP-independent. Mutant p53 was not affected by IAP

antagonist treatment, likely a function of the low expression or absence of Mdm2 in cells that express mutant p53 (Midgley and Lane, 1997). Interestingly, IAP antagonist treatment of MCF-7 cells, which expresses only cIAP1 and not cIAP2, also resulted in downregulation of p53. Since both cIAP1 and cIAP2 can ubiquitinate NIK (Zarnegar et al., 2008), the downregulation of cIAP1 in these cells may be sufficient to activate NF-κB and reduce p53 levels.

Reduction of p53 following downregulation of cIAP2 is mediated by NF-κB

NF-κB, as part of its program to promote survival, can antagonize p53 through multiple ways. Tergaonkar et al. (2002) showed that activation of IKK-β destabilizes p53 by inducing Mdm2 and prevents upregulation of p53 induced by doxorubicin treatment. Additionally, the IκB family member, Bcl-3, is inducible by NF-κB and can bind the Mdm2 promoter to induce its expression, thus decreasing p53 levels (Kashatus et al., 2006). It was later shown that IKK-β can directly phosphorylate p53 at serines 362 and 366, thereby promoting its degradation by β-TrCP (Xia et al., 2009).

Here, we demonstrate that inhibition of the IKKs in MCF-7 cells using the inhibitor BMS-345541 results in an increase in phospho-p53 (Ser15) and total p53 (Figure 3.3A). While treatment using the proteasome inhibitor MG132 alone increased p53 levels with an accumulation of higher molecular weight forms consistent with ubiquintinated p53, co-treatment with both BMS-345541 and MG132 resulted in an increase in non-ubiquitinated p53. This result suggests that the IKKs promote ubiquitination of p53 that leads to its degradation by the proteasome. Additionally, stabilization of total p53 using BMS-345541 was accompanied by an increase in phopsho-p53 (Ser15), consistent with the association of this phosphorylation with

stability of p53 protein (Dumaz et al., 1999; Lakin et al., 1999). Conversely, stable transfection of MCF-7 cells with constitutively active IKKs, particularly IKK-β, resulted in a decrease in phospho-p53 (Ser15) (Figure 3.3C). This supports a role for IKK-β in the regulation of p53 serine 15 phosphorylation. Phosphorylation of p53 at serine 15 is mediated by multiple kinases, including ATM, ATR, PI3K, DNA-PK and CDK5 (Meek and Anderson, 2009). It is possible that activation of IKK-β negatively regulates a kinase that mediates serine 15 phosphorylation on p53. Conversely, the activation of IKK-β may increase the activity of a phosphatase that dephosphorylates p53 at serine 15. Specifically, Wip1 (wild type p53-induced phosphatase 1) is a serine/threonine phosphatase that is overexpressed in several types of cancers, including breast cancer. It exerts oncogenic effects through its negative regulation of the DNA damage response (Lu et al., 2008). For example, Wip1 can decrease p53 (Ser15) phosphorylation by dephosphorylating p53 kinases, including ATM, CHK1 and CHK2 (Fujimoto et al., 2006; Lu et al., 2005; Shreeram et al., 2006), as well as by dephosphorylating p53 itself (Lu et al., 2005). Thus, overexpression of Wip1 decreases stability of p53 protein. Recently, Wip1 was identified as a target of transcriptional activation by NF-κB (Lowe et al., 2010). Activation of IKK-β, resulting from the downregulation of cIAP2, may decrease phospho-p53 (Ser15) through a mediator such as Wip1. Decreased phosphorylation of p53 at serine 15 increases its susceptibility to Mdm2-mediated degradation (Dumaz et al., 1999), thereby resulting in a decrease in total p53 levels.

We demonstrate that inhibition of either IKK-α or -β partially rescued the levels of p53 following cIAP2 downregulation, but inhibition of both kinases was required to fully restore p53 protein to the control level (Figure 3.3D). This suggests that both

kinases contribute individually to the downregulation of p53. Although current literature indicates IKK-β as the inducer of Mdm2 and kinase for p53, it does not exclude the possibility that IKK-α may impact p53 stability through other modifications or intermediates, which will be discussed in a later section. Also, IKK-α directly phosphorylates IKK-β and enhances its activity (O'Mahony et al., 2000; Yamamoto et al., 2000). It is therefore possible that an increase in IKK-α activity exerts its effects, in part, through the activation of IKK-β. Additionally, p53 levels in IAP antagonist-treated MCF-7 cells were rescued by co-treating with the pan IKK inhibitor BMS-345541. This further supports a role for NF-κB in cIAP1/2 downregulation-induced reduction of p53. Of interesting note, BMS-345541 is unable to inhibit NF-κB in MCF-10AT1 cells (data not shown). This "resistance" to BMS-345541 occurs in certain cell types for unknown reasons (Dr. James Burke, Bristol-Myers-Squibb, personal communication).

cIAP2 downregulation promotes post-translational modification of Mdm2 known to increase activity

The regulation of p53 stability is mediated predominantly by its ubiquitin ligase Mdm2 (Kubbutat et al., 1997). This interaction can be disrupted using the small molecule nutlin-3, which results in the stabilization of p53 and increases transcription of its target genes (Vassilev et al., 2004). Transfection with either cIAP1 or cIAP2 siRNA into MCF10-AT1 cells resulted in a downregulation of p53 (Figure 3.4A). Since both cIAPs participate in a complex that ubiquitinates NIK, disruption of either protein has the potential to alter the stoichiometric balance of that complex, resulting in stabilization of NIK and activation of NF-κB. The treatment of cIAP1/2 siRNA-transfected cells with nutlin-3 resulted in a rescue of p53 levels, implicating Mdm2 as the mediator for the

reduction of p53 following cIAP downregulation. The levels of Mdm2 were also increased following nutlin-3 treatment, likely a consequence of transcriptional activation by the stabilized p53.

NF-κB is able to impinge on p53 regulation by directly inducing the expression of Mdm2 (Busuttil et al., 2010) and by increasing its activity (Yang et al., 2010). It is unclear if cIAP2 downregulation-induced NF-κB activation resulted in an induction of total Mdm2 levels. In fact, the levels of Mdm2 appeared to decrease following cIAP downregulation despite an apparent increase in activity. This decrease could be a result of lowered p53 levels, resulting in a reduction in Mdm2 transcription. However, this reduction in Mdm2 levels is most likely a function of the specificity of the Mdm2 antibody. The antibody used in these experiments to detect Mdm2 (SMP14) does not recognize Mdm2 phosphorylated at serine 166 (de Toledo et al., 2000), and may not recognize other forms of Mdm2 modified in the region that contains the epitope for SMP14. Therefore, any changes in Mdm2 levels as detected by SMP14 reflect not only changes in the total level of protein, but also levels of its modified forms. The use of this antibody also made it difficult to assess whether total levels of Mdm2 protein were affected by cIAP downregulation. An additional antibody is required in conjunction with the SMP14 antibody to determine if the activation of NF-κB induced Mdm2 levels. Verification of the antibody is important in the detection of Mdm2 since another antibody, 2A10, has also been shown to lose reactivity when Mdm2 is modified by phosphorylation at serine 395 (Meek and Knippschild, 2003).

Consistent with the idea that the decrease in Mdm2 detected by SMP14 was a loss of epitope recognition, an increase in the levels of Mdm2 phosphorylated at serine 166

was detected following cIAP2 downregulation (Figure 3.4B). Phosphorylation of Mdm2 at serine 166 induces the nuclear translocation of Mdm2, where it can then interact with p53 (Meek and Knippschild, 2003). The antibody used for immunoblotting phospho-Mdm2 (Ser166) detected two bands: one at 95 kDa, the typical detected molecular weight of Mdm2, and an additional band at approximately 120 kDa. During nuclear translocation, and also in the nucleus, Mdm2 can be sumoylated, which redirects its ubiquitin ligase activity towards p53 and inhibits autoubiquitination (Miyauchi et al., 2002). Sumoylation also increases the apparent molecular weight of Mdm2 to approximately 120 kDa when analyzed by western blotting (Xirodimas et al., 2002). Compared to control, the 95 kDa form was decreased when cIAP2 siRNA was transfected, but a significant increase in the 120 kDa form was detected. Based on the reported molecular weight of sumoylated-Mdm2, our results suggest that the downregulation of cIAP2 increases phospho-Mdm2 (Ser166), stimulating nuclear entry, where phospho-Mdm2 (Ser166) then becomes sumoylated. This process would increase the 120 kDa form while decreasing the 95 kDa form of phospho-Mdm2 (Ser166) and Mdm2 (SMP14), which is consistent with our observations. Overall, we propose that the induction of Mdm2 activity through PTMs contributes to the downregulation of p53 following cIAP2 depletion. A proposed model detailing this regulation is presented in Figure 4.1.

Mdm2 phosphorylation at serine 166 is mediated predominantly by Akt (Mayo and Donner, 2001; Zhou et al., 2001). However, under the current experimental conditions, cIAP2 downregulation did not increase levels of phosphorylated Akt (Figure A.6), therefore it is unlikely that it was the kinase responsible for the increase in

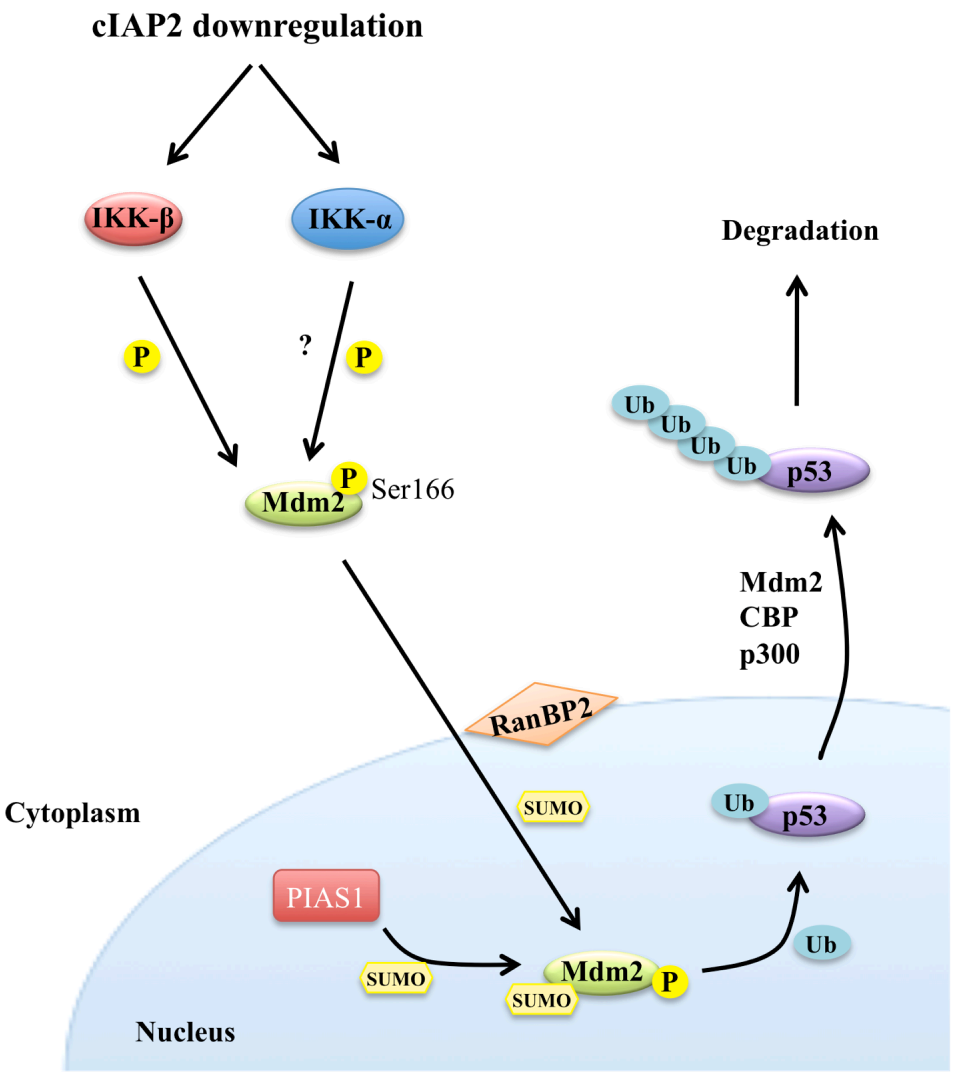


Figure 4.1. Proposed model of Mdm2 modification following cIAP2 depletion
cIAP2 downregulation results in the activation of ERK1/2, IKK-α and IKK-β. Both ERK1/2 and IKK-β have been shown to increase phosphorylation of Mdm2 at serine 166, and it is possible that IKK-α may also contribute to this process. Phosphorylation of Mdm2 serine 166 promotes its nuclear import. During the translocation, Mdm2 can be sumoylated by RanBP2 in the nuclear pore then further sumoylated by PIAS1 in the nucleus. Sumoylation of Mdm2 increases its E3 ubiquitin ligase activity towards p53. Monoubiquitination of p53 by Mdm2 induces its nuclear export. Once in the cytoplasm, p53 is polyubiquitinated and degraded by the proteasome.

phospho-Mdm2 (Ser166) following cIAP2 downregulation. Additional kinases have been identified for this residue of Mdm2. Recently, deletion of IKK-β was found to reduce the phosphorylation of Mdm2 at Ser166, resulting in an increase in p53 levels (Yang et al., 2010). The role of IKK-α was not examined so it is unclear whether it may also impact on Mdm2 Ser166 phosphorylation. Our results indicate that, compared with cells transfected with cIAP2 siRNA alone, concurrent depletion of IKK-α resulted in strong reduction of both 95 kDa and 120 kDa forms of phospho-Mdm2 (Ser166). Since sumoylation of Mdm2 depends on its nuclear translocation (Meek and Knippschild, 2003), a decrease in the phosphorylation of Mdm2 (Ser166) would impact both 95 kDa and 120 kDa forms of Mdm2.

An interesting result was that IKK-α depletion resulted in a similar increase of the 120 kDa form of sumoylated-Mdm2 as did cIAP2 depletion. As shown by Liu et al (2006), IKK-α associates with PIAS1 and this interaction is decreased upon phosphorylation of PIAS1 by activated IKK-α. Our results confirm that in control conditions, IKK-α can be co-immunoprecipitated with PIAS1. Upon activation of IKK-α by cIAP2 downregulation, this association is dramatically decreased (Figure 3.4C). It is possible that when PIAS1 is complexed with IKK-α, its ability to sumoylate some substrates is impaired. Therefore, when IKK-α levels were depleted by siRNA, free PIAS1 can interact with Mdm2, resulting in the increase in the 120 kDa form of Mdm2. Similarly, cIAP2 downregulation increases the 120 kDa form of Mdm2 by increasing the phosphorylation of PIAS1 by IKK-α, thus releasing PIAS1 to result in sumoylation of Mdm2. A proposed model of the regulation of PIAS1 by IKK-α following cIAP2 downregulation is presented in Figure 4.2. In contrast to cIAP2-depleted cells, p53 levels

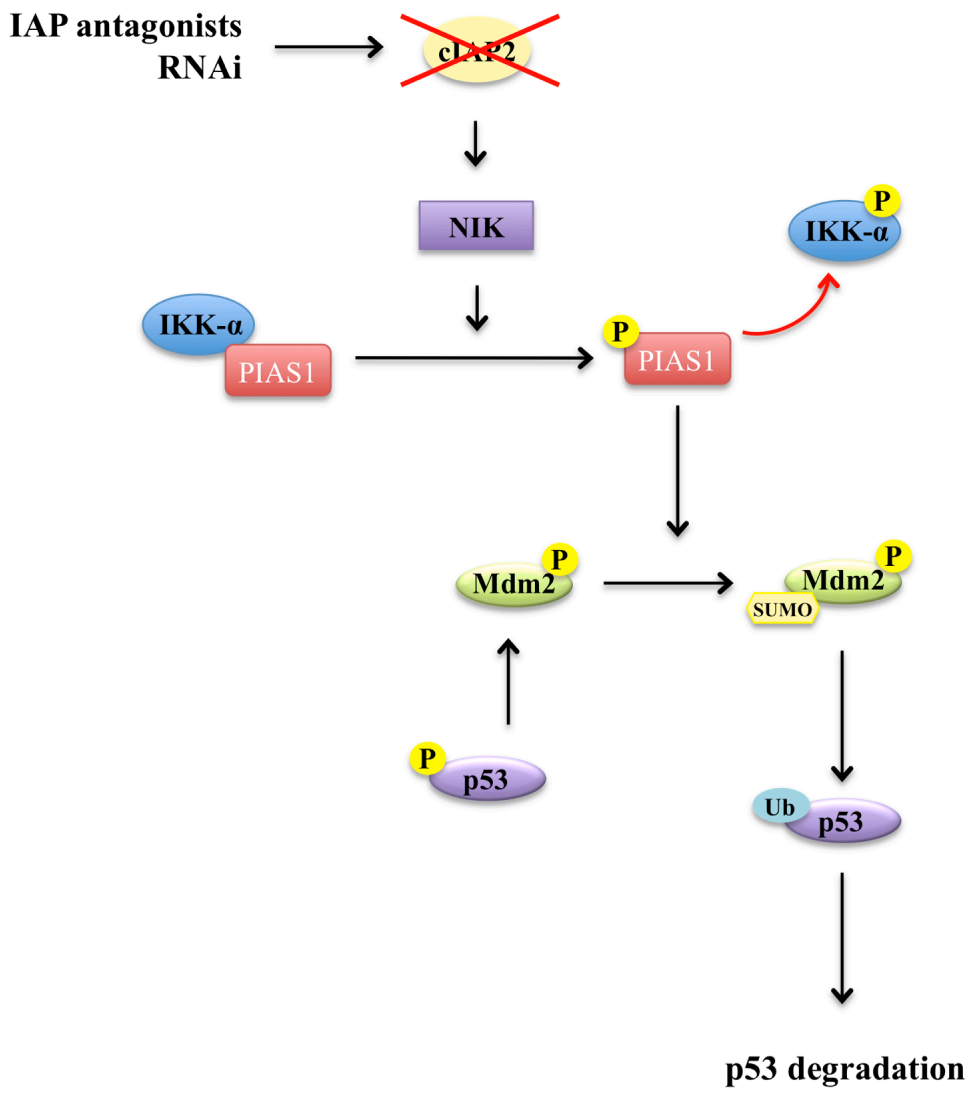


Figure 4.2. Proposed model of cIAP2 downregulation in promoting Mdm2 sumoylation
The sumo ligase PIAS1 is complexed with and inhibited by inactive IKK-α. Downregulation of cIAP2 results in an increase in NIK levels and activity, resulting in the phosphorylation of IKK-α. This releases PIAS1, which then sumoylates Mdm2 in the nucleus. Sumoylation of Mdm2 increases its ubiquitin ligase activity towards p53, resulting in its proteasomal degradation.

were unchanged in IKK-α depleted cells, despite the increase in the 120 kDa form of Mdm2. It is possible that in addition to increasing Mdm2 activity, the downregulation of cIAP2 modulates other signaling pathways, such as activation of NF-κB, that result in an increase in p53 susceptibility to degradation. For example, phosphorylation of serine 20 on p53 by Chk 2 and Chk 1 stabilizes p53 by interrupting the interaction between p53 and Mdm2 (Dumaz et al., 2001). In addition, acetylation of p53 is a stabilizing modification (Ito et al., 2001) and either an increase in acetylation or a decrease in deacetylation would result in a stabilization of p53, regardless of Mdm2 activity.

Together, these results show that both IKK-α and IKK-β may participate in the phosphorylation of phospho-Mdm2 (Ser166), and in particular, IKK-α appears to have an additional role in the regulation of Mdm2 sumoylation through its interaction with PIAS1. Further investigation is required to elucidate the exact role of each IKK in the regulation of Mdm2 activity. Additionally, knockdown studies involving PIAS1 will confirm its involvement in the regulation of Mdm2 activity following cIAP2 downregulation.

In the previous section, we showed that activation of IKK-β decreases the phosphorylation of p53 at serine 15, thus increasing its susceptibility to Mdm2-mediated degradation. Concurrently, activation of IKK-α increases the activity of Mdm2 by promoting its sumoylation. Together, the activation of IKK-α and IKK-β following cIAP2 downregulation co-operates to reduce p53 levels. This proposed mechanism is illustrated in Figure 4.3. The decrease in p53, along with survival signaling activated by NF-κB, contribute to the increase in proliferation following a reduction of cIAP2 levels.

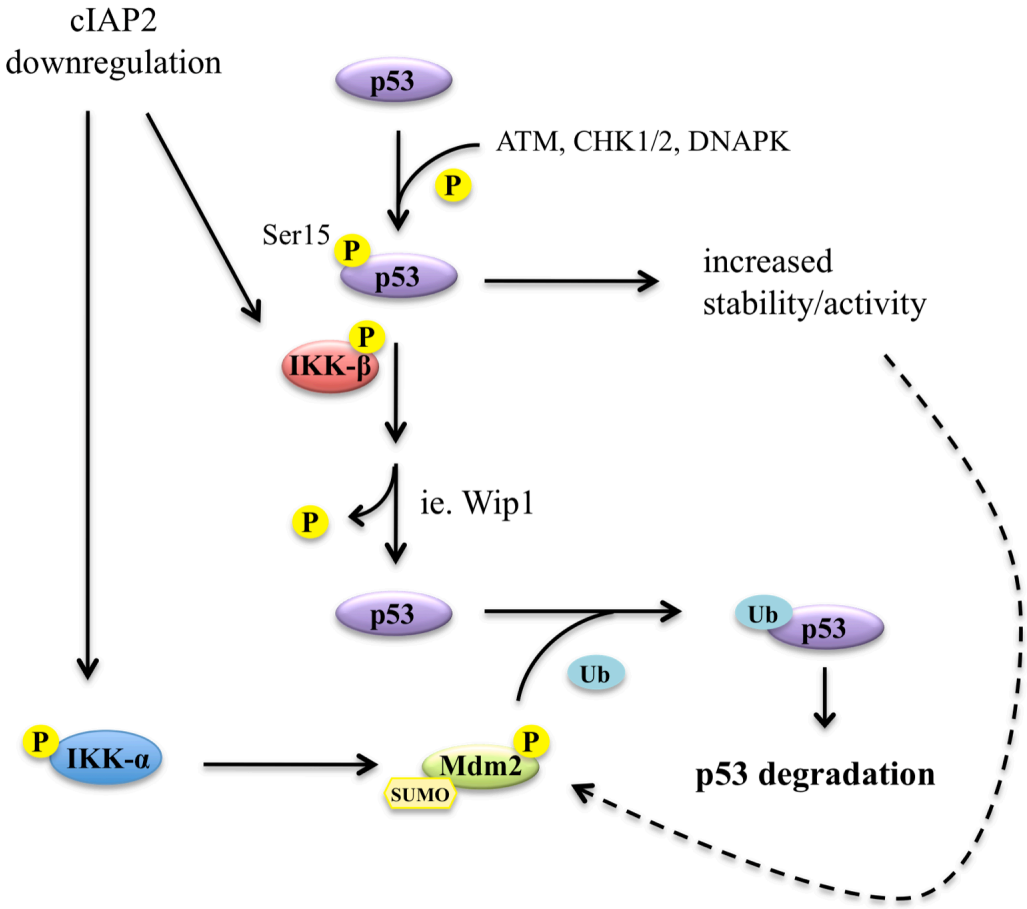


Figure 4.3. Proposed coordinated activities of IKKs in the downregulation of p53
The downregulation of cIAP2 results in activation of IKK-α and IKK-β as previously described. Activated IKK-β decreases the level of phosphorylated p53 at serine 15, a PTM associated with increased stability and activity of p53. The dashed arrow represents a feedback loop where active p53 protein could transcriptionally increase Mdm2 levels. Concurrent activation of IKK-α may increase Mdm2 activity towards p53 by promoting its sumoylation. Together, the increase in p53 susceptibility to degradation and increased Mdm2 activity results in a decrease in p53 levels following cIAP2 downregulation.

cIAP2 downregulation abolishes doxorubicin induction of p53

Doxorubicin is an anthracycline antibiotic shown to be effective in the treatment of breast cancer (Hurley, 2002). It exerts its effects by inducing DNA damage through the formation of DNA adducts, and by inhibiting topisomerase II (Tewey et al., 1984), both of which lead to double strand breaks, activating p53-mediated apoptosis (Burden and Osheroff, 1998). Additional p53-independent mechanisms of action have been proposed for the cytotoxic effects exerted by doxorubicin, including inhibition of DNA and RNA synthesis and production of free radicals (Gewirtz, 1999), which may explain why p53-mutant breast cancer cells are also susceptible to doxorubicin-induced cell death (Manna et al., 2011). Nevertheless, in cells that express wildtype p53, the induction of p53-mediated apoptosis is an important component of cell death caused by doxorubicin (Lowe et al., 1993). Treatment of MCF-10AT1 cells with doxorubicin induced a robust increase in p53 (Figure 3.5). However, when the cells were co-treated with doxorubicin and the IAP antagonist, this upregulation was abolished. This result has significant implications for the evaluation of IAP antagonists in clinical trials since combination therapy involving IAP antagonism may hinder the cytotoxic effects of a chemotherapeutic agent that utilizes the p53 pathway as part of its mechanism of action.

In contrast, reports have shown that overexpression of cIAP2 can confer doxorubicin resistance (Jonsson et al., 2003) and that cIAP2, along with other IAPs, is induced following doxorubicin treatment (Abe et al., 2007). It follows that the inhibition of cIAPs should then sensitize cells to treatment. This has been shown in some cell lines (Awasthi et al., 2011), although most of the cell lines tested expressed mutant p53. Thus the treatment with IAP antagonists in those cells would either have no effect on p53

levels since Mdm2 regulation of p53 is often impaired in p53 mutant cells, and any downregulation of p53 achieved would still likely have no impact on wildtype p53 target genes. The use of IAP antagonists in conjunction with doxorubicin in p53-wildtype cells would likely not augment doxorubicin-induced cell death (as described in the following section), and may, in fact, interfere with its efficacy by downregulating wildtype p53.

While cIAP1 is expressed ubiquitously, expression of cIAP2 appears to be induced following stress stimuli (Liu et al., 2005; Liu et al., 2006). As a tumour progresses and acquires further mutations, including in p53, it is more likely to express and depend on cIAP2 for survival, in a manner similar to "oncogene addition". This concept describes a phenomenon wherein cancer cells are more dependent on a specific gene for survival compared to normal cells because they carry multiple inactivated genes and are thus less adaptable (Weinstein and Joe, 2008). It appears that certain cancer cell lines, such as MDA-MB-231 cells, have become dependent on the overexpression of cIAP2 for survival and are particularly sensitive to its loss. These cells are ideal candidates for treatment with IAP antagonists. However, since many breast cancers retain expression of wildtype p53 (Bertheau et al., 2008), it is important to distinguish between sensitive versus insensitive cells, especially those insensitive cells that express wildtype p53, to ensure that treatment with the IAP antagonists will not exacerbate the disease.

cIAP2 downregulation activates MAPK signaling

In both MCF-10AT1 and MCF-7 cells, cIAP2 downregulation by different methods resulted in an increase in phosphorylated ERK1/2 (Figure 3.7A-B). Since overexpression of NIK has been shown to increase phosphorylation of ERK1/2 (Dhawan and Richmond, 2002), it is likely that the increase in phospho-ERK1/2 was mediated, at

least in part, by the activation of NIK. ERK activation can also occur through the IKK complex. The MEK kinase, TPL-2, is bound to p105 (NF-κB1) and phosphorylation of p105 by IKKα/β results in its proteasomal processing. This releases TPL-2, which then phosphorylates MEK and downstream MAPKs including ERK1/2 (Beinke et al., 2004; Waterfield et al., 2003). Together, these results indicate that following cIAP2 downregulation, NIK and NF-κB can activate MEK-ERK signaling, which generally results in an increase in proliferation (McCubrey et al., 2007).

While cIAP2 plays a role in limiting activation of ERK1/2, ERK1/2 in return also regulates cIAP2 levels in a negative manner (Figure A.7). This establishes a regulatory loop wherein the downregulation of cIAP2 permits activation of ERK1/2, which further maintains low levels of cIAP2. This is unexpected since both are typically associated with survival and the pathways that generally activate ERK1/2 also activate NF-κB (Perona and Sanchez-Perez, 2007), which upregulates cIAP2. For example, Ras has been well established to activate MEK-ERK signaling (Campbell et al., 1998) and also upregulates cIAP2 through a transforming growth factor (TGF)-α-dependent mechanism (Liu et al., 2005). It is puzzling that the same signaling pathways result in opposing effects on cIAP2. It is possible that cIAP2 and ERK1/2 serve to limit the activity of the other protein to prevent excessive activation of survival signaling. The mechanism by which ERK1/2 can regulate cIAP2 is unclear. However, there is evidence to suggest that MEK mediates the upregulation of cIAP1 downstream of fibroblast growth factor 2 (FGF-2) (Pardo et al., 2003). An upregulation of cIAP1 would increase proteasome-mediated degradation of cIAP2, thus lowering its levels following MEK-ERK activation,

resulting in an amplification of ERK activity. These results further support a tumour suppressing function for cIAP2 wherein it limits ERK-activated survival pathways.

DMBA-induction of mammary tumours in cIAP2-null mice

Induction of tumourigenesis in cIAP2-null mice produced results that were consistent with the *in vitro* observations on MCF-10A(Ha-*ras*/cIAP2 shRNA) cells. Analysis of the DMBA-induced tumours, which rarely express mutant p53 (Jerry et al., 1994), revealed that activation of the NF-κB pathway was increased in the absence of cIAP2 as indicated by an increase in phosphorylation of IKK-α (Figure A.3A-C). This observation is in agreement with current literature that indicates the ablation of the cIAPs results in accumulation of NIK, resulting in activation of IKK-α (Zarnegar et al., 2008). This activation was concurrent with strong reductions in phospho-p53 (Ser15) and total p53 (Figure 3.6). Therefore, even in an *in vivo* context, the loss of cIAP2 clearly regulated p53 levels. The reduction of p53 corresponded with an increase in the expression of c-Myc (Figure A.3E), an oncogenic transcription factor that promotes proliferation and is overexpressed in breast cancer (reviewed in (Adhikary and Eilers, 2005). c-Myc expression is normally repressed by p53 (Ho et al., 2005). During tumourigenesis, Myc cooperates with oncogenes such as Ras to induce the expression of genes that contribute to cellular transformation and tumourigenesis (Born et al., 1994). The loss of the tumour suppressor p53 and the resulting deregulation of signaling pathways lead would contribute to an increase in adenocarcinoma development.

Interestingly, the increase in phospho-IKK-α was not observed in normal mammary glands of cIAP2-null mice (Figure A.3D). The activation of IKK-α appears to occur as a result of transformation. Similarly, activation of IKK-β was observed only in

adenocarcinomas, but absent in hyperplasias. Our observations are consistent with the association of activated NF-κB signaling with a cancer phenotype (Karin, 2006). In the absence of an initiating event, the lack of cIAP2 does not appear to be sufficient to induce a cancer phenotype. Together, these results indicate that following transformation, the activation of NF-κB signaling and decrease in p53 contribute to the increase in adenocarcinomas observed in null animals compared to wildtype control mice through affecting multiple gene targets.

DMBA-induced formation of tumours in cIAP2-null mice required a longer latency period compared to control mice (Figure A.1). This was likely attributable to decreased inhibition of apoptosis in the null animals, as indicated by higher levels of TUNEL staining (Figure A.2A). Previous characterization of cIAP2-null mice did not reveal an overt phenotype and mice appeared healthy up to 52 weeks of age. The only documented difference between cIAP2-null and wildtype mice was a resistance to lipopolysaccharide (LPS)-induced endotoxic shock in cIAP2-null mice (Conte et al., 2006). Mammary glands in the cIAP2-null mice also appeared to develop normally (Figure A.5), thus the increase in apoptosis in cIAP2-null tumours was likely a result of decreased ability to counteract the cellular stresses involved in DMBA-induced transformation. This is in line with the expression pattern of cIAP2, which is low in normal cells but stimulated in response to stress stimuli (Liu et al., 2005; Liu et al., 2006). DMBA-induced tumourigenesis is associated with Ras mutations, which contribute to the transforming abilities of DMBA (Bizub et al., 1986; Dandekar et al., 1986). Expression of oncogenic Ras has been reported to upregulate cIAP2 and XIAP to block anoikis, a type of apoptosis that occurs in response to detachment from the

extracellular matrix (Liu et al., 2005). It is likely that in cIAP2-null mice, upregulation of XIAP alone is less efficient in inhibiting apoptosis when challenged with DMBA, given that cIAP2 augments XIAP function by preventing its inhibition by Smac (Hu and Yang, 2003; Wilkinson et al., 2004). These results indicate an important role for cIAP2 in inhibition of apoptosis during carcinogen-induced mammary tumourigenesis that is not sufficiently compensated for by other inhibitor of apoptosis proteins such as XIAP and cIAP1.

Despite the increase in apoptosis, a larger number of highly proliferative and progressed adenocarcinomas ultimately formed in the cIAP2-null mice. This is a key finding, since this result indicates that the increase in proliferative signaling resulting from the absence of cIAP2, including activation of NF-κB and the subsequent downregulation of p53, was sufficient to overcome the increased apoptosis, resulting in more malignant tumours in cIAP2-null mice.

Mutation in p53 impacts cytotoxic response to IAP antagonism

Since IAP antagonist treatment abrogated p53 upregulation by doxorubicin in MCF-10AT1 cells, it was expected that cells treated with IAP antagonist would likely be protected from doxorubicin-induced cell death. However, co-treatment of MCF-7, ZR-75 and MCF-10AT1 cells did not increase cell viability. This may, in part be due to p53-independent mechanisms of cell death such as through the production of reactive oxygen species, which can induce mitochondrial cytochrome c release, cleavage of procaspase-3 and upregulate Bax in the absence of p53 (Tsang et al., 2003). Additionally, it has been reported that doxorubicin activates NF-κB in sarcomas to induce expression of cIAP2 as

a means of resistance (Bednarski et al., 2007). In the absence of cIAP2, cells co-treated with doxorubicin and IAP antagonist would be deficient in survival signaling.

Numerous groups have reported on the ability of IAP antagonists to elicit cell death in cancer cell lines (Lu et al., 2008; Varfolomeev et al., 2007; Vince et al., 2007). These antagonists exert their apoptotic effect by inducing the proteasomal degradation of cIAPs, resulting in a stabilization of NIK and activation of canonical and non-canonical NF-κB signaling. The transcription of TNF-α is induced, which in the absence of the cIAPs, results in the formation of a death complex consisting of FADD and caspase-8 and non-ubiquitinated RIP1 (Wang et al., 2008) (see Figure 1.7). The use of IAP antagonists as a single agent has shown limited success, and these treatments are often combined with other agents such as TNF-α and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to increase cytotoxicity (Cheung et al., 2009). The mechanisms that confer IAP antagonist resistance are unclear. The current results show that IAP antagonist-induced cytotoxicity was most pronounced in the p53-mutant cell line, MDA-MB-231, and to a lesser extent T-47D cells (Figure 3.8A). It has been shown that the induction of TNF-α is required for the cytotoxic effects of IAP antagonists (Gaither et al., 2007; Varfolomeev et al., 2007). Measurement of TNF-α transcript levels following IAP antagonist treatment in a number of cell lines revealed that only p53-mutant MDA-MB-231 cells transcribed significantly higher levels of TNF-α compared to vehicle treatment while the other cell lines, MCF-7, ZR-75 and T-47D cells did not (Figure 3.8E). Therefore, these results are in agreement with reports that TNF-α production is associated with IAP antagonist-induced cytotoxicity.

Augmentation of doxorubicin-induced cytotoxicity by IAP antagonist was also associated with p53-mutant cell lines, which suggested that either the loss of functional wildtype p53, or the expression of a gain-of-function (GOF) mutant p53 may be contributing to the cytotoxic effects of IAP antagonism. Downregulation of mutant p53 in MDA-MB-231 cells resulted in a significant decrease (60%) in the transcription of TNF-α following IAP antagonist treatment (Figure 3.9B). Further experiments are required to determine if the decrease in TNF-α production was sufficient to rescue MDA-MB-231 cells from cell death following IAP antagonism. Although TNF-α is required for the cytotoxic effects of IAP antagonists (Varfolomeev et al., 2007), it is possible that these cells also depend on inhibition of apoptosis by the cIAPs in order to survive.

GOF p53 mutants have been well documented to regulate the transcription of targets distinct from those activated by wildtype p53 that are involved in cell proliferation and tumour progression. It can interact with other transcription factors and aid in the recruitment of transcriptional activators to the promoter region of their target genes to augment transcription (Di Agostino et al., 2006). An example of this is that mutant p53 has been reported to augment NF-κB activation in response to TNF-α (Weisz et al., 2007). Although the mechanism whereby this occurs is unclear, it is possible that mutant p53 interacts with activated NF-κB complexes and augments the signal by recruiting transcriptional activators.

T-47D cells, despite expressing mutant p53, did not transcribe increased levels of TNF-α mRNA in response to IAP antagonist treatment. The p53 mutation harboured by T-47D cells, a leucine to phenylalanine in codon 194, is located in the DNA-binding domain (Mirza et al., 2002). It is possible that this particular p53 mutation does not

augment transactivation of TNF-α. In agreement with this, stable expression of the p53 mutant 173L in MCF-7 cells was unable to induce the transcription of TNF-α in response to IAP antagonist treatment, indicating that not all p53 mutants are able to perform this function (Figure 3.9D). Augmentation of doxorubicin-induced cell death by IAP antagonist treatment in T-47D cells is likely due to the loss of apoptosis inhibition rather than enhancement of TNF-α production. In contrast, MDA-MB-231 cells express an arginine to lysine mutation at codon 280, which has been shown to be a GOF p53 mutant that can activate the transcription of ID4 (inhibitor of DNA binding 4). ID4 is not a target of wildtype p53 and its expression increases the angiogenic potential of cancer cells (Fontemaggi et al., 2009). It is possible that the p53 mutant in MDA-MB-231 cells can augment transactivation TNF-α in conjunction with active NF-κB complexes.

Three tumour-derived p53 mutants were tested for their abilities to increase IAP antagonist-induced TNF-α transcription. The first, p53-173L, is a DNA-binding region mutant (Ludwig et al., 1996) that is transcriptionally inactive. Transfection of MCF-7 cells with this mutant did not augment TNF-α transcription following IAP antagonist treatment (Figure 3.9D). Transfection of HEK293 cells with a second mutant, 175H, a GOF mutant that has previously been shown to induce expression of p100 (NF-κB2) (Scian et al., 2005), unexpectedly did not increase TNF-α transcription following IAP antagonist treatment (Figure 3.9F). Lastly, transfection with p53-143A significantly increased IAP antagonist-induced TNF-α transcription by approximately 2-fold. The p53-143A mutant contains a mutation in the core domain. It is a temperature-sensitive mutant that binds to wildtype p53 consensus sequences at 32°C (Friedlander et al., 1996) and otherwise acts as a dominant negative in cells that express wildtype p53. p53-143A has

also been shown to transactivate at 37°C, which confers the ability to contribute to tumourigenesis (Ludes-Meyers et al., 1996; Scian et al., 2004). Additionally, the transcriptional activity of human endogenous retrovirus (HERV)-1 long terminal repeat (LTR) is inhibited by wildtype p53 but is activated by mutant p53-143A (Chang et al., 2007). It is possible that the TNF-α promoter is repressed by wildtype p53 and may be activated by specific p53 mutants. This would explain why p53 siRNA reduced TNF-α transcript levels in MDA-MB-231 cells and why transfection with the p53-143A mutant augmented TNF-α transcription. Indeed, in addition to being a general inhibitor of NF-κB-mediated transcription, there is evidence to support that wildtype p53 specifically suppresses TNF-α transcription (Yarosh et al., 2000). Therefore, in cells that express wildtype 53, the transcription of TNF-α is repressed, even following IAP antagonist treatment. In cells where p53 is not functional, either through its loss or inactivation by a dominant negative mutant, IAP antagonist-induced NF-κB can transcriptionally activate TNF-α as observed in MDA-MB-231 cells. Lastly, it remains possible that certain p53 mutants, in addition to loss of repression of the TNF-α promoter, may induce its promoter activity. The ability of mutant p53 from MDA-MB-231 cells to bind to the TNF-α promoter is currently being investigated through EMSA and ChIP analyses. A model of the proposed mechanism by which mutant p53 can augment NF-κB-induced transcription of TNF-α is presented in Figure 4.4.

Since cIAP2 plays a tumour suppressing function by maintaining levels of wildtype p53, it would not perform this function in p53-mutant cells. Instead, the loss of cIAPs in p53-mutant cells results in a loss of apoptosis inhibition, as well as an increase in potential to activate non-canonical NF-κB signaling and lead to the production of

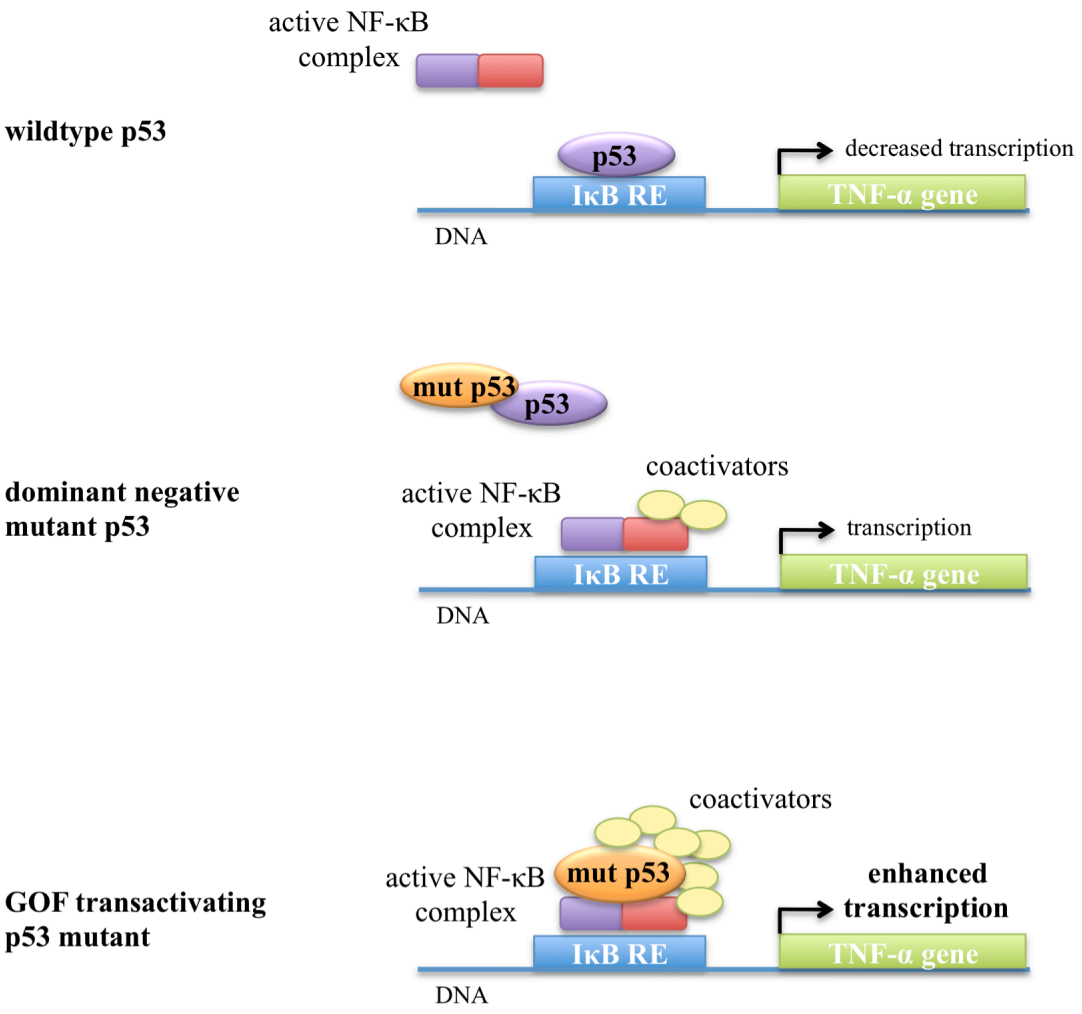


Figure 4.4. Proposed enhancement of NF-κB-induced TNF-α transcription by mutant p53 in response to IAP antagonist treatment

Following treatment with the IAP antagonist, activation of NF-κB activation in cells expressing wildtype p53 results in minimal transcription of TNF-α due to repression of the promoter. In cells expressing a dominant negative p53, inactivation of wildtype p53 allows activated NF-κB to induce transcription of TNF-α. Lastly, in cells that express a gain of function (GOF) transactivating p53 mutant, the p53 mutant may interact with the activated NF-κB complex and tethers to the IκB element where it aids in the additional recruitment of transcriptional coactivators. This results in augmentation of the transcription of TNF-α induced by IAP antagonist treatment.

TNF-α. Additionally, since mutant p53 is not subject to regulation by Mdm2, its levels accumulate in these cells and remain high following IAP antagonism, allowing it to facilitate transcription of TNF-α.

Nuclear IKK-α levels decrease in p53-mutant cells following IAP antagonism

The role of cytoplasmic IKK-α in the activation of canonical and non-canonical NF-κB pathways has been well described (Hayden and Ghosh, 2004). Recent evidence suggests that IKK-α performs additional functions in the nucleus, although these roles have not been extensively characterized. In keratinocytes, nuclear IKK-α plays a tumour suppressor role by promoting cell cycle arrest and differentiation (Sil et al., 2004). However, in other cell types, nuclear IKK-α facilitates the expression of genes that promotes growth and proliferation. IKK-α can perform this function by a few mechanisms, such as phosphorylating histone H3, SMRT, and other transcription factors (Espinosa et al., 2011). In response to IAP antagonist treatment, nuclear levels of IKK-α in p53-mutant cell lines MDA-MB-231 and T-47D decreased, as evaluated by immunofluorescence. In contrast, there was no change in IKK-α staining following IAP antagonist treatment in p53-wildtype cells MCF-7, MCF-10AT1 and ZR-75. Fractionation of cell lysates from MDA-MB-231 and MCF-7 cells treated with the IAP antagonist confirmed that nuclear IKK-α was decreased in MDA-MB-231 cells while it was unchanged in MCF-7 cells (Figure 3.10A-C). Examination of SMRT levels in MCF-7 cells following IAP antagonist treatment revealed a time-dependent decrease in SMRT protein levels while no change was observed in MDA-MB-231 cells (Figure 3.10D). This suggests that the decrease in nuclear IKK-α in MDA-MB-231 cells following IAP antagonist treatment resulted in SMRT remaining on chromatin and was not degraded.

The dissociation of SMRT from chromatin allows binding of active p65/p50 complexes to specific promoters, where they are phosphorylated by IKK-α and stimulate gene expression (Hoberg et al., 2004). This process has been shown to mediate expression of survival genes targeted by NF-κB (Huang et al., 2007), as well as Notch-dependent genes such as hes1 and herp2 (Fernandez-Majada et al., 2007). Therefore, it is possible that the reduction in nuclear IKK-α resulted in a decrease in the transcription of pro-survival genes and partially accounts for the increase in sensitivity to IAP antagonist treatment. It is unclear which genes are induced by the derepression of SMRT in MCF-7 cells. Future experiments may include the use of ChIP-Seq, in which IKK-α is immunoprecipitated and the bound DNA is sequenced to determine the identity of the genes induced by nuclear IKK-α following IAP antagonist treatment. An alternative approach would be to perform a microarray. Although this approach would not identify genes directly affected by IKK-α, it would be informative regardless to determine differential gene expression that contributes to survival or sensitivity following IAP antagonist treatment.

Model of the tumour suppressing role of cIAP2

A model of the proposed tumour-suppressing role of cIAP2 is presented in Figure 4.5. In cells that express cIAP2 and wildtype p53, it performs both pro-survival and tumour suppressing roles. To promote survival, cIAP2 ubiquitinates RIP1 to prevent the formation of a pro-death complex in response to TNF-α, and it sequesters endogenous IAP inhibitors such as Smac. However, it also ubiquitinates and limits the accumulation of NIK. In its absence, NIK is stabilized and activates IKK-α. IKK-α, together with IKK-β can increase Mdm2 levels and activity by transcriptional activation and by promoting its post-translational modfication. This results in ubiquitination and destabilization of

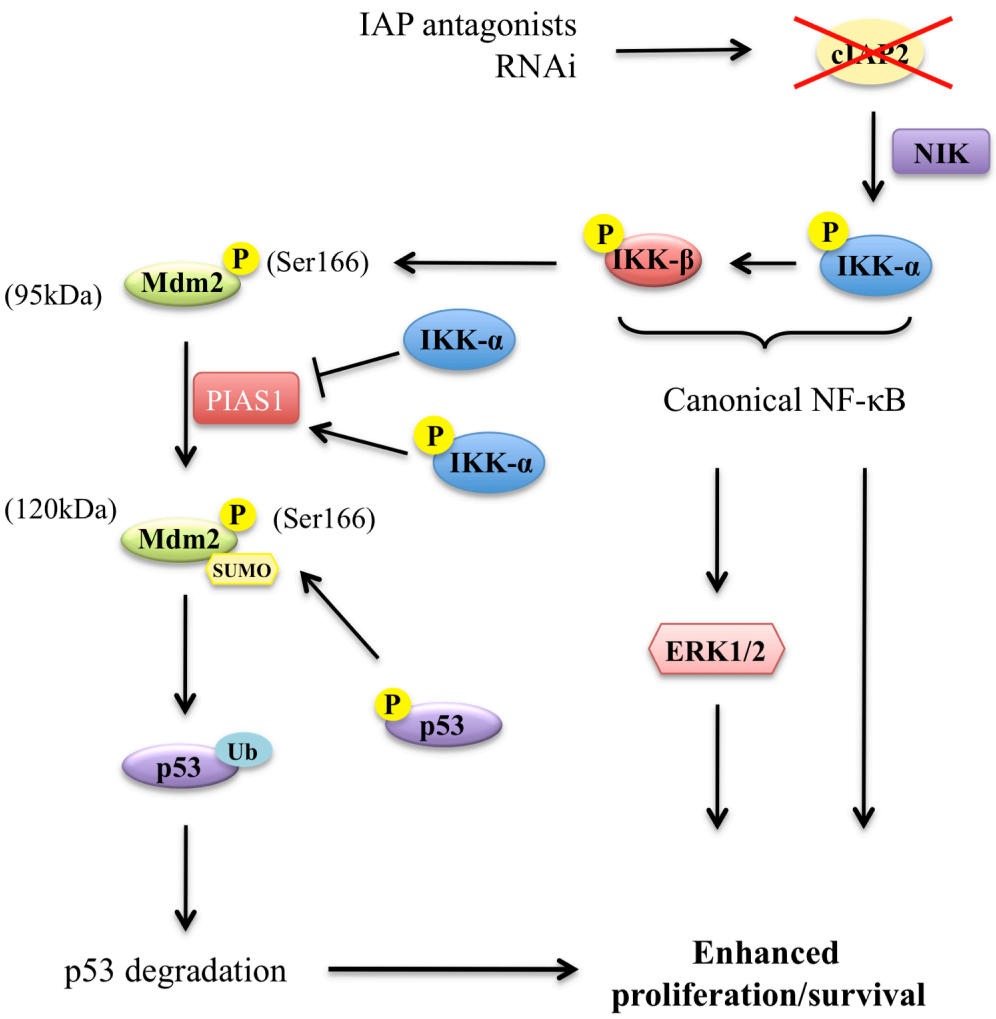


Figure 4.5. Proposed model of cIAP2 tumour suppressor function
In p53-wildtype cells, the loss of cIAP2-mediated repression of NIK leads to an activation of IKK-α. IKK-α can phosphorylate and activate IKK-β, resulting in activation of canonical NF-κB signaling. Canonical NF-κB activates MAPKs while the IKKs contribute to Mdm2 activation as previously described, to negatively regulate p53 levels. Ultimately, the reduction or loss of cIAP2 in p53-wildtype mammary epithelial cells results in an increase in cell proliferation and oncogenic signaling.

p53. Downregulation of cIAP2 also activates MEK-ERK signaling, which contributes to growth and proliferation, thereby augmenting the effects of downregulated p53. The overall consequence of cIAP2 downregulation in wildtype p53 cells is an increase in pro-survival and proliferative signaling, which dominates over the loss of apoptosis inhibition to promote oncogenesis.

Conclusion

The role of cIAP2 in tumourigenesis presents an interesting paradox since both its overexpression and its loss are associated with a proliferative advantage in different cancers. Its function as an inhibitor of apoptosis is contrasted by its role as a suppressor of NF-κB survival signaling and the integration of these signals ultimately determine the outcome of its overexpression or loss. The results presented here define a novel role for cIAP2 in maintaining expression of wildtype p53 in mammary epithelial cells. These observations are reminiscent of the association between the loss of cIAP2 and multiple myeloma, in which constitutive activation of NF-κB contributes to the pathogenesis of the disease (Annunziata et al., 2007; Demchenko et al., 2010; Keats et al., 2007). It is important to note that p53 mutations occur rarely in multiple myeloma (Owen et al., 1997; Paydas et al., 1997).

Given the complexity of signaling pathways and the crosstalk that exists to regulate the many processes in a given cell, it is no surprise that the effects of cIAP2 downregulation are largely context-dependent. The genetic differences present in the cell, such as p53 status and expression of signaling pathway components - ie. TNF-α, have significant impact on the downstream consequences of cIAP2 depletion. The findings presented here highlight the importance of fully characterizing the consequences of

inhibiting a specific protein for therapeutic purposes. In the case of cIAP2, the use of IAP antagonists may be beneficial for the treatment of cancers that express mutant p53 and have become dependent on cIAP2 for survival. However, IAP antagonist treatment of cells with intact p53 function may be ineffective, or worse, could potentiate the disease.

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APPENDIX

SUPPLEMENTARY FIGURES AND TABLE

Please note: breeding/genotyping of cIAP2-null mice and DMBA protocol were performed by Emma Tibbo. The data from those experiments are summarized in figure A.1 and table 1. Minying Niu performed the experiments shown in figures A.2, A.3 and A.5.

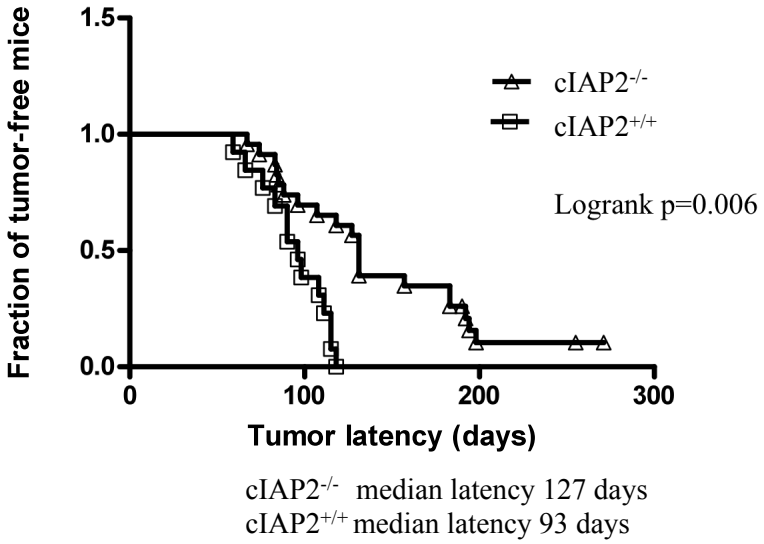

Figure A.1. DMBA-induced tumours develop following a longer latency in cIAP2- null mice

Kaplan-Meier analysis of adenocarcinoma latency in DMBA-induced cIAP2$^{-/-}$ and wildtype mice. The analysis shows significantly longer latency to tumour palpation in cIAP2$^{-/-}$ mice compared with wildtype mice (p=.006, Log rank test)

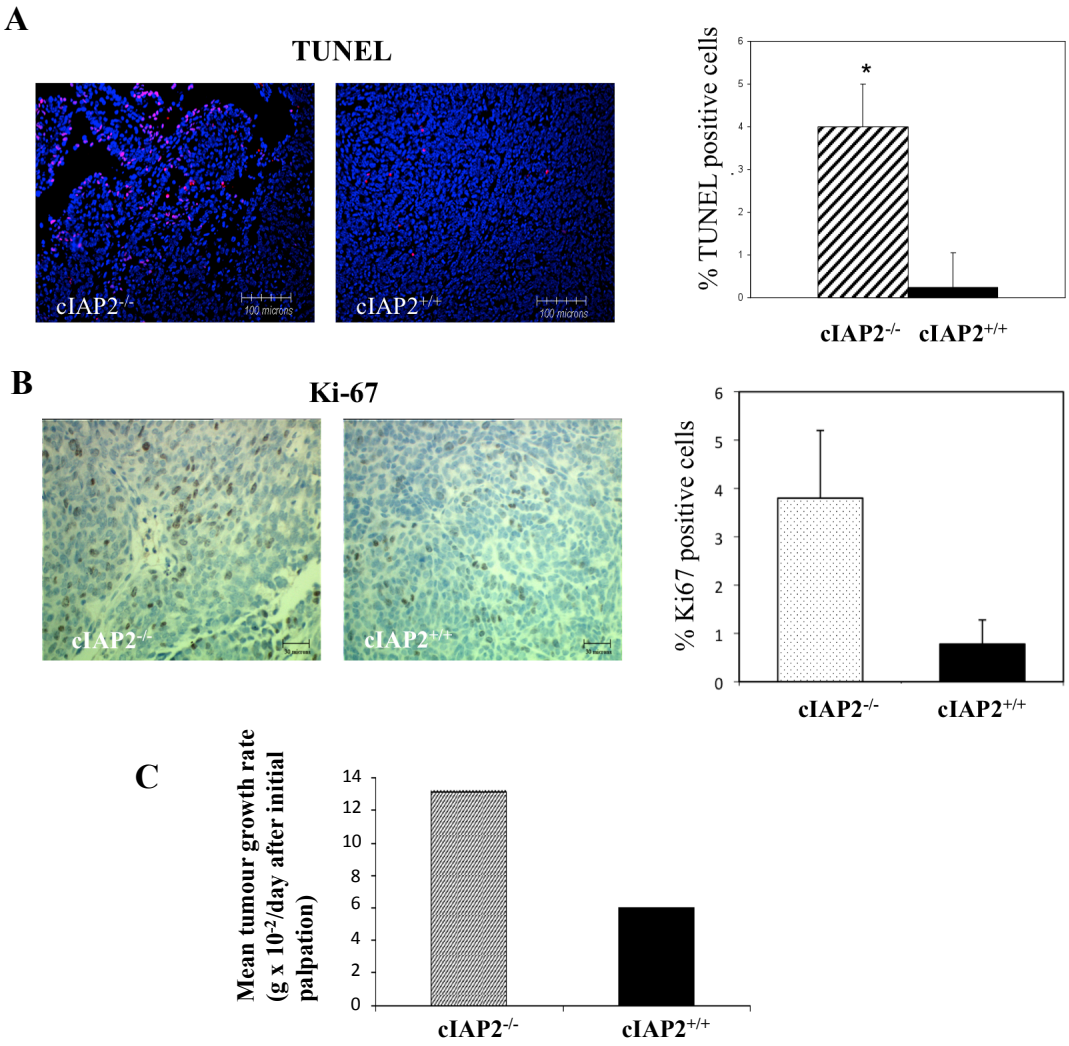

Figure A.2. cIAP2-null tumors contain high rates of apoptosis and proliferation
A, TUNEL staining of sections from tumours derived from wildtype and cIAP2$^{-/-}$ mice. The right panel shows enumeration of apoptotic cells in tumor sections. Three random high power fields of 300 cells were counted from each tumor section and subjected to a Student's T-test (*, p <0.05 for both sets). Tumors: cIAP2$^{-/-}$ sections n=7; cIAP2$^{+/+}$ sections n=6. **B,** Representative Ki67 immunostaining of tumours from cIAP2$^{-/-}$ and wildtype mice. The right panel shows **e**numeration of Ki67-positive mitotic cells in tumors. A total of 500 cells were counted from random microscopic fields of seven different tumors each from cIAP2$^{-/-}$ and control mice. **C,** Histogram showing average daily growth rate of cIAP2$^{-/-}$ and control tumors. Mice were palpated three times weekly and growth rates were calculated by dividing the wet weights of tumors on resection by the number of days from initial palpation to endpoint. Tumor data is derived from controls, n=9 and cIAP2$^{-/-}$, n=10

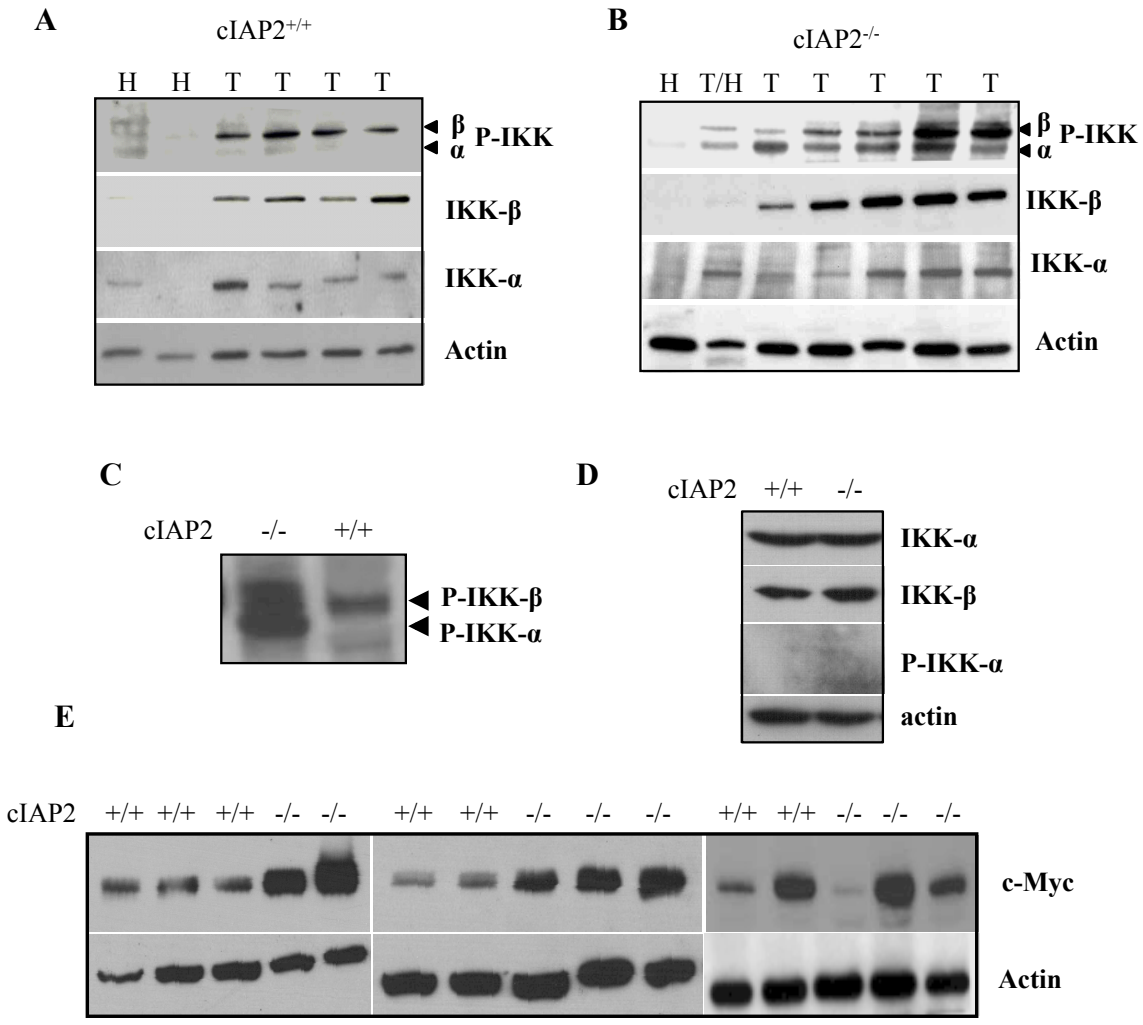

Figure A.3. **IKK-α is differentially activated in cIAP2-null tumors**
Thirty μg of extracts from DMBA-induced tumours (T) or squamous hyperplasias (S) from **A**, cIAP2$^{+/+}$ mice or **B**, cIAP2$^{-/-}$ mice were immunoblotted with an antibody that recognizes both phospho-IKK-α and -β (upper panel), anti-IKK-β, anti-IKK-α. **C,** Immunoblot of tumour extracts from wildtype and cIAP2$^{-/-}$ mice comparing phospho-IKK-α/β on the same blot. **D,** protein extracts from normal mammary glands excised from 10-week old control (+/+) or cIAP2-null (-/-) mice were immunoblotted for IKK-α, IKK-β and phospho-IKK-α. **E,** protein extracts from DMBA-induced tumours from cIAP2$^{+/+}$ or cIAP2$^{-/-}$ mice were immunoblotted for c-Myc. Actin was used as a loading control in all experiments.

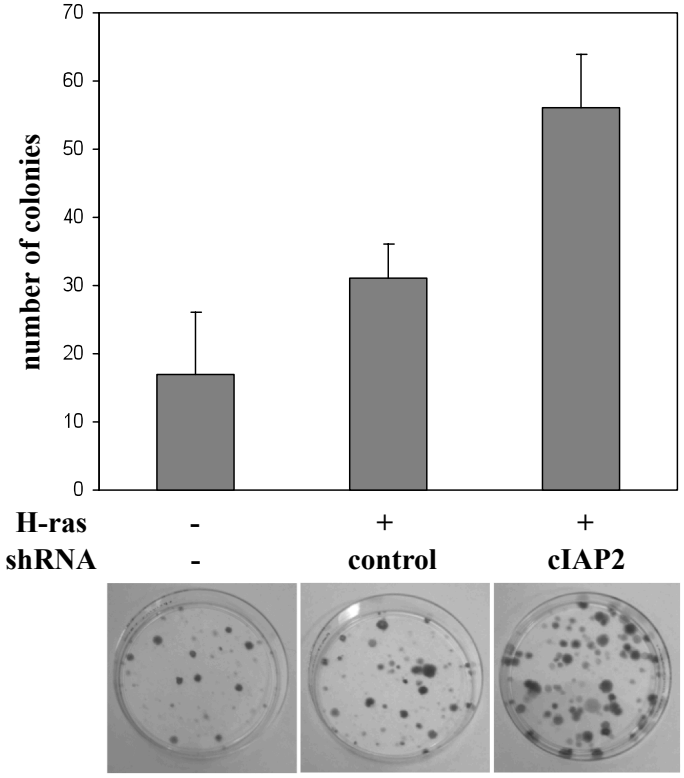

Figure A.4. Reduction of cIAP2 increases colony formation in mammary epithelial cells. MCF-10A human mammary epithelial cells were cotransfected with ^{V12}*H-ras* with either cIAP2 shRNA or a control shRNA or transfected with vector only (pcDNA3). Colonies were selected in G418 in triplicate and stained in 0.2% crystal violet for enumeration and results presented for cells transfected with the indicated plasmids. Bars represent mean ±S.D. of triplicate plates.

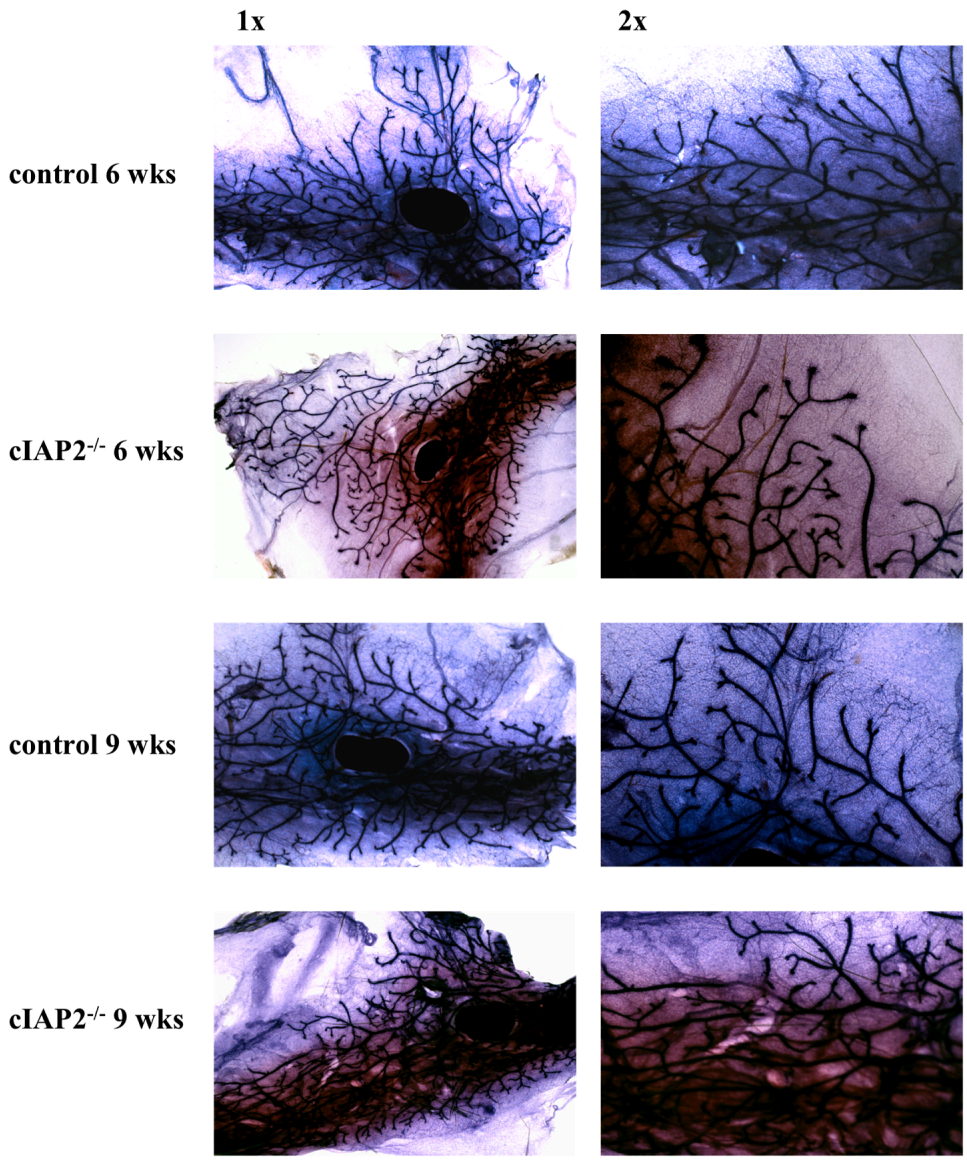

Figure A.5. Absence of cIAP2 does not alter ductal branching or terminal end bud formation

Wholemounts of the fourth inguinal mammary glands from 6- and 9-week old cIAP2 wildtype and null mice were prepared as wholemounts and compared to detect changes in ductal branching, end bud formation and mammary gland size.

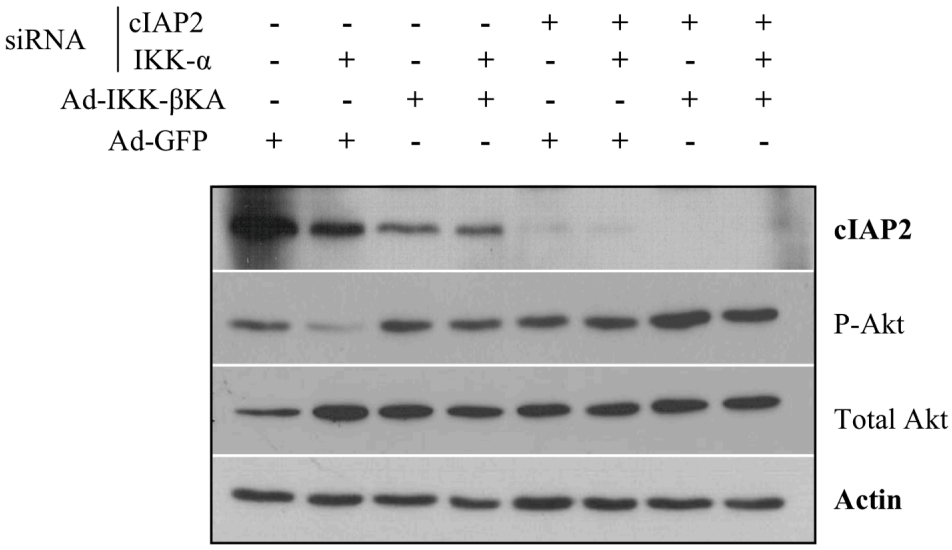

Figure A.6. cIAP2 downregulation does not alter levels of phosphorylated Akt
MCF-10AT1 cells were transfected with siRNA targeted against cIAP2 or IKK-α then infected with an adenovirus expressing a dominant-negative mutant of IKK-β as in figure 3.2F. Protein extracts were immunoblotted for cIAP2, phospho-Akt and total Akt. Actin was used as a loading control.

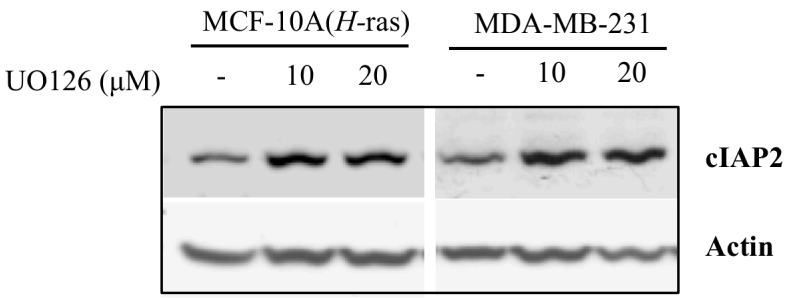

Figure A.7. MAPKs negatively regulate cIAP2 levels
MCF-10A(*H*-ras) and MDA-MB-231 cells were treated with the indicated concentration of UO126 for 16 hours. Total protein extract was immunoblotted for cIAP2. Actin was used as a loading control.

	# carcinomas	# hyperplasias	# lesion free mice	total # mice
cIAP2$^{+/+}$	14	10	0	21
cIAP2$^{-/-}$	23	5	3	24

Table 1. DMBA-induced mammary carcinomas in cIAP2$^{-/-}$ and wildtype mice.
The total number of mammary adenocarcinomas and hyperplasias induced by DMBA in control and cIAP2-null mice are indicated. Initially, 27 female cIAP2$^{-/-}$ mice and 23 wildtype littermates were subjected to a DMBA mammary tumour induction protocol to determine tumour latency and pathology. Of these mice, 3 cIAP2$^{-/-}$ and 2 control mice died without a palpable tumour within 60 days of final gavage due to unrelated causes.