Regulation of Copper Homeostasis by the X-linked Inhibitor of Apoptosis Protein

by

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Foreword

Chapters II and III of this dissertation have been merged into a single manuscript published in *Molecular and Cellular Biology*. Appendix B has been included as part of a manuscript published in *The Journal of Biological Chemistry*.

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List of Abbreviations

AIF, apoptosis-inducing factor
ALS, amyotrophic lateral sclerosis
APAF-1, apoptotic protease activating factor-1
ATM, ataxia-telangiectasia mutant
ATOX1, antioxidant-1
ATP7A, copper-transporting ATPase, α polypeptide
ATP7B, copper-transporting ATPase, β polypeptide
BAK, BCL-2 antagonist killer
BAX, BCL-2 associated X protein
BCL-2, B cell CLL (chronic lymphocytic leukemia)/lymphoma 2
BID, BH3-interacting death domain agonist
BIM, BCL-2 interacting mediator of cell death
BIR, baculovirus inhibitor of apoptosis repeat
BRUCE, BIR-containing ubiquitin-conjugating enzyme
CARD, caspase activation and recruitment domain
CCO, cytochrome *c* oxidase
CCS, copper chaperone for superoxide dismutase
c-IAP, cellular inhibitor of apoptosis
COMMD1, copper metabolism murr1 domain-containing protein 1
CSM, complete supplement mixture
CTR, copper transporter
Cu, copper
DIAP1, *Drosophila* IAP 1
DRONC, *Drosophila* Nedd2-like caspase
DTT, dithiothreitol
DUB, deubiquitinating enzyme
ER, endoplasmic reticulum
FADD, Fas-associated death domain protein
FUS, fused in sarcoma
GFP, green fluorescent protein
GSPT1, G_1 to S phase transition protein 1
GST, glutathione-*S*-transferase
HECT, homologous to the E6-AP carboxy terminus
HEK, human embryonic kidney
HIF, hypoxia-inducible factor
HTRA2, human homolog of *E. coli* HtrA (high temperature requirement A) 2
IAP, inhibitor of apoptosis
IBM, IAP-binding motif
IκB, inhibitor of κB

IKK, IκB kinase
ILP-2, IAP-like protein 2
IMS, intermembrane space
LOX, lysyl oxidase
LRPPRC, leucine-rich PPR motif-containing protein
MDM2, mouse double minute 2 homolog
MEF, mouse embryonic fibroblast
MEKK2, mitogen-activated protein/ERK (extracellular signal-related kinase) kinase kinase 2
ML-IAP, melanoma IAP
MOMP, mitochondrial outer membrane permeabilization
NBT, nitroblue tetrazolium
NEMO, NF-κB essential modulator
NF-κB, nuclear factor-κB
NIK, NF-κB-inducing kinase
NOD, nucleotide-binding oligomerization domain protein
PAM, peptidylglycine α-amidating monooxygenase
PTEN, phosphatase and tensin homolog
PUMA, p53-upregulated modulator of apoptosis
RING, really interesting new gene
RIP, receptor-interacting protein
RNAi, RNA interference
ROS, reactive oxygen species
siRNA, small interfering RNA
SMAC, second mitochondrial activator of caspases
SOD, superoxide dismutase
TAB1, TAK1-binding protein 1
TAK1, TGF-β-activated kinase 1
TDP-43, TAR (*trans*-activating region) DNA binding protein, 43 kDa
TGF-β, transforming growth factor-β
TNF, tumor necrosis factor
TNFR, TNF receptor
TRAF, TNF receptor associated factor
Ub, ubiquitin
UBA, ubiquitin-associated domain
UBC, ubiquitin-conjugating enzyme
UBR, ubiquitin recognition box protein
XIAP, X-linked inhibitor of apoptosis
YNB, yeast nitrogen base

Abstract

Mammalian inhibitor of apoptosis (IAP) proteins were identified as homologs of a baculovirus IAP and were originally thought to function as direct inhibitors of death-inducing proteases known as caspases. More recent work has demonstrated that, in fact, most IAPs are incapable of caspase inhibition, and many IAPs perform essential cellular functions unrelated to apoptosis. IAPs have been shown to play important caspase-independent roles in such diverse cellular processes as receptor-mediated signaling, cytokinesis, innate immunity, and copper metabolism.

Recently, X-linked IAP (XIAP) was found to play a regulatory role in copper homeostasis. Copper is an essential transition metal whose ability to exchange electrons is harnessed by many intracellular and extracellular copper-dependent enzymes to facilitate oxidation-reduction reactions in such processes as peptide amidation, mitochondrial respiration, and dismutation of superoxide. However, this ability of copper to participate in redox reactions makes it highly toxic because it can generate reactive oxygen species and directly oxidize proteins and DNA. For this reason, an elaborate system of transporters and chaperone proteins has evolved to deliver copper to copper-dependent proteins while not allowing free copper to accumulate and damage the cell.

Given the excess copper-buffering capacity of the cytosolic environment, we hypothesized that a copper chaperone might be required to deliver copper to XIAP. As copper trafficking pathways are highly conserved evolutionarily, we performed a targeted

genetic screen in *Saccharomyces cerevisiae* to identify candidate proteins involved in delivering copper to XIAP. Through this genetic screen, we identified the copper chaperone for superoxide dismutase (CCS) as a mediator of copper delivery to XIAP in both yeast and mammalian cells. We also found that XIAP and CCS physically interact in human cells, and that XIAP induced ubiquitination of CCS. Interestingly, XIAP-mediated ubiquitination of CCS did not seem to target CCS for proteasomal degradation. Instead, we found that ubiquitination of CCS by XIAP was an activating event, enhancing the ability of CCS to deliver copper to its physiologic target copper/zinc superoxide dismutase (SOD1). Collectively, our results provide valuable insights into mechanisms of regulation of intracellular copper homeostasis and redox metabolism through the XIAP-CCS complex.

Chapter I

Introduction

One of the central challenges facing living organisms is simply keeping the internal environment stable in a constantly changing external environment. For single-celled organisms this challenge can be met primarily with a system of sensors for temperature, osmolarity, etc., and regulated transporters (for import and export of nutrients and toxins) to ensure that the intracellular environment meets the organism's needs. Multicellular organisms, however, face a unique challenge: not only must they handle a changing external environment, they must maintain the proper numbers and types of cells in the proper orientations, as well as ensure that the individual cells have the necessary nutrients to perform their proper functions. Either loss of essential cells or accumulation of unnecessary cells is potentially fatal to the organism. To deal with this problem, a number of tightly regulated cell death pathways have evolved, all of which can be initiated or prevented by either intracellular or extracellular factors. Similarly, the proper balance of nutrients must be maintained for each cell of the organism. One

particularly problematic nutrient is copper (Cu), which is essential for life in aerobic organisms but which is also highly toxic. An elegant set of mechanisms for uptake, transport, and export has evolved to ensure that copper is available to all cells but is not allowed to accumulate to toxic levels. This dissertation will focus on one pathway of programmed cell death, apoptosis, and how its regulation is unexpectedly tied to cellular copper homeostasis. The intersection of these two critical homeostatic systems provides insight into the complexity of multicellular organisms and will be explored in further detail.

Apoptosis: Non-inflammatory programmed cell death

The best-characterized programmed cell death pathway is an evolutionarily conserved protease-dependent process of cellular fragmentation termed apoptosis (from the Greek for "falling off") (Kerr et al., 1972). Apoptosis is a coordinated process by which the nuclear membrane is broken down, nuclear DNA is fragmented, and the cell is broken down into membrane-coated particles called blebs. These membrane blebs are recognized and destroyed by phagocytic cells so that the dying cell can be disposed of without affecting neighboring cells (Cox et al., 1995; Fadok et al., 1998). By contrast, another form of cell death, necrosis, results in spilling of cellular contents and activation of an inflammatory response in the surrounding area. The key mediators of apoptosis are a family of highly conserved proteases called caspases (cysteine-dependent aspartate-specific proteases) (Fuentes-Prior and Salvesen, 2004; Riedl and Shi, 2004). The highly similar caspases -3 and -7 are generally considered to be the "executioner" caspases because their activation is the common endpoint of the two apoptotic cascades: the

intrinsic pathway (initiated by cellular stress) and the extrinsic pathway (initiated by external signals) (Fig. 1.1).

The intrinsic apoptotic pathway

As described above, two basic mechanisms exist for initiation of apoptosis in multicellular organisms: the intrinsic and extrinsic pathways. The intrinsic pathway is initiated by caspase-9, which directly activates the executioner caspase-3 by proteolytic cleavage (Slee et al., 1999; Bratton et al., 2001; Yin et al., 2006). However, caspase-9 on its own is not an active protease. Caspase-9 is only activated by formation of the apoptosome, an evolutionarily conserved complex consisting of caspase-9, apoptotic protease-activating factor-1 (APAF-1), adenosine triphosphate (ATP), and cytochrome *c*, which resides in the mitochondrial intermembrane space and is an essential component of the mitochondrial respiratory chain (Liu et al., 1996; Li et al., 1997; Ow et al., 2008). APAF-1 normally exists as a monomer in the cytosol but oligomerizes in the presence of cytochrome *c* and dATP, allowing the recruitment and proximity-induced activation of pro-caspase-9 (Zou et al., 1999; Acehan et al., 2002; Pop et al., 2006). Thus, the critical event in triggering the intrinsic apoptotic pathway is mitochondrial outer membrane permeabilization. It is not surprising, then, that mitochondrial outer membrane permeabilization is very tightly regulated.

Proper regulation of mitochondrial outer membrane permeabilization is accomplished via a large family of proteins termed the B-cell lymphoma-2 (BCL-2) family, which consists of both positive and negative regulators of mitochondrial outer membrane permeabilization (Chipuk et al., 2006; Chipuk and Green, 2008). The pro-

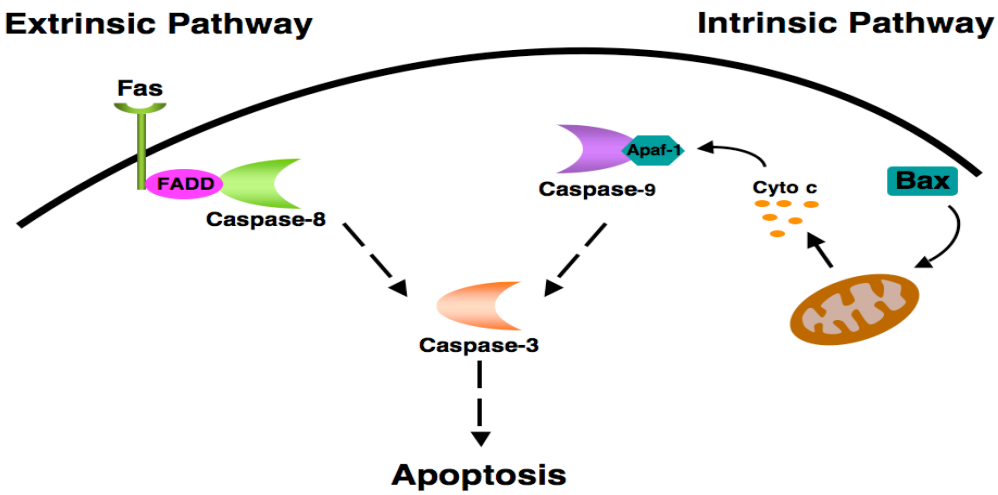

Figure 1.1. The intrinsic and extrinsic apoptotic pathways. Apoptosis can be induced by either intracellular stress or extracellular signals. The intrinsic pathway is initiated by Bax and other pro-apoptotic BCL-2 family members, which permeabilize the outer mitochondrial membrane to release cytochrome *c* and allow formation of the apoptosome. The extrinsic pathway is induced by death receptors such as Fas, which engage and activate caspase-8 through receptor-associated adaptors such as FADD.

apoptotic family members include, but are not limited to, BAX and BAK, which function by actively forming pores in the mitochondrial outer membrane to release pro-apoptotic proteins from the IMS (Chittenden et al., 1995; Kiefer et al., 1995). The founding member of the family, BCL-2, is an antagonist of BAX and BAK and functions to prevent mitochondrial outer membrane permeabilization by sequestering BAX and BAK, as well as other pro-apoptotic family members such as BID and BIM that activate BAX and BAK (Oltvai et al., 1993; Yang et al., 1997; Luo et al., 1998; O'Connor et al., 1998; Cheng et al., 2001). A number of BCL-2-related anti-apoptotic molecules antagonize the pro-apoptotic BCL-2 family members to maintain the proper life-death balance by ensuring that mitochondria remain intact in healthy cells but can be rapidly permeabilized

to induce apoptosis if necessary (Boise et al., 1993; Kozopas et al., 1993; Gibson et al., 1996). Indeed, the importance of proper mitochondrial outer membrane permeabilization regulation is illustrated by the observation that a number of cancers, especially hematologic malignancies, are characterized by deregulated expression of anti-apoptotic BCL-2 family members (Tsujimoto et al., 1985; Oltersdorf et al., 2005; Del Gaizo Moore et al., 2007).

Initiation of mitochondrial outer membrane permeabilization by pro-apoptotic BCL-2 family members is activated by cellular stress due to growth factor withdrawal, ultraviolet radiation, chemotherapeutics, or a host of other causes. Perhaps the most important regulator of apoptosis in response to intracellular stress signals is p53, a transcription factor that initiates cell cycle arrest, apoptosis, or senescence in response to DNA damage and other signals (Levine, 1997; Vousden and Lane, 2007; Green and Kroemer, 2009). Post-translational regulation of p53 activity is quite complex, involving ubiquitination, methylation, acetylation, and phosphorylation. The E3 ubiquitin ligase MDM2 normally keeps p53 protein levels very low via constitutive ubiquitination and degradation of p53. However, in the presence of DNA damage p53 is stabilized via phosphorylation by the DNA damage-sensing kinase ataxia-telangiectasia mutant (ATM), resulting in p53-mediated transcriptional activation of pro-apoptotic genes encoding the BCL-2 antagonists p53-upregulated modulator of apoptosis (PUMA), NOXA, and others (Banin et al., 1998; Canman et al., 1998; Villunger et al., 2003; Jeffers et al., 2003). Thus, cellular stress is transduced to induce mitochondrial outer membrane permeabilization and the intrinsic apoptotic pathway through p53 and the BCL-2 family.

The extrinsic apoptotic pathway

In addition to apoptosis induced by intrinsic cellular stress, apoptosis can also be induced by extracellular factors. The extrinsic apoptotic pathway is also known as the death receptor pathway, because it is induced by engagement of cell surface receptors by death-inducing ligands that may be soluble, in the plasma membrane of neighboring cells, or in the extracellular matrix. A wide variety of death receptors have been described, with the types and quantities of expressed death receptors varying widely among different cell types and developmental stages. Prominent among plasma membrane death receptors are the members of the tumor necrosis factor (TNF) receptor superfamily. Within this group, the Fas death receptor is a particularly potent inducer of receptor-mediated apoptosis (Itoh et al., 1991; Suda et al., 1993). Engagement of Fas by its cognate ligand (FasL) results in initiation of the caspase cascade through the receptor-associated apical caspase-8 (Los et al., 1995; Enari et al., 1996). The precise mechanism of caspase-8 activation is still controversial and will be discussed in greater detail later. Once activated, caspase-8 activates the executioner caspases -3 and -7 to initiate cell death.

Cross-talk between the intrinsic and extrinsic pathways

Although the intrinsic and extrinsic apoptotic pathways are generally considered to be distinct, they can intersect and regulate each other at various points. This is perhaps best illustrated by Fas-mediated apoptosis, whose precise mechanism varies depending on the cell type. In some cells, Fas-mediated caspase-8 activation leads directly to caspase-3 activation and apoptosis (type I cells), but in other cells Fas-mediated caspase-

8 activation cannot directly activate caspase-3 to initiate apoptosis (type II cells). In type II cells an additional amplification step involving the intrinsic apoptotic pathway is necessary for Fas-induced apoptosis. BH3-interacting domain death agonist (BID) is cleaved by caspase-8, and truncated BID (tBID) activates BAX and BAK to induce mitochondrial outer membrane permeabilization, leading to cytochrome *c* release, formation of the apoptosome, and caspase-3 activation by caspase-9 (Luo et al., 1998; Li et al., 1998). In type I cells, overexpression of BCL-2 or other anti-apoptotic BCL-2 family members does not protect the cells from Fas-mediated apoptosis, whereas in type II cells BCL-2 overexpression blocks Fas-mediated apoptosis (Barnhart et al., 2003).

In addition to mitochondrial amplification of receptor-mediated apoptosis, evidence exists for amplification of intrinsic apoptosis through the caspase-8 pathway. Although the mechanism for this amplification loop is not well understood, it seems that caspases -3 and -7 activate caspase-6, which then activates caspase-8 by proteolysis (Slee et al., 1999; Slee et al., 2001; Inoue et al., 2009).

IAPs: inhibitors of apoptosis

Among the regulators of intrinsic and extrinsic apoptosis, the inhibitor of apoptosis (IAP) family is particularly important. The first IAP was identified in the baculovirus *Cydia pomonella* (Cp-IAP) (Crook et al., 1993; Clem and Miller, 1994), and *Iap* genes were discovered in higher organisms later based on sequence homology (Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996; Rothe et al., 1995). Although the name suggests that all IAP family members inhibit apoptosis, in fact only a subset of them do; the defining characteristic of the IAP family is not inhibition of apoptosis but

the presence of one or more ~65 amino acid baculovirus IAP repeat (BIR) domains (Hinds et al., 1999) (Fig. 1.2).

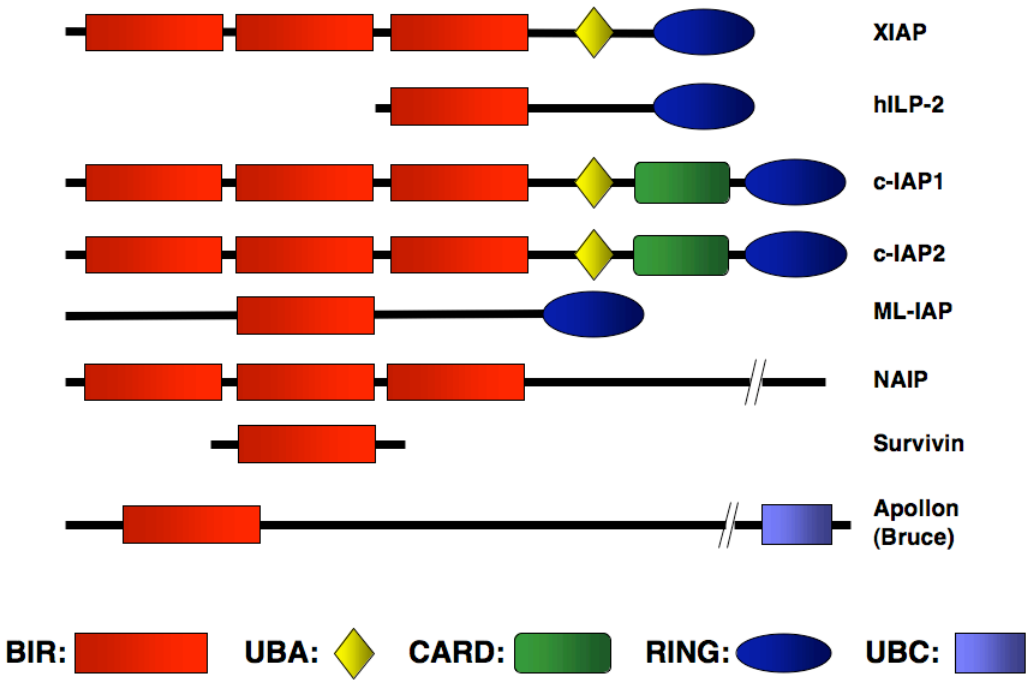

Figure 1.2. Mammalian IAPs. A schematic showing mammalian IAP proteins and their shared domain structures. The BIR domains are thought to mediate most protein-protein interactions, while the RING domains mediate autoubiquitination and ubiquitination of other proteins.

The BIR domain is a zinc-binding domain that essentially functions as a protein-protein interaction domain. Several IAPs also contain a RING domain that possesses E3 ubiquitin ligase activity (Yang et al., 2000). RING-containing IAPs can catalyze post-translational modification of themselves and other proteins by covalent attachment of the small modifier protein ubiquitin in a manner that will be discussed in greater detail later.

Generally, RING-containing IAPs bind to their ubiquitination substrates via their BIR domain(s), while the RING domain directs attachment of ubiquitin to the substrate (Vaux and Silke, 2005).

IAPs and caspase inhibition

The contributions of IAPs to regulation of intrinsic and extrinsic apoptosis have been under intense study since the first IAP was identified and remain somewhat controversial. It was originally thought that a primary function of BIR domains in baculovirus and other IAPs was direct inhibition of caspase activity (Uren et al., 1996; Hawkins et al., 1996), but more recent work has demonstrated that this is not the case (Eckelman and Salvesen, 2006; Vucic et al., 2005; Shin et al., 2005; Marusawa et al., 2003). The prototype IAPs from baculoviruses, Cp-IAP from *Cydia pomonella* granulosis virus and Op-IAP from *Orgyia pseudotsugata* nuclear polyhedrosis virus, potently block apoptosis in virus-infected insect cells (Crook et al., 1993; Clem and Miller, 1994; Birnbaum et al., 1994). However, although these IAPs inhibit apoptosis, they do so by a mechanism that does not involve directly inhibiting caspases (Manji et al., 1997; Seshagiri and Miller, 1997; Wilkinson et al., 2004; Wright et al., 2005). Similarly, the *Drosophila melanogaster* IAP DIAP1 was originally thought to be a caspase inhibitor but actually protects cells from apoptosis via non-degradative, inactivating polyubiquitination of the caspase DRONC (Ditzel et al., 2008). Other IAPs, such as mammalian survivin and the yeast IAP Bir1, function in cytokinesis rather than apoptosis regulation (Li et al., 1998; Li et al., 2000; Yoon and Carbon, 1999).

Although a substantial subset of IAPs can block apoptosis, and several within this subset are capable of binding to caspases, only one IAP is a physiologic inhibitor of caspase proteolytic activity. The mammalian X-linked IAP (XIAP) is a direct inhibitor of caspases -3/-7 and -9 via its BIR2 and BIR3 domains, respectively (Deveraux et al., 1997; Riedl et al., 2001; Scott et al., 2005; Shiozaki et al., 2003). Physical interaction with caspases -3 and -7 is mediated by residues within XIAP that are shared with other IAPs, but inhibition of the caspase active site is determined by residues in the region immediately amino-terminal to BIR2 that are unique to XIAP (Eckelman and Salvesen, 2006). Similarly, XIAP binds to caspase-9 via conserved residues within BIR3, but it is residues immediately carboxy-terminal to BIR3 that are unique to XIAP and bind to the caspase-9 dimerization domain, maintaining the caspase in an inactive conformation (Sun et al., 2000; Shiozaki et al., 2003). The mammalian IAPs cellular IAP 1 and 2 (c-IAP1 and c-IAP2) were originally thought to be direct caspase inhibitors, but it now seems that their roles in apoptosis are independent of caspase inhibition and, at least in the case of c-IAP1, may involve ubiquitination and degradation of the effector caspases -3 and -7 (Choi et al., 2009).

IAP antagonists

The delicate balance between pro-apoptotic and anti-apoptotic factors in the cell is such that, in order for efficient initiation of apoptosis in the appropriate circumstances, IAP-mediated protection is removed by pro-apoptotic proteins that antagonize IAPs. Generally, these proteins are released from the mitochondrial IMS along with cytochrome *c*, although the IAP antagonist G_1 to S phase transition protein 1 (GSPT1) is released

from the endoplasmic reticulum (ER) (Hegde et al., 2003). Most prominent among this class of pro-death molecules is the second mitochondrial activator of caspases (SMAC) (Du et al., 2000; Verhagen et al., 2000). Like GSPT1 and the other mitochondrial IAP antagonists, SMAC binds to IAPs by mimicking the amino-terminus of activated caspases, the IAP-binding motif (IBM) (Wu et al., 2000; Liu et al., 2000). Other known and putative IAP antagonists include the serine protease Omi/HtrA2 (Suzuki et al., 2001; Hegde et al., 2001), the RNA-binding protein LRPPRC (also known as LRP130), and glutamate dehydrogenase, among others (Verhagen et al., 2007). These IAP antagonists all contain amino-terminal IBMs and either target IAPs for degradation or simply sequester them to prevent them from binding to caspases and inhibiting apoptosis. There is evidence to suggest that some IAPs may actually function to inhibit apoptosis by sequestering IAP antagonists rather than through direct interaction with caspases (Wilkinson et al., 2004). Although they are highly evolutionarily conserved, the precise role of IAP antagonists in apoptosis regulation is not yet clear. Many researchers have speculated that IAP antagonists may play a role in inhibiting IAP function in a non-apoptotic context as well, as there is some evidence to suggest that proteins such as SMAC may be released from the intermembrane space in the absence of apoptosis (Deng et al., 2003; Csomos et al., 2009).

IAPs as signaling molecules

In addition to their potentially important role in degradation of effector caspases, c-IAP1 and c-IAP2 play key roles in modulating receptor-mediated apoptosis at the level of the cell surface receptor. The c-IAPs are generally found in complexes with TNF

receptor associated factors (TRAFs), which localize to the cytoplasmic tails of TNF receptor (TNFR) superfamily members via their TRAF domains (Rothe et al., 1995; Rothe et al., 1994).

In cultured cells, engagement of TNFR1 by TNF-α activates the canonical nuclear factor-κB (NF-κB) signaling pathway, which proceeds via phosphorylation of the inhibitor of κB α (IκBα) by an enzymatic complex consisting of IκBα kinase α (IKKα), IKKβ, and IKKγ (also known as NEMO) (Mercurio et al., 1997; Yamaoka et al., 1998). Phosphorylated IκBα is ubiquitinated and degraded, allowing release of active NF-κB dimers (consisting usually of RelA and p50), nuclear translocation, and NF-κB-mediated transcription of pro-survival genes (Hayden and Ghosh, 2008). Thus, TNF-α induces apoptosis only when transcription or translation of NF-κB target genes is inhibited pharmacologically. c-IAP1 and c-IAP2 enhance activation of NF-κB by TNF-α, and loss of either protein has been reported to enhance sensitivity to TNF-mediated apoptosis in some circumstances (Mahoney et al., 2008; Varfolomeev et al., 2008). Several TNFR superfamily members activate an alternative or non-canonical NF-κB signaling pathway, which proceeds via IKKα-mediated phosphorylation, ubiquitination, and partial degradation of the inactive p100 precursor to release the active p52 subunit (Xiao et al., 2004; Amir et al., 2004). Activation of this NF-κB pathway is inhibited by the c-IAPs, and pharmacologic targeting of the c-IAPs with small molecules that mimic the amino-terminal IBM of SMAC (SMAC mimetics) potently induces this pathway in tumor cells (Vince et al., 2007; Varfolomeev et al., 2007; Petersen et al., 2007; Gaither et al., 2007). The role of the c-IAPs in modulating TNF signaling to both NF-κB pathways is thought

to primarily involve their differential effects on ubiquitination of key signaling molecules through their RING domains in a manner that will be described in greater detail later.

In addition to modulating NF-κB activation by TNFR superfamily members, the c-IAPs seem to regulate the apoptotic threshold by inhibiting the formation of pro-death signaling complexes after TNFR engagement. Indeed, as described above, treatment of tumor cells with SMAC mimetics profoundly sensitizes them to TNF-mediated apoptosis. The mechanism for this anti-apoptotic activity of the c-IAPs is still unclear but it seems that in the absence of c-IAP1 and c-IAP2, engagement of TNFR1 by TNF-α triggers caspase-8 activation rather than NF-κB signaling (Wang et al., 2008; Bertrand et al., 2008).

XIAP has also been reported to be a signaling molecule, although its cellular roles independent of caspase inhibition are still unclear. It has been shown to modulate transforming growth factor-β (TGF-β) and NF-κB signaling (Birkey Reffey et al., 2001; Lewis et al., 2004; Lu et al., 2007; Jin et al., 2009), and it regulates immunity to *Listeria monocytogenes* in a manner that seems to be independent of caspase inhibition (Bauler et al., 2008). Furthermore, XIAP regulates cellular copper homeostasis through ubiquitination and degradation of the copper regulatory protein COMMD1 (Burstein et al., 2004), although the mechanistic details of how XIAP modulates copper metabolism remain unclear.

Copper: an essential but toxic nutrient

Copper is a critical cofactor for many biological processes in eukaryotes. The genome of the simplest eukaryote, the baker's yeast *Saccharomyces cerevisiae*, encodes

three specific copper import proteins – Ctr1, Ctr2, and Ctr3. Ctr1 and Ctr3 are localized to the plasma membrane (Dancis et al., 1994b; Dancis et al., 1994a; Pena et al., 2000), while Ctr2 is thought to be localized primarily to the vacuolar membrane (Rees et al., 2004). Interestingly, although extracellular copper exists primarily as a divalent cation (Cu(II)), all three Ctr proteins are capable of transporting only monovalent copper ions (Cu(I)). It is thought that divalent copper is reduced by metalloreductase enzymes residing adjacent to Ctr1-3 at the plasma or vacuolar membrane to allow transport of monovalent copper into the cytosol (Hassett and Kosman, 1995; Georgatsou et al., 1997; Rees and Thiele, 2007) (Fig. 1.3).

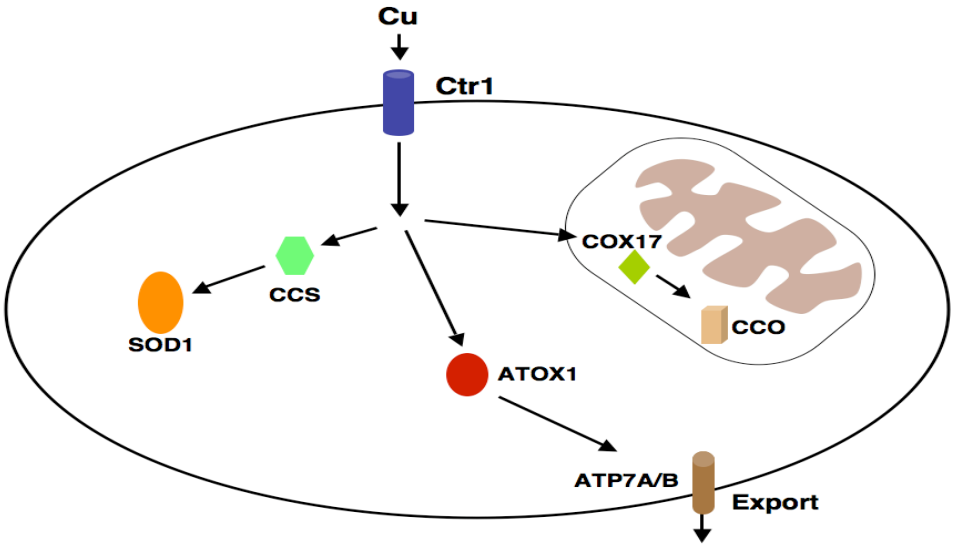

Figure 1.3. Mammalian copper transporters and chaperones. A basic schematic of mammalian copper trafficking: copper is imported by the specific high-affinity transporter Ctr1 and acquired by cytosolic copper chaperones CCS and ATOX1 for distribution to copper transporters and copper-dependent proteins. Mitochondrial copper is required for activation of cytochrome *c* oxidase by COX17 and other copper chaperones, but the mechanisms by which copper is transported to mitochondria are poorly understood. Not shown: ATP7A/B-mediated copper transport into the Golgi apparatus for insertion into secreted proteins such as lysyl oxidase and ceruloplasmin.

The human genome contains only two genes encoding copper import proteins, *CTR1* and *CTR2*. While the functions of mammalian copper importers are thought to be generally analogous to their yeast counterparts, *Ctr1* deficiency in mice leads to embryonic lethality (Lee et al., 2001; Nose et al., 2006). This is in contrast to yeast, in which loss of all three copper importers produces yeast that have a defect in respiration but are nevertheless viable (Portnoy et al., 2001; Rees et al., 2004).

The different challenges facing yeast and mammals are illustrated by their differential requirements for copper. Copper-dependent proteins play critical roles in aerobic respiration, pigment formation, iron metabolism, peptide amidation, neurotransmitter synthesis, connective tissue formation, and protection from reactive oxygen species (Madsen and Gitlin, 2007; Kim et al., 2008; Turski and Thiele, 2009). While yeast can survive without these copper-dependent processes, mice and humans absolutely require copper for survival. At the same time, mammals face the difficult challenge of ensuring that, while cells get the copper they need, excess copper is not allowed to accumulate.

Human disorders of copper metabolism

The consequences of copper deficiency in multicellular organisms are illustrated by the human disorder Menkes disease, which results from mutation in the *ATP7A* gene. *ATP7A* encodes a copper-transporting P-type ATPase that is homologous to the *S. cerevisiae* copper transporter Ccc2. ATP7A is essential for moving copper from the digestive tract to other organs, and inactivating mutations in *ATP7A* result in copper deficiency in all organs but the intestine and kidney. Copper incorporation into copper-

dependent proteins is severely compromised, leading to defects in pigmentation, musculoskeletal and vascular abnormalities, neurodegeneration, and typically death within the first decade of life (Llanos and Mercer, 2002). Treatment of Menkes disease with copper supplementation may be effective in some patients, but only if it is initiated within the first few weeks of life (Kaler et al., 2008).

Conversely, the copper accumulation disorder Wilson disease results from inactivating mutations in *ATP7B*, which like *ATP7A* encodes a copper-transporting P-type ATPase. ATP7A and ATP7B are highly similar proteins, and indeed either can compensate for Ccc2 deficiency in yeast (Hung et al., 1997; Payne and Gitlin, 1998). The differences between ATP7A and ATP7B, and the reason why mutations in their respective genes paradoxically cause diseases with essentially opposite phenotypes, lie in their different tissue distributions and intracellular trafficking patterns (Linz and Lutsenko, 2007). ATP7B is essential for excretion of hepatic copper into the bile, and as a result loss of ATP7B function causes accumulation of copper first in the liver and eventually other tissues, including the brain. For patients whose Wilson disease is recognized early enough, therapeutic reduction of copper levels using long-term chelation therapy has proven to be fairly effective in managing the disease (Brewer, 2000; Gitlin, 2003).

Copper-dependent enzymes and human disease

Of particular importance to multicellular organisms is aerobic respiration, which can be bypassed in some unicellular organisms through alternative processes such as fermentation. Respiration requires transfer of electrons through mitochondrial respiratory

complexes I-IV and finally to molecular oxygen, the final electron acceptor in the respiratory chain. Copper is essential for the final steps in the chain, transfer of an electron from cytochrome *c* to complex IV (cytochrome *c* oxidase) and then to oxygen. Cytochrome *c* oxidase is an enzymatic complex that consists of thirteen distinct polypeptides, including peptides encoded in both the mitochondrial and the nuclear genomes. Assembly of cytochrome *c* oxidase requires the coordinated activity of a host of chaperone proteins, among them copper chaperones that mediate insertion of two critical copper ions (Cobine et al., 2006). Without copper, cytochrome *c* oxidase is enzymatically inactive, and thus mitochondrial respiration is severely impaired. In humans, defects in cytochrome *c* oxidase assembly, and indeed defects in any component of the respiratory chain, result in a disease known as Leigh syndrome. There are a variety of genetic causes of Leigh syndrome, in both nuclear and mitochondrial genes, but all result in a fundamental defect in mitochondrial respiration (Shoubridge, 2001; Finsterer, 2008). Although the symptoms and course vary among subtypes of Leigh syndrome, generally organs with high oxygen requirements (eg. brain, heart, muscle, etc.) are most affected.

Other mammalian copper-dependent enzymes include tyrosinase, hephaestin, ceruloplasmin, peptidylglycine α-amidating monooxygenase, dopamine β-hydroxylase, lysyl oxidase, and copper/zinc superoxide dismutase (SOD1). Of these, only the last two will be explored in detail here. The others have been reviewed elsewhere (Kim et al., 2008).

Lysyl oxidase is an extracellular enzyme that plays a critical role in collagen and elastin cross-linking in connective tissue development. It receives copper from ATP7A

in the secretory pathway, and thus inactivating mutations in *ATP7A* result in the defective connective tissue formation seen in Menkes disease through loss of lysyl oxidase activity (Royce et al., 1980; Kaler, 1998). Other mutations in *ATP7A* that result in production of a partially active copper transporter are responsible for the connective tissue disorder cutis laxa (Ehlers-Danlos syndrome type IX), in which lysyl oxidase function is impaired but other signs and symptoms of copper deficiency are less severe than those seen in Menkes disease (Byers et al., 1980; Kaler et al., 1994).

Because of its role in remodeling connective tissue, lysyl oxidase has also been shown to play a critical role in metastasis of some solid tumors. The potential role of lysyl oxidase in regulating tumor cell metastasis was first suggested by studies demonstrating its upregulation in invasive versus non-invasive breast cancer cells (Kirschmann et al., 1999; Kirschmann et al., 2002). In a subsequent study, ATP7A was also found to be upregulated in invasive cells, suggesting that both increased expression and increased copper loading of lysyl oxidase are important in determining the invasive phenotype (Nagaraja et al., 2006). Subsequently, lysyl oxidase expression was found to be upregulated by hypoxia in a hypoxia-inducible factor (HIF)-dependent manner and essential for hypoxia-induced metastasis of tumor cells (Erler et al., 2006). Collectively these studies demonstrate a critical role for the copper-dependent enzyme lysyl oxidase in mediating tumor metastasis and suggest that copper chelation might be an effective therapy for some tumors. Indeed, several studies have found that copper chelators are effective in treating solid tumors, although the mechanism by which copper chelation inhibits tumor growth remains unclear (Pan et al., 2003; Juarez et al., 2006; Juarez et al., 2008; Turski and Thiele, 2009).

Superoxide dismutase: a cytosolic copper-dependent enzyme

SOD1 is a highly conserved copper-dependent enzyme that converts superoxide in the cytosol or mitochondria to hydrogen peroxide, which is then converted to water and oxygen by catalase. Cytosolic SOD1 exists primarily as a homodimer, with each subunit containing one zinc and one copper ion. The copper ion is coordinated by histidine residues within SOD1 and is critical for enzymatic activity (Culotta et al., 2006). Curiously, SOD1 is primarily a cytosolic protein, whereas most superoxide is produced in the mitochondria, where manganese-dependent superoxide dismutase (SOD2) resides along with a small amount of SOD1. Nevertheless, although *Sod1*-deficient mice are viable, they display increased sensitivity to superoxide-generating chemicals and reduced female fertility (Wong et al., 2000).

Unlike other copper-dependent proteins such as hephaestin and lysyl oxidase that receive copper via ATP7A as they are processed in the secretory pathway, SOD1 is synthesized as a mature protein in the cytosol in the absence of copper. Delivery of copper to copper-free (apo-) SOD1 is necessary to produce the active holo-SOD1 enzyme. However, in contrast to the secretory pathway, free copper is effectively unavailable in the cytosol due to the presence of cytosolic chelators and scavengers that buffer copper. Therefore, a chaperone protein, the copper chaperone for superoxide dismutase (CCS), is required to specifically deliver copper to SOD1 (Culotta et al., 1997; Rae et al., 1999; Wong et al., 2000).

Indeed, the requirement for copper chaperones seems to be universal for proteins outside of the secretory compartment and is conserved from *S. cerevisiae* to humans. As mentioned above, cytochrome *c* oxidase requires a set of chaperones to mediate insertion

of copper ions into its two copper-binding sites. The copper transporters ATP7A and ATP7B, which move copper from the cytosol into other cellular compartments, require the copper chaperone antioxidant-1 (ATOX1) to bring copper ions to them for transport (Klomp et al., 1997; Hamza et al., 1999; Hamza et al., 2001; Hamza et al., 2003). It remains unclear, however, how these copper chaperones are able to acquire copper in the cytosolic environment.

CCS is a highly conserved protein that contains two copper-binding domains, one of which is absolutely required for insertion of copper into the SOD1 copper-binding pocket (Fig. 1.4). Docking of CCS to SOD1 is accomplished via a SOD1-like segment of CCS that allows formation of a heterodimer in a manner analogous to SOD1 homodimerization (Schmidt et al., 1999; Schmidt et al., 2000). Once CCS and SOD1 have docked, copper is transferred from CCS to SOD1 by a mechanism that remains largely obscure but proceeds efficiently in the presence of excess copper chelators, suggesting that the copper ion is directly inserted into SOD1 and is not available for exchange with other copper-binding molecules (Rae et al., 1999). Proper activation of SOD1 also requires formation of an intramolecular disulfide linkage, which is mediated by CCS in an oxygen-dependent fashion via a mechanism that is also unclear but requires formation of an intermediate intermolecular disulfide linkage between CCS and SOD1 (Brown et al., 2004; Furukawa et al., 2004). Many mechanistic aspects of SOD1 activation are still unresolved, including how CCS acquires copper, as it too is synthesized in the cytosol as a copper-free mature protein. The kinetics of copper delivery to apo-SOD1 are so rapid that some have speculated that CCS might receive

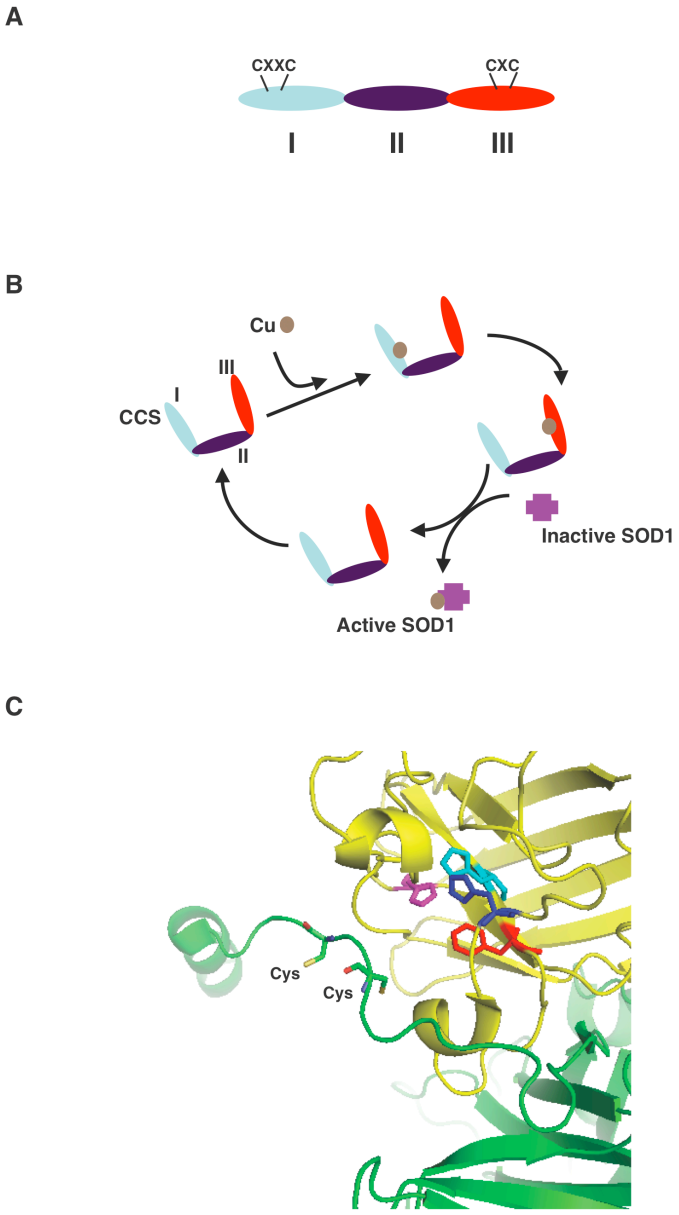

Figure 1.4. CCS delivers copper to SOD1. *(A)* CCS consists of three domains that facilitate copper acquisition (mediated by the CXXC copper-binding motif in domain I), binding to SOD1 (mediated by domain II), and delivery of copper to the SOD1 active site (mediated by the CXC copper-binding motif in domain III). A schematic of copper acquisition and delivery to SOD1 by CCS is shown in *(B)*. Precisely how copper is transferred from CCS domain I to domain III for delivery to SOD1 is not known. *(C)* The crystal structure of *S. cerevisiae* Sod1 (yellow) in complex with Ccs1 (the yeast homolog of human CCS; green) shows the proximity of the CCS domain III copper-binding site (consisting of two cysteine (Cys) residues in a CXC motif) to the SOD1 active site (Lamb et al., 2001). Once transferred to SOD1, copper is coordinated by four histidine residues – H46 (cyan), H48 (red; substituted with a phenylalanine residue in this crystal structure to stabilize the Sod1-Ccs1 complex), H63 (blue), and H120 (magenta).

copper directly from the copper importer CTR1 (Bartnikas and Gitlin, 2003; Caruano-Yzermans et al., 2006), although no physical interaction between CCS and CTR1 has yet been demonstrated. In addition, the step-by-step mechanism of copper transfer and disulfide formation remains unclear, as no copper ion is present in the crystal structure of the Sod1-Ccs1 complex, as shown in Fig. 1.4C (Lamb et al., 2001).

SOD1 plays a prominent role in the pathogenesis of a large subset of the familial cases of amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disease characterized by loss of upper and lower motor neurons. Mutations in SOD1 are thought to lead to production of a mutant protein that is susceptible to formation of neurotoxic aggregates (Valentine and Hart, 2003). Interestingly, mutations in the genes encoding TDP-43 and FUS also cause ALS, possibly via a common mechanism of neurotoxic aggregate formation (Sreedharan et al., 2008; Kabashi et al., 2008; Vance et al., 2009; Kwiatkowski et al., 2009). Indeed, aggregate formation seems to be a common mechanism for a number of neurological diseases, including Huntington's disease, Parkinson's disease, Alzheimer's disease, and others (Davies et al., 1997; Scherzinger et al., 1997; DiFiglia et al., 1997; Spillantini et al., 1997; Selkoe, 1980; Selkoe et al., 1982; Bucciantini et al., 2002; Chiti and Dobson, 2006).

The role of SOD1 metallation in ALS pathogenesis is still controversial, but several disease-causing mutations result in impaired copper binding (Hayward et al., 2002). *In vitro* and *in vivo* studies have demonstrated that copper-free and reduced SOD1 (ie. SOD1 without the CCS-catalyzed intramolecular disulfide bond) are more prone to aggregation than wild-type SOD1 (Furukawa and O'Halloran, 2006; Furukawa et al., 2008), but *Ccs* deficiency does not seem to affect disease progression in mouse models of

ALS (Subramaniam et al., 2002). In contrast, overexpression of CCS led to accelerated disease in some models but not in others (Son et al., 2007; Son et al., 2009). Thus, while it is generally accepted that SOD1 aggregation plays a role in ALS pathogenesis, how copper delivery and activation of SOD1 by CCS modulates aggregate formation and disease progression is unclear. A fuller understanding of how SOD1 activation proceeds mechanistically and how it is regulated to meet cellular requirements will be invaluable to the study of ALS and other diseases of protein aggregation.

The ubiquitin-proteasome system

An essential component of cellular homeostasis, and one that is thought to malfunction in diseases caused by toxic aggregate formation, is the regulated destruction of proteins that are no longer needed by the cell. This function is carried out in eukaryotic cells by the ubiquitin-proteasome system, in which proteins are "tagged" for degradation by covalent attachment of the small (~9 kDa) modifier protein ubiquitin and degraded by the multicomponent proteolytic machinery known as the proteasome. Recently, it has become increasingly evident that ubiquitination of target proteins can also lead to a wide variety of outcomes independent of proteasomal degradation; non-degradative ubiquitination will be explored in more detail later.

Ubiquitin-dependent protein degradation is a highly conserved system in eukaryotes, and a rudimentary system for ubiquitin-like modification and degradation of target proteins has been described in prokaryotes as well (Pearce et al., 2008). In eukaryotes, ubiquitin is attached via its carboxy-terminus to ε-amino groups of lysine residues on target proteins by the coordinated action of a ubiquitin-activating enzyme

(E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (Fig. 1.5). Formation of an amide bond between the terminal carboxyl group of ubiquitin and the ε-amino group of a lysine residue on a target protein results in a covalent modification that is reversible only by specific deubiquitinating enzymes (DUBs) (Singhal et al., 2008).

After attachment of a single ubiquitin moiety to a target protein, a ubiquitin chain can be formed by sequential addition of multiple ubiquitin monomers using lysine residues within ubiquitin itself. Alternatively, in some circumstances a ubiquitin chain

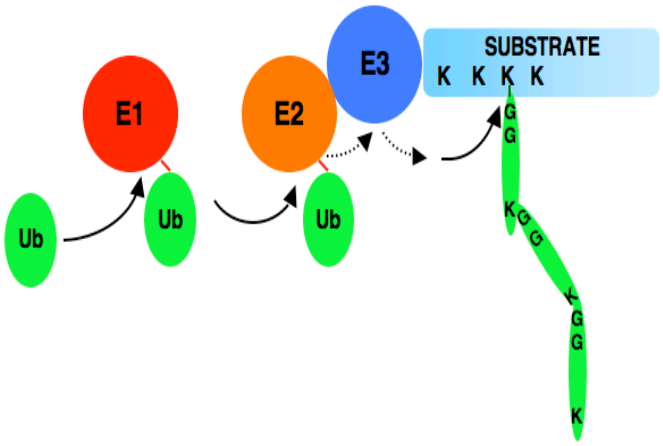

Figure 1.5. Ubiquitination requires E1, E2, and E3 enzymes. The ubiquitin monomer is activated by an E1 enzyme before being conjugated by an E2 enzyme. Substrate specificity is determined by the E3 ligase, which is either covalently linked to the ubiquitin monomer before transfer to the substrate (HECT domain E3s) or facilitates transfer of ubiquitin directly from the E2 to the substrate (RING domain E3s). The first ubiquitin monomer is covalently attached to a lysine residue of the substrate protein via the terminal carboxyl group at glycine 76. Ubiquitin chain formation proceeds by addition of subsequent ubiquitin monomers to lysine residues within ubiquitin itself.

may be preformed before attachment to a lysine residue of the target protein. Human ubiquitin has seven lysine residues that are available for chain formation, with different lysine linkages ultimately resulting in chains of different shapes. For example, chains utilizing exclusively lysine 63 (K63) are linear in structure, whereas chains utilizing

lysine 48 (K48) are branched. Chains of different shapes are thought to lead to different functional outcomes, with K48-linked chains typically targeting proteins for proteasomal degradation (Ikeda and Dikic, 2008; Li and Ye, 2008).

Ubiquitination: specificity and functional consequences

Specificity in the ubiquitin system is generated at three points: the E2, the E3, and the ubiquitin receptor. The specificity in selecting a target protein for ubiquitination is ultimately determined by the E3, which binds to both the E2 and the target protein and brings them together for the ubiquitination event. Depending on the type of E3 ligase, ubiquitin may be transferred to the E3 and then to the substrate, or the E3 may serve merely as a scaffold to allow sufficient proximity so that the E2 transfers ubiquitin directly to the substrate. HECT domain E3 ligases utilize the former mechanism, whereas RING domain E3s utilize the latter (Scheffner et al., 1993; Lorick et al., 1999; Fang and Weissman, 2004).

It remains unclear precisely how the type of ubiquitin chain to be formed on the substrate is determined, but it is thought that the E2 plays a major role in this process. The human genome encodes more than ten different E2 enzymes, each of which participates in a unique subset of ubiquitination events in the cell. It is likely that each E2 is structurally constrained such that it favors formation of certain types of ubiquitin chains over others. For example, the E2 enzyme Ubc13 seems to preferentially form K63-linked chains on target proteins, whereas UbcH5 is more promiscuous (VanDemark et al., 2001; Kim et al., 2007). Ultimately, the unique structural configuration of a particular E2-E3-substrate complex likely determines the probability of forming a given

type of ubiquitin chain. Indeed, the process of ubiquitin chain formation seems to be somewhat stochastic, with ubiquitin chains of mixed linkages often formed on substrate proteins (Ikeda and Dikic, 2008).

In order for ubiquitination to lead to a functional consequence such as degradation or relocalization of the target protein, the ubiquitin signal must be "read" by the cell. This function is carried out by ubiquitin receptors that bind to target proteins and/or their attached ubiquitin chains. Recognition of monoubiquitin and ubiquitin chains can be mediated by a variety of protein domains, among them the ubiquitin-associated (UBA) domain, which is shared by several members of the IAP family. The mechanisms and requirements for recognition of ubiquitinated proteins are still unclear, but it seems that a great deal of specificity can be conferred by slight structural differences between ubiquitin chains (Komander et al., 2009). Proteins that are targeted for degradation are recognized by ubiquitin receptors associated with the proteasome, where the ubiquitin monomers may be recycled as the ubiquitinated protein is degraded (Deveraux et al., 1994; Husnjak et al., 2008; Schreiner et al., 2008). Likewise, polyubiquitin chains that target proteins to associate with receptor-associated signaling complexes must be recognized by components of these complexes with ubiquitin-binding domains. Consistent with this model, a recent report demonstrated a critical role for the IAP UBA domain in IAP-mediated NF-κB activation (Komander et al., 2009). Precisely how ubiquitin-binding proteins are able to discriminate among the wide variety of ubiquitin chains is only now beginning to be elucidated.

IAPs and ubiquitination

Several members of the IAP family are able to carry out ubiquitination of target proteins through a shared RING domain, which is located at the extreme carboxy-terminus of RING-containing IAPs. Among mammalian IAPs, c-IAP1, c-IAP2, XIAP, ILP-2, and ML-IAP contain RING domains with E3 ubiquitin ligase activity. Additionally, BRUCE/Apollon contains a ubiquitin-conjugating (UBC) domain that possesses both E2 and E3 activity (Hao et al., 2004; Bartke et al., 2004).

c-IAPs: signaling through ubiquitination

The highly homologous proteins c-IAP1 and c-IAP2 have been shown to participate in receptor-mediating signaling by inducing ubiquitination of a number of components of the canonical and non-canonical NF-κB pathways. In particular, the c-IAPs have been identified as E3 ligases for receptor-interacting protein-1 (RIP1), RIP2, NEMO, TRAF2, and NF-κB-inducing kinase (NIK), among others (Park et al., 2004; Bertrand et al., 2008; Bertrand et al., 2009; Jin et al., 2009; Li et al., 2002; Varfolomeev et al., 2007). Through ubiquitination of these and other target proteins, the c-IAPs participate in receptor-mediated canonical and non-canonical NF-κB signaling (through RIP1, NEMO, TRAF2, and NIK) as well as cytosolic innate immune signaling via NOD-mediated recognition of common bacterial structural motifs known as pathogen-associated molecular patterns (through RIP2).

In addition to ubiquitinating target proteins, the c-IAPs are capable of triggering their own degradation via autoubiquitination. Variants of these proteins containing critical RING domain point mutations are greatly stabilized relative to the wild-type

proteins, suggesting that autoubiquitination is a primary mechanism for regulating cellular levels of the c-IAPs (Yang et al., 2000). The intrinsic ability of the c-IAPs to autoubiquitinate is greatly enhanced by binding to natural IAP antagonists such as SMAC or synthetic SMAC mimetics, and treatment of cells with synthetic IAP antagonists leads to autoubiquitination and rapid proteasomal degradation of the c-IAPs (Yang and Du, 2004; Petersen et al., 2007; Vince et al., 2007; Varfolomeev et al., 2007; Gaither et al., 2007). Loss of c-IAPs has been shown to induce apoptosis in a variety of tumor cells in a manner that seems to involve deubiquitination of both NIK and RIP1. When the c-IAPs are degraded, NIK levels rise and lead to activation of the non-canonical NF-κB pathway, inducing production and secretion of TNF-α. When the secreted TNF-α binds to TNFR1 on the cell surface, deubiquitinated RIP1 forms a pro-death complex with caspase-8, whereas ubiquitinated RIP1 favors formation of a pro-survival complex with TAK1 to activate canonical NF-κB signaling (Bertrand et al., 2008). Thus, the c-IAPs maintain a delicate life-death balance through RING-mediated autoubiquitination and ubiquitination of target proteins and present promising therapeutic targets in cancer cells.

XIAP and ubiquitination

Although primarily known for its role in protecting cells from apoptosis by direct inhibition of caspases -3, -7, and -9, XIAP has been shown to ubiquitinate a wide variety of cellular targets. The role of ubiquitination by XIAP in inhibiting apoptosis has been controversial and will not be discussed in detail here. Evidence has been found to suggest that XIAP is autoubiquitinated during apoptosis, and that RING-deficient XIAP provides enhanced protection against apoptosis in some situations (Yang et al., 2000).

More recently, another group found that XIAP lacking its RING domain (ΔRING) was defective in protecting from apoptosis and was comparable to complete loss of the protein (Schile et al., 2008). This latter finding would suggest that the caspase-inhibitory properties of XIAP are dispensable for blocking apoptosis, and that ubiquitination of caspases and/or IAP antagonists is the primary mechanism for XIAP-mediated protection. However, while other IAPs have been demonstrated to play important roles in caspase ubiquitination (Ditzel et al., 2008; Choi et al., 2009), previous studies have suggested that direct caspase inhibition is a key component of the anti-apoptotic function of XIAP, and that the importance of its E3 ligase function is less clear (Takahashi et al., 1998; Duckett et al., 1998; Lewis et al., 2004).

The non-apoptotic functions of ubiquitination by XIAP are better defined mechanistically, although their physiologic significance largely remains unclear. XIAP has been shown to ubiquitinate apoptosis-inducing factor (AIF), TAK1, MEKK2, PTEN, and COMMD1 independent of apoptosis regulation (Wilkinson et al., 2008; Kaur et al., 2005; Winsauer et al., 2008; Van Themsche et al., 2009; Burstein et al., 2004). Of these, TAK1, PTEN, and COMMD1 are targeted for degradation by XIAP, while AIF is a target for non-degradative ubiquitination, and the effect of XIAP-mediated ubiquitination on MEKK2 is unclear. Ubiquitination of AIF by XIAP seems to enhance AIF-mediated protection from reactive oxygen species, although the mechanism by which ubiquitination enhances AIF function is unknown. XIAP-mediated ubiquitination of TAK1, MEKK2, and COMMD1 enhances NF-κB signaling via distinct mechanisms, with TAK1 and MEKK2 activating NF-κB upstream of the IKK complex, whereas COMMD1 regulates the stability of the NF-κB subunit RelA in the nucleus. The

significance of XIAP-mediated ubiquitination in NF-κB activation remains controversial, as some groups have found the E3 ligase function of XIAP to be essential, whereas others have suggested it is dispensable for NF-κB signaling (Lewis et al., 2004; Jin et al., 2009). An alternative model has been described whereby XIAP monomers dimerize via their BIR1 domains, leading to association with TAB1 and activation of TAK1 to trigger canonical NF-κB activation through the IKK complex (Lu et al., 2007). Similarly, although the role of COMMD1 in targeting RelA for proteasomal degradation is well established, the physiological importance of XIAP in sustaining NF-κB signaling by stabilizing nuclear RelA via COMMD1 ubiquitination is uncertain.

XIAP and copper homeostasis

In parallel with its identification as an XIAP-interacting protein, the gene encoding COMMD1 was cloned as the gene responsible for a copper toxicosis disorder affecting Bedlington terriers. In these dogs, a deletion in *Commd1* results in complete loss of functional Commd1 expression from the affected locus. Dogs carrying two non-functional *Commd1* alleles have no functional Commd1 and accumulate hepatic copper in a manner similar to Wilson disease in humans (van De Sluis et al., 2002). The mechanism by which COMMD1 promotes hepatic copper excretion is unresolved, but it physically interacts with ATP7B, suggesting that Wilson disease and *Commd1* mutations may ultimately result in copper accumulation via a common mechanism (Tao et al., 2003; de Bie et al., 2007).

As XIAP-mediated ubiquitination targets COMMD1 for proteasomal degradation, overexpression of XIAP in cultured cells results in decreased COMMD1 levels and

copper accumulation. Conversely, reduction in XIAP expression leads to accumulation of COMMD1 and enhancement of copper export, and cells and tissues from *Xiap*-deficient mice have significantly reduced copper levels compared to littermate controls (Burstein et al., 2004). Thus, XIAP-mediated ubiquitination of COMMD1 may play an important role in limiting copper export to maintain proper copper stores.

Recently, an analysis of tissues from animals and humans with copper toxicosis disorders revealed an unexpected reciprocal relationship between XIAP and copper. Tissues from animals and humans with pathological copper accumulation show reduced XIAP expression compared to healthy controls, and treatment of cultured cells with excess copper reduces XIAP expression at the protein level without affecting *XIAP* transcription (Mufti et al., 2006). Copper-mediated destabilization of XIAP is accomplished by direct binding to cysteine residues in XIAP, which triggers a distinct conformational change. Copper binding does not result in cleavage or oxidative modification of XIAP, as it is reversible *in vitro* by copper chelators but not reducing agents. The mechanism by which copper binding destabilizes XIAP is unknown, but it is hypothesized that the copper-induced conformational change may trigger autoubiquitination or allow recognition by another cellular E3 ligase that targets XIAP for proteasomal degradation.

Goals of this dissertation

XIAP is a multifunctional protein with important roles in apoptosis regulation, NF-κB signaling, protection from reactive oxygen species, copper homeostasis, and other processes. The recent identification of XIAP as a copper-binding protein and regulator of

cellular copper metabolism is intriguing and suggests a role for XIAP as a cytosolic copper sensor, allowing COMMD1 to accumulate and stimulate copper export when copper levels rise and destabilize XIAP. However, a still unresolved question is how XIAP can bind to copper *in vivo*, given the excess copper-chelating capacity of the cytosolic environment. As described earlier, free copper is unavailable in the cytosol, and proteins that need copper for activity have specific chaperones to deliver copper to them. At very high copper levels, such as in uncontrolled Wilson disease, free copper might overwhelm the buffering capacity of the cell and bind directly to XIAP in the cytosol. However, the relatively rapid (24-36 hours) delivery of copper to XIAP in cells cultured under conditions of mildly elevated copper (25-50 μM) suggests that diffusion of free copper to XIAP is unlikely to be a primary mechanism for XIAP-copper binding. We hypothesized that copper delivery to XIAP might be mediated directly by a copper chaperone or copper transporter as for other known copper-binding proteins. This dissertation will explore further the mechanistic basis of copper delivery to XIAP and the role of XIAP in copper metabolism through the following Specific Aims:

Specific Aim 1 (Chapter II): How is copper delivered to XIAP in cells?

Specific Aim 2 (Chapter III): How does XIAP-mediated ubiquitination regulate the copper chaperone CCS?

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Chapter II

CCS Mediates Copper Delivery to XIAP

Summary

In order to balance the cellular requirements for copper with its toxic properties, an elegant set of mechanisms has evolved to regulate and buffer intracellular copper. The X-linked inhibitor of apoptosis (XIAP) protein was recently identified as a copper binding protein and regulator of copper homeostasis, although the mechanism by which XIAP binds copper in the cytosol is unclear. Here we describe the identification of the copper chaperone for superoxide dismutase (CCS) as an XIAP-interacting protein and mediator of copper delivery to XIAP in mammalian cells, providing mechanistic insight into regulation of copper homeostasis through XIAP.

Introduction

Copper is a required cofactor for critical steps in many biological processes, including aerobic respiration, iron metabolism, pigment formation, peptide amidation, neurotransmitter synthesis, connective tissue development, and protection from reactive oxygen species (Madsen and Gitlin, 2007; Kim et al., 2008; Turski and Thiele, 2009). Although it is an essential nutrient, the ability of copper ions to easily exchange electrons makes copper highly toxic, so an elaborate system of transporters, chaperones, and chelators has evolved to control the intracellular and extracellular trafficking of copper. Thus, defects in copper uptake or export, either at the cellular or organismal level, result in pathologic copper deficiency or accumulation, respectively.

The importance of copper in mammalian biology is illustrated by the diseases caused by mutations in the genes encoding the copper-transporting ATPases ATP7A and ATP7B. Menkes disease is caused by mutations in the gene encoding ATP7A, which is essential to bring copper from the digestive tract to other organs. Loss-of-function mutations in *ATP7A* result in severe copper deficiency in all organs but the intestine and kidney, leading to musculoskeletal defects, vascular abnormalities, neurodegeneration, and usually death within the first decade of life (Llanos and Mercer, 2002). Conversely, the copper toxicosis syndrome Wilson disease is caused by mutations in the gene encoding ATP7B, which is highly similar to ATP7A but differs in its intracellular trafficking patterns and tissue distribution (Linz and Lutsenko, 2007). Patients with Wilson disease accumulate copper first in the liver and later in other organs, eventually

leading to liver cirrhosis and damage to other organs if copper levels are not reduced therapeutically (Brewer, 2000; Gitlin, 2003).

A number of other copper accumulation disorders have also been described, although their genetic and biochemical mechanisms are generally less understood. One of the less characterized disorders of copper accumulation occurs in a subset of Bedlington terriers that lack a functional *Commd1* gene (van De Sluis et al., 2002). COMMD1 is a ~20 kDa protein that has been implicated in wide variety of pathways, including nuclear factor-κB (NF-κB) signaling, response to hypoxia, sodium regulation, and copper homeostasis (de Bie et al., 2006; Maine et al., 2007; van de Sluis et al., 2007; Biasio et al., 2004). At least in canines, COMMD1 is required for proper hepatic copper excretion, and it can physically interact with ATP7B, suggesting a common mechanism for human Wilson disease and canine *Commd1* deficiency through ATP7B-mediated copper export (Tao et al., 2003; de Bie et al., 2007).

COMMD1 was independently identified as an interacting partner of the X-linked inhibitor of apoptosis (XIAP) (Burstein et al., 2004). Mammalian inhibitors of apoptosis (IAPs) were originally identified as homologs of a baculovirus IAP and, as the name suggests, were thought to be primarily involved in regulation of apoptosis (Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996). Since their initial characterization, it has become clear that IAPs regulate a wide variety of cellular processes including mitosis, receptor-mediated signaling pathways, and copper metabolism (Srinivasula and Ashwell, 2008; O'Riordan et al., 2008). XIAP binds to and ubiquitinates COMMD1 in cells, targeting it for degradation by the proteasome (Burstein et al., 2004). Through this

mechanism, XIAP is thought to raise intracellular copper through degradation of a key copper export protein.

Surprisingly, while XIAP regulates copper homeostasis through its interaction with COMMD1, XIAP is in turn regulated by intracellular copper levels. Elevated intracellular copper leads to direct binding of copper to cysteine residues within XIAP, resulting in a distinct conformational change (Mufti et al., 2006). This conformational change leads to an altered electrophoretic mobility of XIAP even under denaturing, reducing conditions, and more importantly it decreases the stability of XIAP and impairs its ability to inhibit caspases. Thus, XIAP seems to participate in a regulatory loop, promoting its own degradation by raising intracellular copper levels. However, the mechanism by which XIAP binds to copper in the cell remains unclear. Because of its toxicity, intracellular copper is tightly controlled so that free copper is unavailable even when total copper is elevated (Rae et al., 1999). Given that copper-dependent proteins require specific copper chaperones to deliver copper to them, we hypothesized that a chaperone protein might be necessary to mediate copper delivery to XIAP as well.

Through a yeast genetic screen designed to identify candidate proteins involved in delivering copper to XIAP, we identified the copper chaperone for superoxide dismutase (CCS) as an XIAP-interacting protein. We find that CCS is important for copper delivery to XIAP and that the two proteins physically interact in mammalian cells. Interestingly, our data indicate that interaction of CCS with XIAP is mediated by the same CCS domain that mediates docking with its physiologic target superoxide dismutase (SOD1), suggesting that copper transfer from CCS to XIAP and SOD1 may proceed via a common mechanism.

Materials and methods

Plasmids, oligonucleotides, and transfections: pEBB, pEBG, pEBB-XIAP, and all pEBB-derived XIAP expression plasmids have been described previously (Duckett et al., 1998; Lewis et al., 2004; Burstein et al., 2004; Mufti et al., 2006). pEBB-CCS-FLAG was generously provided by Drs. Prim de Bie and Leo Klomp (University Medical Center Utrecht, Utrecht, Netherlands) and was used to generate pEBB-CCS-GST. CCS deletion constructs were derived by PCR cloning using primers spanning the appropriate domain(s) containing nested restriction sites. The *S. cerevisiae* expression vector pTW437 is a *CEN/URA3* variant of pTW435, which has been described previously (Daley et al., 2005). Untagged human XIAP was cloned into pTW437 by gap-repair cloning in *S. cerevisiae* using standard protocols. Briefly, the coding sequence of human XIAP was PCR-amplified using pEBB-XIAP as a template, and the XIAP amplicon was co-transformed into wild-type *S. cerevisiae* with SmaI-linearized pTW437. The recombined pTW437-XIAP plasmid was recovered by electroporation into *Escherichia coli*. All siRNA oligonucleotides were obtained from Qiagen and used according to the manufacturer's instructions. For all experiments using siRNA oligos, an oligo targeting GFP was used for control transfections. Double-stranded siRNA oligos were designed to target the following sequences: 5'-AACTGCAACAGCTGTGGGAAT-3' (CCS coding sequence nucleotides 418-438); 5'-AATGGAGGATGAGCAGCTGAA-3' (CCS coding sequence nucleotides 546-566); 5'-AAGACCCGCGCCGAGGTGAAG-3' (GFP coding sequence nucleotides 322-342). All transfections were performed using a standard calcium phosphate method with the following exception: for transfection of siRNA

oligos, 25 μM chloroquinone was added to the culture medium 10-15 minutes before transfection to enhance transfection efficiency, and the medium was changed to chloroquinone-free medium six hours post-transfection.

Yeast strains and conditions: *S. cerevisiae* parental strain BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and the indicated deletion mutants (obtained from Open Biosystems) were transformed with pTW437-XIAP using standard protocols. Transformed yeast strains were cultured in copper-free minimal selective medium, which was prepared with yeast nitrogen base (YNB) without amino acids, with ammonium sulfate, and lacking copper, iron, zinc, and manganese (US Biological). Synthetic medium (10X) deficient in only copper was prepared by adding iron sulfate, zinc chloride, and manganese chloride tetrahydrate to a mixture of YNB and uracil-free complete supplement mixture (CSM-URA; Qbiogene) in sterile water to achieve final concentrations of 2 mg/L iron, 4 mg/L zinc, and 4 mg/L manganese in the 10X YNB/CSM mixture. The final copper-free synthetic medium consisted of 1X YNB/CSM and 2% dextrose in sterile water. Yeast were grown at 30 °C in selective medium without copper overnight with shaking, then diluted at least ten-fold to an optical density (OD_{600}) of ~0.10 and allowed to grow to an OD_{600} of ~0.25 before addition of copper, additional growth with shaking, and harvest. Prior to analysis by western blot, cells were washed with sterile water followed by 300 mM sodium hydroxide, then lysed by boiling in NuPAGE LDS sample buffer (Invitrogen) with DTT.

Cell lines and culture conditions: HEK 293 and 293T cells and mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37 °C in

an atmosphere of 5% CO_2. Generation of *Ccs*-deficient and wild-type control MEFs has been described (Caruano-Yzermans et al., 2006). For copper treatments, copper sulfate ($CuSO_4$) was obtained from Sigma, and copper solutions were freshly prepared for each experiment.

Cell lysis and SDS-PAGE conditions: Unless otherwise noted, cells were harvested with Triton X-100 lysis buffer (1% Triton X-100 (Sigma), 10% glycerol, 25 mM HEPES pH 7.9, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, supplemented with protease inhibitors) on ice. Lysates were cleared of cellular debris by centrifugation at 20,000×*g* for 10 minutes at 4 °C, and protein content was determined by the Bradford method. For western blot analysis, proteins were resolved using 4-12% gradient bis-tris NuPAGE gels (Invitrogen).

GST pulldowns, western blot conditions, and antibodies: GST pulldowns were performed with GSH-Sepharose (GE Healthcare) as described (Burstein et al., 2004). All western blot analysis was performed under reducing, denaturing conditions using Invitrogen NuPAGE 4-12% gels as described previously (Mufti et al., 2006). The following primary antibodies were used for western blot analysis: anti-XIAP (BD Transduction Labs), anti-CCS (Santa Cruz), anti-β-actin (Sigma), anti-FLAG (unconjugated and HRP-conjugated; Sigma), and anti-GST (Santa Cruz). In most cases, and in all cases where quantitation of western blots was performed, blots were scanned using a Li-Cor Odyssey infrared scanner after incubation with the appropriate infrared dye-conjugated secondary antibody (Li-Cor). Band quantitation was performed with Li-Cor Odyssey software (version 2.1) according to the manufacturer's instructions. For western blots developed with enhanced chemiluminescence (ECL) and film, HRP-

conjugated secondary antibodies and ECL were obtained from GE Healthcare and used with Kodak XAR film.

Results

Copper delivery to XIAP is mediated by the copper chaperone Ccs1 in *S. cerevisiae*.

Given the excess copper-chelating capacity of the cytosolic environment, we reasoned that simple diffusion of copper to XIAP was unlikely to be a primary mechanism for XIAP-copper binding *in vivo*. We introduced human XIAP into *Saccharomyces cerevisiae* in order to carry out a targeted genetic screen to identify candidate proteins involved in delivering copper to XIAP. Copper trafficking pathways are remarkably conserved between yeast and mammalian cells, and *S. cerevisiae* has proven to be a useful model for studying mammalian copper homeostasis (Rees and Thiele, 2004). Consistent with this, we observed that copper was efficiently delivered to human XIAP expressed ectopically in wild-type *S. cerevisiae* (Fig. 2.1A). Similar to the studies described previously, the copper-bound form of XIAP was distinguished from native XIAP by its faster migration on SDS-PAGE under denaturing, reducing conditions (Mufti et al., 2006).

To identify proteins that might affect copper trafficking to XIAP, we introduced human XIAP into sixteen *S. cerevisiae* deletion strains in which genes encoding individual copper chaperones and transporters, or other proteins with putative roles in copper biology, have been deleted by homologous recombination. Interestingly, in the majority of the tested strains, copper was delivered to XIAP with comparable efficiency to the wild-type strain in the presence of 5 μM copper sulfate (Fig. 2.1B). However, yeast lacking the metal-responsive transcription factor Ace1, the metalloenzyme Sod1, or the copper chaperone Ccs1 (the yeast homolog of human CCS) demonstrated reduced

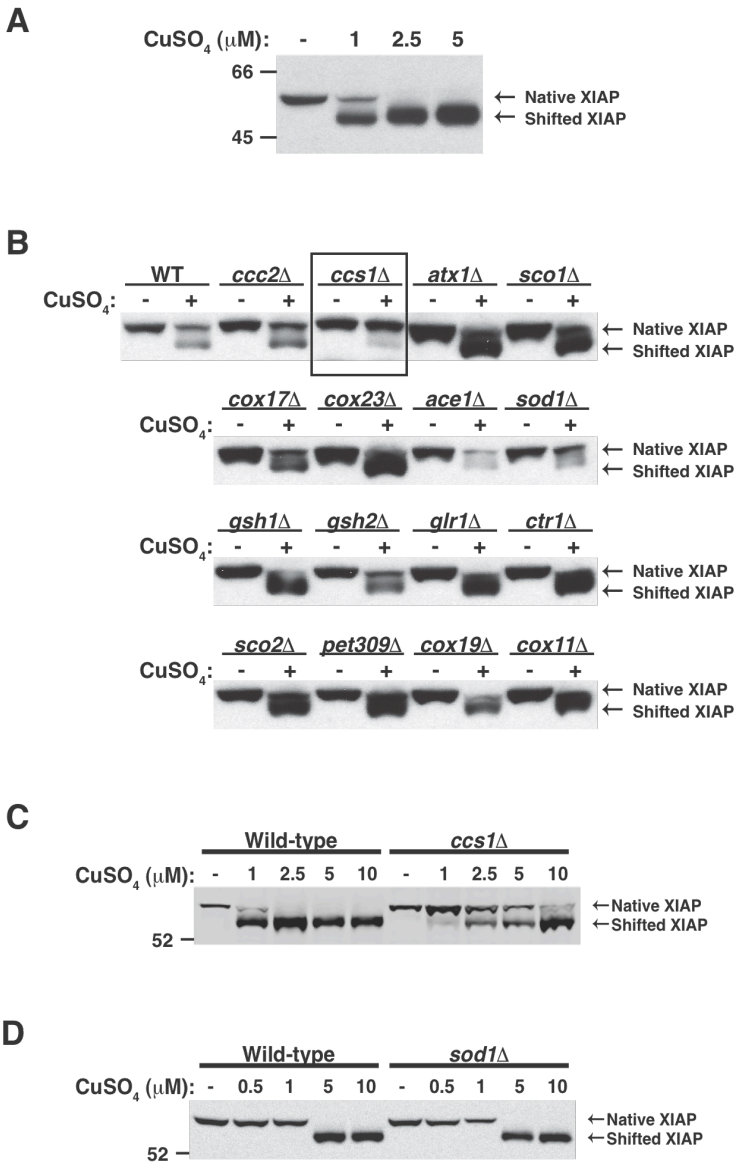
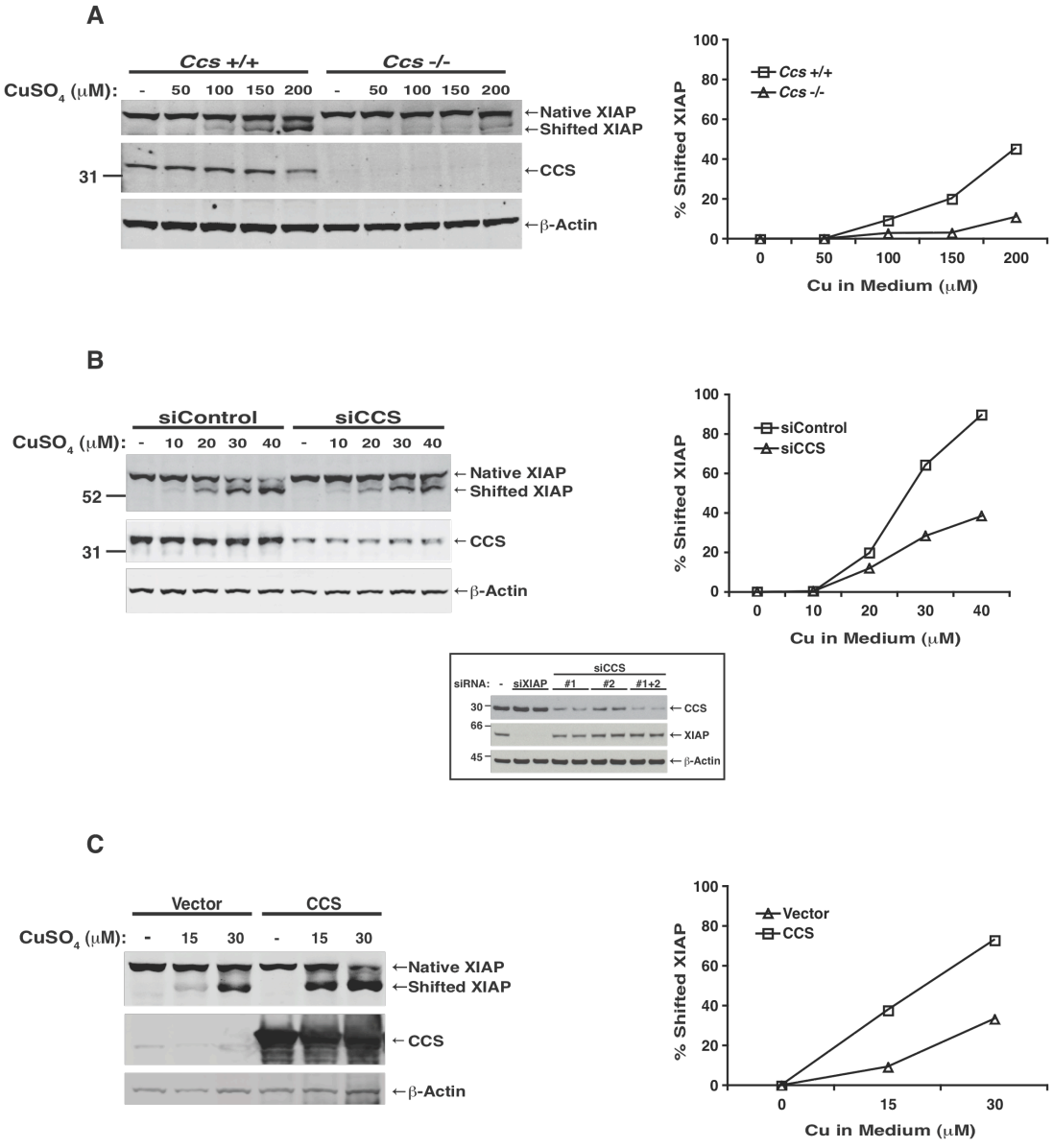


Figure 2.1. Ccs1 mediates copper delivery to human XIAP expressed in *S. cerevisiae*. *(A)* Wild-type *S. cerevisiae* were transformed with a plasmid containing the coding sequence of human XIAP, grown in selective medium, and treated with 0-5 μM $CuSO_4$ for 2 hours. Copper delivery to XIAP was determined by SDS-PAGE and western blot analysis with an antibody directed against human XIAP. Note that copper-bound ("shifted") XIAP migrates faster than native XIAP under denaturing, reducing SDS-PAGE conditions. *(B)* Wild-type yeast and the indicated deletion strains were transformed with human XIAP and treated with 0 or 5 μM $CuSO_4$ for 2 hours prior to analysis by western blot as in *(A)*. *(C)* and *(D)* Wild-type yeast and yeast lacking *CCS1 (C)* or *SOD1 (D)* were transformed with a human XIAP expression vector and treated with 0-10 μM $CuSO_4$ for 1 hour.

copper delivery to XIAP compared to wild-type and the other strains tested. Ace1 is required for metallothionein gene transcription, and thus the *ace1*Δ strain is exquisitely sensitive to copper (Thiele, 1988; Buchman et al., 1989). As a result, very few viable *ace1*Δ cells were recovered for western blot analysis after exposure to elevated extracellular copper. Thus, while we cannot exclude a direct role for Ace1 and metallothioneins in delivering copper to XIAP, the apparent defect in the *ace1*Δ strain is likely due to its drastically reduced fitness under conditions of elevated copper. In contrast, the *ccs1*Δ strain did not exhibit a growth defect in the presence of copper, and its defect in copper delivery to XIAP was confirmed by direct comparison with the wild-type strain in the presence of 0-10 μM copper (Fig. 2.1C). Although some variability was observed in the absolute copper concentration required to produce the electrophoretic mobility shift of XIAP, the difference between the wild-type and *ccs1*Δ strains was consistent across multiple experiments, and in all cases comparisons were made between samples that were treated and analyzed concurrently.

In yeast and mammals, Ccs1/CCS is essential to deliver copper to Sod1 but does not participate in copper uptake, export, or copper delivery to other metalloenzymes (Culotta et al., 1997; Wong et al., 2000). Given that the role of Ccs1 in copper trafficking is limited to Sod1 metallation, its effect on copper delivery to XIAP was likely to be either direct or via Sod1. In contrast to the *ccs1*Δ strain, side-by-side comparison of the wild-type and *sod1*Δ strains did not reveal any differences in copper delivery to XIAP (Fig. 2.1D). Thus, efficient copper delivery to human XIAP expressed ectopically in yeast was found to require endogenous yeast Ccs1, and furthermore the role of Ccs1 in copper delivery to XIAP was found to be independent of Sod1 activation.

Figure 2.2. CCS mediates copper delivery to XIAP in mammalian cells. *(A)* Embryonic fibroblasts derived from either wild-type or *Ccs*-deficient mice were treated with 0-200 μM $CuSO_4$ for 48 hours. Copper delivery to Xiap was determined by western blot and quantitated using Li-Cor Odyssey software as described in Materials and Methods. *(B)* HEK 293T cells were transfected with a control siRNA oligonucleotide or a combination of two oligonucleotides targeting CCS, incubated for 48 hours, and treated with 0-40 μM $CuSO_4$ for 24 hours prior to lysis and analysis by western blot. *Inset:* cells were transfected with siRNA oligonucleotides targeting XIAP or CCS as indicated. Cell lysates were analyzed by western blot with antibodies targeting CCS, XIAP, and β-actin. *(C)* HEK 293 cells were transfected with a plasmid encoding human CCS or empty vector, then treated with 0-30 μM $CuSO_4$ for 48 hours and analyzed by western blot as in *(A)* and *(B)*. The quantitative data shown graphically in *(A)*, *(B)*, and *(C)* were derived from the immunoblots displayed and are representative of at least three independent experiments in each case.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Ccs +/+
Ccs -/-
CuSO4 (μM): - 50 100 150 200 - 50 100 150 200
←Native XIAP
←Shifted XIAP
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CCS mediates copper delivery to XIAP in mammalian cells.

To determine whether CCS might be involved in delivering copper to XIAP in mammalian cells, embryonic fibroblasts derived from *Ccs*-deficient mice were treated with excess copper and compared to fibroblasts derived from a littermate control. In agreement with the results observed in *S. cerevisiae*, copper delivery to endogenous Xiap was substantially reduced in mouse embryonic fibroblasts (MEFs) lacking *Ccs* (Fig. 2.2A). Similarly, suppression of endogenous CCS expression by transfection of double-stranded small interfering RNA (siRNA) oligonucleotides reduced copper delivery to XIAP compared to control transfection in human embryonic kidney (HEK) 293T cells (Fig. 2.2B). In order to achieve maximal suppression of CCS, two different oligonucleotides that moderately reduced CCS expression when transfected separately (Fig. 2.2B, *inset*) were pooled and transfected together. Conversely, overexpression of CCS lowered the threshold for copper delivery to endogenous XIAP (Fig. 2.2C). Although the effect of CCS overexpression was modest, this may be partly a reflection of transfection efficiency, as this assay detected copper binding to endogenous XIAP in a pool of transfected cells (with transfection efficiency being generally ~70% with the calcium phosphate method in our hands). Thus, in mammalian cells absent or decreased CCS expression reduced, whereas CCS overexpression enhanced, copper delivery to XIAP. Taken together, our data suggest that, although it is not absolutely required, CCS plays a major role in delivery of copper to XIAP.

Of note, greater concentrations of copper were required to produce a mobility shift in XIAP in mammalian cells as compared to *S. cerevisiae*, which may be due to intrinsic differences in efficiency of copper uptake between mammalian and yeast cells;

alternatively, the difference may be attributable to the greater chelating capacity of the serum-containing medium in which the mammalian cells were grown.

XIAP physically interacts with CCS.

To address whether the role of CCS in copper delivery to XIAP might involve a physical interaction between the two proteins, co-precipitation studies were performed with ectopically expressed proteins. CCS or the indicated CCS domain(s) (Fig. 2.3A) with a carboxy-terminal glutathione-*S*-transferase (GST) epitope tag was expressed with amino-terminally hemagglutinin (HA) tagged XIAP in HEK 293 cells. Full-length CCS and deletion constructs containing the SOD1-like second domain of CCS efficiently precipitated XIAP from cell lysates, whereas domains 1 and 3 in isolation did not bind to XIAP (Fig. 2.3B). The observation that domain 2 of CCS was required for interaction with XIAP is interesting, as this domain mediates CCS homodimerization as well as binding to SOD1 (Schmidt et al., 1999). A similar experiment was carried out with full-length CCS and domain deletions of XIAP fused in-frame with an amino-terminal GST tag (Fig. 2.3C). We found that interaction with CCS was mediated by the most carboxy-terminal baculovirus IAP repeat (BIR3) domain of XIAP, as this domain was necessary and sufficient for precipitation of CCS. It is not clear why the BIR 1-2-3 construct did not efficiently precipitate with CCS as BIR3 alone did, but this result was consistent despite adequate expression of the GST-BIR 1-2-3 construct. Collectively, these data suggest that the second domain of CCS and the third BIR domain of XIAP are necessary and sufficient to mediate the XIAP-CCS interaction.

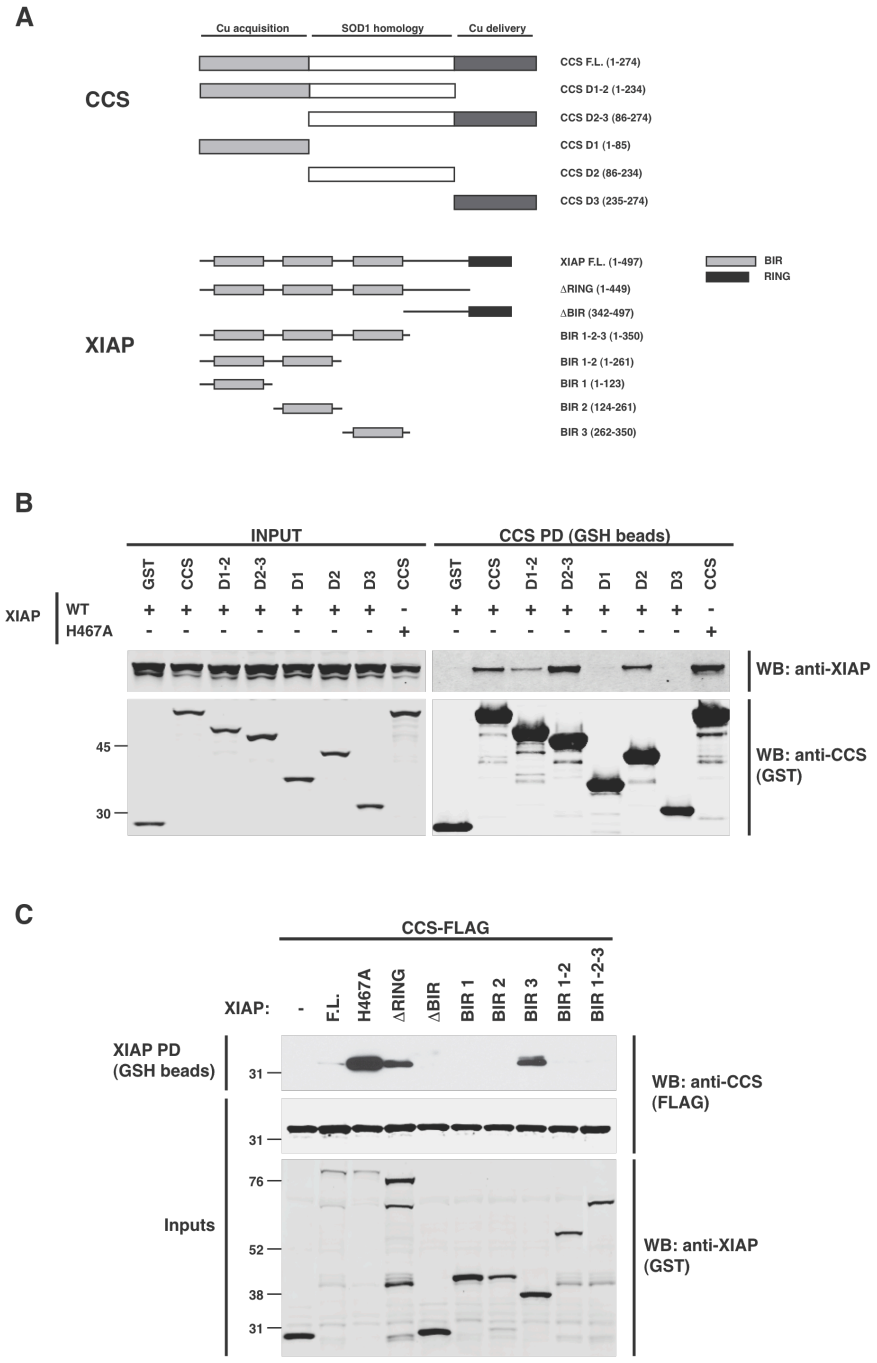

Figure 2.3. CCS domain 2 and XIAP BIR3 mediate the XIAP-CCS interaction. *(A)* Schematic representations of CCS and XIAP domains and deletion constructs. *(B)* Full-length CCS-GST and the indicated CCS deletion constucts were co-expressed in HEK 293 cells with either wild-type XIAP or a point mutant of XIAP that lacks E3 ubiquitin ligase activity (H467A). CCS was precipitated from whole cell lysates with glutathione (GSH) beads, and pulldown samples were analyzed by western blot. *(C)* GST-tagged wild-type XIAP and the indicated XIAP mutants were expressed with FLAG-tagged CCS, precipitated from cell lysates with GSH beads, and analyzed by western blot.

Interestingly, a deletion mutant of XIAP lacking its carboxy-terminal RING domain (ΔRING) and full-length XIAP containing a critical RING domain point mutation (H467A), both of which lack E3 ubiquitin ligase activity (Yang et al., 2000), consistently co-precipitated with CCS more efficiently than wild-type XIAP (Fig. 2.3B and 2.3C). Based on previous studies of XIAP and its ubiquitination targets (Burstein et al., 2004; Wilkinson et al., 2008; Winsauer et al., 2008), this observation suggested that disruption of the E3 activity of XIAP might capture what would otherwise be a transient XIAP-CCS complex, and that CCS might be a target for XIAP-mediated ubiquitination.

Discussion

The recent characterization of XIAP as a copper-binding protein provided a novel link between copper homeostasis and apoptosis regulation, but the mechanism and regulation of XIAP-copper binding remains unclear. Here, we used a yeast genetic screen to identify a novel physical and functional interaction between XIAP and the copper chaperone CCS, providing further insight into how intracellular copper and the apoptotic machinery are regulated.

The interaction of XIAP with CCS is an intriguing and potentially important nexus between copper metabolism and apoptosis. We hypothesize that as intracellular copper levels rise, copper-bound CCS accumulates and delivers copper to XIAP. We did not observe any XIAP in the copper-bound state in the absence of supplemental copper, suggesting that XIAP does not compete effectively for copper with SOD1. Based on this observation, it is likely that CCS only delivers copper to XIAP when the cytosolic pool of copper-free (apo-) SOD1 is essentially depleted. Interestingly, in yeast lacking Sod1, copper delivery to XIAP did not seem to be enhanced compared to wild-type yeast, perhaps because of cytosolic copper scavengers or other proteins that compete effectively with XIAP for copper bound to Ccs1.

It is not clear whether the constraints on CCS-dependent copper delivery to XIAP are primarily kinetic or thermodynamic, but a recent report from Banci and colleagues (Banci et al., 2010) sheds some light on this question. A systematic analysis of copper-binding proteins showed the dissociation constant (K_D) of the human CCS-copper(I) complex to be 2.42×10^{-15} M, based on competition for copper(I) with increasing

concentrations of DTT. At concentrations of DTT between 10 and 20 mM, ~50% of CCS-bound copper was liberated by DTT. In contrast, in our laboratory we find that copper binding to XIAP (endogenous, overexpressed, or recombinant) is not appreciably altered in the presence of up to 250 mM DTT (Mufti et al., 2006). Similarly, we find that, while both XIAP and CCS exhibit altered mobility on denaturing SDS-PAGE when treated with copper *in vitro*, only XIAP consistently exhibits this altered mobility when lysates from copper-treated cells are analyzed (Fig. 2.2 and data not shown), suggesting that copper dissociates from CCS more readily than from XIAP under these conditions. Thus, while the affinity of XIAP for copper is not known, these data suggest that it is likely to be greater than that of CCS, allowing copper transfer from CCS to XIAP to be thermodynamically favorable. However, the affinity of SOD1 for copper ($K_D = 2.3 \times 10^{-16}$ M) is greater than that of CCS, and the relative abundance of SOD1 molecules would kinetically favor copper acquisition by SOD1 as well (Rae et al., 1999). Thus, while we cannot compare directly the thermodynamics of copper transfer from CCS to XIAP versus SOD1, it is likely that the latter would be strongly favored from a kinetic perspective. In this scenario, only when the pool of apo-SOD1 is depleted does copper transfer to XIAP become kinetically favorable. Collectively, the data from yeast and mammalian cells suggest that copper delivery to XIAP is a relatively inefficient process in comparison to SOD1 metallation and requires accumulation of copper-bound CCS above basal levels.

Interestingly, the residual copper-bound XIAP observed under high-copper conditions in *ccs1*Δ *S. cerevisiae* (Fig. 2.1C) and *Ccs*-deficient MEFs (Fig. 2.2A) suggests the existence of a CCS-independent pathway of copper delivery to XIAP. This

is consistent with previous work by Culotta and colleagues demonstrating CCS-independent metallation of SOD1 via a pathway that requires reduced glutathione (Carroll et al., 2004; Leitch et al., 2009a; Leitch et al., 2009b; Jensen and Culotta, 2005). It is unclear which protein(s) might be involved in the alternate pathway for copper loading of XIAP. Physical interaction between XIAP and other copper-binding proteins such as CTR1, SOD1, ATP7B, and ATOX1 was not detectable under conditions where a CCS-XIAP interaction was detected (data not shown). It is possible that a non-proteinaceous carrier such as glutathione might be involved, as is the case for SOD1. Indeed, XIAP has been shown to bind to copper-bound glutathione *in vitro* (Mufti et al., 2006), suggesting that glutathione might be a possible alternative copper source for XIAP. Regardless of the mechanism, our data indicate that CCS-independent copper delivery to XIAP requires substantially higher copper levels as compared to the CCS-dependent pathway, suggesting that CCS-mediated copper delivery is likely to be the predominant pathway for copper loading of XIAP *in vivo*.

CCS-dependent copper delivery to XIAP may present an opportunity for therapeutic intervention for copper toxicosis disorders or tumors overexpressing XIAP. In the setting of Wilson disease and other copper toxicosis syndromes, hepatic copper levels rise gradually over time, eventually reaching toxic levels and causing local and systemic damage. We hypothesize that an early event in copper toxicosis diseases, before copper levels have risen sufficiently to damage cells directly, may be the accumulation of copper-bound CCS and copper delivery to XIAP. Based on our findings, copper binding to XIAP would be expected to happen when copper levels are only slightly elevated above physiologic levels. Pro-apoptotic insults due to metabolic

toxins and other sources might induce apoptosis in these otherwise healthy cells due to reduced XIAP expression, meaning that liver pathology might begin even when copper accumulation is relatively mild. Similarly, patients undergoing copper-reducing therapy might still be at risk for liver damage via XIAP depletion if hepatic copper levels remain mildly elevated. In this setting, therapeutic targeting of the CCS-XIAP interaction to preserve XIAP expression could be beneficial in protecting hepatocytes from indirect copper-mediated toxicity.

Interestingly, elevated XIAP expression has been demonstrated in a variety of tumor types, and targeting of XIAP with antisense oligonucleotides and small molecules has proven effective in preclinical studies (Amantana et al., 2004; LaCasse et al., 2006; McManus et al., 2004). Further study of the XIAP-CCS interaction may provide structural insights that can be used for rational design of small molecules that can bind copper ions and mimic the XIAP-binding region of CCS to deliver copper to XIAP and trigger its degradation.

Finally, the observation that RING-deficient mutants of XIAP were able to co-precipitate with CCS more efficiently than wild-type XIAP suggests that CCS might be targeted for ubiquitination by XIAP. This observation is intriguing and could provide a mechanism by which XIAP could regulate its own stability by ubiquitination of its copper chaperone.

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Chapter III

XIAP-mediated CCS Ubiquitination Regulates SOD1 Activation

Summary

In Chapter II, I identified a novel interaction between XIAP and CCS. I found that CCS could mediate copper delivery to XIAP in yeast and mammalian cells and that the two proteins could physically interact. In this chapter I found that CCS is a target of the E3 ubiquitin ligase activity of XIAP, although interestingly ubiquitination of CCS by XIAP did not trigger its proteasomal degradation. Rather, ubiquitination by XIAP led to enhancement of CCS's chaperone activity toward its physiologic target superoxide dismutase-1 (SOD1). Collectively, these results reveal a novel link between apoptosis, copper metabolism, and redox regulation through the XIAP-CCS complex.

Introduction

Through a yeast genetic screen designed to identify candidate proteins involved in delivering copper to XIAP, I identified the copper chaperone for superoxide dismutase (CCS) as an XIAP-interacting protein in Chapter II. I found that CCS was important for copper delivery to XIAP in mammalian cells and that CCS and XIAP could physically interact. Interestingly, variants of XIAP lacking E3 ubiquitin ligase activity were able to co-precipitate with CCS more efficiently than wild-type XIAP, suggesting that CCS might be a target for XIAP-mediated ubiquitination.

Here I report that XIAP targets CCS for ubiquitination through the E3 ligase activity of its RING domain. The effect of XIAP expression on CCS ubiquitination was similar to that of treating cells with copper, which has been previously shown to lead to ubiquitination of CCS (Caruano-Yzermans et al., 2006). Surprisingly, ubiquitination of CCS by XIAP differs from copper-induced CCS ubiquitination in that it seems to be proteasome-independent and, rather than triggering degradation of CCS, enhances its ability to deliver copper to its physiologic target superoxide dismutase-1 (SOD1).

Materials and methods

Plasmids, oligonucleotides, and transfections: pEBB, pEBB-XIAP, and all pEBB-derived XIAP expression plasmids have been described previously (Duckett et al., 1998; Lewis et al., 2004; Burstein et al., 2004; Mufti et al., 2006). For production of lentiviruses, a cassette containing the histone H1 promoter and a sequence directing expression of an RNA hairpin targeting either XIAP or green fluorescent protein (GFP) was cloned into the FG12 vector, kindly provided by Dr. David Baltimore (Qin et al., 2003). Human ubiquitin with an amino-terminal histidine tag (pEBB-His_6-Ubiquitin) and an empty expression vector with a carboxy-terminal biotinylation sequence tag (pEBB-cTB) were generously provided by Drs. Gabriel Maine and Ezra Burstein (University of Texas-Southwestern, Dallas, TX). pEBB-CCS-FLAG was generously provided by Drs. Prim de Bie and Leo Klomp (University Medical Center Utrecht, Utrecht, Netherlands) and was used to generate untagged pEBB-CCS and pEBB-CCS-cTB. CCS mutants were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). To generate pEBB-SOD1-HA, the SOD1 coding sequence was PCR-amplified from a human T-cell leukemia cDNA library with the Advantage HF2 high-fidelity PCR kit (Clontech) and cloned into pEBB-(carboxy-terminal)HA. All siRNA oligonucleotides were obtained from Qiagen and used according to the manufacturer's instructions. For all experiments using siRNA, an oligo targeting GFP was used for control transfections. Double-stranded siRNA oligos were designed to target the following sequences: 5'-AAGTGGTAGTCCTGTTTCAGC-3' (XIAP coding sequence nucleotides 111-131); 5'-AAGACCCGCGCCGAGGTGAAG-3' (GFP coding sequence nucleotides 322-342).

Sequences of RNA hairpins for stable suppression of XIAP and GFP were the same as the oligos described above. All transfections were performed using a standard calcium phosphate method with the following exception: for transfection of siRNA oligos, 25 μM chloroquinone was added to the culture medium 10-15 minutes before transfection to enhance transfection efficiency, and the medium was changed to chloroquinone-free medium six hours post-transfection.

Cell lines and culture conditions: HEK 293 and 293T cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37 °C in an atmosphere of 5% CO_2. Stable cell lines were generated by lentiviral infection using FG12 expression vectors containing the relevant hairpin sequence (XIAP or GFP) and packaging plasmids as described previously (Galbán et al., 2009). For copper treatments, copper sulfate ($CuSO_4$) was obtained from Sigma, and copper solutions were freshly prepared for each experiment.

Mice: *Xiap*-deficient mice were backcrossed in the C57BL/6 strain for at least 12 generations (Harlin et al., 2001). All mice were housed under specific pathogen-free conditions within the animal care facility at the University of Michigan. All animal experiments were approved by the University of Michigan Committee on the Use and Care of Animals.

Denaturing and native PAGE conditions: Unless otherwise noted, cells were harvested with Triton X-100 lysis buffer (1% Triton X-100 (Sigma), 10% glycerol, 25 mM HEPES pH 7.9, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, supplemented with protease inhibitors) on ice. Lysates were cleared of cellular debris by centrifugation at 20,000×*g* for 10 minutes at 4 °C, and protein content

was determined by the Bradford method. For western blot analysis, proteins were resolved using 4-12% gradient bis-tris NuPAGE gels (Invitrogen). For SOD activity assays, lysates were loaded into 4-12% gradient tris-glycine gels (Invitrogen) and run for one hour at room temperature under native conditions. Gels were soaked in deionized water for 30-45 minutes and then stained for SOD activity according to the method of Beauchamp and Fridovich with slight modification (Beauchamp and Fridovich, 1971). Briefly, gels were soaked individually in 100 mL 0.2 mg/mL nitroblue tetrazolium (NBT; Sigma) for 15 minutes. After rinsing briefly in water, gels were incubated in 100 mL of 50 mM KPO_4 pH 7.8 supplemented with 0.1 mg/mL riboflavin (Sigma) and 1 μL/mL TEMED (Boehringer Mannheim) for 15 minutes in the dark. After rinsing briefly, gels were developed by exposure to ambient fluorescent light. SOD gels were imaged on a conventional scanner (Epson), and band intensity was quantitated using ImageJ software (http://rsbweb.nih.gov/ij/).

Immunoprecipitation, western blot conditions, and antibodies: Immunoprecipitations were performed with an anti-HA antibody (Roche) as described (Burstein et al., 2004). For ubiquitination assays, cells were lysed at room temperature under denaturing conditions (8M urea, 50 mM Tris pH 8.0, 300 mM NaCl, 50 mM Na_2HPO_4, 0.5% NP-40, 1 mM PMSF, supplemented with protease inhibitors), and ubiquitinated material was recovered by rotation with nickel-coated agarose beads (Invitrogen) as described previously (Burstein et al., 2004; Burstein et al., 2005; Maine et al., 2007). Unless otherwise noted, cells were treated with 40 μM MG132 (Sigma) for four hours prior to lysis to increase recovery of ubiquitinated proteins. All western blot analysis was performed under reducing, denaturing conditions using Invitrogen NuPAGE 4-12% gels

as described previously (Mufti et al., 2006). The following primary antibodies were used for western blot analysis: anti-XIAP (BD Transduction Labs), anti-CCS (Santa Cruz), anti-β-actin (Sigma), anti-FLAG (unconjugated and HRP-conjugated; Sigma), anti-GST (Santa Cruz), and anti-HA (Covance). In most cases, blots were scanned using a Li-Cor Odyssey infrared scanner after incubation with the appropriate infrared dye-conjugated secondary antibody (Li-Cor). For western blots developed with enhanced chemiluminescence (ECL) and film, HRP-conjugated secondary antibodies and ECL were obtained from GE Healthcare and used with Kodak XAR film.

Two-step ubiquitin/biotin pulldown and mass spectrometry: Ubiquitinated and biotinylated CCS was produced in HEK 293 cells by co-transfection of pEBB-CCS-cTB, pEBB-His_6-Ubiquitin, and pEBB-XIAP, and purified via a protocol modified from that developed by Kaiser and colleagues (Tagwerker et al., 2006). A full description of the two-step purification scheme will be published elsewhere (G. Maine and E. Burstein, personal communication). Briefly, cells were transfected as described above and treated with exogenous biotin (4 μM, Sigma) and MG132 (40 μM) to enhance recovery of biotinylated, ubiquitinated CCS. As for other His_6-Ubiquitin pulldowns, the cells were lysed with 8M urea lysis buffer and sonicated. Lysates were rotated sequentially in disposable drip columns (Bio-Rad) packed with nickel-coated and then streptavidin-coated agarose beads. After the final elution step, proteins were resolved by SDS-PAGE and stained with colloidal Coomassie (Invitrogen) prior to mass spectrometric analysis at the University of Michigan Department of Pathology Proteomics Facility.

In-gel digestion with trypsin: Protein bands corresponding to unmodified and modified CCS were excised, and destained with 30% methanol for 3 hours. Cysteines were

reduced and carbamidomethylated by incubating the gel slices with DTT (10 mM) followed by iodoacetamide (50 mM) at room temperature for 30 minutes each. Gel slices were crushed, dried using a vacufuge and re-swollen in 30 μL of ammonium bicarbonate buffer containing 500 ng of sequencing grade, modified trypsin (Promega). After a 5 minute incubation on ice, 40 μL of ammonium bicarbonate buffer was added and digestion was carried out at 37 °C, overnight. Additional 250 ng of trypsin was added 2 hours prior to the extraction of peptides. Peptides were extracted once each with 60% acetonitrile/0.1% TFA and acetonitrile/0.1% TFA. All extracts were pooled and concentrated using a vacufuge to a final volume of 30 μL.

Protein identification by LC-tandem MS: Ten μL of the digest was resolved on a reverse phase column (Aquasil C18, 15 μm tip x 75 μm i.d. x 10 cm, Picofrit column, New Objectives, Woburn, MS) using acetonitrile/1% acetic acid gradient system (5-60% acetonitrile over 40 minutes followed by 95 % acetonitrile wash for 5 minutes) at a flow rate of ~300 nL/min. Eluted peptides were directly introduced into an ion-trap mass spectrometer (LTQ-XL, ThermoFisher) equipped with a nano-spray source. The mass spectrometer was operated in data-dependent MS/MS mode to acquire a full MS scan (400-2000 m/z) followed by MS/MS on the top 5 ions from the full MS scan (relative collision energy ~35%). Dynamic exclusion was set to collect 2 MS/MS spectra on each ion and exclude it for further 2 minutes. Raw files were converted to mzXML format and searched against human IPI database (v 3.41, 72,254 entries) appended with decoy (reverse) database using X!Tandem with *k-score* plug-in, an open-source search engine developed by the Global Proteome Machine (www.thegpm.org). Search parameters included a precursor peptide mass tolerance window of 2 Da and fragment mass tolerance

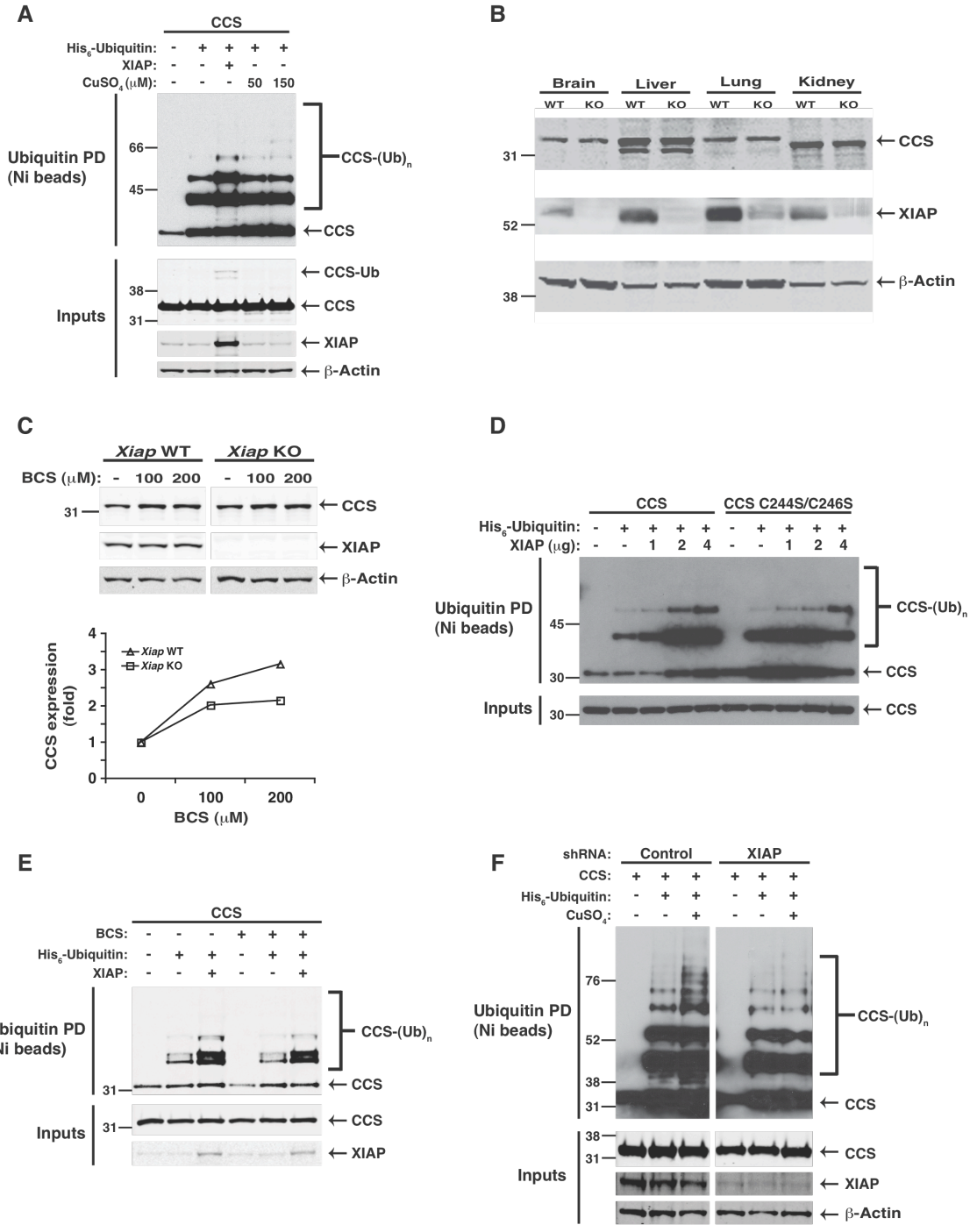
of 0.5 Da. Oxidation of methionine (+16 Da), carbamidomethylation of cysteines (+57 Da) and ubiquitination of lysines (+114 Da) were considered as variable modifications. Search was restricted to tryptic peptides with only one missed cleavage tolerated. Results of the X!Tandem search were analyzed using the Trans-Proteomic Pipeline (TPP) containing PeptideProphet (Keller et al., 2002) and ProteinProphet (Nesvizhskii et al., 2003). Proteins fulfilling a ProteinProphet probability score of >0.9 (error rate <1%) were considered *bona fide* identifications. MS/MS spectra corresponding to peptides that were identified as ubiquitinated with a PeptideProphet score of >0.95 were manually verified.

Results

XIAP induces CCS ubiquitination.

The possibility that XIAP might ubiquitinate CCS was examined by co-transfection of HEK 293 cells with CCS and human ubiquitin carrying an amino-terminal histidine tag in the absence or presence of XIAP. When CCS was expressed alone, low basal levels of CCS ubiquitination were observed, which were increased by co-transfection of XIAP (Fig. 3.1A). The XIAP-induced increase in CCS ubiquitination required the RING domain of XIAP, consistent with the hypothesis that CCS is a direct ubiquitination target of XIAP. The effect of XIAP transfection was greater than that of treating cells with excess copper, which was previously reported to result in proteasomal degradation of CCS (Bertinato and L'Abbe, 2003; Caruano-Yzermans et al., 2006). However, Ccs levels were found to be unchanged in tissues from *Xiap*-deficient mice as compared to tissues from wild-type mice (Fig. 3.1B), suggesting that XIAP is not a primary regulator of CCS stability. This is in contrast to the effect of copper, whose levels have been shown to inversely correlate with CCS levels (Prohaska et al., 2003; Bertinato et al., 2003; Bertinato and L'Abbe, 2003; Caruano-Yzermans et al., 2006). Consistent with previous reports, we found that CCS expression was enhanced in cells treated with the copper(I) chelator bathocuproine disulfonic acid (BCS), and this effect did not require XIAP (Fig. 3.1C). In addition, XIAP expression, but not copper treatment, induced the accumulation of an apparently monoubiquitinated form of CCS in the whole cell lysate (Fig. 3.1A), further suggesting that XIAP-induced ubiquitination and copper-induced ubiquitination of CCS might represent distinct ubiquitination

Figure 3.1. XIAP ubiquitinates CCS. *(A)* CCS was co-expressed in HEK 293 cells with histidine-tagged ubiquitin (Ub), and cells were treated by either co-transfecting XIAP or adding copper to the culture medium for 24 hours. Ubiquitinated material was recovered from whole cell lysates by incubation with nickel-coated beads and analyzed by anti-FLAG western blot. *(B)* The indicated tissues were harvested from C57BL/6 *Xiap*-deficient ("knockout") and wild-type mice. Tissue lysates were analyzed by SDS-PAGE and western blot with antibodies targeting CCS, XIAP, and β-actin. WT, wild-type; KO, knockout. *(C)* MEFs derived from *Xiap*-deficient (KO) and wild-type (WT) mice were treated with the copper chelator BCS for 48 hours and analyzed by SDS-PAGE and western blot as indicated. For each condition, CCS protein expression was normalized to β-Actin using the Li-Cor Odyssey software as described in Materials and Methods. CCS expression in BCS-treated cells was then quantitated relative to that in untreated cells within each genotype (wild-type and *Xiap*-deficient). *(D)* Wild-type CCS or a copper-resistant mutant (C244S/C246S) was co-expressed in HEK 293 cells with histidine-tagged ubiquitin (Ub) and increasing amounts of XIAP. Ubiquitinated material was recovered from whole cell lysates by incubation with nickel-coated beads and analyzed by anti-FLAG western blot as in *(A)*. *(E)* CCS with FLAG epitope tag was co-expressed in HEK 293T cells with histidine-tagged ubiquitin (Ub) in the presence or absence of XIAP. Cells were either left untreated or incubated with 150 μM BCS for 24 hours prior to harvest and analysis by nickel pulldown and western blot. *(F)* HEK 293 cells with stably suppressed XIAP (shXIAP) were derived by lentiviral infection as described in Materials and Methods. The shXIAP cells and control cells were tranfected with plasmids encoding human CCS and human ubiquitin, then treated with 50 μM $CuSO_4$ for 24 hours prior to analysis by nickel pulldown and western blot.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CCS
His6-Ubiquitin: - + + + +
XIAP: - - + - -
CuSO4 (μM): - - - 50 150
Ubiquitin PD (Ni beads)
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CCS-(Ub)n
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CCS-Ub
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XIAP
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B
Brain
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52
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BCS: - - - + + +
His6-Ubiquitin: - + + - + +
XIAP: - - + - - +
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CCS
XIAP
β-Actin

pathways. Indeed, XIAP was able to ubiquitinate a mutant of CCS that is insensitive to copper (C244S/C246S) with comparable efficiency to wild-type CCS (Fig. 3.1D) (Caruano-Yzermans et al., 2006). Similarly, treatment with BCS did not alter XIAP-mediated ubiquitination of CCS, suggesting that copper-free CCS may be the primary ubiquitination substrate for XIAP (Fig. 3.1E). However, both basal ubiquitination and copper-induced ubiquitination of CCS were reduced in HEK 293 cells in which XIAP expression was stably suppressed by a short hairpin RNA (Fig. 3.1F), suggesting a possible role for XIAP in copper-induced ubiquitination of CCS as well. Taken together, these data suggest that XIAP is an E3 ligase for CCS, and that the mechanism and consequences of ubiquitination may be determined by whether or not CCS is bound to copper.

Lysines 76, 189, 216, and 241 of CCS are ubiquitinated in cells.

To examine further the mechanistic basis of CCS ubiquitination, the ubiquitinated lysine residues of CCS were mapped by mass spectrometry. We utilized a two-step affinity purification scheme developed by Burstein and colleagues (G. Maine and E. Burstein, personal communication) to obtain a purified pool of ubiquitinated CCS for analysis (Fig. 3.2A). Human CCS with a carboxy-terminal biotinylation sequence tag was expressed in HEK 293 cells with histidine-tagged ubiquitin. To increase the total cellular pool of ubiquitinated CCS, untagged XIAP was co-expressed with CCS and ubiquitin, and the cells were treated with the proteasome inhibitor MG132 for four hours before lysis. A pure preparation of ubiquitinated CCS was obtained by passing the lysate

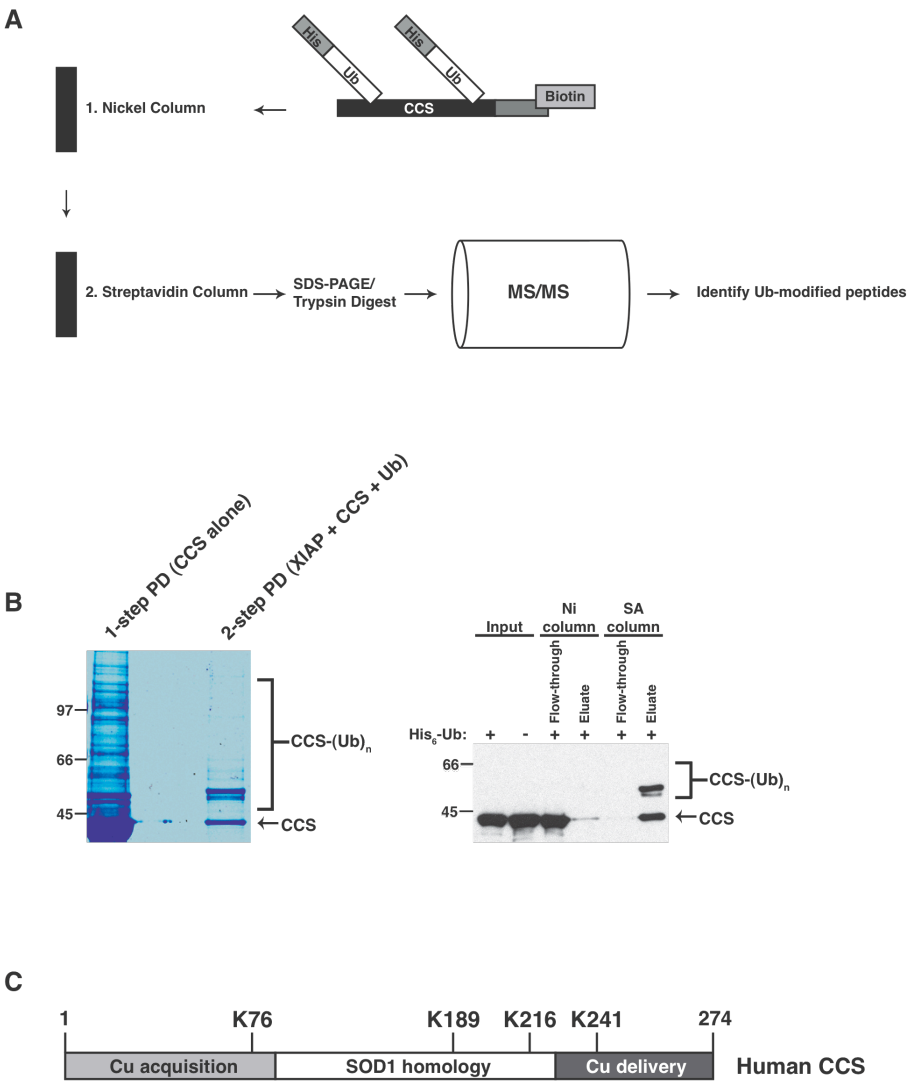


Figure 3.2. Identification of ubiquitinated lysine residues in CCS. *(A)* Ubiquitinated CCS was isolated from HEK 293 cells for mass spectrometric analysis by a 2-step purification scheme using His_6-Ubiquitin and biotinylated CCS. *(B)* Analysis of eluates from 1-step (streptavidin column only) and 2-step (nickel column followed by streptavidin column) pulldowns by SDS-PAGE and colloidal Coomassie stain (left panel) and analysis of samples before and after each step of the purification scheme by western blot with an antibody directed against CCS (right panel). *(C)* The four lysines that were identified as ubiquitinated residues by mass spectrometry are shown in the context of CCS domains 1-3.

through a nickel column followed by a streptavidin column, both in the presence of 8M urea (Fig. 3.2B). The tagged CCS construct was also expressed separately and purified with the streptavidin column to allow visualization of the unmodified CCS species. Of the seven lysines in human CCS, four were represented in the mass spectrometric data, and all four were ubiquitinated (Fig. 3.2C). Thus, while only lysines 76, 189, 216, and 241 were observed to be ubiquitinated in this study, it is possible that the remaining three lysines in CCS could be ubiquitinated as well.

XIAP preferentially ubiquitinates CCS at lysine 241.

In order to investigate the relative contributions of the four identified lysine residues to ubiquitin-dependent regulation of CCS, the four lysine sites were changed to arginines by site-directed mutagenesis. Substitution of all four lysines with arginine residues substantially reduced CCS ubiquitination overall and abrogated XIAP-mediated and copper-induced ubiquitination (Fig. 3.3A). Analysis of individual CCS lysine mutants in the absence of supplemental copper revealed that substitution of lysine 241 with arginine substantially reduced ubiquitination of CCS by XIAP compared to wild-type CCS and the other individual lysine mutants (Fig. 3.3B). A similar experiment with copper-treated cells did not reveal a preferred residue for copper-induced CCS ubiquitination (Fig. 3.3C), supporting the hypothesis that XIAP and copper act on CCS in two distinct ubiquitination pathways. Remarkably, restoration of lysine 241 in the quadruple lysine mutant of CCS rescued ubiquitination by XIAP to levels comparable to wild-type CCS (Fig. 3.3D), suggesting that lysine 241 of CCS is the preferred ubiquitination site for XIAP. Interestingly, lysine 241 is located very close to the copper-

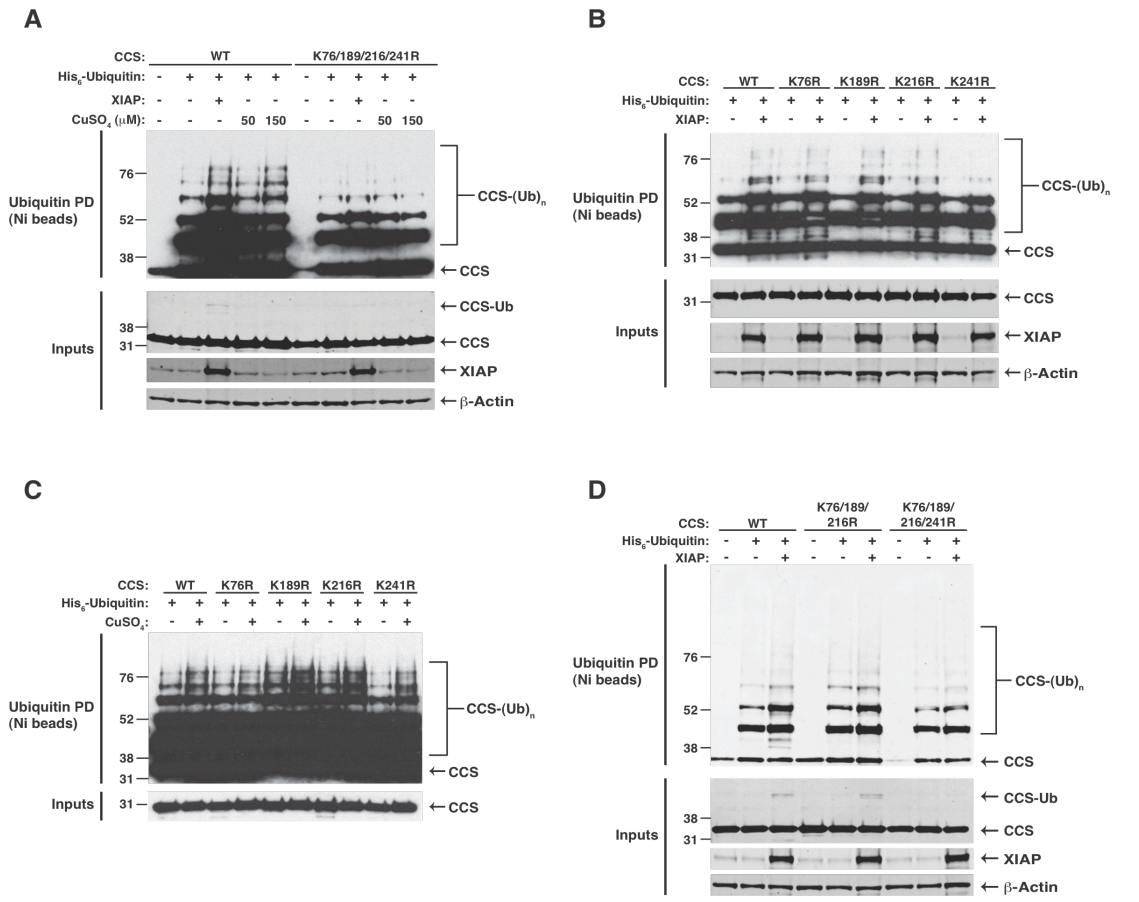


Figure 3.3. Preferential ubiquitination of CCS at lysine 241 by XIAP. *(A)* Wild-type CCS or a mutant of CCS in which lysines 76, 189, 216, and 241 were replaced with arginines was expressed in HEK 293 cells with His_6-Ubiquitin. The effects of XIAP and copper treatment on CCS ubiquitination were determined by western blot analysis of nickel pulldowns with an anti-FLAG antibody. *(B)* The effect of XIAP on wild-type CCS and individual lysine mutants was determined as in *(A)*. *(C)* Wild-type CCS and the individual lysine mutants were co-expressed in HEK 293 cells with histidine-tagged ubiquitin (Ub). Cells were either left untreated or incubated with 150 μM copper sulfate for 24 hours prior to harvest and analysis. *(D)* The effect of XIAP on the K76/189/216R mutant of CCS was compared directly to wild-type CCS and the quadruple lysine mutant of CCS as in *(A)* and *(B)*.

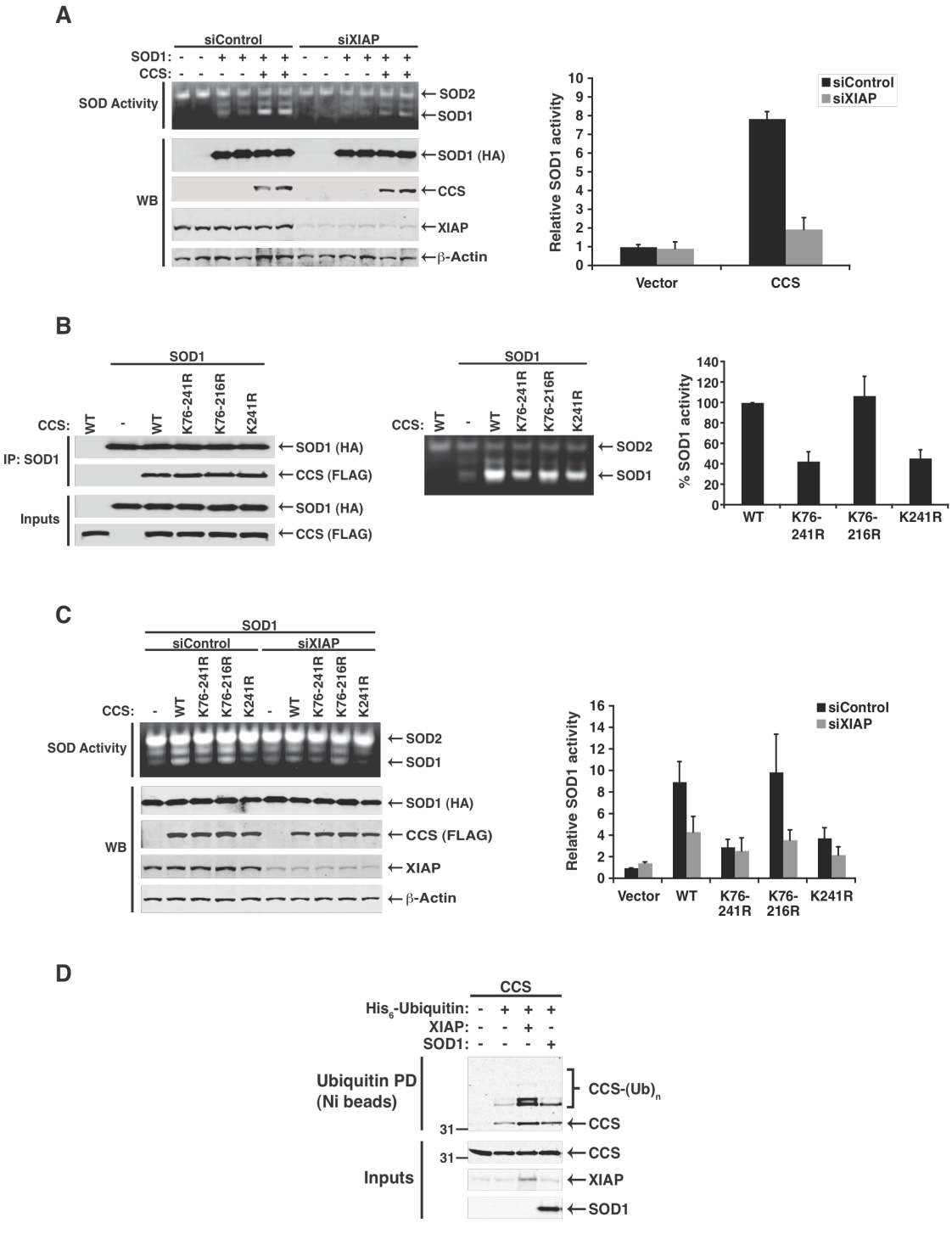
binding site in the third domain of CCS, which is required for physiologic metallation of SOD1 by CCS (Lamb et al., 2001; Schmidt et al., 1999), suggesting that XIAP-mediated ubiquitination of CCS might regulate SOD1 activation.

XIAP positively regulates activation of SOD1 by CCS.

To determine whether ubiquitination of CCS by XIAP could regulate activation of SOD1, SOD1 and CCS were co-expressed in cells in which XIAP expression was reduced by transient transfection of siRNA. Surprisingly, suppression of endogenous XIAP substantially reduced SOD1 activation, even when CCS was overexpressed (Fig. 3.4A). This finding suggests that ubiquitination of CCS by XIAP does not inhibit its activation of SOD1 or trigger its proteasomal degradation. Rather, ubiquitination of CCS by XIAP seems to be an activating event, whereby CCS is less efficient in delivering copper to SOD1 in the absence of XIAP.

Consistent with this notion, the quadruple lysine mutant of CCS (K76-241R) was substantially impaired in its ability to activate SOD1, although binding to SOD1 was unaffected (Fig. 3.4B). Substitution of lysine 241 with arginine in wild-type CCS (K241R) reproduced this defect, while restoration of lysine 241 in the quadruple mutant (K76-216R) restored efficient SOD1 activation. Although we cannot exclude an alternative role independent of ubiquitin for lysine 241 in copper delivery to SOD1, the effect of arginine substitution was blunted when XIAP expression was reduced by siRNA transfection (Fig. 3.4C), suggesting that the role of lysine 241 is as an acceptor site for an XIAP-mediated ubiquitination event that enhances SOD1 activation. In contrast, we could not detect a reciprocal effect of SOD1 on CCS ubiquitination, suggesting that the CCS-SOD1 interaction does not directly regulate CCS ubiquitination (Fig. 3.4D). Collectively, our data support a model in which XIAP-mediated ubiquitination of CCS, primarily at lysine 241, enhances the ability of CCS to activate SOD1, and SOD1 activation is reduced substantially by removal of either XIAP or lysine 241 of CCS.

Figure 3.4. XIAP-mediated ubiquitination of CCS positively regulates SOD1 activation. *(A)* Human SOD1 was expressed alone or with human CCS in HEK 293T cells. One day prior to transfection of plasmid DNA, cells were transfected with a control siRNA oligonucleotide or an oligonucleotide targeting XIAP. Cell lysates were analyzed for SOD1 activity by in-gel NBT staining, and band intensity was quantitated using ImageJ software. Data are presented as mean ± SD of duplicate transfections and are representative of several independent experiments (n = 7). *(B)* Human SOD1 was expressed in HEK 293T cells with wild-type CCS or the indicated lysine mutants of CCS. Two days after transfection, SOD1 was precipitated with an antibody directed against the HA tag, and CCS was detected in the immunoprecipitate with an anti-FLAG antibody (left panel). Whole lysates were analyzed for SOD1 activity by in-gel NBT staining and quantitated as in *(A)* (middle and right panels). *(C)* CCS and the indicated CCS mutants were expressed together with SOD1 in cells that had been transfected the previous day with a control siRNA oligonucleotide or an oligonucleotide targeting XIAP, and SOD1 activity was determined as in *(A)* and *(B)*. Data in *(B)* and *(C)* are presented as mean ± SEM of pooled data from several independent experiments (n ≥ 4), with representative SOD gels and western blots shown. In each case, SOD1 activity was determined relative to the appropriate control (ie. mock transfection or wild-type CCS) within each experiment before data were pooled. All data obtained for CCS and the CCS lysine mutants with the control oligonucleotide in *(C)* were included in the pooled data presented in *(B)*. *(D)* CCS with FLAG epitope tag was co-expressed in HEK 293T cells with histidine-tagged ubiquitin (Ub) in the presence or absence of XIAP or HA-tagged SOD1. After harvest and lysis, ubiquitinated material was recovered from whole cell lysates by incubation with nickel-coated beads, and CCS ubiquitination was analyzed by anti-FLAG western blot.


A
siControl
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SOD1:
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siControl
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Vector
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SOD1
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K76-216R
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SOD1 (HA)
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siXIAP
SOD Activity
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CCS (FLAG)
XIAP
β-Actin
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Vector
D
CCS
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31

Discussion

Copper delivery to the metalloenzyme SOD1 by CCS is essential for SOD1 activation and cellular antioxidant protection (Wong et al., 2000). Proper metallation of SOD1 is also critical to prevent its misfolding and aggregation, which is thought to be the initiating event in a large subset of familial amyotrophic lateral sclerosis (ALS) (Furukawa and O'Halloran, 2005). Apo-SOD1 is more prone to aggregation than copper-bound (holo) SOD1, and several ALS-associated mutations in SOD1 lead to impaired copper binding (Hayward et al., 2002). The role of copper delivery to SOD1 in ALS pathogenesis is unclear, however, as *Ccs* deficiency does not affect disease progression in mouse models of ALS (Subramaniam et al., 2002), whereas CCS overexpression leads to accelerated progression in some models but not in others (Son et al., 2007; Son et al., 2009). Thus, while the CCS-SOD1 interaction is critical for proper intracellular copper homeostasis and redox regulation, the mechanisms by which copper delivery to SOD1 is dynamically regulated, and how this process impacts ALS pathogenesis, are not clear. Here we describe post-translational regulation of the CCS-SOD1 interaction through ubiquitination of CCS by XIAP, which could play a regulatory role in maintaining proper folding and activation of SOD1.

Several studies have implicated dynamic post-translational regulation, including ubiquitination and phosphorylation, of copper chaperones and transporters in maintaining cellular copper homeostasis (Ooi et al., 1996; Petris et al., 2003; Liu et al., 2007; Caruano-Yzermans et al., 2006; de Bie et al., 2007; Bartee et al., 2009). A better

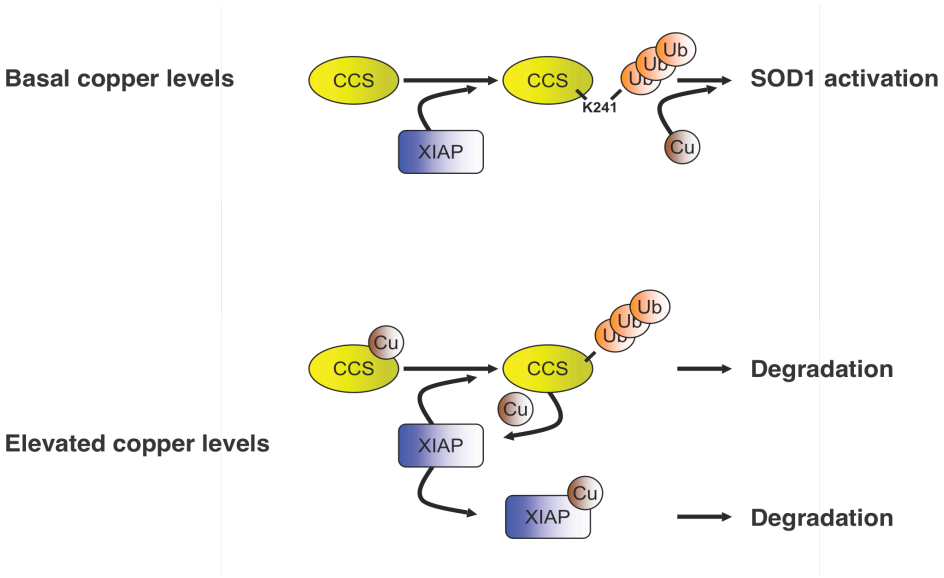

Figure 3.5. Proposed model for regulation of CCS by distinct ubiquitination pathways. Under basal conditions *(top panel)*, XIAP-mediated ubiquitination of CCS at lysine 241 enhances CCS-mediated activation of SOD1, perhaps by enhancing the ability of CCS to acquire copper. Under conditions of elevated intracellular copper *(bottom panel)*, copper-bound CCS accumulates and transfers copper to XIAP, which is then degraded. Copper-bound CCS is ubiquitinated by XIAP or other cellular E3 ligases and targeted for proteasomal degradation.

understanding of how copper trafficking from transporters to chaperones to copper-dependent proteins is dynamically regulated in response to changing cellular requirements will be essential in developing improved therapies for disorders of deregulated copper metabolism.

Post-translational regulation of CCS by ubiquitination and proteasome-dependent degradation has been described previously (Bertinato and L'Abbe, 2003; Caruano-Yzermans et al., 2006). Our data are consistent with the previous reports of copper-induced CCS degradation and suggest the existence of two distinct pathways of CCS ubiquitination (Fig. 3.5): ubiquitination of presumably copper-free CCS mediated by XIAP that leads to enhanced copper acquisition and delivery to SOD1, and ubiquitination of copper-bound CCS by XIAP or another E3 ligase that leads to CCS degradation.

Taken together, our data and other previous reports of copper-induced CCS degradation are consistent with a model in which interaction of copper-free CCS and XIAP results in non-degradative ubiquitination of CCS, whereas copper-bound CCS would transfer copper to XIAP and be ubiquitinated and targeted for proteasomal degradation.

The roles of ubiquitin and ubiquitin-like proteins in proteasome-independent regulation of protein function have become increasingly evident in recent years (Aguilar and Wendland, 2003; Chen and Sun, 2009; Hochstrasser, 2009). Addition and removal of monoubiquitin and various types of ubiquitin chains play a critical role in dynamic regulation of many intracellular processes and signaling events. Here we describe a novel role for the ubiquitin system in regulation of intracellular redox metabolism through XIAP, CCS, and SOD1. The mechanism by which ubiquitination of CCS might enhance its ability to deliver copper to SOD1 is unclear, but it seems to be independent of CCS-SOD1 binding. Instead, ubiquitination of CCS might trigger its trafficking to upstream source(s) of copper or modify its affinity for copper, thereby enhancing its acquisition or release of copper to SOD1. Regardless of the precise mechanism by which ubiquitination enhances CCS activity, the effect of lysine 241 ubiquitination on CCS function provides new insights into dynamic regulation of transition metal metabolism and the wide reach of the ubiquitin system in maintaining intracellular homeostasis.

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Chapter IV

Conclusions

Since their initial characterization as homologs of baculovirus IAPs, it has becoming increasingly clear that mammalian IAP proteins perform many cellular roles independent of apoptosis regulation. In particular, while XIAP is a potent caspase inhibitor and can protect cells from a variety of apoptotic stimuli, it also participates in multiple cellular signaling pathways.

In this dissertation I have focused on the role of XIAP in intracellular copper homeostasis. Evidence for a role of XIAP in copper metabolism first emerged via the identification of COMMD1 as a target of the E3 ubiquitin ligase activity of XIAP (Burstein et al., 2004). XIAP was subsequently found to bind copper directly and to be deregulated in a number of copper toxicosis disorders in humans and canines (Mufti et al., 2006). However, the mechanism by which XIAP acquires copper *in vivo* remains elusive, as intracellular copper is tightly regulated so that free copper is unavailable to bind to proteins (Rae et al., 1999).

Through a genetic screen in *S. cerevisiae* designed to identify proteins that play a role in delivering copper to ectopically expressed human XIAP, I identified yeast Ccs1 as

a major regulator of XIAP-copper binding. I went on to find that CCS played an important role in delivering copper to endogenous XIAP in mammalian cells. Furthermore, I found that XIAP and CCS can physically interact in human cells, and that XIAP expression induces ubiquitination of CCS. XIAP-mediated ubiquitination of CCS was found to proceed via a mechanism distinct from copper-induced CCS ubiquitination and to occur primarily at lysine 241 of CCS. Interestingly, I found that suppression of XIAP expression using RNAi decreased CCS-mediated activation of SOD1, suggesting that ubiquitination of CCS by XIAP was required for efficient SOD1 metallation. Indeed, substitution of CCS lysine 241 with arginine produced a similar defect in SOD1 activation in control cells but did not further reduce SOD1 activation in cells in which XIAP expression was suppressed. Collectively, these data support a model in which XIAP and CCS reciprocally regulate one another post-translationally to cooperatively modulate cellular copper homeostasis and redox metabolism.

CCS-mediated copper delivery to XIAP

In chapter II of this dissertation I utilized a yeast genetic screen to identify a novel physical and functional interaction between CCS and XIAP in mammalian cells. I found that yeast and murine cells lacking *ccs1* and *Ccs*, respectively, exhibited a defect in copper delivery to XIAP. In human cells, RNAi-mediated suppression of CCS decreased copper delivery to XIAP, whereas overexpression of CCS led to enhanced copper delivery. Taken together, these data suggest that CCS mediates copper delivery to XIAP *in vivo*.

Interestingly, in the absence of CCS copper can be delivered to XIAP via an alternative pathway. While this does not change my conclusions with regard to the role of CCS, it is interesting and would be consistent with previous studies demonstrating CCS-independent metallation of SOD1 (Carroll et al., 2004; Jensen and Culotta, 2005; Carroll et al., 2006). The mechanism of CCS-independent copper delivery to XIAP is unclear but could potentially proceed via reduced glutathione as was demonstrated for SOD1. Indeed, XIAP has been shown to bind to copper-bound glutathione *in vitro* (Mufti et al., 2006), suggesting that glutathione could deliver copper to XIAP in cells lacking CCS.

The physiologic importance of CCS-dependent and -independent copper delivery to XIAP is unclear, but copper-mediated degradation of XIAP has been suggested to play a role in the pathophysiology of copper toxicosis disorders (Mufti et al., 2006; Mufti et al., 2007). Therapeutic targeting of the XIAP-CCS interaction to preserve XIAP expression might be useful in combination with copper-lowering therapy for human Wilson disease, either via drugs that specifically prevent association of XIAP and CCS or drugs that antagonize CCS. It might be possible to reduce CCS expression by designing small molecules that would bind to CCS and induce a conformational change similar to that triggered by copper binding, thereby causing CCS to be ubiquitinated and degraded (Caruano-Yzermans et al., 2006; Kim et al., 2008). As described in Chapter II of this dissertation, XIAP does not seem to compete effectively with SOD1 for copper-bound CCS, and copper delivery to XIAP seems to be an inefficient process compared to SOD1 metallation. Therefore, a therapeutic window may exist whereby targeting of CCS could preserve expression of copper-free XIAP while still allowing for activation of SOD1.

XIAP-mediated ubiquitination of CCS

In Chapter III of this dissertation I found that coexpression of XIAP induced ubiquitination of CCS, suggesting that XIAP could regulate activation of SOD1 by CCS. I found that XIAP-mediated ubiquitination of CCS could be distinguished from copper-induced CCS ubiquitination in three ways: dependence on CCS lysine 241, accumulation of ubiquitinated CCS in the absence of proteasome inhibition, and accumulation of an apparently monoubiquitinated species of CCS in the whole cell lysate. In addition, XIAP readily ubiquitinated a variant of CCS that is insensitive to copper-induced ubiquitination (Caruano-Yzermans et al., 2006).

Surprisingly, I found that ubiquitination of CCS by XIAP enhanced CCS-mediated activation of SOD1. Substitution of CCS lysine 241 reduced activation of SOD1 by >50%, similar to the effect of XIAP suppression by RNAi. The effects of XIAP suppression and loss of lysine 241 were not additive; in fact, the effect of lysine 241 substitution was negligible in the setting of XIAP suppression. These results, together with the observation that CCS K241R could still bind to SOD1 and be ubiquitinated in response to copper treatment, suggest that substitution of arginine for lysine 241 does not substantially alter the structure of CCS or its copper-binding site. Instead, these data support a model in which lysine 241 serves as an acceptor site for XIAP-mediated ubiquitination. This ubiquitination event enhances the ability of CCS to deliver copper to SOD1 through a mechanism that remains unclear. Ubiquitinated CCS could be better able to acquire copper or to transfer copper to SOD1 than unmodified CCS. For example, ubiquitinated CCS might be targeted to acquire copper directly from

the copper importers CTR1 and CTR2. The ubiquitin moiety conjugated to CCS via lysine 241 might facilitate docking with the copper transporter itself or with a transporter-associated adaptor protein in a manner analogous to the well-described role of ubiquitin chains as scaffolds for assembly of multiprotein complexes in NF-κB signaling. Alternatively, ubiquitination at lysine 241 might facilitate positioning of the CCS copper-binding site for transfer of copper after CCS has docked with SOD1 via its SOD1 homology domain. Regardless of the precise mechanism, XIAP-mediated ubiquitination of CCS seems to enhance SOD1 activation and may be one mechanism by which efficient SOD1 activation is maintained when copper is relatively scarce.

XIAP as a sensor for copper and copper chaperone activity

The previous studies describing XIAP as a regulator of copper homeostasis and copper-binding protein, together with the data presented in this dissertation, suggest a role for XIAP as a cellular copper sensor. COMMD1 stimulates copper export via ATP7A/B, and under normal circumstances its expression is limited by XIAP-mediated ubiquitination (Burstein et al., 2004; Maine et al., 2009). When copper levels rise, the cytosolic pool of apoSOD1 is depleted, allowing copper-bound CCS to accumulate and transfer copper to XIAP. Copper-bound XIAP is destabilized, allowing COMMD1 to accumulate and stimulate increased copper export (Fig. 4.1). In this model, cellular copper levels are controlled through the CCS-XIAP-COMMD1 axis. In the setting of Wilson disease or canine *Commd1* deficiency, copper export is compromised, leading to depletion of XIAP and sensitization to apoptotic stimuli.

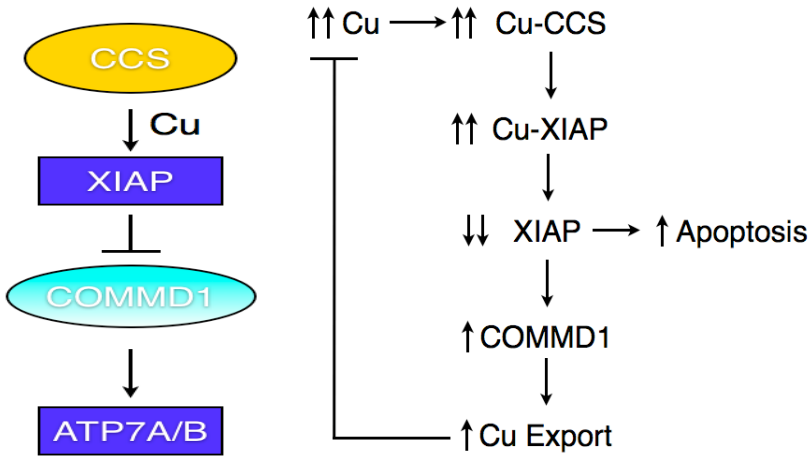

Figure 4.1. Regulation of copper homeostasis through the CCS-XIAP-COMMD1 axis. The schematic on the left shows the relationship among CCS, XIAP, and COMMD1 in cellular copper metabolism. In the absence of COMMD1 or ATP7B, copper-mediated degradation of XIAP does not lead to increased copper export but instead triggers depletion of XIAP and lowering of the apoptotic threshold.

Interestingly, the available evidence suggests an additional level of regulation through ubiquitination of CCS. Under conditions of relative copper deficiency, the majority of cellular CCS is thought to be copper-free (Caruano-Yzermans et al., 2006). In this setting, interaction of CCS with XIAP would not result in copper transfer to XIAP but would instead result in ubiquitination of CCS as described in Chapter III of this dissertation. I propose that binding to copper-free CCS is interpreted as a sign of relative copper deficiency, and thus this is an activating ubiquitination event that enhances copper delivery to SOD1 by CCS (Fig. 4.2). Copper-free XIAP then remains stable and constitutively targets COMMD1 for proteasomal degradation to keep copper export rates low. Conversely, under conditions of elevated intracellular copper, copper-bound CCS accumulates and transfers copper to XIAP, targeting XIAP for degradation and allowing COMMD1 levels to rise and stimulate copper export. Copper-bound CCS is also targeted

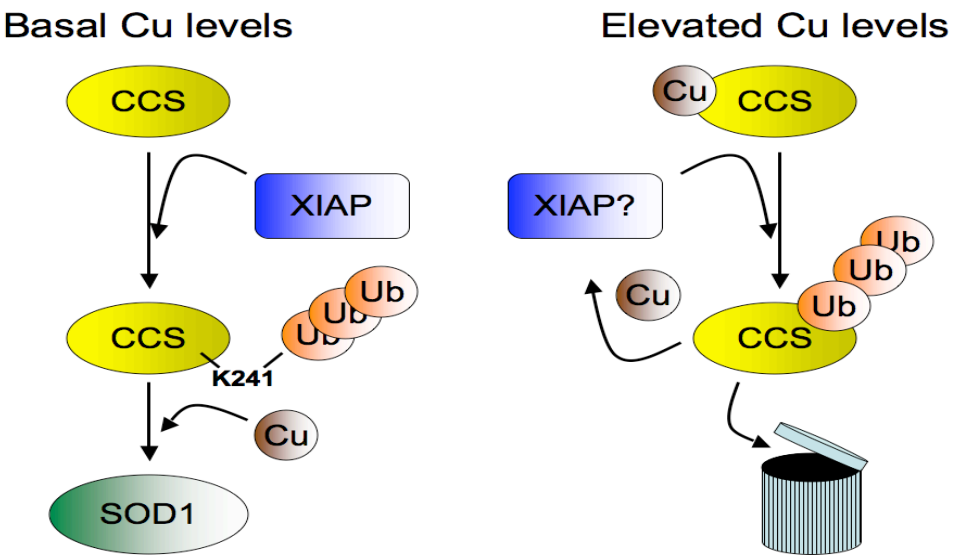

Figure 4.2. CCS is regulated by distinct ubiquitination pathways. Under usual circumstances, the majority of CCS in the cytosol is copper-free. Copper-free CCS is ubiquitinated by XIAP at lysine 241, enhancing its ability to acquire and deliver copper to SOD1. When cellular copper is elevated, the cytosolic pool of apo-SOD1 is depleted, and copper-bound CCS accumulates. CCS transfers copper to XIAP and is ubiquitinated and targeted for proteasomal degradation, possibly by XIAP or another E3 ligase.

for degradation, although it is not clear whether XIAP is the primary E3 ligase for this ubiquitination event. Degradation of copper-bound CCS by XIAP or another E3 might serve to limit copper delivery to XIAP and allow accumulation of copper-free XIAP as the cell stabilizes copper levels through COMMD1 and ATP7A/B. An integrated view of the roles of CCS, XIAP, and COMMD1 in cellular copper homeostasis is presented in Fig. 4.3.

Roles and regulation of IAPs

It is increasingly evident that IAP proteins perform many important roles in maintaining cellular homeostasis outside of regulating apoptosis. An emerging theme in

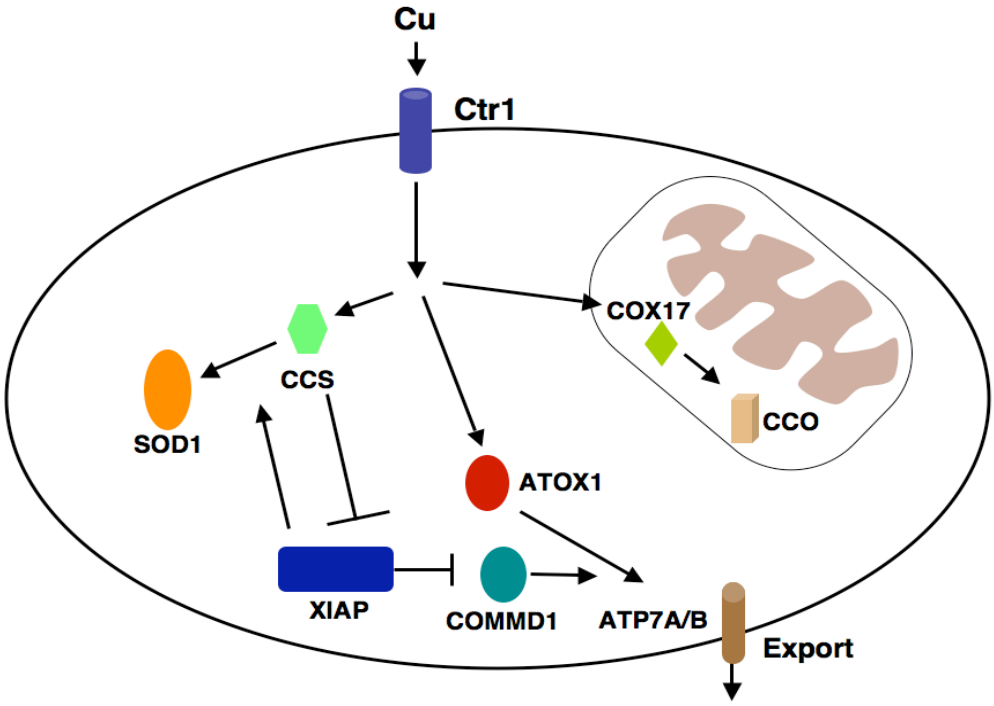

Figure 4.3. An integrated view of the role of XIAP in copper homeostasis. XIAP participates in the post-translational regulation of CCS and COMMD1, and is in turn regulated by CCS-mediated copper delivery.

the IAP field is protein-protein interactions with many partners via the shared BIR domains and regulation of interacting proteins via RING-mediated ubiquitination. Many interaction partners are common to several IAP family members because of the high degree of homology among analogous BIR domains of different IAP proteins. It is likely that many important functions can be carried out by several IAP proteins, such that loss of one IAP does not necessarily produce an overt phenotypic defect. A better understanding of the cellular roles of IAPs and their relative contributions will be invaluable as IAP-targeting therapies are developed for the treatment of malignancies and other diseases.

The work presented in this thesis advances and provides mechanistic insight into the previous description of XIAP as a copper-binding protein. Destabilization of XIAP by binding to copper is just one example of the many different modes of post-translational regulation of IAPs. Most prominent among these is autoubiquitination by RING-containing IAPs (Yang et al., 2000). Curiously, while the c-IAPs are unstable proteins that are greatly stabilized by mutation of their RING domains (Csomos et al., 2009b; Csomos et al., 2009a), the significance of XIAP autoubiquitination is less clear, as RING-mutated XIAP is not stabilized relative to wild-type XIAP (S. Galbán, G. Brady, and C. Duckett, unpublished data). Post-translational regulation of XIAP may be rather more complex and seems to be dynamically regulated by, among other factors, metal ion availability and reactive oxygen species rather than constitutive autoubiquitination (Makhov et al., 2008; Tsang et al., 2009).

Closing remarks

In this dissertation I have attempted to elucidate the mechanistic basis by which XIAP participates in regulation of intracellular copper homeostasis. The mechanism by which XIAP acquires copper in the cytosol is still unclear but seems to rely primarily on the cytosolic copper chaperone CCS. Although all BIR domains and the RING of XIAP can bind to copper ions *in vitro*, the relevant copper-binding site(s) *in vivo* is unknown. Ubiquitination of CCS at lysine 241 by XIAP would suggest that copper could be transferred from domain 3 of CCS to the RING of XIAP, but this hypothesis has not been tested experimentally.

Regulation of CCS by XIAP-mediated ubiquitination suggests a mechanism by which efficient SOD1 activation could be maintained under conditions of adequate copper and relative copper deficiency. It also illustrates the wide variety of processes regulated by the ubiquitin system and by IAP proteins through their RING domains. As IAPs are actively studied as therapeutic targets in human disease, a fuller understanding of their cellular roles and relative contributions to cellular homeostasis will be critical in order to achieve the desired treatment outcomes while minimizing undesirable side effects.

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Appendix A

Biochemical Screen for CCS Interacting Proteins

Summary

In Chapters II and III I described a novel physical and functional interaction between XIAP and the copper chaperone CCS, whereby CCS transfers copper to XIAP and is itself ubiquitinated by XIAP. This ubiquitination event enhances activation of SOD1 by CCS; however, how ubiquitination might affect CCS activity is not clear. One possibility is that XIAP might enhance acquisition of copper by CCS by altering its trafficking patterns or affinity for upstream copper source(s). To explore this possibility, we conducted a biochemical screen for proteins that interact with CCS. Here we report several novel CCS interacting partners, including several proteins involved in formation and breakdown of ubiquitin chains. These findings support our hypothesis that ubiquitination and deubiquitination of CCS may play an important role in dynamic regulation of CCS activity to meet changing cellular requirements for SOD1 activity. Precisely how ubiquitination modulates CCS activity remains unclear and is an exciting area for future study.

Introduction

In Chapters II and III I described a novel physical and functional interaction between XIAP and CCS. Under conditions of elevated copper levels, CCS transfers copper to XIAP. Copper binding to XIAP leads to its degradation and sensitization of cells to apoptosis (Mufti et al., 2006). Conversely, XIAP reciprocally regulates CCS via its E3 ubiquitin ligase activity, specifically ubiquitinating CCS at lysine 241; this ubiquitination event does not seem to result in proteasomal degradation of CCS, but rather to enhancement of copper delivery by CCS to SOD1. However, how CCS-mediated metallation of SOD1 might be enhanced by ubiquitination is not clear.

CCS-mediated metallation and activation of SOD1 consists of four principle steps: (1) copper acquisition by CCS, (2) docking of CCS to SOD1, (3) copper transfer from CCS to SOD1, and (4) CCS-catalyzed formation of an intramolecular disulfide bond within SOD1. While XIAP-mediated ubiquitination of CCS could potentially regulate any of these four components, we could not detect an effect of mutating the lysine residue within CCS that is preferentially targeted by XIAP (lysine 241) to arginine on binding of CCS to SOD1 (Chapter III). Therefore, it seems unlikely that the effect of XIAP is via enhancement of CCS docking to SOD1. Similarly, while the final steps in the process (copper transfer and disulfide bond formation) could potentially be enhanced by ubiquitination of CCS, we could not detect an effect of copper chelation on XIAP-mediated CCS ubiquitination, suggesting that copper binding to CCS is not required for ubiquitination by XIAP and that copper-bound CCS is not the primary target for XIAP. Therefore, we hypothesize that ubiquitination of CCS by XIAP is likely to occur early in

the SOD1 activation process, before acquisition of copper by CCS; indeed, others have hypothesized that under basal copper conditions the vast majority of CCS in the cell is copper-free (Caruano-Yzermans et al., 2006). We reasoned that XIAP-mediated ubiquitination of CCS could enhance its ability to acquire copper by altering its intracellular trafficking or enhancing its affinity for upstream copper sources such as CTR1. However, a very limited number of CCS-interacting proteins have been reported, none of which would be likely to participate in ubiquitin moiety-mediated trafficking of CCS. To explore the possibility that other proteins might interact with CCS and participate in ubiquitin-mediated trafficking, we conducted an unbiased biochemical screen to identify CCS-associated proteins.

Materials and Methods

Cell culture, plasmids, and transfections: HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine and maintained at 37 ˚C in an atmosphere of 95% air, 5% CO_2. Plasmids pEBB, pEBB-cTB, and pEBB-CCS-cTB are described in Chapters II and III. Cells were transfected with plasmids using a standard calcium phosphate transfection protocol.

Purification of CCS-associated proteins: HEK 293T cells were seeded in 15-cm dishes and, after 24 hours, transfected with 10 μg of either pEBB-CCS-cTB or empty vector (pEBB-cTB). After 18 hours the culture medium was supplemented with 4 μM biotin (Sigma) to optimize biotinylation of the CCS bait protein. One day later, cells were washed with PBS and split for lysis and protein purification under two different conditions: (1) native conditions and (2) protein cross-linking with formaldehyde and denaturing conditions (Tagwerker et al., 2006). Native lysis was performed on ice using Triton X-100 lysis buffer as described in Chapter II. For denaturing conditions, cells were treated with 1% formaldehyde in DMEM for 10 minutes at room temperature prior to lysis, in order to cross-link associated proteins in an attempt to capture proteins transiently associated with CCS. The cross-linking reaction was quenched by addition of 125 mM glycine for 5 minutes. The cells were then washed with PBS and lysed under denaturing conditions with lysis buffer containing 8M urea as described in Chapter III. CCS-associated proteins were purified by incubation with streptavidin-coated agarose beads (Invitrogen) essentially as described in Chapter III. For purification under native conditions, all wash steps were performed with Triton X-100 lysis buffer; for denaturing

conditions, initial washes were performed with buffer containing 8M urea, with the final wash performed in buffer containing 50 mM Tris pH 8.0, 0.5 mM EDTA, and 1 mM DTT. For all samples, the beads were resuspended in loading buffer containing LDS and DTT prior to loading into NuPAGE 4-12% Bis-Tris gels for Coomassie staining and mass spectrometric analysis as described in Chapter III. To reduce the impact of artifacts associated with the epitope tag, a control purification and mass spectrometric analysis with empty vector was conducted for each of the purification conditions. Proteins present in the empty-vector control sample were subtracted from the experimental data sets prior to analysis.

Cluster analysis of CCS-associated proteins: Cluster analysis of CCS-associated proteins according to Gene Ontology (GO) functional classification was performed using the web-based DAVID software tool (http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003).

Results and Discussion

Given the effect of XIAP-mediated ubiquitination of CCS on copper delivery by CCS to SOD1, we hypothesize that the ubiquitination status of CCS might affect its intracellular trafficking by modulating its relative affinity for interaction partners. However, relatively few CCS-interacting proteins have been described. We conducted an unbiased biochemical screen to identify proteins interacting with CCS that might participate in CCS trafficking and ubiquitin-dependent regulation of CCS activity.

Given the relative paucity of known CCS-interacting proteins, we reasoned that some interactions with CCS might be transient and/or low-affinity interactions. To increase the sensitivity of our study for detection of transient interactions, we performed a biochemical screen for CCS-interacting proteins using two different purification schemes. Cells were transfected with epitope-tagged CCS and either (1) harvested and lysed under native conditions to preserve stable CCS-containing complexes or (2) cross-linked with formaldehyde to preserve transient or unstable complexes, then lysed under denaturing conditions as described in Materials and Methods. After SDS-PAGE and trypsin digestion, CCS-associated proteins were collected via affinity purification and subjected to mass spectrometric analysis for identification (Fig. A1).

Proteins purified with CCS were subjected to cluster analysis using the web-based DAVID software tool and grouped according to Gene Ontology (GO) functional classification, the results of which are shown in Tables A1 and A2. Unique sets of proteins were isolated with CCS under the two different purification conditions, and the overall number of proteins recovered after cross-linking and denaturing was greater than

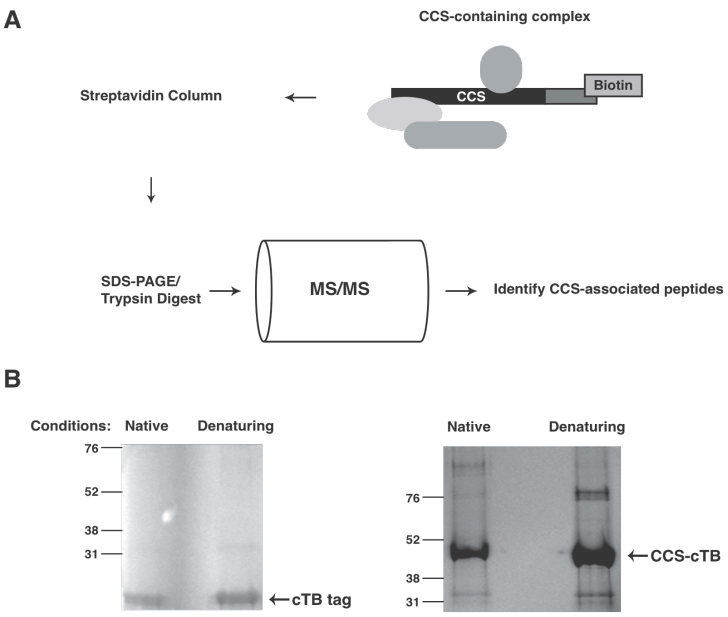

Figure A.1. Identification of CCS-associated proteins. *(A)* CCS-associated proteins were recovered from HEK 293T cells for mass spectrometric analysis by an affinity purification scheme using biotinylated CCS and streptavidin-coated beads. Analysis of pulldowns by SDS-PAGE and colloidal Coomassie stain is shown in *(B)*. The left panel shows the cTB tag expressed alone and purified under native (Triton buffer) and denaturing (urea buffer) conditions; expression and purification of tagged CCS is shown in the right panel.

that recovered under native conditions. Under both conditions, several proteins involved in ubiquitin conjugation and regulation of ubiquitin ligase activity were purified with CCS. Proteins involved in ubiquitin conjugation were particularly enriched in the denatured data set ($p < 0.003$), while those involved in regulation of ubiquitin ligase activity were highly enriched in the native data set ($p < 0.0002$). Other prominent classifications enriched in both data sets included cellular stress response, regulation of apoptosis, and proteins present in the nuclear lumen or involved in nucleotide binding. A particularly strong association was found with proteins involved in macromolecule catabolism ($p < 0.000003$), which was unique to the denatured purification scheme.

Table A1. Functional Clusters – Proteins associated with CCS (denaturing conditions).

GO Functional Classification	Number of Proteins	Enrichment Score	p-value
cellular macromolecule catabolic process	22	3.59	0.0000024
ubl conjugation pathway	12	3.59	0.0023
nuclear lumen	27	2.87	0.00026
nucleotide binding	35	2.73	0.0042
cellular response to stress	17	2.51	0.000059
enzyme binding	16	2.07	0.00016
regulation of ubiquitin-protein ligase activity	4	1.28	0.035
regulation of apoptosis	12	0.76	0.12
metal ion binding	26	0.01	1.0

Table A2. Functional Clusters – Proteins associated with CCS (native conditions).

GO Functional Classification	Number of Proteins	Enrichment Score	p-value
nuclear lumen	18	3.11	0.005
nucleotide binding	20	2.44	0.0011
regulation of ubiquitin-protein ligase activity	6	2.42	0.00016
ubl conjugation	7	1.23	0.089
response to oxidative stress	3	0.9	0.29
regulation of apoptosis	8	0.46	0.28
metal ion binding	26	0.12	0.67

Overall, these data suggest that the cellular role of CCS might be more complex than previously thought and provide interesting avenues for future study.

A selection of specific proteins purified with CCS is shown in Table A3. As expected, SOD1 and XIAP were both isolated with CCS; the latter was only present in the cross-linked sample, suggesting that the XIAP-CCS interaction may be transient. Interestingly, many of the other proteins purified with CCS were proteins involved in assembly and disassembly of ubiquitin chains. Particularly highly represented was ubiquitin C-terminal hydrolase L1 (six unique peptides), a deubiquitinating enzyme that

has recently been linked to both lymphoma and Parkinson's disease (Hussain et al., 2010; Maraganore et al., 2004; Liu et al., 2009).

To our knowledge this is the first biochemical screen conducted to identify proteins that interact with CCS. Collectively, our data reveal a large set of novel CCS interaction partners, which are involved in a wide variety of cellular processes. Notably

Table A3. Selected proteins associated with CCS.

Protein Name	Conditions	# of peptides
Superoxide dismutase-1	Both	11
Ubiquitin C-terminal hydrolase L1	Denaturing	6
Parkinson disease 7	Native	5
NEDD8-conjugating enzyme Ubc12	Denaturing	4
Peroxiredoxin-1	Native	4
Peroxiredoxin-5	Denaturing	3
Fragile X protein 1	Denaturing	2
Ubiquitin-conjugating enzyme E2 K	Denaturing	2
Ubiquitin C-terminal hydrolase 5	Denaturing	1
Ubiquitin thioesterase OTU1	Denaturing	1
X-linked inhibitor of apoptosis	Denaturing	1

absent in this data set is a viable candidate as an upstream copper source for CCS such as CTR1. This could be because our experimental conditions were not sufficiently sensitive to detect a CCS-CTR1 interaction; alternatively, CCS might acquire copper through an intermediate such as glutathione rather than interacting directly with CTR1.

The substantial number of proteins in this data set that are involved in assembly and disassembly of ubiquitin chains is intriguing and suggests the possibility that dynamic ubiquitination and deubiquitination might play a role in regulation of CCS. This

would be consistent with our finding that XIAP-mediated ubiquitination of CCS substantially enhanced CCS activity (Chapter III), although it remains unclear exactly how CCS activity might be regulated by ubiquitination. Further study will be necessary to validate the CCS interaction partners described here and determine the mechanism by which ubiquitination modulates acquisition and/or delivery of copper by CCS.

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Appendix B

TRAF2 Stabilizes c-IAP1 and Protects Cells from Apoptosis

Summary

The cellular inhibitor of apoptosis (c-IAP) proteins play critical roles in receptor-mediated signaling to NF-κB through association with TNF receptor associated factor (TRAF) proteins. However, the roles of the c-IAP-TRAF interaction in regulation of apoptosis remain unclear. Here we report that coexpression of TRAF2 stabilizes c-IAP1 by inhibiting its autoubiquitination. Stabilized c-IAP1 potently protects cells against both intrinsic and extrinsic apoptotic stimuli. Interestingly, we found that TRAF2-mediated stabilization of c-IAP1 did not require a direct physical interaction, suggesting that these two RING-containing E3 ubiquitin ligases may compete for common components of the cellular ubiquitination machinery.

Introduction

The cellular inhibitor of apoptosis (c-IAP) proteins were originally identified via a biochemical screen as proteins associated with the type 2 TNF receptor (TNFR2) (Rothe et al., 1995). As described in Chapter I of this dissertation, c-IAPs play important roles in signaling to both canonical and non-canonical NF-κB pathways through RING-mediated ubiquitination of RIP1, NEMO, NIK, and perhaps other signaling molecules. However, through constitutive autoubiquitination the c-IAP proteins are kept at low levels even when expressed ectopically in cultured cells (Clem et al., 2001; Csomos et al., 2009). Thus, mechanistic study of the roles of c-IAPs in signaling and apoptosis has proved difficult.

Here we report that forced expression of TRAF2 stabilized c-IAP1 post-translationally in a manner similar to disruption of the c-IAP1 RING domain. Stabilized c-IAP1 potently protected cells from apoptosis induced by either extrinsic or intrinsic stimuli. Interestingly, stabilization of c-IAP1 did not require direct interaction with TRAF2, suggesting that TRAF2 may compete with c-IAP1 for a common component of the ubiquitination machinery rather than directly inhibiting c-IAP1 autoubiquitination. We also found that the roles of c-IAP1 in blocking apoptosis via these two pathways could be distinguished by the requirement for the c-IAP1 RING domain, suggesting that c-IAP1 can inhibit apoptosis via at least two distinct mechanisms. Collectively, these studies provide valuable insights into the mechanisms of post-translational regulation of c-IAP1 and c-IAP1-mediated protection from apoptosis.

Materials and Methods

Plasmids: pEBB HA-c-IAP1 was subcloned from pEBG c-IAP1 (Duckett et al., 1998). Site-directed mutagenesis to generate c-IAP1 H588A and c-IAP1 E64A/R65A was performed using the QuikChange site-directed mutagenesis kit (Stratagene). To generate c-IAP1 ΔBIR1, an internal *Bam HI* site was used to subclone the c-IAP1 coding sequence lacking nucleotides 1-363 (corresponding to residues 1-121) into pEBB-HA. Unless otherwise noted, other plasmids and all siRNA oligonucleotides used for this study have been described previously (Duckett et al., 1998; Duckett and Thompson, 1997; Wilkinson et al., 2004).

Cell Culture and Transfections: HEK293 cells and MEFs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine and maintained at 37 ˚C in an atmosphere of 95% air, 5% CO_2. Cells were transfected with plasmids and siRNA oligonucleotides using a standard calcium phosphate transfection protocol with one exception: MDA-MB-231 cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer's instructions.

Caspase Activity Assays: Adherent and floating cells were harvested and caspase-3 assays were performed using the Caspase assay kit (BIOSOURCE) according to the manufacturer's instructions. AFC released was measured over time at 37 ˚C using a Cytofluor 4000 multi-well plate reader (Applied Biosystems). A total of 20 measurements at 3 minute intervals were taken for AFC release with an excitation wavelength = 400 nm and an emission wavelength = 508 nm.

Cell Lysate Preparation and Immunoblot Analysis: Cell lysates were prepared with RIPA lysis buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors, for 30 min on ice to ensure complete lysis unless noted otherwise. Protein quantification was determined by Bradford assay (Bio-Rad). RIPA cell lysates of equal protein concentrations were prepared in LDS sample buffer, separated on denaturing NuPAGE 4-12% polyacrylamide gradient gels, and transferred to 0.45 μm nitrocellulose membranes (Invitrogen). Membranes were blocked in 5% milk in TBS, followed by incubation with the indicated antibodies in 2.5% milk with 0.02 - 0.2% Tween 20 (Bio-Rad) for 1 hour at room temperature or overnight at 4 ˚C. After washing with TBS containing 0.02 - 0.2% Tween, membranes were incubated with secondary antibodies for 1 hour at room temperature. Western blots were developed by scanning on the Li-Cor Odyssey infrared scanner or with enhanced chemiluminescence (GE Healthcare) and Kodak XAR film after incubation with the relevant secondary antibodies.

Immunoprecipitations: Cell lysates were prepared in RIPA lysis buffer, normalized for protein content, and incubated with an anti-HA antibody for 2 hours at 4 ˚C. Protein G-coupled agarose beads were then added to the lysates and incubated for an additional 1 hour. Centrifugation was performed to recover agarose beads, followed by washing in RIPA lysis buffer. Precipitated proteins were eluted by adding LDS sample buffer (Invitrogen) and heating the samples for 10 min at 95 ˚C. Recovered proteins were subsequently separated by electrophoresis, and immunoblot analysis was performed as described above.

Results

TRAF2 enhances c-IAP1 expression.

The association of c-IAP proteins with TNFR superfamily members through interaction with TRAFs is well-established, but the effects of TRAF proteins on c-IAP expression and c-IAP-mediated apoptosis inhibition remain unclear. We hypothesized that association with TRAF proteins might affect the post-translational regulation of c-IAP1. Remarkably, we found that c-IAP1 was nearly undetectable in *TRAF2*-deficient MEFs (Fig. B.1A), suggesting that the presence of TRAF2 enhances c-IAP1 expression. Consistent with this finding, we observed that suppression of TRAF2 expression in human cells produced a similar reduction in c-IAP1 expression (Fig. B.1B and B.1C).

To assess whether TRAF2 affected c-IAP1 expression at the level of protein or mRNA, the two proteins were expressed together ectopically. Consistent with the results observed with the endogenous proteins, coexpression of TRAF2 greatly enhanced expression of c-IAP1 in a manner that was comparable to disruption of the c-IAP1 RING domain (Csomos et al., 2009). Importantly, we found that TRAF2 expression did not increase the levels of c-IAP1 mRNA, suggesting that the effect of TRAF2 on c-IAP1 was post-translational.

Stabilization of c-IAP1 does not require interaction with TRAF2.

As the effect of TRAF2 expression was similar to the effect of disruption of the c-IAP1 RING, we hypothesized that TRAF2 might directly inhibit c-IAP1 autoubiquitination. We generated mutants of c-IAP1 that have been previously shown to

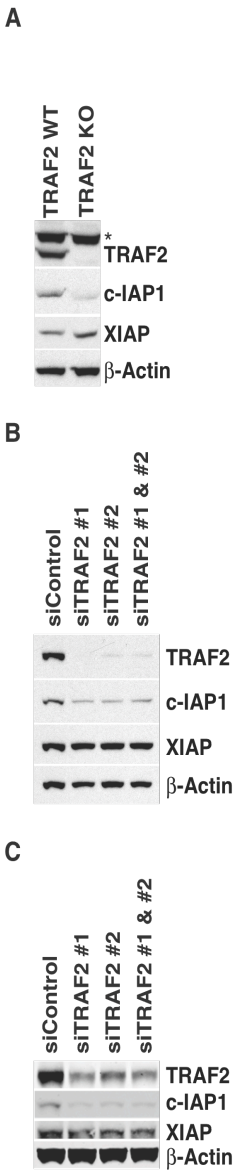

Figure B.1. Reduction of c-IAP1 in TRAF2-deficient cells. (*A)* TRAF2-deficient MEFs were harvested and immunoblot analysis was performed for endogenous TRAF2, c-IAP1 and XIAP. The *asterisk* indicates a nonspecific protein recognized by the TRAF2 antibody. HEK293 cells *(B)* and MDA-MB-231 cells *(C)* were transfected with double-stranded scrambled siRNA oligonucleotides or two different siRNA oligonucleotides targeting TRAF2. Forty-eight hours following transfection, cells were harvested and immunoblot analysis was performed for endogenous TRAF2, c-IAP1 and XIAP.

be incapable of binding to TRAF2 (Samuel et al., 2006; Varfolomeev et al., 2006). Surprisingly, we found that substitution of two critical residues (Glu 64 and Arg 65) in the most amino-terminal (BIR1) domain of c-IAP1 abolished TRAF2 binding but did not prevent TRAF2-mediated stabilization of c-IAP1 (Fig. B.2A). To determine whether stabilization of c-IAP1 E64A/R65A was due to residual TRAF2 binding that was undetectable in our immunoprecipitation assay, we generated a c-IAP1 construct lacking the entire BIR1 domain. Consistent with previous studies, c-IAP1 ΔBIR1 was incapable of binding to TRAF2. However, c-IAP1 ΔBIR1 was robustly expressed in the absence or presence of TRAF2, suggesting that different elements within c-IAP1 determine TRAF2 binding and stabilization.

Stabilized c-IAP1 protects cells from apoptosis.

To assess whether TRAF2-stabilized c-IAP1 could protect cells from apoptosis, the two proteins were coexpressed with Bax or Fas, to induce the intrinsic or extrinsic apoptotic pathways, respectively, and induction of apoptosis was assessed by a quantitative assay of caspase-3 activity. We found that, when expressed alone, c-IAP1 did not protect cells from either Bax- or Fas-induced apoptosis (Fig. B.2B and B.2C). However, coexpression of TRAF2 led to robust protection comparable to the potent caspase inhibitor XIAP. Consistent with the results observed in Fig. B.2A, c-IAP1 E64A/R65A was able to protect cells from both apoptotic stimuli in a TRAF2-dependent manner. However, it was not capable of protecting cells to the same degree as wild-type c-IAP1, suggesting a role for direct interaction with TRAF2 in enhancing the anti-apoptotic properties of c-IAP1. Interestingly, RING-deficient (H588A) c-IAP1 and c-

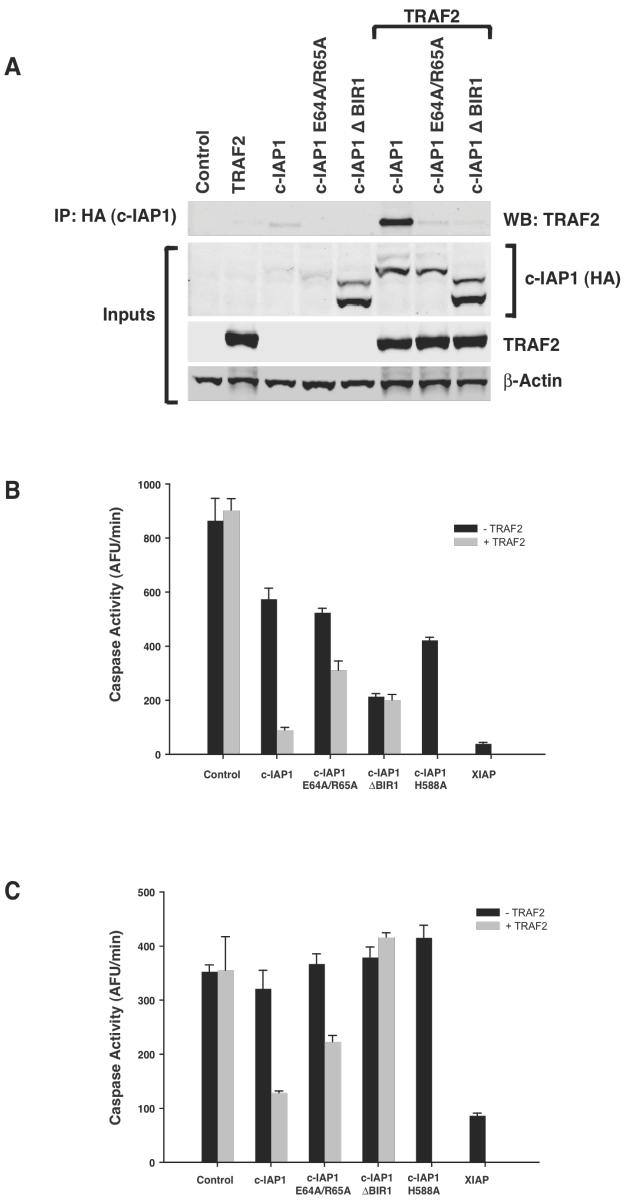

Figure B.2. Stabilization of c-IAP1 does not require direct interaction with TRAF2. *(A)* HEK293T cells were transfected with the indicated c-IAP1 plasmids in the absence or presence of TRAF2. Two days following transfection, cells were harvested and c-IAP1 was immunoprecipitated with an antibody against the amino-terminal HA tag. The presence of TRAF2 in the immunoprecipitate was determined by immunoblot analysis. *(B)* HEK293T cells were transfected with Bax and the indicated c-IAP1 plasmids in the absence or presence of TRAF2. Sixteen hours following transfection, caspase-3 activity was evaluated by incubation with the fluorogenic substrate, DEVD-AFC. *(C)* HEK293T cells were transfected with Fas and the indicated c-IAP1 plasmids in the absence or presence of TRAF2. Twenty-four hours after transfection, caspase-3 activity was evaluated as in *(B)*. The data shown are derived from multiple independent samples and are representative of at least two independent experiments.

IAP1 ΔBIR1 potently protected against Bax-mediated apoptosis but provided no protection from Fas-mediated apoptosis. This finding suggests that c-IAP1 can protect cells via at least two distinct mechanisms, one that does not require an intact RING or BIR1, and one that requires both the RING and BIR1.

Discussion

The roles of c-IAP proteins in modulating NF-κB signaling and the apoptotic threshold have begun to be elucidated recently by a number of elegant studies using genetic, RNAi, and pharmacologic approaches (Rumble et al., 2008; Vince et al., 2007; Varfolomeev et al., 2007; Petersen et al., 2007; Gaither et al., 2007; Mahoney et al., 2008; Varfolomeev et al., 2008; Wang et al., 2008; Bertrand et al., 2008). However, the mechanisms of post-translational regulation of c-IAPs are still largely unclear. Here we describe robust stabilization of c-IAP1 by TRAF2 in a manner that occurred post-translationally yet did not require direct interaction between the two proteins. Stabilization of c-IAP1 by TRAF2 led to potent inhibition of both intrinsic and extrinsic apoptotic pathways, and studies using c-IAP1 mutants revealed two distinct mechanisms by which c-IAP1 could block apoptosis.

As coexpression of TRAF2 resulted in stabilization of c-IAP1 in a manner analogous to disruption of the c-IAP1 RING domain, we initially hypothesized that interaction with TRAF2 might result in a distinct conformational change in c-IAP1 that prevented the c-IAP1 RING from carrying out autoubiquitination. Alternatively, binding of TRAF2 to BIR1 of c-IAP1 might block autoubiquitination at a critical lysine residue that determined proteasomal degradation of c-IAP1. Surprisingly, disruption of TRAF2 binding by mutation of two critical BIR1 residues did not prevent TRAF2-mediated stabilization of c-IAP1. This finding suggests two mechanisms for stabilization of c-IAP1 by TRAF2. One possibility is that c-IAP1 E64A/R65A is capable of binding to TRAF2 to a degree that allows stabilization but is below the detection threshold of our

immunoprecipitation assay. The results observed in Fig. B.2B and B.2C seem to support this model, as coexpression of TRAF2 enhanced the anti-apoptotic properties of c-IAP1 E64A/R65A, but not to the same extent as wild-type c-IAP1. Alternatively, TRAF2 might compete with c-IAP1 for a common component of the cellular ubiquitination machinery such as an E2 enzyme. This model would allow for TRAF2-mediated stabilization of c-IAP1 without direct interaction but would not explain the difference between wild-type and E64A/R65A in protection from apoptosis. It is possible that full stabilization of c-IAP1 by TRAF2 involves a combination of both direct interaction and sequestration of other molecules; indeed, while TRAF2 could robustly stabilize c-IAP1 E64A/R65A, we found that it was generally not stabilized to the same extent as wild-type c-IAP1 (Fig. B.2A and data not shown).

It is surprising that removal of the entire BIR1 domain, which is required for interaction with TRAF2, resulted in robust c-IAP1 expression in the absence or presence of TRAF2. Taken together with the data demonstrating TRAF2-mediated stabilization of c-IAP1 E64A/R65A, this finding indicates that distinct regions within c-IAP1 BIR1 determine TRAF2 binding and c-IAP1 stability. It is likely that a region in the amino-terminus of c-IAP1 makes it intrinsically unstable, as either removal of BIR1 or fusion with a large amino-terminal epitope tag results in stabilization of c-IAP1 (Fig. B.2A of this study and S. Galbán and C. Duckett, unpublished data). The N-end rule pathway is an evolutionarily conserved mechanism for protein degradation that proceeds via recognition of destabilizing amino-terminal residues by a unique family of E3 ubiquitin ligases known as UBRs (Tasaki and Kwon, 2007). Interestingly, the *Drosophila* IAP DIAP1 is a bona fide substrate of this degradation pathway (Ditzel et al., 2003; Ditzel et

al., 2008), and c-IAP1 has recently been proposed to be a substrate as well (Wickliffe et al., 2008). Indeed, an unbiased biochemical screen for c-IAP1 interacting proteins identified members of the N-end rule ubiquitin ligase family (S. Galbán and C. Duckett, unpublished data), suggesting that this pathway may play a role in regulation of constitutive c-IAP1 degradation. In this scenario, TRAF2 might stabilize c-IAP1 by sequestering N-end rule pathway ubiquitin ligases rather than E2 enzymes required for c-IAP1 autoubiquitination.

The differential effects of the c-IAP1 E64A/R65A mutations and removal of BIR1 on the ability of c-IAP1 to block apoptosis suggest that c-IAP1 can protect cells via distinct mechanisms. Protection from Bax-mediated apoptosis did not require either BIR1 or the RING of c-IAP1, suggesting that binding to SMAC or caspases was sufficient to protect cells under these conditions. Indeed, expression of increasing amounts of c-IAP1 H588A was found to sequester SMAC away from XIAP in a manner similar to that previously described for Op-IAP (Csomos et al., 2009; Wilkinson et al., 2004), which could allow endogenous XIAP to protect cells from Bax-induced death. This model would explain the protection provided by both ΔBIR1 and H588A c-IAP1, which can both bind to SMAC but are incapable of binding to TRAF2 and catalyzing ubiquitination of target proteins, respectively.

The complete lack of protection from Fas-mediated apoptosis provided by ΔBIR1 and H588A c-IAP1 suggests a distinct mechanism for c-IAP1-mediated inhibition of extrinsic apoptosis. A recent study demonstrated RING-mediated ubiquitination of caspases -3 and -7 by c-IAP1 (Choi et al., 2009), which could explain the deficiency of c-IAP1 H588A in protection from Fas-mediated apoptosis. However, BIR1 is not required

for binding to caspases, suggesting that another mechanism must account for the effect of BIR1 deletion. A model that could reconcile these observations is one in which c-IAP1 is recruited via BIR1-mediated interaction with TRAF2 or other TRAFs to the Fas receptor, where its RING-mediated ubiquitination of other receptor-associated proteins is required to protect cells from apoptosis. Ubiquitination of RIP1 by c-IAPs has recently been shown to protect cells from TNF-mediated apoptosis (Bertrand et al., 2008), suggesting a common mechanism for c-IAP1-mediated protection via RIP1 ubiquitination. The effect of c-IAP1 E64A/R65A on Fas-induced apoptosis is difficult to reconcile with this model, as TRAF2 is thought to serve as a scaffolding molecule to facilitate c-IAP-mediated RIP1 ubiquitination (Park et al., 2004; Bertrand et al., 2008). However, it remains possible that c-IAP1 retains residual TRAF2-binding capacity that allows it to associate with RIP1 and protect cells from Fas-mediated apoptosis. Alternatively, c-IAP1 might be able to associate with RIP1 in a manner that does not absolutely require binding to TRAF2 but does require a different element within the BIR1 domain. This model would be consistent with a previous report demonstrating that interaction of c-IAP2 with RIP1 required c-IAP2 BIR1 and was enhanced by, but did not absolutely require, TRAF2 (Park et al., 2004).

Taken together, the results presented in this study suggest that c-IAP1 is dynamically regulated at the post-translational level by a number of processes including autoubiquitination, TRAF2 binding, and possibly degradation by the N-end rule pathway. We propose that efficient protection from apoptosis by c-IAP1 depends on either its E3 ligase activity or its capacity to bind to cytosolic SMAC, depending on the apoptotic

stimulus. These findings provide novel insights into the post-translational control of c-IAP proteins and their roles in protecting cells from apoptosis.

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