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Huang, Yafen

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REGULATION OF APOPTOSIS BY UBIQUITINATION: THE FUNCTIONAL CONSEQUENCES AFTER LIVIN ASSOCIATES WITH SMAC/DIABLO; ARF COMPLEXES WITH COMMD1

HUANG YAFEN

SCHOOL OF BIOLOGICAL SCIENCES

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REGULATION OF APOPTOSIS BY UBIQUITINATION: THE FUNCTIONAL CONSEQUENCES AFTER LIVIN ASSOCIATES WITH SMAC/DIABLO; ARF COMPLEXES WITH COMMD1

HUANG YAFEN

School of Biological Sciences

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ABSTRACT

Ubiquitination is a posttranslational modification that the substrate proteins are covalently conjugated with an ubiquitin or a polyubiquitin chain. Ubiquitination regulates a wide range of cellular progresses including cell cycle progression, transcription and antigen processing in both proteasome dependent and independent manner. Recent findings also substantiate many essential roles of ubiquitination in the regulation of apoptosis. In this thesis, I report my studies on how ubiquitination controls the stability and functions of proteins involved in apoptosis.

Livin is a member of the IAP (Inhibitor of apoptosis protein) family. It has been reported that Livin inhibits caspase-3 and -7 *in vitro* and caspase-9 *in vivo* and is negatively regulated by Smac/DIABLO (Second mitochondria derived activator of caspases/Direct IAP binding protein with low PI). However the detailed mechanism underlying its anti-apoptotic function has not yet been fully characterized. In the first part of my thesis, the anti-apoptotic function of Livin through its ability to ubiquitinate and degrade death inducer Smac is presented. In summary, it was found that Livin can act as an E3 ubiquitin ligase to target Smac or itself for degradation by the proteasome. RING domain mediated E3 ligase activity is essential for the autoubiquitination and rapid degradation of Livin under normal state. However upon apoptotic stimulation, Livin promotes degradation of its antagonist Smac by accelerating the ubiquitination process.

Both BIR domain and RING finger domain of Livin are required for the ubiquitination of Smac *in vivo* and *in vitro*. Mutation in BIR domain nullifies the association of Livin and Smac and thus results in a reduced anti-apoptotic function of Livin. Together, these findings provide a novel function of Livin: it exhibits E3 ubiquitin ligase activity to degrade the pivotal apoptotic regulator Smac through the ubiquitin–proteasome pathway.

The second part of this thesis is mainly focused on the studies of the functional and physical interaction between tumor suppressor ARF (Alternate reading frame) and COMMD1 (Copper metabolism gene *MURR1* domain containing protein 1). In summary, it was found that ARF associates with COMMD1 and promotes K63 mediated polyubiquitination of COMMD1 in a p53 independent way. I found that ARF interacts with COMMD1 *in vivo*. Deletion analysis of ARF suggests that N-terminal amino acids 15-45 are important for its interaction with COMMD1. In addition, endogenous ARF redistributes from nucleolus to nucleoplasm and interacts with COMMD1 when DNA is damaged by actinomycin D. Interestingly, ARF promotes polyubiquitination of nuclear COMMD1 through the K63 of ubiquitin but not K48, which does not target COMMD1 for proteasomal proteolysis. ARF induced K63 ubiquitination stabilizes COMMD1 in a proteasome independent manner. Conversely, knockdown of ARF by RNAi results in decreased K63 mediated polyubiquitination of COMMD1 and destabilizes COMMD1. Moreover, ARF mutants lacking the interacting domains with COMMD1 did not promote

COMMD1 ubiquitination, indicating that physical association is a prerequisite condition for the ubiquitination process. Together, these data suggest that the ability to promote K63 mediated polyubiquitination of COMMD1 is a novel property of ARF independent to p53.

(491 words)

LIST OF ABBREVIATIONS

aa	amino acid
Apaf-1	Apoptosis protease activating factor-1
ARF	Alternate reading frame
ARF/BP1	ARF/binding protein 1
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bik	Bcl-2 interacting killer
BIR	Baculoviral IAP repeat
bp	base pair
BRUCE	BIR repeat-containing ubiquitin-conjugating enzyme
cDNA	complementary deoxyribonucleic acid
CDK	Cyclin dependent kinase
CHX	Cycloheximide
CIP	Calf intestine phosphatase
COMMD1	Copper metabolism gene *MURR1* domain containing protein 1
CREB	cAMP response element-binding protein
DAPI	4',6-diamidino-2-phenylindole
DIAP1	Drosophila IAP member 1
DMEM	Dulbecco's modified eagle's medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DP1	DRTF1 polypeptide 1
DR6	Death receptor 6
ECS^{SOCS1}	Ubiquitin ligase containing Elongins B/C, Cul2 and SOCS1
EDTA	Ethylene diamine tetra-acetic acid
FADD	Fas-associated protein with death domain
FAK	Focal adhesion kinase
FasL	Fas ligand
FBS	Fetal bovine serum
GFP	Green fluorescence protein
HID	Head envolution defective protein
HIF1-α	Hypoxia-inducible factor 1-α
HRP	Horseradish peroxidase

HtrA2/Omi	High temperature requirement A serine peptidase 2
IBM	IAP binding motif
ILP2	IAP-like protein 2
IKK	IκB kinase
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ISMs	Intersegmental muscles
IκB	Inhibitor of NF-κB
KD	Kilodalton
LB	Luria-Bertani
mAb	monoclonal antibody
Mcl-1	Myeloid cell leukemia sequence 1
MDM2	Mouse double minute 2
MDMX	MDM2 like p53 binding protein X
MEFs	Mouse embryo fibroblasts
mg	milligram
ml	milliliter
ML-IAP	Melanoma IAP
mM	millimole
MW	Molecular weight
NIAP	Neuronal inhibitor of apoptosis protein
NF-κB	Nuclear factor κB
NMR	Nuclear magnetic resonance
NoLS	Nucleolar localization signal
NPM	Nucleoplasmin
pAb	polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PVDF	Polyvinylidnene fluoride
p300/CBP	E1A binding protein p300/ CREB binding protein
Rad6	Radiation sensitivity protein 6
RB	Retinoblastoma protein
RHD	Rel homology domain
RHG	Reaper-Hid-Grim
RING	Really interesting new gene
RIP3	Receptor-interacting protein

RNA	Ribonucleic acid
RNAi	RNA interfence
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcription-polymerase chain reaction
SCF	Skp-Cullin-F box
S.D.	Standard Derivation
SDS	Sodium dodecyl sulfate
siRNA	small interfering RNA
Smac/DIABLO	Second mitochondria derived activator of caspases/Direct IAP binding protein with low PI
SUMO	Small ubiquitin like modifier
TBP1	Tat binding protein 1
TNF	Tumor necrosis factor
TNFR1	TNF receptor 1
TRAF	TNF receptor associated factor
Tris	Tris (hydroxmethyl)-aminomethane
tsBN75	Temperature-sensitive x-linked mutants of the BHK21 cell line 75
Ub	Ubiquitin
Ubl	Ubiquitin like protein
WRN	Werner helicase
wt	wild type
XIAP	X-chromosome-linked inhibitor of apoptosis protein
YY1	Yin-Yang 1
µg	microgram
µM	micromole
µl	microliter

Chapter 1 General introduction

1.1Apoptosis

A feature common to all multicellular organisms is the ability to control cell number. During developmental and adult phase, metazoans need to get rid of cells in excess or potentially dangerous. For this purpose, they use a delicate molecular mechanism known as apoptosis or in some situation called programmed cell death to get rid of these unwanted cells [2]. Malfunction or dysregulation of apoptosis has been found in various diseases ranging from autoimmune and immunodeficiency disease to neurodegenerative disease and cancer [3, 4]. Therefore underscoring the mechanism of this process has always been considered an essential issue in cell biology and clinic research.

Apoptosis proceeds in a highly regulated manner. A variety of extracellular or intracellular stimuli can activate this process. Once initiated, the signal is transduced by some conserved molecules through a series of protein-protein interactions. Ultimately, caspases, the executioners of apoptotic pathway cleave key substrates such as DNA repair enzymes, lamins, actin regulatory proteins and Focal adhesion kinase (FAK) which subsequently result in chromatin cleavage, organelles breakdown and final cell death [2]. As cell death is irreversible, it needs to be kept at very stringent control. To date a wide range of cellular factors that regulate the apoptotic pathway have been identified and characterized. These include Bax (Bcl-2 associated X protein), Apaf-1 (Apoptosis protease activating factor-1), p53, RB (Retinoblastoma protein) and $p21^{Cip\text{-}1}$ which have important pro-apoptotic activities; Bcl-2 (B-cell lymphoma

2), Mcl-1 (Myeloid cell leukemia sequence 1), IAPs and NF-κB (Nuclear factor κB) which are well known for their anti-apoptotic functions. The counterbalance between these pro- and anti-apoptotic factors determines cells' fate: to live or to die.

1.2 Ubiquitination

Ubiquitin is a small protein containing only 76 amino acids. It is ubiquitously expressed in all eukaryotic species and considered one of the most highly conserved proteins through evolution [5]. Ubiquitin can be covalently conjugated to the substrate protein, resulting in formation of an isopeptide bond between the C-terminal glycine (G76) of ubiquitin and a lysine residue in the substrate. The reaction needs sequential actions of three enzymes: E1 (ubiquitin activating enzyme) that activates the G76 of ubiquitin; E2 (ubiquitin conjugating enzyme) which transiently carries activated ubiquitin; E3, the ubiquitin protein ligase that recruits E2 and the substrate together and transfers the activated ubiquitin from E2 to the substrate [5, 6]. Successive rounds of ubiquitination lead to formation of polyubiquitin chain on the substrate. Proteins modified in this way are finally recognized and degraded by the proteasome, a multicomplex protease [7]. Typically, polyubiquitin chain that targets protein for proteasomal degradation is linked through the lysine 48 (K48) of ubiquitin. Ubiquitin-proteasome pathway regulates a wide range of cellular functions including progression of cell cycle, induction of inflammatory response, apoptosis and antigen presentation [8]. Selective degradation of key

regulatory proteins controls the occurrence and direction of these cellular affairs. Not surprisingly, dysregulation of the ubiquitin proteasome pathway has been suggested as a causative factor in cancer and some inherited diseases.

Although ubiquitin is best characterized for promoting protein degradation by proteasome, evidences revealed that some of its functions are proteolysis independent. Polyubiquitination through K63 linkage does not promote protein degradation, but was demonstrated to be involved in protein kinase activation, DNA repair and vesicle trafficking. Similarly, monoubiquitination at single or multiple lysine sites could be a signal for receptor internalization, vesicle sorting, DNA repair, gene silencing and protein translocation [5]. For example, monoubiquitination of PCNA (Proliferating cell nuclear antigen) was reported to be important for DNA spontaneous mutagenesis. Upon DNA damage by UV or other chemicals, PCNA is monoubiquitinated by E2 Rad6 (Radiation sensitivity protein 6) and a RING domain containing E3 Rad18 (Radiation sensitivity protein 18). The monoubiquitinated PCNA activates translesion DNA synthesis, resulting in DNA damage induced mutagenesis [9, 10]. Monoubiquitinated PCNA can also be further polyubiquitinated through the K63 linkage catalyzed by E2 Ubc13/Mms2 (Ubiquitin conjugating enzyme 13) and E3 ligase Rad5. The K63 mediated polyubiquitination of PCNA is essential for the error-free repair for damaged DNA [11, 12]. Thus two different types of ubiquitin modification on a single lysine residue in PCNA can each control a different outcome in mutagenesis and DNA repair.

1.3 Regulation of apoptosis by ubiquitination

Recently, ubiquitination has gained more attention for its critical roles in the regulation of apoptosis. Evidences that link ubiquitination and apoptosis came from the study of developmentally programmed atrophy and degeneration of the intersegmental muscles (ISMs) in hawkmoth [13, 14]. Northern blot analysis showed that expression of ubiquitin increases dramatically before apparent morphological changes of apoptosis appear. Later report from other system also indicated that enzymes of ubiquitination machinery are activated and the quantity of ubiquitin protein conjugates and proteasome is largely increased during apoptosis [15]. Antisense oligonucleotides of ubiquitin could inhibit γ irradiation induced protein ubiquitination and apoptosis in lymphocytes, implying that ubiquitination is essential for the lymphocytes apoptosis [16]. Inhibitors of proteasome effectively blocked apoptosis induced by DNA damage reagents and glucocorticoid in lymphocytes, suggesting that ubiquitination also plays an important role in regulating the mitochondria apoptotic pathway [15, 17].

Now it becomes clear that a number of proteins involved in the programmed cell death are regulated by the ubiquitin-proteasome pathway. p53 is a potent tumor suppressor that induces cell cycle arrest, apoptosis and senescence in response to various kinds of cellular stress. For its effective action after stimulation, p53 needs to be controlled in a very stringent manner. One of these mechanisms is achieved by controlling the stability of the protein at the

posttranslational stage. MDM2 is a RING domain containing protein that act as an E3 ligase to promote ubiquitination and proteasomal degradation of p53. In unstimulated cell, ubiquitination of p53 by MDM2 facilitates nuclear export of p53 from nucleus to cytoplasm, causing p53 to have no transactivation activity in the nucleus. However, upon hyperprolifreative signals such as oncogene activation, cellular factors such as MDMX (MDM2 like p53 binding protein X) and RB can stabilize p53 by binding to MDM2-p53 complex and inhibit the E3 ligase activity of MDM2. Stabilized p53 then activates the downstream effector genes [18-20]. Another example for explaining the role of ubiquitin in regulation of programmed cell death is the IAP. The majority of IAP family proteins possess a RING domain which enables them to serve as E3 ligases to promote polyubiquitination and degradation of proteins physically associated with them. Most of their substrates are pro-apoptotic proteins such as caspases and caspase activator Smac/DIABLO [21]. Thus IAP family proteins protect cells from cell death partially through its function to degrade either caspases that could be spontaneously activated or Smac/DIABLO which is incidentally released from the mitochondria.

For cell death induced by genotoxic insults or other apoptotic stimuli, rapid depletion of anti-apoptotic factors by ubiquitin-proteasome pathway may also be essential for the initiation of apoptosis. Proteasomal degradation of Mcl-1, an anti-apoptotic member of Bcl-2 family triggered by genotoxic stress has been demonstrated to be required for the translocation of Bax and Bcl-X_L to

mitochondria and subsequent cytochrome C release and caspase activation [22]. In drosophila, Grim, Reaper, and Hid proteins, the homologues of Smac/DIABLO induce apoptosis by binding to and promoting rapid degradation of Drosophila IAP member 1 (DIAP1), further facilitating the activation of apoptosis [23].

Interestingly, although most of the studies to date are focused on how ubiquitin-proteasome pathway regulates apoptosis through controlling the degradation of key regulatory factors, recent evidences revealed that the proteasome itself could be negatively regulated by caspases during apoptosis. Sun *et al.* found that certain subunits of 26s proteasome like S6', S1 and S5a are cleaved by caspases at the early stage of apoptosis induced by various stimuli, which subsequently leads to the inability of the proteasome to recognize and degrade polyubiquitinated proteins [24]. Caspases mediated cleavage of proteasome subunits results in stabilization of both ubiquitin dependent substrates like Smac/DIABLO and ubiquitin independent substrates such as nithine decarboxylase, and thus accelerates the process of apoptosis. Based on these findings, the role of the ubiquitin-proteasome machinery in the regulation of programmed cell death could possibly be explained as: at early stage, proteasome promotes rapid degradation of anti-apoptotic factors such as Mcl-1 and IAP proteins, which removes the threshold of apoptosis. Once apoptosis is initiated, proteasome would be disabled to allow accumulation of pro-apoptotic factors that direct cells to the irreversible death [24, 25]. However,

under certain circumstances, the net balance between anti-apoptotic and pro-apoptotic factors, the availabilities and affinities of various E2 and E3 for these substrates during apoptosis would also determine whether proteasome complex facilitates or prevents cell death [24, 25].

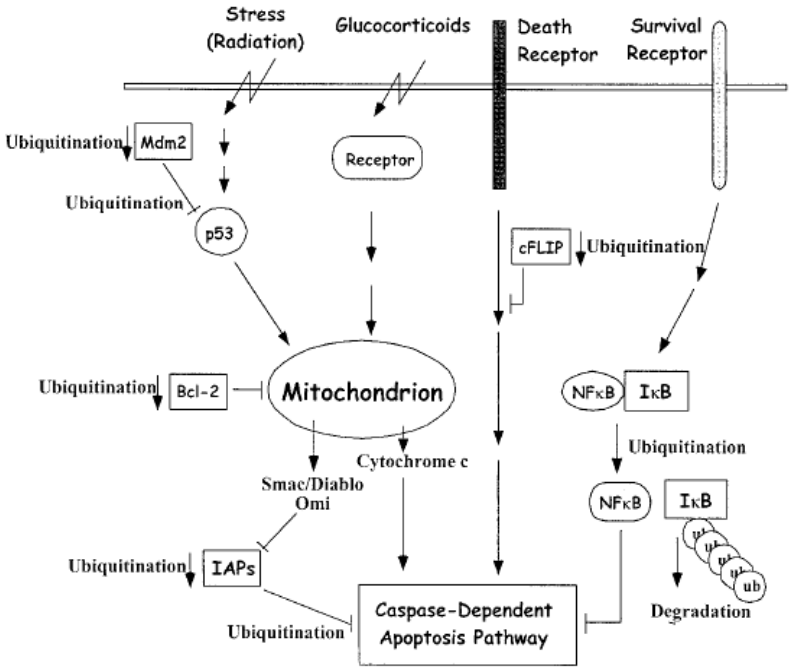


Figure1.1 The role of ubiquitination in regulation of cell death pathways. Reproduced from [26].

Chapter 2 Livin promotes Smac/DIABLO degradation by ubiquitin-proteasome pathway

2.1 Introduction

The IAP proteins were initially identified in baculovirus and characterized as containing 1-3 tandems of BIR motifs [27, 28]. Later, IAP homologues were found in a wide range of eukaryote species including yeast, *c.elegan*, drosophila, and mammalian species such as mouse, rat, chicken, pig and human [29]. To date, eight IAP proteins, NIAP (Neuronal inhibitor of apoptosis protein), XIAP (X-chromosome linked IAP), c-IAP1, c-IAP2, BRUCE (BIR repeat-containing ubiquitin-conjugating enzyme), ILP2 (IAP like protein 2), Survivin and Livin have been identified in human. Overexpression of these IAP proteins was reported to suppress apoptosis induced by a variety of stimuli including Tumor necrosis factor (TNF), Fas, menadione, staurosporine, etoposide, taxol and growth factor withdrawal [30-32]. However, the expression of IAP proteins *in vivo* appears to be regulated differentially, suggesting that these proteins might be activated in response to particular cellular or environmental signals [29].

2.1.1 Functional domains of IAPs

All IAP proteins contain at least one BIR domain which was reported to be important for their anti-apoptotic activities. BIR domain was originally identified as a sequence of around 70 amino acids which folds into a structure with a hydrophobic centre that includes a C_2HC motif [33]. This C_2HC motif contains several conserved residues $\underline{C}X_2\underline{C}X_6WX_3DX\underline{H}X_6\underline{C}$ (X could be any amino acid), representing a novel zinc binding domain. On the surface are both hydrophobic and hydrophilic residues, which enable BIR core a protein

interacting region. IAPs were reported to interact and inhibit caspases specifically through BIR domain [34-37]. Some evidences showed that although sequences flanking BIR core, but not BIR core itself appear to contribute most to IAP protein interactions, conserved residues of the core may also be required for the anti-apoptotic activities of IAPs [38, 39]. In addition to caspases, IAP antagonists in insects such as Reaper, Grim, Hid, sickle and Jafrac2) and mammals such as Smac/DIABLO and HtrA2/Omi (High temperature requirement A serine peptidase 2) also interact with IAPs through BIR domain [40-44]. Interactions of IAPs and these antagonists were demonstrated to relieve the inhibition of caspases imposed by IAPs.

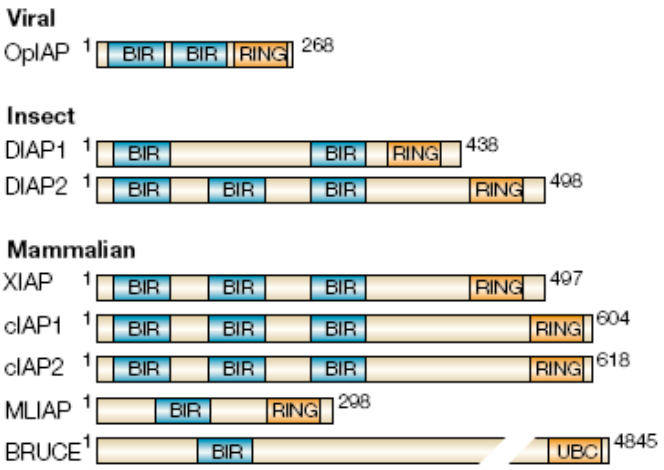


Figure 2.1.1 Schematic representation of IAP proteins in different species. Reproduced from [1].

In addition to BIR domain, most IAP proteins also possess a C-terminal RING domain. This domain was initially found in the *RING1*(*Really interesting new gene 1*) gene and therefore named RING finger [45]. The common feature of RING finger proteins is the presence of a C_3HC_4 motif, which can be defined as a sequence $\underline{C}X_2\underline{C}X_{9\text{-}39}\underline{C}X_{1\text{-}3}\underline{H}X_{2\text{-}3}\underline{C}X_2\underline{C}X_{4\text{-}48}\underline{C}X_2\underline{C}$ (X could be any amino acid).

The seven cysteines and one histidine form a cross-brace structure for binding two zinc atoms. Till now, more than 380 RING motifs have been identified in human [46]. RING domain has been shown to mediate a crucial step in the ubiquitination pathway that targets protein for degradation by 26s proteasome [21]. Three enzymes are involved in the pathway and RING finger protein has been classified as ubiquitin ligase because it can recruit E2 and the substrate together which subsequently leads to the ubiquitin transferred from E2 to the substrate.

2.1.2 IAPs as caspase inhibitors

How do IAP proteins achieve their anti-apoptotic activities? One effective way is to bind and inhibit caspases. Caspases is a family of cysteine proteases produced as catalytically inactive zymogens. Effector caspases such as caspase-3 and -7 are activated by initiator caspases caspase-8 and -9. Caspase-8 and -9 are the most important initiating caspases in the Fas/TNF death receptor pathway and mitochondrial Apaf-1/cytochrome c pathway respectively. Recruitment of plasma membrane receptor complexes such as Fas can trigger the autoactivation of procaspase-8 [47, 48]. Once initiated, caspase-8 activates procaspase-3 and other downstream caspases which execute cell death by cleaving essential cellular components in cells. In the mitochondria apoptotic pathway, caspase activation involves a protein termed Apoptosis protease activating factor-1(Apaf-1) [49, 50]. Apaf-1 is bond by Cytochrome c after it is released from mitochondria during apoptosis. The Apaf-1/Cytochrome c

complex associates with procapspase-9 and results in auto-processing and activation of caspase-9. Caspase-9 then activates caspapse-3 and other caspase cascades.

At least six family members including XIAP, c-IAP1, c-IAP2 , NAIP, survivin and Livin have been found to bind and potently inhibit caspase-3, -7 and procaspase-9, but not caspase-1, -6, -8 or -10 [2]. Although IAP proteins do not directly bind caspase-8, they can bind its substrate caspase-3, thus blocking the cascade of caspases and protecting cell from death receptor induced apoptosis [36, 37]. In the mitochondria pathway, Overexpresssion of IAP proteins has been demonstrated to block caspase activation and apoptosis induced by Bax, Bik (Bcl-2 interacting killer), Bak (Bcl-2 homologous antagonist killer), and other Bcl-2 family proteins which target mitochondria and initiate release of Cytochrome c [36]. IAPs provide protection by binding directly to procaspase-9 and caspase-3, preventing their autoproteolysis and activation induced by Cytochrome c [36]. Compared with other IAP proteins, XIAP is a very potent inhibitor for caspases. Its inhibitory constants (*Ki*) is equivalent to that of the baculovirus p35 caspapse inhibitor [51]. The *Ki* of c-IAP1 and c-IAP2 are 50-100 folds higher than that of XIAP, suggesting that these two IAP proteins are far less potent [35].

2.1.3 Smac/DIABLO, antagonist of IAPs

In understanding the anti-apoptotic roles of IAP proteins in regulating caspase

activity, a number of IAP binding proteins that can suppress the activities of IAPs have been discovered. One example is the Second mitochondria derived activator of caspases (Smac), also known as direct IAP binding protein with low PI (DIABLO) in mouse. Smac is encoded in the nucleus and localized to the inter-membrane space of mitochondria [43, 52]. It is synthesized as a 239 amino acids precursor molecule. The 55 residues at N-terminus are responsible for the mitochondrial targeting and proteolytically removed after import, leaving the remaining 184 amino acids as the mature form of Smac [43, 52]. Upon apoptotic stimulation, when Cytochrome c is released from the mitochondria and forms complex with Apaf-1 to activate procaspase-9 and caspase-3, mature Smac is also released to the cytosol and alleviates the interaction between caspases and IAP proteins by binding to the same pockets in the IAP proteins which are used to bind caspases. Caspases are then displaced and activated to execute cell death [53]. The N-terminus of mature Smac begins at residue 56, as 1-55 residues are removed after translocation to the mitochondria. Structure studies showed that residues 56-59 (Ala-Val-Pro-Ile) of Smac are homologous to the exposed N-terminal motif that is used by caspase-9 to bind BIR domain of XIAP [54, 55]. Smac also binds to the same pocket of XIAP and thus it can displace caspase from the complex. A synthetic peptide that consists of 56-59 residues of Smac could effectively mimic the inhibitory activity of mature Smac on IAP proteins, raising the possibility of using small peptide mimics to treat cancer cells [54, 55].

In Drosophila *melanogaster*, three proteins Hid, Grim and Reaper were found to be the functional homologues of mammalian Smac/DIABLO. These proteins interact with DIAP1 and relieve its inhibitory effect on caspases [40-44]. Structure analysis revealed that these proteins share notably similarity in the N-terminus with Smac and that N-terminal sequences from Hid/Grim/Reaper recognize the same surface groove of IAP proteins [55]. The N-terminal motif was then defined as IAP binding motif (IBM) or Reaper-Hid-Grim (RHG) motif and thought to be conserved through evolution. This is further supported by the observation that drosophila IBM containing proteins can bind to c-IAP1 in mammals and suppress the ability of c-IAP1 to inhibit caspase-7 [56].

2.1.4 IAPs as E3 ligases: target antagonists or themselves?

Most IAP family proteins including XIAP, c-IAP1, c-IAP2 and Livin contain a RING domain at their Carboxyl terminus. The most prominent role of RING domain plays in IAPs has been attributed to its E3 ubiquitin ligase activity: i.e., it promotes degradation of target proteins or themselves through the ubiquitin-proteasome pathway. It was found that XIAP, c-IAP1 and c-IAP2 are degraded by proteasomes in thymocytes upon apoptotic stimulation with glucocorticodes or etoposide and proteasome inhibitors could effectively block the thymocyte apoptosis [57]. All these IAP proteins undergo autoubiquitination *in vivo* and *in vitro* due to their RING domain dependent E3 ligase activity. Therefore autoubiquitination and degradation of these IAP proteins was suggested to lower the threshold of survival and allow cells to commit suicide

upon apoptotic stimulation [57].

In some circumstances, the E3 ligase activity of IAPs also enables them to promote degradation of proteins that directly bind to them. c-IAP2 was found to ubiquitinate caspase-7 and caspase-3 but not caspase-1 *in vitro* [58]. XIAP, a potent caspase-3 inhibitor was shown to induce degradation of caspase-3 *in vivo* and both RING domain and interaction between XIAP and caspase-3 are required for the fulfillment of ubiquitination [59]. In addition to caspases, IAP proteins also target IAP antagonists such as Smac or Reaper, Hid and Grim in drosophila for proteasome dependent degradation. In drosophila, DIAP-1 mediated ubiquitination and degradation of Reaper has been described and a non-ubiquitinatable form of Reaper was found to be more effective than the wild type for inducing cell death [60]. Mammalian c-IAP1 and c-IAP2 were reported to stimulate the ubiquitination of Smac both under *in vivo* and *in vitro* condition, leading to the proteasomal degradation of Smac [61]. The E3 ligase activity is mediated by the RING domain and associations of c-IAP1, c-IAP2 with Smac are required. Strikingly, although Smac could be a substrate for XIAP mediated ubiquitination, the ubiquitination does not target Smac for degradation, suggesting that Smac undergo either multi-monoubiquitination or non-K48 ubiquitination that may have some distinct roles in regulating the function of Smac [62, 63].

Although most antagonist proteins are targeted by IAPs for proteasomal

degradation, evidences revealed that sometimes antagonists may conversely regulate the turnover of IAP by affecting the ubiquitination process. Reaper, Grim and Hid in drosophila were found to induce ubiquitination and degradation of DIAP-1 in a proteasome dependent way [64-66]. Moreover, RING domain dependent E3 ligase activity is essential for the degradation of DIAP-1 promoted by these antagonists and degradation of DIAP-1 was found to be required for the proceeding of cell death in drosophila. In mammalian cells, Smac was reported to induce autoubiquitination and rapid degradation of c-IAP1 and c-IAP2 *in vivo* [67]. Smac could also promote ubiquitination of XIAP and Livin, but failed to target these proteins for rapid degradation by the proteasomes [67]. Therefore the mechanisms by which Smac regulate the ubiquitination and degradation of IAPs is still inconclusive.

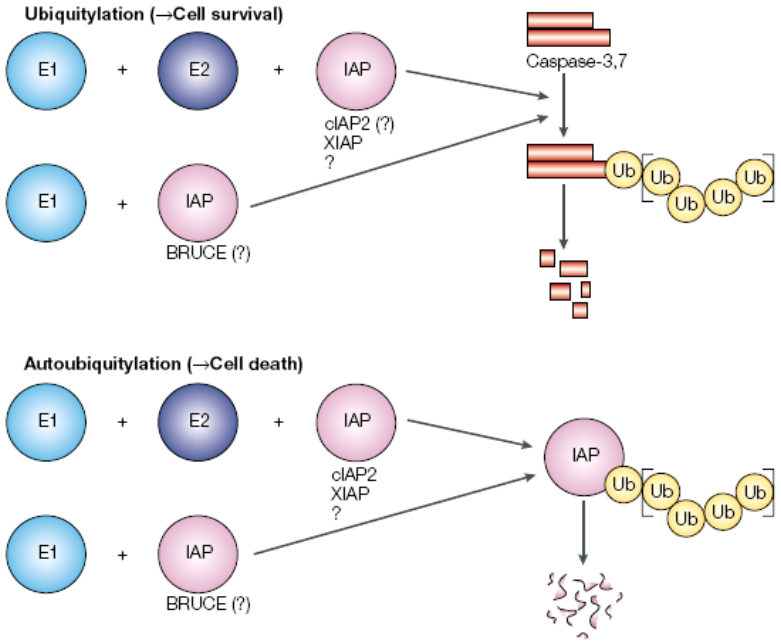


Figure 2.1.2 RING domain containing IAPs act as E3 ligases to promote polyubiquitination of their substrates or themselves. Reproduced from [1].

2.1.5 Livin, a novel IAP family member

Livin, also known as Melanoma IAP (ML-IAP) is a novel IAP family protein that contains a single BIR domain and a RING domain at C-terminus [68, 69]. The Livin gene is localized to the chromosome 20q13.3, a region frequently amplified in melanomas and diverse carcinomas. Northern blot analysis revealed that the expression of Livin within normal tissues could be detected during fetal development and then restricted in a few adult tissues including heart, testis, ovary, thymus and spleen [69, 70]. Elevated level of Livin was observed in cancer lines, especially melanoma, colon, prostate carcinomas, suggesting that Livin may endow cancer cells with a survival advantage during their tumor progressions [69, 70].

Overexpresson of Livin could inhibit apoptosis induced by a wide range of apoptotic stimuli including DR6 (Death receptor 6), FADD (Fas associated protein with death domain), RIP3 (Receptor interacting protein 3) and Bax [68, 69]. Conversely, knockdown of Livin by RNAi was found to induce apoptosis in HeLa cells [69]. Like other IAP family members, Livin is able to bind caspases and inhibit the proteolysis activation of caspase-3, -7 and -9 [68, 69]. The inhibitory function of Livin is also regulated by Smac/DIABLO. It was demonstrated that Livin physically interacts with Smac through its BIR domain [71]. Expression of Smac could disrupt the binding of Livin and processed caspase-9 and thus attenuate the anti-apoptotic activity of Livin. Compared with XIAP, Livin is much less potent for inhibiting caspases. However, Livin has a

high binding affinity to Smac. Overexpression of Livin could result in formation of Livin/Smac complex and disruption of interaction between XIAP and Smac [72]. Therefore in addition to directly inhibiting caspases, Livin may regulate apoptotic pathway by sequestering Smac and preventing it from antagonizing XIAP mediated inhibition of apoptosis.

2.1.6 Aim of this project

Previously Livin was reported to effectively inhibit apoptosis induced by a wide range of stimuli. It prevents cell death partially by inhibiting caspases through its BIR domain. However, compared with XIAP, Livin is a poor caspase inhibitor. This raises the possibility that Livin may exert its anti-apoptotic properties by as yet uncharacterized mechanisms that are independent of inhibition on caspases. In addition to BIR domain, RING domain dependent E3 ligase activity has also been implicated in conveying the anti-apoptotic functions of IAP proteins. As well as regulating target proteins, IAP proteins themselves are controlled by the ubiquitin-proteasome pathway. Livin contains a RING domain at C-termini. Therefore the purpose of this project is to identify how Livin exerts its protective function through RING domain mediated E3 ligase activity. The questions need to be addressed are (a) whether autoubiquitination catalyzed by RING domain is essential for the rapid turnover of Livin under normal state; (b) upon apoptotic stimulation, whether Livin could ubiquitinate and degrade the death inducer Smac/DIABLO and thus protect cells from programmed cell death. We believe that resolving these

questions would contribute to the understanding of biochemical mechanisms by which Livin regulates the process of apoptosis.

2. 2 Materials and methods

2.2.1 Plasmid construction

Materials

Enzymes

Pfu and *Tag* DNA polymerase, restriction enzymes, T4 ligase and calf intestine phosphatase (CIP) were purchased from NEB, MA, USA.

Kits

PCR purification kit, gel extraction kit, plasmid mini, midi preparation kits were purchased from Qiagen, Hilden, Germany.

E. Coli strains

DH5α

Method

pCDNA3-Flag-Livin

Primers:

P1: 5'CGGAATTCCATGGGACCTAAAGACAGTGCCAAG3'

P2: 5'CGGTCGACCTAGGACAGGAAGGTGCGCACGCG3'

Full-length Livin gene fragment was amplified from HeLa cDNA library by PCR using primer pair P1/P2. The amplified fragment was inserted into EcoRI/XhoI sites of pCDNA3-Flag vector. The plasmid has been verified by DNA sequencing.

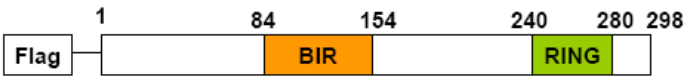


Figure 2.2.1 Scheme representation of pCDNA3-Flag-Livin construct.

pCDNA3-Flag- Livin-ΔRING

Primers:

P1: 5'CGGAATTCCATGGGACCTAAAGACAGTGCCAAG3'

P3: 5'CGGTCGACCTACACATCCCTGGCTCCTGGGGGCTC3'

cDNA fragment coding for Livin-ΔRING (residues1–239) was generated by PCR reaction by using primers P1/P3. PCR product was digested with restriction enzymes EcoRI/XhoI and subcloned into pcDNA3-Flag vector.

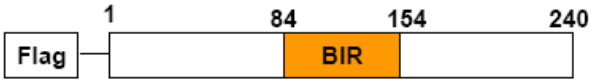


Figure 2.2.2 Scheme representation of Flag- Livin-ΔRING.

pCDNA3-Flag-Livin-C124A, -W134A, H144A, -C252A

Primers:

P1: 5'CGGAATTCCATGGGACCTAAAGACAGTGCCAAG3'

P2: 5'CGGTCGACCTAGGACAGGAAGGTGCGCACGCG3'

P4: 5'CAGGACAAGGTGAGGGCCTTCTTCTGCTATGGGGGC3'

P5: 5'AGCAGAAGAAGGCCCTCACCTTGTCCTGATG3'

P6: 5' GCAGAGCGCGAAGCGCGGGGACGACC3'

P7: 5'CCCCGCGCTTCGCGCTCTGCAGGCCCCCATA3'

P8: 5'GGACGGAGGCTGCCAAGTGGTTCCCCAG

P9: 5'TGGCAGCCTCCGTCCAGGGGTCG3'

P10: 5'GCAGGAGGAGAGGACGGCCAAGGTGTGCCTGGACCGC3'

P11: 5'CCAGGCACACCTTGGCCGTCCTCTCCTCCTGCAGCCGCCG3

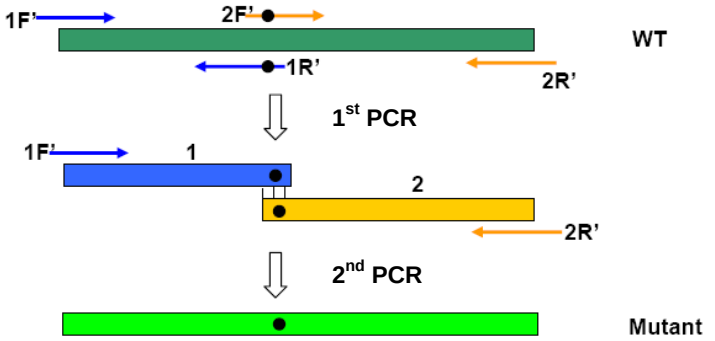


Figure 2.2.3 Strategy for PCR-mediated mutagenesis. ● denotes the mutation site.

PCR mediated mutagenesis method was used to generate Livin point mutants including Livin-C124A, -W134A, -H144A, and -C252A. As shown in Figure 2.2.3, DNA fragment 1 and 2 are generated by first step PCR with primers 1F'/1R' and 2F'/2R' respectively (primers 1R' and 2F' carry the mutation site). DNA fragment encoding Livin mutant was then generated by the second step PCR using primers 1F'/2R'. The primer pairs for generating each mutant are: P1/P5 and P4/P2 for Livin-C124A; P1/P7 and P6/P2 for Livin-W134A; P1/P9 and P8/P2 for Livin-H144A; P1/P11 and P10/P2 for Livin-C252A. The mutation sites in each primer pairs were underlined. Fragments carrying each one single site mutation were then subcloned into pCDNA3-Flag vector. All the mutated genes had been verified by DNA sequencing before the following experiments were carried out.

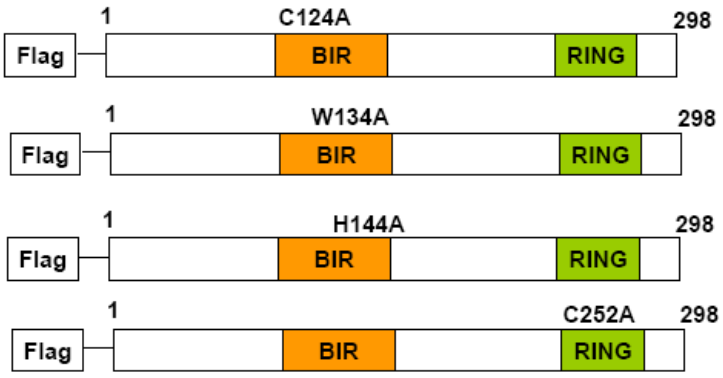


Figure 2.2.4 Scheme representation of Livin point mutants Livin-C124A, -W134A, -H144A and -C252A.

2.2.2 Mammalian cell culture

Materials

Cell line

HeLa cell line used in this study was kindly provided by Dr. Liu Ding Xiang's lab.

Media

Plain Dulbecco's modified eagle's medium (DMEM) was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1×nonessential amino acids, 1×MEM sodium pyruvate, 100mg/ml penicillin and 100mg/ml streptomycin to make the complete media. All were purchased from Invitrogen, Carlsbad, CA, USA.

Reagents for stimulating cell

- Etoposide (Sigma, St.Louis, MO, USA)
- Taxol (Sigma, St.Louis, MO, USA)

Transfection reagent

- Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA)

Method

HeLa cells were incubated in humidified atmosphere containing 5% CO_2 at 37^{o}C and subcultured 2-3 times a week.

Transfection of cells with various mammalian expression vectors by Lipofectamine 2000 was according to the method described by the manufacture's instruction.

2.2.3 Western blotting

Materials

Antibodies

- anti-Actin pAb (Santa Cruz Biotechnology, CA, USA)
- anti-Ubiquitin pAb (Calbiochem, La Jolla, CA, USA)
- anti-Flag pAb (Sigma, St.Louis, MO, USA)
- anti-PARP (poly ADP-ribose polymerase) pAb (Upstate, Lake Placid, NY, USA)
- anti-Smac/DIABLO mAb (Chemicon, USA)
- anti-Flag M2 mAb (Sigma, St.Louis, MO, USA)
- anti-GFP (Green fluorescence protein) mAb (MBL, Nagoya, Japan)
- anti-Smac/DIABLO mAb (Cell Signaling Technology, USA)
- anti-Caspase-9 mAb (Immunotech, France)
- anti-Livin mAb (IMGENEX, San Diego, CA, USA)

Secondary antibodies

HRP (Horseradish peroxidase) conjugated anti-mouse or anti-rabbit secondary antibodies were purchased from Amersham, UK. HRP conjugated anti-goat secondary antibody was purchased from Santa Cruz Biotechnology, CA, USA.

Membrane

Hybond ECL Nitrocellulose and Hybond-P PVDF (Polyvinylidnene fluoride) used in this study were purchased from Amersham, UK.

Detection kit

ECL advanced western blotting detection kit (Amersham, UK)

Method

1. Preparation of cell lysates

Cells were first lysed in lysis buffer for 5 min at room temperature. Cell lysate was harvested and kept on ice for further 30 min. Lysate was then centrifuged at 12000g for 5 min to clear the debris. Supernatant was collected in fresh tubes, mixed with proper volume of SDS sample buffer and boiled at 95^{o}C for 5 min. Denatured sample was kept on ice or stored at -20^{o}C for further use.

2. Proteins separation by SDS/PAGE

Protein samples were loaded into the wells of gel and separated under 200V for 45-60 min.

3. Membrane transblotting

“Sandwich" used for transblotting was assembled according to Bio-Rad’s instruction. Membrane transfer was done at 100V for 1 hour. After transferring,

membranes were washed with TBST for 5 min.

4. Membrane blocking and antibody incubation

Membrane was blocked with 10% non-fat milk in TBST for 1 hour. After blocking, Membrane was incubated with primary antibody diluted in 10% non-fat milk for 3 hours at room temperature or overnight at 4^{o}C. Membrane was then washed 10min with TBST for three times. After washing, membrane was incubated with HRP conjugated secondary antibody diluted in 10% non-fat milk for 1 hour followed by washing with TBST for 3 times.

5. Detection

Detection by ECL kits was performed according to manufacturer's instruction.

2.2.4 Immunoprecipitation

Materials

Beads

- Ultralink immobilized protein A/G (Pierce Biotechnology, IL, USA)
- Flag-M2 affinity gel (Sigma, St.Louis, MO, USA)

Buffer

- Lysis buffer: 1% Triton X-100, 10% glycerol, 150mM NaCl, 20mM Tris-HCl, pH 7.5, 1× Protease inhibitor cocktail
- Wash buffer: 1% Triton X-100, 150mM NaCl, 20mM Tris-HCl, pH7.5

Method

Cells were first lysed in a Triton X-100 based lysis buffer for 30 min at 4^{o}C.

Cellular debris was cleared by centrifugation. Cell lysate was incubated with primary antibody for 6 hours at 4^{o}C. Protein A/G agarose was then added to the lysate and incubation was continued for another 2 hours. After that, immunoprecipitates were washed five times with lysis buffer and proteins were recovered by boiling beads in SDS sample buffer and analyzed by Western blot.

2.2.5 Protein stability assay

Materials

- MG132 (N-CBZ-leu-leu-leucinal) (Calbiochem, La Jolla, CA, USA)
- ALLN (Nacetyl-leu-leu-norleucinal) (Sigma, St.Louis, MO, USA)
- Cycloheximide (Sigma, St.Louis, MO, USA)

Method

HeLa cells were seeded and cultured in 24-well plates overnight at 37^{o}C. Proteasome inhibitor MG132 or ALLN was added to treat cells for 1 hour. After incubation of cells with proteasome inhibitor for 1 hour, media was replaced with fresh media containing 20μg/ml cycloheximide. Cells were harvested at 0, 2, 4, 6, 8, 10 hours and analyzed by Western blotting.

2.2.6 Cell death assay

Material

- Hoechst 33342

Method

The abilities of Livin and Livin mutants to affect cell viability were assayed by transfecting HeLa cells in 24-well plates (2×10^4 cells/well) with various Livin constructs. In order to compare the protein level and count the dead cells more accurately, same concentration of plasmids under same conditions used for transfection was strictly employed. 24 hours after transfection, cells were incubated with drug for the desired period of time and the viability of cells was measured by counting round GFP-positive cells characterized with aberrant nuclei stained by Hoechst 33342. Data are expressed as percentages of control and are means of three independent experiments.

2.2.7 Protein purification

Materials

E. coli strain

- *BL21-DE3*

Beads

- Ni-NTA magnetic agarose beads (Qiagen, Hilden, Germany)
- Glutathione sepharose^{TM} 4B (Amersham, UK)

Buffers

For purification of GST-tag protein

- PBS: 20mM Tris-HCl, 150mM NaCl, pH7.6
- 1×Protease inhibitors cocktail (Roche, Switzerland)

- Elution buffer: 10mM reduced glutathione in PBS

For purification of His-tag protein

- Lysis buffer: 50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, pH8.0
- Wash buffer: 50mM NaH_2PO_4, 300mM NaCl, 80mM imidazole, pH8.0
- Elution buffer: 50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, pH8.0
- 1× Protease inhibitor cocktail

Method

Purification of GST tagged protein

Fresh transformant colony carrying plasmid that encodes GST fusion protein was inoculated into 5ml LB/Amp media and incubated overnight. Next day, 1ml cell culture was transferred to fresh 1L LB/Amp media and kept shaking for 4-6 hours until OD_{600} reached ~0.6. For expression of GST-Livin and GST-LivinC124A, cell cultures were induced by 1mM IPTG (Isopropyl-beta-D-thiogalactopyranoside) overnight at 16^{o}C. For expression of GST-Livin-ΔRING, cells were induced by 1mM IPTG at 30^{o}C for 6 hours. After induction, bacteria cells were harvested and resuspended in cold PBS containing protease inhibitors cocktail, followed by sonication at amplitude 60, pulse 2s×30cycle for 2 times. Cell extracts were cleared by centrifuge at 15000g for 15 min. Supernatants were collected and incubated with 50µl glutathione sepharose beads at 4^{o}C for 2 hours. Beads were washed with cold PBS for 3 times. GST tagged proteins were then eluted with 10mM reduced glutathione for two times. Elution fractions were finally combined together and

stored at 4°C or -80°C for further use.

Purification of His tagged protein

BL21 cell cultures carrying plasmid pET20a-His_6-Smac were inoculated into 1L LB/Kan media and kept shaking until OD_{600} reached 0.6. Expression of His_6-Smac was induced by 1mM IPTG at 30°C for 4 hours. Cell cultures were then harvested by centrifuge at 5000g for 20 min and resuspended in 5ml cold lysis buffer. Cells were lysed by sonication at amplitude 60, pulse 3s x 20 cycles for 3 times, followed by centrifuge at 15000g for 15 min to clear the cell debris. 1ml Ni-NTA agarose slurry was added to and incubated with the supernatant for 1 hour at 4°C. After incubation, agarose beads were washed with wash buffer for 3 times. His_6-smac was then eluted from the beads by elution buffer for three times. Different elution fractions were analyzed SDS-PAGE gel and fraction with the highest purity of His_6-Smac was kept for further use.

2.2.8 *In vitro* ubiquitination assay

Materials

- E1, His_6-ubiquitin, Mg-ATP were purchased from Boston Biochem, MA, USA. UBCH5b was kindly provided by Dr. Peter Cheung.
- Reaction buffer: 50mM Tris-HCl, pH 7.6.

Method

40µl cold 50mM Tris-HCl, pH7.6 was added a fresh 1.5ml tube and kept on ice.

Following materials were then mixed into cold Tris-HCl buffer: 100nM rabbit E1, 400nM E2 UbcH5b, 20μM ubiquitin, 200nM GST-Livin or Livin mutants, 400nM His_6-Smac/DIABLO and 2mM Mg-ATP. Tubes were kept shaking for 90 min at 30^{o}C. SDS sample buffer was added to stop the reaction. Polyubiquitination of Smac/DIABLO was analyzed by Western blotting using anti-Smac antibody.

2.3 Results

2.3.1 Livin is a short-lived protein, which is subjected to proteasome dependent degradation

Livin, a member of the IAP family was reported to be an unstable protein [69]. Therefore we determined the half life of endogenous Livin in the presence of cycloheximide (CHX), an inhibitor of protein translation. Incubation of CHX was continued for the indicated periods of time before cell extracts were collected and analyzed by Western blotting with anti-Livin antibody. As shown in Figure 2.3.1, in the presence of CHX, endogenous Livin protein levels decreased quickly and approximately half of Livin protein was shown to be degraded after 4 hours CHX treatment. Nearly no Livin was detectable after 8 hours treatment of cells with CHX, indicating that in the absence of *de novo* protein synthesis, the half life of endogenous Livin is approximately 4 hours. The faint band above Livin marked as Livin* is expected to be its modified form, although this requires further characterization.

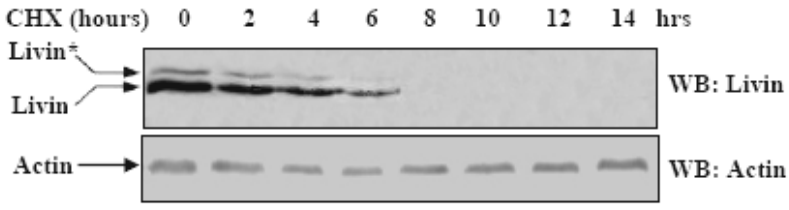


Figure 2.3.1 Determination of the half life of Livin. HeLa cells were treated with 20μg/ml CHX for the indicated period of time before soluble extract were collected. The protein levels of Livin were analyzed by immunoblotting with anti-Livin antibody. Endogenous protein levels of Actin were used as loading controls. Livin* is expected to be the modified form of Livin.

Next we examined whether rapid degradation of Livin is mediated by the ubiquitin-proteasome machinery. HeLa cells were pretreated with proteasome inhibitor MG132 or ALLN for 1 hour and further treated with CHX for the indicated period of time. As shown in Figure 2.3.2, in the absence of *de novo* synthesis, degradation of Livin could be blocked by MG132 or ALLN, suggesting that degradation of Livin is specifically mediated by the proteasome. In addition, accumulation of endogenous Livin following proteasome blockade was also observed in the absence of CHX (Figure 2.3.3). Together these data demonstrate that Livin is inherently unstable under normal state and subjected to ubiquitin-proteasome mediated degradation.

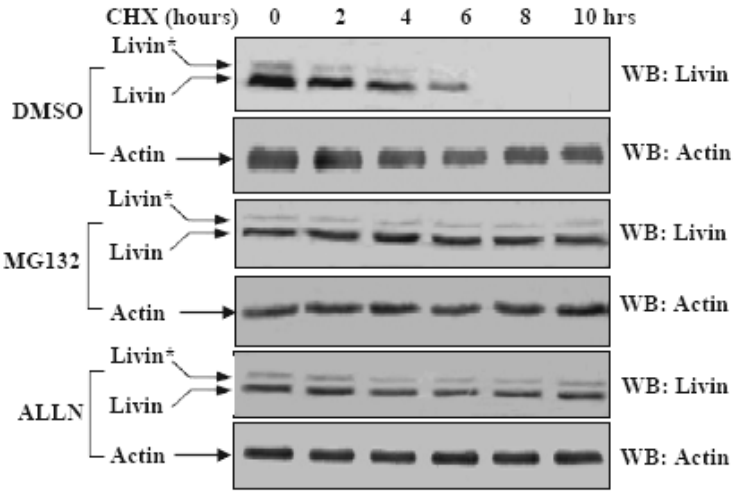


Figure 2.3.2 Livin is subjected to proteasome dependent degradation. HeLa cells were pretreated with vehicle DMSO, 20μM MG132 and 100μM ALLN respectively for 1 hour and further incubated with 20μg/ml CHX for the indicated periods of time. The protein levels of endogenous Livin were then analyzed by Western blotting with anti-Livin antibody. Protein levels of Actin were used as loading controls. Livin* is expected to be the modified form of Livin.

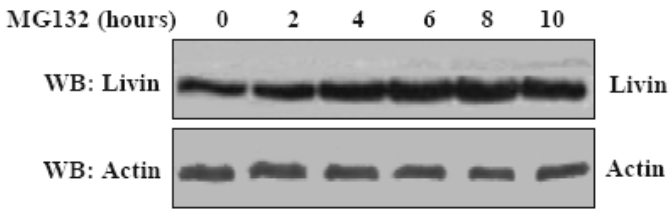


Figure 2.3.3 Livin is stabilized in the presence of proteasome inhibitor. HeLa cells were treated with 20 μM MG132 for 0, 2, 4, 6, 8 and 10 hours individually. The protein levels of Livin were analyzed by Western blotting using anti-Livin antibody. Protein levels of Actin were used as loading controls.

2.3.2 Livin undergoes autoubiquitination *in vivo*

Most IAPs proteins possess C-terminal RING finger domain that was reported to mediate autoubiquitination and proteasomal degradation of these proteins under physiological situation [57]. To explore whether autoubiquitination is essential for the rapid turnover of Livin under normal state, we determined autoubiquitination of Livin *in vivo* and the effects of RING domain and BIR domain of Livin on its autoubiquitination. After transient transfection with Flag control, Flag-Livin, BIR domain mutant Flag-Livin-C124A or RING domain deletion mutant Flag-Livin-ΔRING respectively, HeLa cells were treated with MG132 for the further 12 hours. Cell lysates were then collected and immunoprecipitated with anti-Flag antibody. As shown in Figure 2.3.4 lane 2, a high molecular weight smear characteristic of polyubiquitinated product was detected in cells expressing Flag-Livin, suggesting that Livin undergoes autoubiquitination *in vivo*. Polyubiquitination was also observed in cell transfected with Flag-Livin-C124A (lane 3), but not in cells transfected with

Flag-Livin-ΔRING (lane 4), indicating that RING domain but not BIR domain is required for the autoubiquitination of Livin.

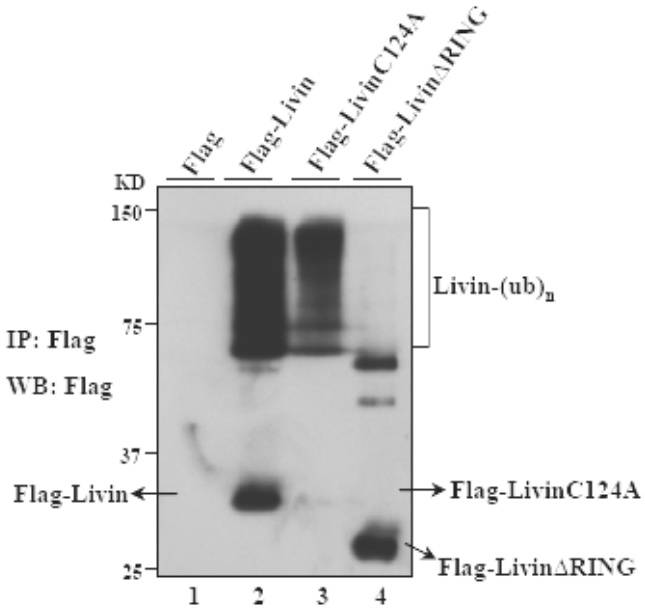


Figure 2.3.4 Autoubiquitination of Livin *in vivo*. HeLa cells were transfected with plasmids encoding Flag, Flag-Livin, Flag-Livin-C124A or Flag-Livin-ΔRING individually. 24 hours after transfection, cells were treated with MG132 for further 12 hours before cell lysates were prepared and immunoprecipitated with mouse anti-Flag M2 bound protein A/G agarose. Immunoprecipitated Flag tagged proteins were then analyzed by Western blotting with rabbit anti-Flag antibody.

To further confirm the role of RING domain in controlling the stability of Livin, we tested the effect of proteasome inhibitor MG132 on the protein level of Flag-Livin, Flag-Livin-ΔRING, Flag-Livin-C124A and Flag-Livin-C252A respectively. In addition to Livin-ΔRING, we also chose Cys252 as a mutation site for creating RING domain mutant. Cys252 is one of the zinc binding residues in RING domain $\underline{C}X_2CX_{9-39}CX_{1-3}HX_{2-3}CHX_2CX_{4-48}CX_2C$ and mutation at Cys252 was reported to abrogate the ubiquitination of Livin [67,

73]. As shown in Figure 2.3.5, the protein level of wt-Livin or Livin-C124A was considerably elevated in the presence of MG132 (lanes 2, 6). In contrast, no obvious change of Livin-ΔRING and Livin-C252A protein levels was observed before and after MG132 treatment (lanes 3, 4, 7, 8), indicating that RING domain, but not BIR domain is responsible for the proteasomal degradation of Livin.

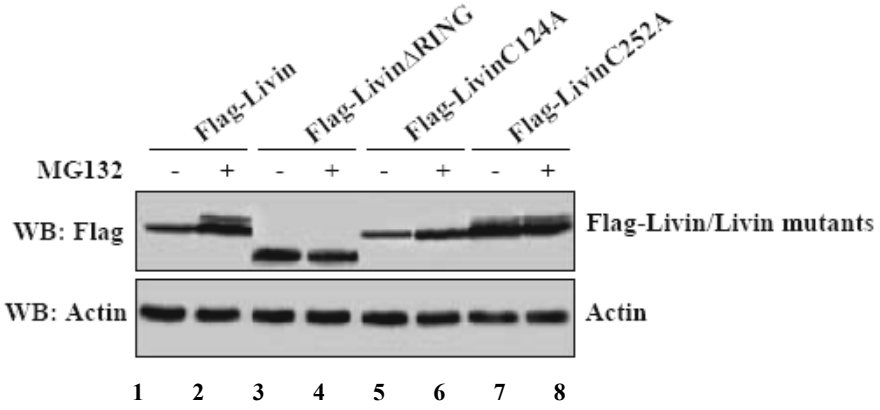


Figure 2.3.5 Protein stabilities of Livin RING domain mutants are inert to proteasome inhibition. HeLa cells were transfected with plasmid encoding Flag-Livin, Flag-Livin-ΔRING, Flag-Livin-C124A or Flag-Livin-C252A individually. 24 hours after transfection, cells in each well were split into two equal parts. One part was treated with 20μM MG132 and the other part was left untreated for 12 hours. Expression levels of different proteins were analyzed by Western blotting using anti-Flag antibody. Protein levels of Actin were used as loading controls.

2.3.3 Mutations in BIR domain result in reduced stability and anti-apoptotic activity of Livin

We noticed that either in the absence or presence of proteasome inhibition, protein level of Livin-C124A was always much lower than that of wt-Livin

Figure 2.3.5, lane 1, 2, 5, 6). This result suggests that BIR domain may have some essential role in controlling the stability of Livin in a proteasome independent way. Previously studies from nuclear magnetic resonance (NMR) indicated that the structure of BIR2 domain of XIAP resembles a classical zinc finger in which amino acids C200 and H220 are important for chelating zinc and stabilizing protein folding [74]. To further explore the function of BIR domain in controlling the stability of Livin in the term of resistance to degradation, we constructed two additional Livin BIR domain point mutants, namely Livin-W134A and Livin-H144A. Trp134 of Livin is highly conserved in IAP BIRs and His144 of Livin corresponds to His220 of XIAP. We then compared the stability of wt-Livin with that of Livin BIR mutants. As shown in Figure 2.3.6, the steady-state levels of each BIR domain mutants were much lower than that of wt-Livin in the absence of MG132 (lanes 1, 3, 5, 7, marked with asterisks), implying that Livin BIR mutants are less stable than wt-Livin. Upon treatment with MG132, the protein levels of wt-Livin and Livin BIR mutants were all largely increased (lanes 2, 4, 6, 8,), but levels of each BIR mutants (lanes 2, 4, 6) were still much lower than that of wt-Livin (lane 8). This result further demonstrates that the critical role of BIR domain in controlling the stability Livin does not involve effect on the ubiquitination process; BIR domain may be required for stabilizing correct protein folding of Livin molecule and thus disruption of BIR domain leads to destabilization of Livin in a proteasome independent way.

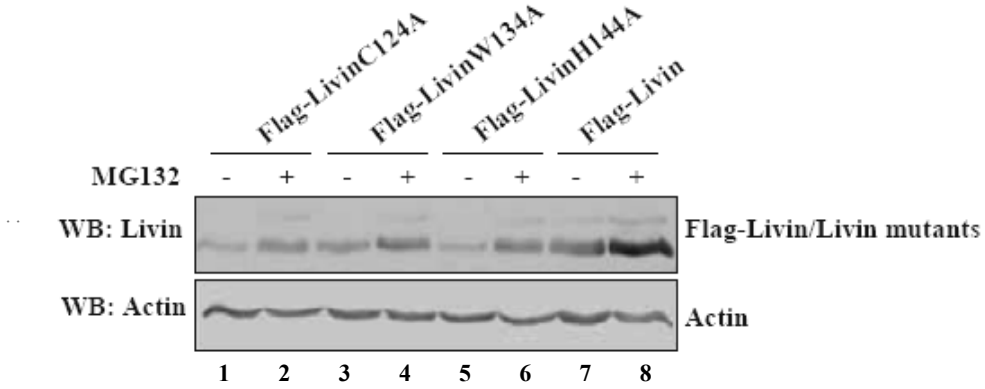
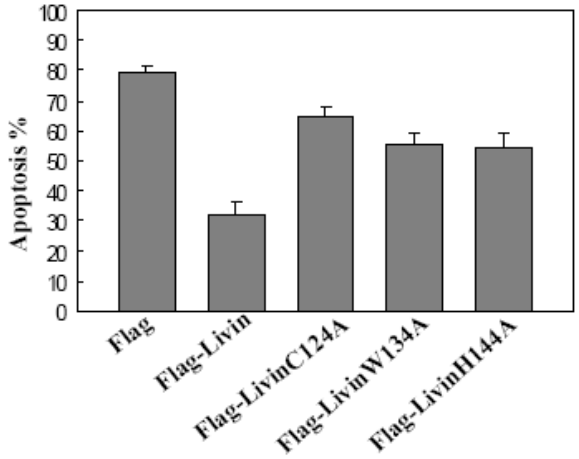


Figure 2.3.6 Mutation in BIR domain destabilizes Livin in a proteasome independent way. HeLa cells were transfected with Flag-Livin, Flag-Livin-C124A, Flag-Livin-W134A or Flag-Livin-H144A individually. 24 hours later, cells were split into two equal parts. One part was treated with MG132 and the other part was left untreated for another 12 hours. Cells were then collected and analyzed by Western blotting using anti-Flag antibody. Actin protein levels were used as loading controls.

Previously BIR domain was demonstrated to be critical for the anti-apoptotic functions of most IAP family proteins [34-37]. Hence we reasoned that if the single BIR domain that Livin possesses is required for its anti-apoptotic function, then cells expressing BIR mutant of Livin will be more prone to apoptosis than cells expressing wt-Livin. Because Livin has been demonstrated to be able to prevent HeLa cells from apoptosis induced by RIP3, we compared the protective ability of wt-Livin and Livin BIR domain mutants upon RIP3 stimulation. HeLa cells were cotransfected with pEGFP-C1-RIP3 plus wt-Livin, Livin-C124A, Livin-W134A or Livin-H144A respectively (the protein expression of each construct has been normalized to the same level). Cells were then stained with Hoechst 33342 and the viability of cells was scored and

compared by plotting. Bisbenzimidazole dye Hoechst 33342 can penetrate the plasma membrane and stain the DNA in cells without permeabilization. In contrast to normal cells, apoptotic cells have highly condensed chromatin that can be uniformly stained by Hoechst 33342. Thus after short exposure to Hoechst 33342, apoptotic cells would have stronger blue fluorescence compared to non-apoptotic cells. As shown in Figure 2.3.7 a, wt-Livin (32.3%) significantly reduced RIP3-induced apoptosis compared with vector control (79.2%). In contrast, BIR domain mutants Livin-C124A (57.41%), -W134A (49.42%) and -H144A (48.48%) showed obvious decreased anti-apoptotic activity, indicating that intact BIR domain is essential for Livin's anti-apoptotic function. In Figure 2.3.7 b, the execution of apoptosis in cells transfected with different mutants was also confirmed by cleavage of procaspase-9 and RARP.

a.



b.

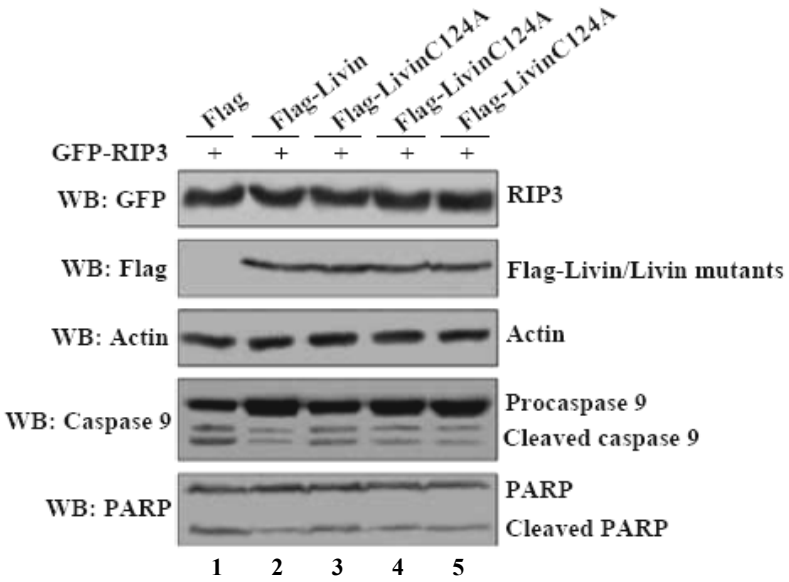


Figure 2.3.7 Mutation in BIR domain attenuates the anti-apoptotic activity of Livin (a). After cotransfection with pEGFP/C1-RIP3 along with Flag control, Flag-Livin, Flag-Livin-C124A, Flag-Livin-W134A or Flag-Livin-H144A for 24 hours, HeLa cells were stained with 5μg/ml Hoechst 33342. The viability of cells was determined by counting both living and dead GFP-positive cells. Five different microscopic fields containing 100–150 cells from each well were chosen at random for counting samples. (b). Expression of different constructs was analyzed by Western blotting with anti-Flag antibody. The cleavage of pro-caspase-9 or RARP was detected by anti-caspase-9 or anti-PARP antibody respectively.

2.3.4 Livin interacts with Smac/DIABLO *in vivo*

In addition to inhibiting caspases, IAP proteins may also exert anti-apoptotic function through negatively regulating the IAP antagonists such as Smac/DIABLO [61-63]. Livin was reported to physically interact with Smac *in vivo* [71]. It is possible that decrease in the anti-apoptotic activity of Livin BIR mutants is due to the loss of their ability to bind and inhibit Smac. Therefore first we determined whether Livin interacts with Smac under physiological situation. As shown in Figure 2.3.8, under normal state, no association between Livin and Smac could be detected (lane 1). However, upon apoptotic stimuli such as Taxol or etoposide treatment, when Smac could be released from mitochondria to the cytosol, endogenous Livin was readily be detected in Smac precipitates, suggesting that Livin specifically interacts with Smac in response to apoptotic stimulation.

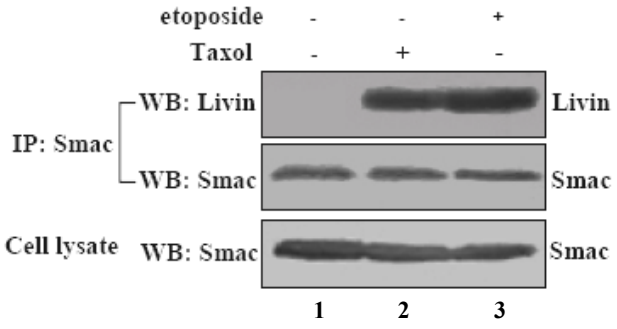


Figure 2.3.8 Livin interacts with Smac/DIABLO upon apoptotic stimulation. HeLa cells were treated with 200nM Taxol, 100μg/ml etoposide or left untreated for 24 hours. Cell lysates prepared from each sample were immunoprecipitated with anti-Smac antibody. The immunoprecipitates were then analyzed by Western blotting with anti-Livin or anti-Smac antibody.

Next we examined whether Livin associates with Smac through its BIR domain. As shown in Figure 2.3.9, in the absence of Taxol treatment, no association of Smac and wt-Livin or any Livin mutants could be observed. However upon Taxol stimulation, wt-Livin and Livin-ΔRING could be pulled down together with endogenous Smac. In contrast, none of Livin BIR mutants could be detected in the Smac precipitates, indicating that BIR domain is responsible for the association of Livin and Smac.

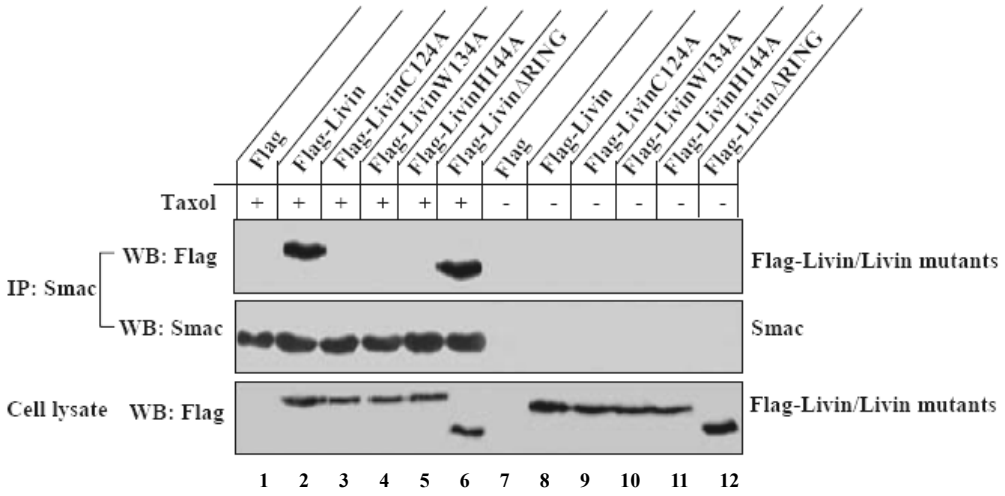


Figure 2.3.9 Livin interacts with Smac/DIABLO through its BIR domain. HeLa cells were transfected with wt-Livin or various Livin mutants for 24 hours before each transfectant was split into two equal parts. One part was treated with 200nM Taxol for 24 hours and the other part was left untreated. Cell lysates prepared from each transfectant were immunoprecipitated with anti-Smac antibody and analyzed by Western blotting using anti-Flag or anti-Smac antibodies.

2.3.5 Smac/DIABLO is an ubiquitination substrate for Livin

We have demonstrated that Livin physically interacts with Smac through its BIR domain. Because Livin contains RING domain which endows itself E3 ligase activity, we proposed that upon apoptotic stimulation, Livin would

possibly target its antagonist Smac for proteasomal degradation in favor of survival. To test it, protein levels of endogenous Smac in the presence of ectopically expressed wt-Livin or various Livin mutants were analyzed. As shown in Figure 2.3.10 a, in the absence of MG132, protein level of Smac significantly decreased in cells expressing Flag-Livin compared with that of cells expressing empty vector. In contrast, cells expressing Flag-Livin-ΔRING or Flag-Livin-C124A did not show obvious decrease in the protein level of Smac. In the presence of MG132, when Smac could be stabilized by proteasome blockade, protein levels of Smac became comparable among cells expressing wt-Livin and Livin mutants. This result suggests that Livin promotes proteasome dependent degradation of Smac in response to DNA damage stimulation; both RING domain and BIR domain of Livin are required for the accelerated degradation process.

A reduced level of Smac is expected to decrease its apoptotic potential. Therefore apoptosis induced by etoposide was measured in each of the four above-mentioned transfected cells and the corresponding results are plotted in Figure 2.3.10 b. Apoptosis of transfectants induced by etoposide ordered increasing is as follows: cells expressing Flag-Livin (37.17%), Flag-Livin-ΔRING (49.43%) and Flag-Livin-C124A (56.27%). This result is in agreement with the hypothesis that reduced degradation of Smac by Livin mutant could result in increased apoptosis.

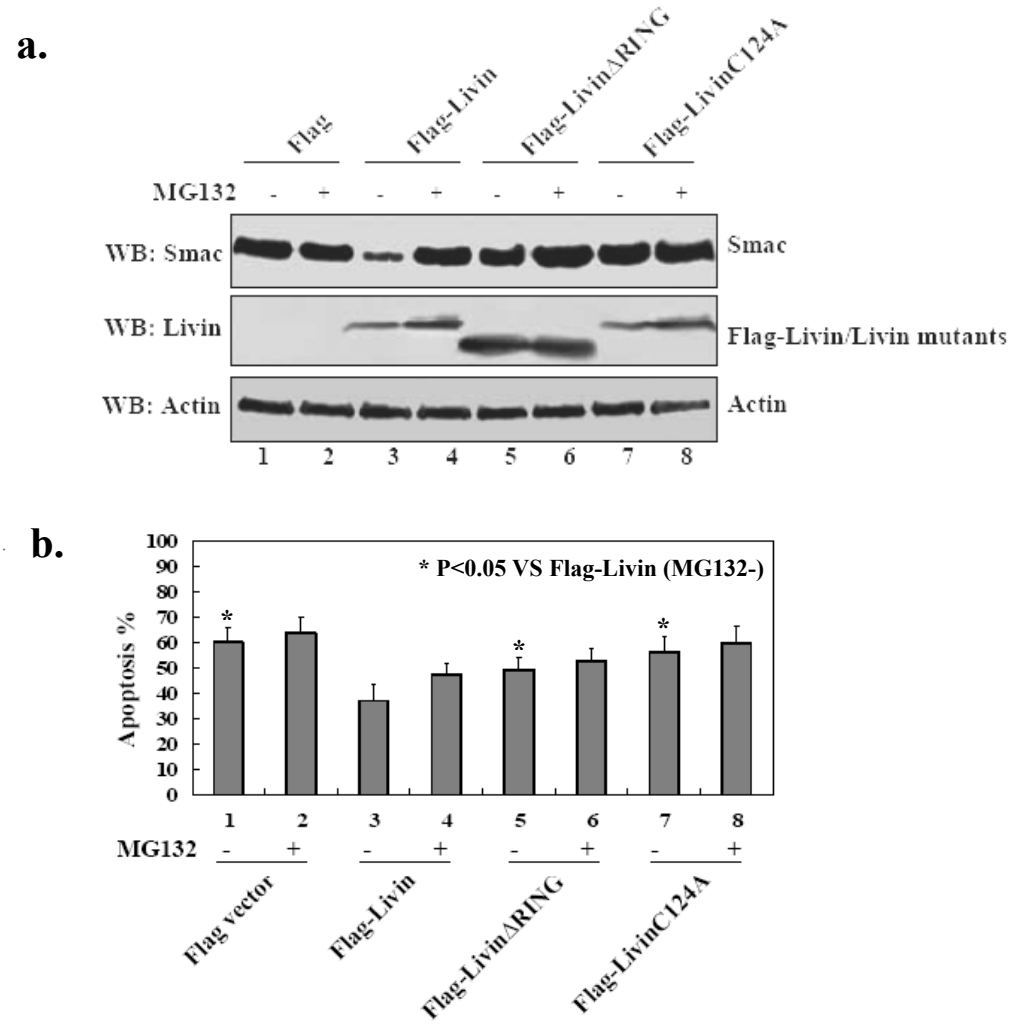


Figure 2.3.10 Livin accelerates proteasomal degradation of Smac and results in decreased apoptotic potential of Smac upon apoptotic induction. (a.) HeLa cells were transiently transfected with Flag, Flag-Livin, Flag-Livin-ΔRING or Flag-Livin-C124A. One day later, cells in each well were split into two equal parts and pretreated with 100μg/ml etoposide for 24 hours, followed by treatment with or without 20μM MG132 for 12 hours. Protein levels of Smac were analyzed by Western blotting using anti-Smac antibody. (b.) Cells were stained with Hoechst 33342 and both live and dying/dead cells were scored and the data were plotted as percent apoptosis. Each bar represents the mean from three independent experiments. Statistical analysis was by t-test with *P<0.05.

Next we examined whether Livin destabilizes Smac by promoting polyubiquitination of the protein *in vivo*. After transiently transfection with

Flag-Livin, Livin-ΔRING or Livin-C124A, cells were stimulated with etoposide for 12 hours followed by proteasome inhibition for further 12 hours. Endogenous Smac was then immunoprecipitated by anti-Smac antibody. As shown in Figure 2.3.11, ectopic expression of wt-Livin led to great recovery of polyubiquitinated Smac, whereas cells expressing either Livin BIR mutant Flag-Livin-C124A or RING mutant Flag-Livin-ΔRING showed little, if any background ubiquitination of Smac. The ubiquitination of Smac in the presence of wt-Livin was also confirmed by Western blotting using anti-Smac antibody (Figure 2.3.11, right panel). Together, these data indicate that Livin promotes polyubiquitination and proteasomal degradation of Smac upon apoptotic stimulation; the ubiquitination process depends on the presence of both intact Livin RING finger domain and functional BIR domain.

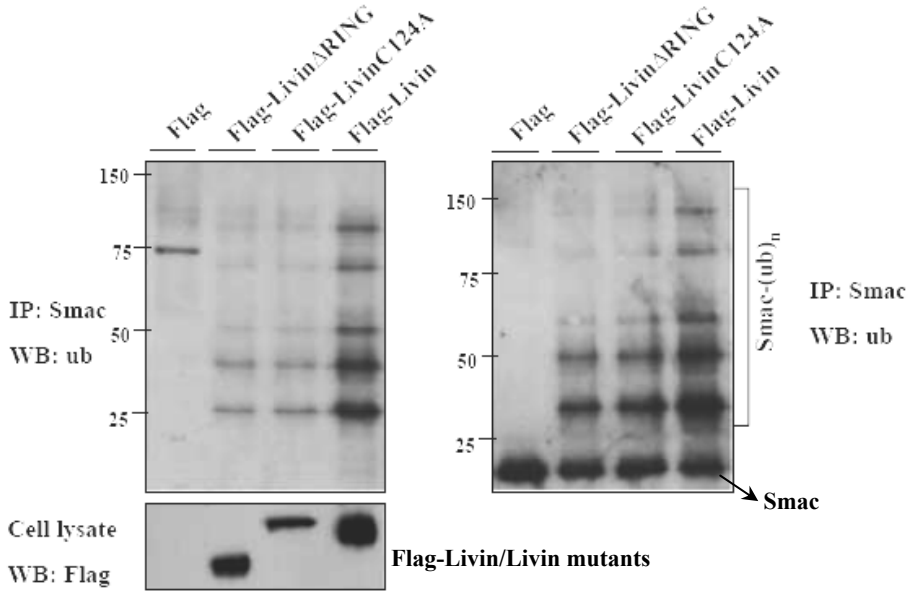


Figure 2.3.11 Livin promotes proteasome dependent polyubiquitination of Smac. HeLa cells were transiently transfected with Flag, Flag-Livin-ΔRING, Flag-Livin-C124A or Flag-Livin. 24 hours after transfection, cells were treated with 20μM etoposide for 12 hours followed by treatment with or without 10μM MG132 for further 12 hours. Cell lysates were immunoprecipitated with anti-Smac antibody and the immunoprecipitates were analyzed by Western blotting using anti-ubiquitin or anti-Smac antibody.

Lastly, to provide more direct evidence that Livin serves as an E3 ligase to ubiquitinate Smac, an *in vitro* ubiquitination assay was performed. As shown in Figure 2.3.12, Smac was found to be ubiquitinated by GST-Livin (lane 8), but not GST-Livin-ΔRING or GST-Livin-C124A (lanes 6, 7), indicating that Livin acts as E3 ligase to bridge E2 and Smac together and promotes Smac ubiquitination *in vitro*. BIR domain and RING domain are essential for Livin's E3 ligase activity under *in vitro* condition, which is in accordance with the result of *in vivo* ubiquitination assay.

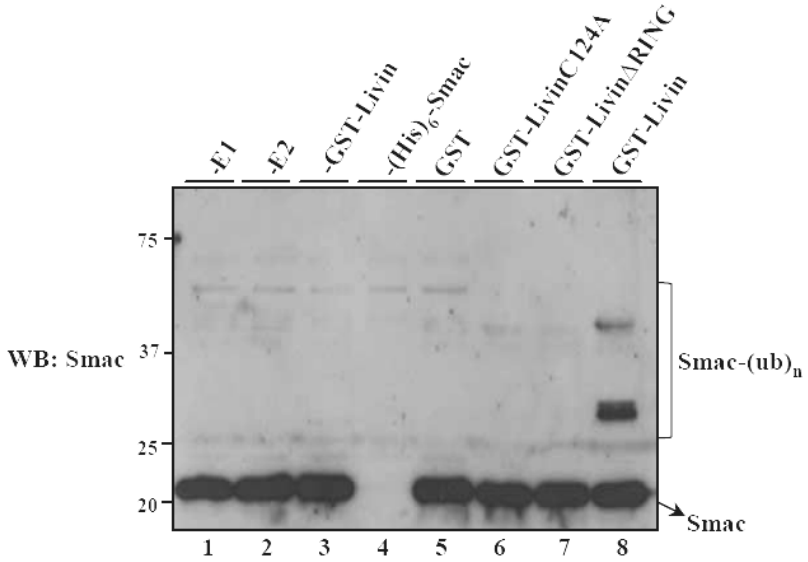


Figure 2.3.12 Livin ubiquitinates Smac/DIABLO *in vitro*. Assays were performed in the presence of all the other assay components, but in the absence of purified recombinant $(His)_6$-Smac, E1, E2, GST-Livin for 90 min (lanes1–4). The effect of purified GST, GST-Livin, GST-Livin-C124A or GST-Livin-ΔRING on the ubiquitination of recombinant $(His)_6$-Smac are shown in lane 5–8. SDS was added to stop the reaction followed by Western blotting analysis using anti-Smac antibody.

2.4 Discussion

2.4.1 Autoubiquitination of Livin

It has been demonstrated that XIAP, c-IAP1 and c-IAP2 undergo autoubiquitination mediated degradation both *in vivo* and *in vitro* [57]. RING domain was reported to be essential for the E3 ligase activity and autoubiquitination of these IAP proteins. We found that like other IAP proteins, Livin undergoes autoubiquitination and rapid degradation by 26s proteasome. However, RING domain deletion mutant of Livin failed to autoubiquitinate itself and its degradation is inert to the proteasome inhibition treatment. These results demonstrate that autoubiquitination is critical for the rapid degradation of Livin under normal state; RING domain mediated E3 ligase activity is required for the autoubiquitination of Livin *in vivo*.

2.4.2 BIR domain is essential for the stability and anti-apoptotic activity of Livin

We found that Livin BIR mutant Livin-C124A showed much less stability compared with that of wt-Livin. The reason as we initially proposed may be that mutation at BIR domain accelerates autoubiquitination and proteasomal degradation of the protein. However, destabilization of Livin-C124A was not sensitive to proteasome inhibitor treatment and similarly Livin-C124A exhibited much less autoubiquitination than that of wt-Livin, suggesting that Livin BIR mutant is degraded by an as yet uncharacterized mechanism that is independent of proteasome. Vucic *et al.* reported that Livin-C124A cannot

block Fas and TNFR1 induced apoptosis, as C124 together with H144 are predicted to chelate zinc ions to stabilize the overall structure of Livin [68]. We demonstrated that two other BIR domain point mutants Livin-W134A and Livin- H144A are degraded more rapidly than wt-Livin. Therefore it is possible that intact BIR domain is crucial for stabilizing protein folding or alternatively suppresses the degradation of Livin by some uncharacterized mechanism.

All three Livin BIR domain mutants showed less effectiveness in inhibiting RIP3-induced apoptosis, supporting the conclusion that Livin's anti-apoptotic activity is dependent on the functional BIR domain. Till now, no obvious role has been reported for the amino acid W67 in Survivin or W210 in XIAP according to their anti-apoptotic activities. We firstly demonstrated that the amino acid W134 of Livin which corresponds to W67 in Survivin and W210 in XIAP is functionally important. The Livin BIR domain point mutant Livin-W134A not only decreases its ability to protect cells from RIP3-induced apoptosis, but also abrogates interaction of Livin and mitochondria death inducer Smac. It suggests that the amino acid W134, like C124 and H144, is a critical residue for Livin to bind Smac. Taken together, these results indicate that integrity of BIR domain stabilizes Livin and enables the protein to exert anti-apoptotic activity in response to apoptotic stimulation.

2.4.3 To live or to die: Livin VS Smac/DIABLO

Under some circumstances, RING domain mediated E3 ligase activity enables

IAP proteins to promote polyubiquitination and degradation of proteins that physically interact with them. Caspases are inactivated when they bind to IAPs or antagonists of IAPs such as Smac, whereas Hid/Grim/Reaper in *Drosophila melanogaster* activates caspases by alleviating the interaction between IAPs and caspases. XIAP and DIAP1 were reported to induce proteasome dependent polyubiquitination of Reaper which subsequently led to a significant impact on Reaper's ability to initiate apoptosis [60]. BRUCE functions as an E2-Ubc for both Smac and caspase-9 that displays an essential cytoprotection function in preventing Smac induced apoptosis [75]. In addition, the DIAP1 RING finger in *Drosophila melanogaster* was demonstrated to mediate ubiquitination of Dronc which does not have obvious function in regulating apoptosis [76]. Livin interacts with caspase-3, -7 and -9 and proteins containing IAP-interacting motif such as Smac/DIABLO and Omi/HtrA2. Smac is a negative regulator of Livin in response to apoptotic stimulation. Wilkinson *et al.* reported that IAPs could inhibit apoptosis through neutralization of IAP antagonists, such as Smac. In addition to directly inhibiting caspases, Livin is believed to sequester Smac , which prevents Smac from antagonizing XIAP-mediated inhibition of caspases [72]. However, whether Livin antagonizes Smac through its RING domain dependent ubiquitin ligase activity has not yet been characterized. We demonstrate that ectopically expression of wt-Livin, but not of Livin RING domain or BIR domain mutant could promote polyubiquitination and degradation of Smac upon DNA damage treatment. Wt-Livin protects cells

from etoposide induced cell death more effectively than either RING deletion mutant or BIR domain point mutant. Ubiquitination of Smac by Livin requires both functional BIR and RING domain *in vivo* and *in vitro*. Livin binds to Smac through its BIR domain and their interaction is the prerequisite for targeting Smac degradation by proteasome. Equally important, Livin RING finger domain is also essential for promoting ubiquitination of Smac. However, it should be noted that the smeared ubiquitin-modified Smac bands in the anti-ubiquitin or anti-Smac blots were still detected in the presence of Livin-C124A or Livin-ΔRING. This is not unexpected as Smac is also subjected to polyubiquitination by other endogenous IAP factors, such as BRUCE, cIAP-1 and cIAP-2.

The struggle between cell survival and death is ubiquitous and is maintained in balance under intricate regulations including the mechanism of proteasome ubiquitination. On the one hand, by catalyzing its own ubiquitination, Livin lowers the threshold for inducing apoptosis and makes cells more perceptible for cell death. On the other hand, Livin targets death inducer Smac/DIABLO for degradation through the ubiquitin-proteasome pathway, promoting cell survival. Understanding the detailed mechanisms underlying the dual function of Livin in regulating cellular apoptosis and survival requires more extensive future research.

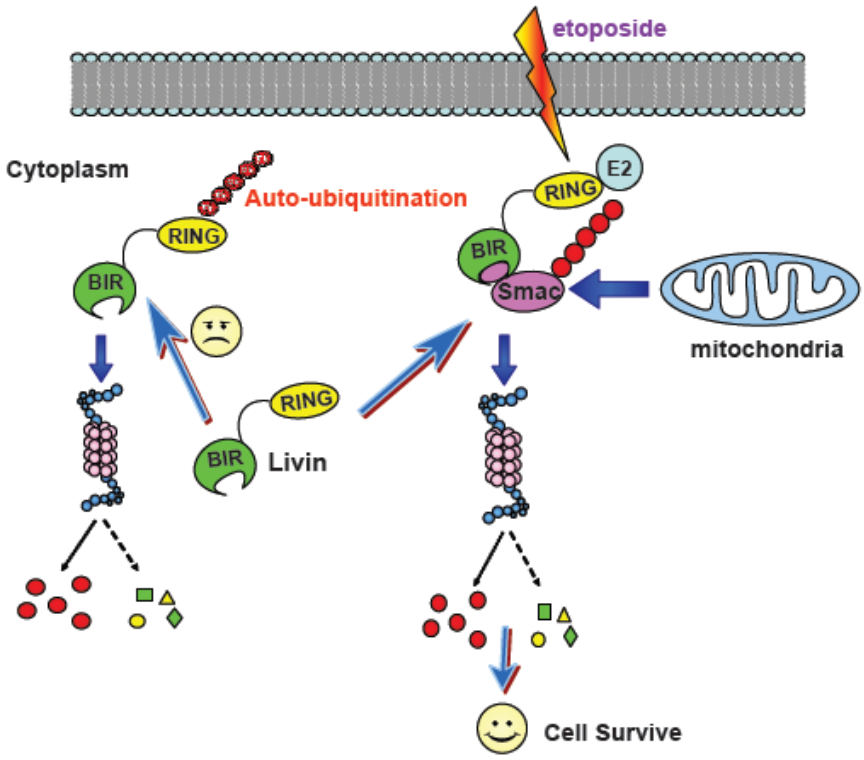


Figure 2.4.1 Livin acts as E3 ligase to promote ubiquitination dependent degradation of its target Smac/DIABLO or itself. Under non-stressed state, RING domain mediated E3 ligase activity promotes auto-ubiquitination of Livin and thus lowers the threshold for inducing apoptosis. However, upon apoptotic stimulation, Livin can target death inducer Smac/DIABLO for ubiquitin-proteasome dependent degradation and thus promotes cell survival.

Chapter 3 Tumor suppressor ARF promotes non-classic proteasomal independent ubiquitination of COMMD1

3.1 Introduction

3.1.1 MDM2/p53 checkpoint

The tumor suppressor p53 is always described as the genome guardian because of its important role in maintaining the genome integrity through arresting cell cycle progression or inducing apoptosis in response to various hyperproliferative stimulation [77]. Genetic alteration of *p53* gene has been seen in close to 50% of human cancers and people carrying one altered *p53* gene are highly prone to spontaneous and carcinogen induced tumors [77]. p53 is a short lived protein (half life<15min) and is often undetectable in normal cells. However, upon treatment with DNA damage reagent, hypoxia or oncogenic signals, p53 is activated through posttranslational modifications that increase its stability and activity. Activated p53 is a DNA sequence specific DNA binding transcription factor. A series of target genes of p53 have been identified which include $p21^{cip\text{-}1}$, *cyclin B1* and *14-3-3σ* which are involved in cell cycle arrest; *Bax*, *Fas* and *DR5* which have been implicated for p53 mediated apoptosis signaling [78].

Rapid p53 turnover under normal state is largely due to MDM2, a RING finger containing E3 ligase. It specifically binds p53 and promotes ubiquitin conjugation to p53, resulting in proteasomal degradation of the protein [79, 80]. In addition to promoting degradation of p53, MDM2 also facilitates nuclear export of the protein under nonstressed state [81]. Early works indicated that MDM2 induced nuclear export of p53 relies on its ability to promote ubiquitin

conjugation to p53 [82, 83]. As MDM2 induced polyubiquitination is also required for proteasomal degradation of p53, it was then believed that p53 is mainly degraded in the cytoplasm. However, this dogma was challenged by the observation that when p53 and MDM2 are forced to stay within the nucleus, nuclear proteasome can effectively degrade p53 in a MDM2 dependent manner [84]. More recently, Li *et al.* demonstrated that inhibition effect of MDM2 on p53 depends on the amount of MDM2 [19]. When MDM2 is abundant, it promotes polyubiquitination and degradation of p53 in the nucleus. However, when MDM2 is scarce, it only promotes monoubiquitination and nuclear export of p53. What's the functional consequence of each type of p53 ubiquitination? Li *et al.* further proposed that both options may contribute to the inactivation of p53: in non-stressed cells where MDM2 level is very low, monoubiquitination mediated nuclear export of p53 makes it have no transcriptional activity in the nucleus; However upon DNA damage stimulation, when p53 is needed to act at the first line of defense, cytoplasmic p53 may undergo rapid deubiquitination and reenter into nucleus to activate downstream effector genes. After successful completion of DNA damage repair, cells may want to eliminate large amount of p53 immediately. At this stage MDM2 is abundant enough to induce p53 polyubiquitination and quick degradation in the nucleus [19].

The precise mechanism by which p53 is activated in response to a wide spectrum of cellular stresses is still not entirely clear. Upon stimulation with chemotherapeutic agent or irradiation, phosphorylation of p53 prevents its

interaction with MDM2 and leads to inhibition of MDM2 mediated degradation [85]. Under certain circumstances, such as oncogene activation, MDM2 associating protein ARF can bind to the MDM2-p53 complex and stabilize p53 through inhibition on MDM2 [86-88]. Overexpression of MDM2 homologue MDMX was also reported to compete with MDM2 for binding with p53 and thus inhibit MDM2 mediated ubiquitination [89]. Together, these evidences suggest that stabilization of p53 through inhibition on MDM2 is critical for p53 activation in response to various stress stimulation.

3.1.2 NF-κB signaling pathway

NF-κB is a pleiotropic transcriptional factor that controls the expression of genes involved in multiple cellular processes including immune response, inflammation, infection, apoptosis and cell cycle progression. In mammals, the NF-κB subunits are encoded by five genes (*RELA*, *RELB*, *REL*, *NFKB1* and *NFKB2*) [90]. RelA is the major transactivating unit of NF-κB. All subunits share an N-terminal ~300 amino acid region of homology termed Rel homology domain (RHD) that mediates DNA binding and dimerization. The *NFKB1* and *NFKB*2 encode large precursor proteins p105 and p100, which are cleaved into mature p50 and p52 subunits respectively [90].

In nonstressed cells, NF-κB is normally sequestered in the cytoplasm in a transcriptional inactive form through interaction with Inhibitor of NF-κB (IκB) proteins. A small amount of RelA could also be detected in the nucleus as a

result of constitutive degradation of IκB. This preexisting nuclear Rel-A may be important for the expression of certain target genes involved in maintaining normal cellular functions [91]. In response to a variety of stimuli, IκB proteins are phophorylated by IκB kinases (IKK), which subsequently lead to their ubiquitination by the SCFb-TrCP ubiquitin ligase [92, 93]. Quick removal of IκB allows nuclear translocation of NF-κB to occur. Nuclear NF-κB complex then binds to the specific DNA sequences and induces gene expressions. In addition to nuclear translocation, transcriptional activation also requires recruitment of co-activators and displacement of co-repressors which are mediated through posttranslational modifications of NF-κB subunits such as phosphorylation and acetylation. The termination of NF-κB mediated transcription is controlled by the nuclear export of NF-κB. Through a negative feedback loop NF-κB induces the synthesis of IκB proteins. Resynthesized IκB then binds to NF-κB again and triggers nuclear export of NF-κB and termination of transcription.

NF-κB plays an important role in tumorigenesis. Considerable evidences suggest that NF-κB has both oncogenic and tumor suppressor properties. Aberrantly activated NF-κB has been reported to be required for the transforming property of many oncogenes including Bcr-Abl, Ras and Tax [94-96]. The anti-apoptotic activity of NF-κB is critical after stimulation with cytokines such as TNF-α [97]. Moreover, NF-κB is activated by chemotherapeutic agents and ionizing irradiation, where its anti-apoptotic

activity has inhibitory effect on the efficacy of these treatments [98, 99]. The protective function of NF-κB could be explained by the fact that NF-κB activates the transcription of a number of anti-apoptotic genes including those encoding IAP proteins, TRAF (TNF receptor associated factor), Bcl-xL and A20 [100]. However, in some circumstances, NF-κB target genes also include those that encode pro-apoptotic factors such as Fas, FasL (Fas ligand), DR4 and DR6 [100]. Previously Rel-A was reported to be required for apoptosis induced by serum starvation [101]. Enforced expression of Rel-A induces apoptosis in breast cancer cell and pro-B cells [102, 103]. Moreover, both antagonistic and co-operative functions of Rel-A have been demonstrated for p53 mediated cell death in different cell types or in response to different stimuli [104]. Therefore whether NF-κB prevents or facilitates apoptosis is still a matter of controversy and sometimes is dependent on stimuli and cell type used in the study.

3.1.3 Tumor suppressor ARF

3.1.3.1 *INK4A/ARF* locus

The *INK4A/ARF* at chromosome 9p21 is among the most frequent sites of genetic loss in human cancer [105]. Deletion of this locus is often found in a variety of malignant cell lines including glioblastoma, melanoma, pancreatic adenocarcinoma, non-small lung cancer, bladder carcinoma and oropharyneal cancer. Therefore it is suggested to be of second or equivalent importance to the tumor suppressor p53 [105]. The *INK4A/ARF* locus encodes two potent and

distinct tumor suppressors, $p16^{INK4A}$ and p14ARF (p19ARF in mouse), which regulates the growth suppressive activity of RB and p53 respectively.

RB protein is a regulator of G1 cell cycle checkpoint. It is dephosphorylated and activated at the end of G1 phase. Activated RB then binds to and inhibits E2Fs mediated activation of genes that are needed for DNA synthesis and nucleotide metabolism. However, if it is time for entry into S phase, the ability of RB to inhibit E2F could be suppressed through phosphorylation of RB, a process catalyzed by Cyclin dependent kinases (CDKs). $p16^{INK4A}$ is an inhibitor of cyclin dependent kinase CDK4 and CDK6, so it can maintain RB in its hypophosphorylated and growth-suppressive state through inhibiting the activity of CDK4 and CDK6 [106]. Inactivation of $p16^{INK4A}$ by deletion, point mutation or promoter methylation occurs in many human cancers, suggesting that it is an important tumor suppressor at 9p21 [107].

The discovery of ARF was surprising. Sherr's lab first found that an alternative first exon of *INK4a* could be transcribed at the locus, producing an alternative reading frame, from which ARF takes its name [108]. This alternative reading frame has its own promoter and first exon (exon1β), but shares the second and third exon with $p16^{INK4A}$ (Figure 3.1.1). $p16^{INK4A}$ and ARF are not isoforms and have no amino acid homology, but both of them were found to play critical roles in prevention of tumorigenesis. Evidences that ARF is also a tumor suppressor came from the observation that the protein was a potent cell cycle

inhibitor [108]. Mice lacking exon 1β of ARF were highly prone to spontaneous or carcinogen induced tumors which is similar to the loss of $p16^{INK4A}$ [109]. Although ARF protein level is not detectably in normal cell tissues, stimulation with hyperproliferative signals such as Myc, E2F1 and Ras can induce the transcription of ARF. Activated ARF subsequently activates p53 mediated cell cycle arrest or apoptosis through inhibition on p53 negative regulator MDM2 [110-113]. The ARF/MDM2/p53 therefore serves as a checkpoint that protects cells from oncogene induced tumorigenesis.

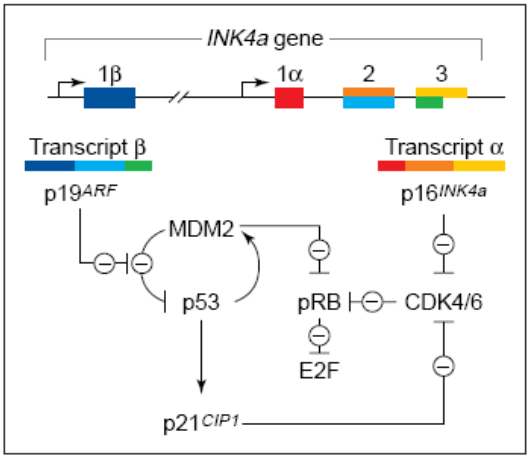


Figure 3.1.1 The *INK4a/ARF* locus utilizes alternative first exons and common downstream exons to encode two distinct products. Reproduced from [114].

Till now, the ARF gene has been identified in several species including human (132aa, p14), mouse (169aa, p19), rat (160aa, p19), gray opossum (169aa, p19) and chicken (60aa, p7). Human ARF shares only 49% and 44% amino acid sequence identity with ARF in mouse and opossum respectively. Chicken ARF has only 60 amino acids encoded by exon1β but still possesses the ability to bind MDM2 and stabilize p53 [115]. Surprisingly, there is no alternative

reading frame and no $p16^{INK4A}$ homologue either in chicken. Such a high degree of sequence divergence suggests that ARF may not be evolved to perform function for cell survival and development [18]. In fact many established cell lines have homozygous deletion of *INK4A/ARF* locus and mice deficient for ARF developed tumor in their early life but exhibited no obvious developmental defects [107, 116]. The MDM2 gene, best identified target of ARF has been identified in frog, zebrafish and asicidan, suggesting that it evolved earlier than ARF. If go back further, MDM2 major target, p53 gene can be found in the drosophila *melanogaster* genome. It therefore implicates that the ARF-MDM2-p53 pathway may be evolved by the sequential addition of an upstream regulator during evolution [18].

3.1.3.2 ARF-MDM2-p53 pathway

The initial clue to ARF's function in regulating p53 pathway came from the observation that overexpression of ARF in wild type but not p53-/- cells caused cell cycle arrest, suggesting that ARF acts upstream of p53 [117]. Upon hyperproliferavtive signals such as oncogenic activation, ARF was found to be transcriptional activated and led to increased level of p53 and p53 target gene $p21^{Cip-1}$ [117]. Further evidences from a number of studies revealed that ARF activates p53 through physically associating with MDM2 and relieving its inhibition effect toward p53 [86, 118, 119].

The exact mechanism by which ARF inhibits MDM2 is still a matter of

controversy. In some circumstances, ARF was found to relocalize MDM2 in the nucleolus. A portion of MDM2 was found in nucleolus in HeLa cells co-expressing ARF or in aging MEFs (Mouse embryo fibroblasts) where both MDM2 and ARF protein levels are elevated [88, 120]. Relocalization of MDM2 in nucleolus was then suggested to dissociate MDM2-p53 complex and release p53 from MDM2 mediated degradation and transcriptional inhibition. However, till now dissociation of MDM2-p53 complex by ARF has not been observed. Expression of ARF mutants lacking main Nucleolar localization signal (NoLS) still induced p53 stabilization and transcriptional activity *in vivo*, indicating that ARF could activate p53 through a mechanism that does not require nucleolar sequestration of MDM2 [121, 122]. However, in certain circumstances, relocalization of MDM2 by ARF in nucleolus may contribute to the stabilization and activation of p53 [88].

In addition to sequestering MDM2 in the nucleolus, ARF may stabilize p53 and block nuclear export of p53 by inhibiting the E3 ligase activity of MDM2. It was demonstrated that MDM2 mediated nuclear ubiquitination facilitates either nuclear export of p53, causing p53 to have no transactivation activity or proteasomal degradation of p53 in nucleus [82, 83]. Binding with ARF could inhibit p53 specific E3 ligase activity of MDM2 both *in vivo* and *in vitro* [122, 123]. Therefore it is possible that ARF could activate p53 pathway through inhibiting p53 ubiquitination in the nucleus and thus preventing p53 nuclear export and degradation of p53 by the proteasome [122, 123].

3.1.3.3 Subcellular localization

ARF is expressed at high level and predominantly nucleolar in *p53* deficient cancer cell lines [119]. In human fibroblast, the level of ARF is undetectable under normal state, but the gene expression and nucleolar localization of ARF can be induced in response to oncogene stimuli or ionize irradiation [88]. However，it should be noted that ARF is not only a nucleolar protein. In some circumstances, a significant fraction of the protein is also detected in nucleoplasm and sometimes it is predominantly nucleoplasmic without nucleolar localization [87, 124]. Predominantly nucleoplasmic localization of ARF could be detected in human cervical carcinomas, aggressive B-cell lymphomas and tumors in nervous system [125-128]. Interestingly, under DNA damage treatment with UV, actinomycin D or cisplatin, nucleolar ARF redistributes immediately into the nucleoplasm in tumor cell lines [129, 130]. DNA damage also disrupts the interaction of ARF with nucleolar protein B23 and enhances its association with MDM2 in the nucleoplasm, suggesting that ARF could serve as a link between nucleolar stress response and downstream events that activate the p53 pathway [129]. Thus ARF is only restricted in the nucleolus but exhibits a more dynamic nucleolar-nucleoplasmic shuttling localization.

3.1.3.4 Functional domains

ARF proteins are highly basic proteins which contain ~20% arginine. With isoelectric point greater than 12, they are very distinguishable among cellular

signaling factors. Exon 1β of *INK4a/ARF* locus encodes the first 64 and 63 amino acids of human and mouse ARF respectively, while exon 2 encodes the C-terminal domain. The extreme N-terminal regions of human and mouse ARF are very similar (17/29 identity; 21/29 similarity) and possess a repeated motif that contains hydrophobic residues flanked by the arginine residues [131]. Secondary structure predictions for ARF proteins suggest that there is a common β-sheet strand at N-terminus. In accordance with this prediction, the random conformation of mouse ARFN37 strikingly changes to a β-strand secondary structure when the protein is mixed with MDM2 *in vitro*, suggesting that unless it complexes with some proteins, ARF alone could be probably unstructured *in vitro* or in living cells.

Importantly, peptides containing conserved N-terminal segments of human and mouse ARF have been shown to possess tumor suppressor function which is similar or equivalent to that of wild type ARF [87, 108, 132]. For instance, a peptide containing N-terminal 37 amino acids of mouse p19ARF is able to bind and sequester MDM2 in the nucleolus and thus activate p53 dependent cell cycle arrest [88]. Additionally, a N-terminal 20 amino acids peptide from human p14ARF has a prominent inhibition effect on MDM2 mediated ubiquitination of p53, leading to stabilization and activation of p53 [122]. Further study of ARF N-terminus showed that two distinct domains 1-14 and 26-37 of mouse p19ARF bind individually to MDM2 and are both required for the normal function of ARF [133]. Human p14ARF also makes multiple

contacts with MDM2 that the motifs beyond the first 20 amino acids contribute to the interaction with MDM2 and p53 stabilization [134].

The NoLS of mouse and human ARF is specified by the sequence RRPR [131]. NoLS in mouse p19ARF was found between residues 31-34. Interestingly, when p19ARF binds to MDM2, the ARF NoLS is masked and nucleolar colocalization of ARF/MDM2 relies on the exposure of NoLS in the RING domain of MDM2 [131]. The NoLS in human p14ARF is between residues 87-90 within the polypeptide encoded by exon 2. Point mutation or small deletion of this region was found to impair the nucleolar localization of p14ARF [121]. However, in addition to nucleolar localization, C-terminal residues 65-132 have been shown to have weak or no contribution to the MDM2 binding and p53 response [87, 108, 132].

3.1.3.5 p53 independent functions of ARF

ARF has anti-proliferative activities that are independent of p53. Mice lacking *ARF*, *MDM2* and *p53* developed a much wider spectrum of tumors than mice lacking *ARF* or *p53* alone: These triple dropout mice frequently developed multiple primary tumors at independent sites, where they could involve mesenchymal, epithelial, hematopoietic or neural cells [135]. Overexpression of ARF prevented proliferation of MEF cells that lack p53 function, although their arrests occurred at much slower rate than those with wild type p53 function [136]. Analysis of temporal gene expression pattern identified *ARF* responsive

genes whose induction could be both p53 dependent and p53 independent. The latter includes four members of the B-cell translocation gene family (*Btg1*, *Btg2*, *Btg3*, and *Tob1*) that were demonstrated to inhibit cell proliferation in primary MEFs lacking functional p53 [137]. Recently, ARF was reported to regulate the activity of NF-κB subunit RelA in a MDM2 and p53 independent way [138, 139]. This effect is partially achieved by increased association of Rel-A and histone deacetylase HDAC1 induced by ARF. A p53 independent role of ARF in control of ribosome biogenesis has also been proposed. ARF was reported to delay rRNA processing in the nucleolus [140-142]. Moreover, ARF regulates the ribosome biogenesis through association with and inhibition on nucleolar protein B23, which is involved in maturation of preribosomal particles [141]. Together, these data suggest that ARF has MDM2 and p53 independent role as a tumor suppressor.

3.1.3.6 Identification of novel ARF interacting proteins

Till now at least 30 proteins have been identified as ARF binding partners by using co-immunoprecipitation, GST-pull down or yeast two hybrid assay (Table3.1.1). ARF interacts with and destabilizes S phase inducing transcription factors E2F1, E2F2 and E2F3 which in turn results in reduced transactivation activity of these proteins. In addition to antagonizing p53's negative regulator MDM2, ARF also stabilizes p53 through association and inhibition on ARF/binding protein 1 (ARF/BP1) which has p53 specific E3 ligase activity [143]. Other proteins that associate with ARF include spinophilin, a

phosphotase binding protein, topisomerase I, MDMX, HIF1-α (Hypoxia inducible factor 1-α, Tat binding protein 1 (TBP 1) and DTF1 polypeptide 1 (DP1). The physiological relevancies of these interactions in living cells are still largely unknown. Some of them are implicated in conveying p53 and MDM2 independent functions of ARF.

Table 3.1.1 ARF interacting proteins

ACNO	Binds histone deacetylases and inhibits transcription by nuclear receptors	[144]
ARF/BP1	HECT-containing E3 ubiquitin ligase	[143]
ATR, ATM	Protein kinases that are activated by DNA damage	[139]
CARF	A collaborator of ARF, no known biochemical function	[145]
CTBP1	C-terminal binding protein; an anti-apoptotic transcriptional co-repressor that is destabilized by p14ARF	[146]
Cyclin G1	Canonical p53-induced gene product that recruits PP2A to dephosphorylate MDM2	[147]
E2F1, DP1	Transcription factors that are required for DNA replication	[148, 149]
FOXM1B	Transcription factor from the forkhead box (Fox) family	[150]
LZAP	Leucine zipper-containing ARF-binding protein that antagonizes the inhibition by ARF of MDM2 E3 ligase activity	[151]
HIF1α	Transcription factor that is induced by hypoxia	[152]
MDM2	An E3 ubiquitin ligase for p53	[118]
MYC	Transcription factor capable of the transactivation and transrepression of many target genes	[153]
NPM	Nucleolar phosphoprotein that is implicated in ribosome biogenesis centrosome, duplication and the DNA-damage response	[141]
p120E4F	Cellular transcriptional repressor of the adenovirus E4 gene that is antagonized by adenovirus E1A oncoprotein and increases ARF-induced arrest	[154]
Spinophilin	Type-1 phosphatase binding protein	[144]
TBP1	HIV Tat-binding protein	[155]
Topoisomerase I	Relaxes DNA supercoils	[156]
UBC9	An E2 ligase required for sumoylation	[157]
WRN	Werner helicase mutated in premature-ageing syndrome	[158]
YY1	Transcription factor; a co-factor of MDM2 and negative regulator of p53	[159]

Reproduced from [160].

3.1.3.7 Ubiquitination and sumoylation induced by ARF

Human p14ARF lacks lysine residue and mouse p19ARF contains only one lysine which is not conserved among ARF proteins from different species. It was then suggested that ubiquitin-proteasome pathway does not regulate ARF turnover. However, this dogma was challenged by the observation that in tsBN75 cell line carrying a temperature-sensitive ubiquitin E1-activating enzyme, both p19ARF and p14ARF were found to accumulate at the non-permissive temperature [161]. Like wild-type p19ARF, an engineered lysine-less p19ARF K26R mutant, and human p14ARF which contains no lysine residue were each found to undergo polyubiquitination and proteasome mediated degradation, implicating that the primary N-terminal amino group of ARF proteins may serve as the site for ubiquitin modification [161].

ARF also regulates the turnover of its binding partners by affecting the ubiquitination process. ARF inhibits the function of B23, a nucleolar endoribonuclease involved in the 28sRNA maturation through promoting B23 polyubiquitination and proteasome dependent degradation [141]. In some circumstances, overexpression of ARF destabilizes endogenous MDM2, E2F1 and DP1 by facilitating polyubiquitination of the proteins [86, 149, 158, 162], which subsequently results in reduced oncogenic function of these proteins.

Strikingly, in addition to promoting ubiquitin conjugation, ARF also targets proteins associated with it for small ubiquitin like modifier (SUMO)

modification. SUMO is an ubiquitin like (Ubl) protein that can be conjugated to and alter the functions of a large number of proteins involved in different cellular functions [163]. The pathway of sumoylation is mechanistically analogous to ubiquitination. Like ubiquitin, SUMO is covalently attached to the substrate protein through isopeptide bond between C-terminal glycine in SUMO and lysine residue in the substrate. The sumoylation process also involves E1 activating enzyme, E2 conjugating enzyme, but till now UBC9 is the only known E2 enzyme for sumoylation. To date, a number of E3 enzymes have been identified to promote SUMO transfer from E2 to the specific substrate. They are suggested to be important for the selection of substrate *in vivo* [163]. ARF was demonstrated to induce sumoylation of MDM2, B23, Werner helicase (WRN), HIF-1α and E2F1 that directly interact with it [157, 158, 164]. The extensive effect of protein sumoylation induced by ARF may include control of protein stability, formation of subnuclear structure and regulation of transcriptional activities in a p53 and MDM2 independent manner [163, 165].

3.1.4 COMMD1

COMMD1 (copper metabolism gene *MURR1* domain containing protein 1, previously known as MURR1) is a multifunctional protein. It was initially characterized to be involved in the regulation of copper homeostasis in mammals. Homozygous deletion in *COMMD1* gene led to copper toxicosis in Badlington terriers [166]. COMMD1 was demonstrated to be able to interact

with copper transporter ATP7B [167]. However, the detail mechanism by which COMMD1 regulates the copper metabolism is still inconclusive.

COMMD1 contains 190 amino acids and is highly conserved through evolution. It contains a conserved domain known as copper metabolism gene *MURR1* (COMM) domain. The COMM domain is lecine rich and present in other nine human genes (*COMMD2-10*) [168]. The COMMD family proteins can form heteromeric complex that is mediated by the COMM domain, but the exact composition of these COMMD complexes *in vivo* still remains to be determined. In addition to its role in copper metabolism, COMMD1 was reported to repress NF-κB mediated transcription from endogenous and viral promoters [169]. It associates with several subunits of NF-κB as well as NF-κB inhibitor I-κB, suggesting that COMMD1 directly participates in the NF-κB signaling pathway. Several other COMMD proteins such as COMMD-2, -7, -9, -10 also interact with NF-κB subunits and strongly inhibit NF-κB mediated transcription [168]. Therefore, these COMMD proteins not only share a common domain, but also have similar functional properties in regulation of transcriptional activity of NF-κB.

3.1.4.1 COMMD1 is regulated by E3 ligase XIAP

Association between COMMD1 and XIAP was first identified by a yeast two hybrid screen and was then confirmed *in vivo* by co-immunoprecipitation assay with endogenous proteins [170]. Deficiency of *COMMD1* was found to result in

copper accumulation in cultured cells, which is consistent with the notion that COMMD1 plays a role in the regulation of copper metabolism. Surprisingly, increased expression level of XIAP led to copper accumulation in several cell models and liver tissue of *XIAP-/-* mice displayed a reduced copper content [168, 170]. This result indicates that the role of XIAP in regulating copper homeostasis might be a functional consequence of COMMD1/XIAP association under physiological situation. Reduction of XIAP level by RNAi increased the cellular level of COMMD1. Moreover, XIAP was found to destabilize COMMD1 through facilitating polyubiquitination and degradation of COMMD1 [170]. Collectively, these data suggest a novel function of XIAP in the regulation of copper metabolism through its ability to negatively regulate the cellular protein level of COMMD1.

3.1.4. 2 COMMD1 as a regulator of NF-κB pathway

As previously described, COMMD1 was initially identified in yeast two hybrid system by binding to XIAP [170]. XIAP is an activator of NF-κB signaling. Hence this observation led to the study of COMMD1's effect on NF-κB, which later indicated that COMMD1, as wells as other COMMD family members are potent inhibitors of NF-κB activation induced by various stimuli [168, 169]. COMMD1 is able to bind several NF-κB subunits and NF-κB inhibitor IκB, suggesting that the protein may directly participate in the NF-κB signaling pathway [169].

It was demonstrated that siRNA mediated knockdown of COMMD1 leads to decreased level of IκB. Ubiquitination and degradation of IκB upon activation of classic NF-κB pathway is mediated by the Skp-Cullin-1-F-box (SCF) ubiquitin ligase complex. An interaction between COMMD1 and SCF subunit Cullin-1 has also been detected, suggesting that COMMD1 protects IκB from proteasomal degradation by preventing the ubiquitination of IκB by SCF complex [169]. Based on this hypothesis, overexpression of COMMD1 would result in elevation of IκB and retain NF-κB in the cytoplasm upon TNF stimulation. However, in the presence of ectopically expressed COMMD1, no impaired nuclear translocation of NF-κB was observed in response to TNF stimulation [168]. This suggests that other mechanisms of NF-κB inhibition by COMMD1 may exist. One possibility is that COMMD1 may directly function in the nucleus by regulating the association of NF-κB and chromatin. COMMD1 is recruited to the κB responsive promoter regions and affects the binding of NF-κB to the specific promoter sites after TNF stimulation [168]. Gabriel Maine *et al.* further elucidated that COMMD1 terminates RelA and chromatin interaction by accelerating the ubiquitination and degradation of RelA through its interaction with a multimeric ubiquitin ligase ECS^{SOCS1}, a Cullin containing complex[171]. Collectively, these data demonstrate that regulation of the stability of NF-κB subunits and κB inhibitors by COMMD1 may be a key mechanism to repress κB mediated transcription in response to various stimuli.

3.1.5 Aim of this project

ARF has both p53 dependent and p53 independent tumor suppressive activities. On the one hand, ARF antagonizes MDM2 and activates the p53 mediated transcription, leading to either cell cycle arrest or apoptosis. On the other hand, ARF was implicated to be involved in regulating cellular progresses including cell cycle progression, apoptosis and ribosome biogenesis in a p53 independent way. At a biochemical level, such effects rely on the functional and physical association of ARF with target proteins other than MDM2. Till now at least 25 ARF binding proteins have been discovered by yeast two hybrid, co-immunoprecipitation or GST pull down assay. Therefore for a better understanding of ARF's multiple biological functions, a yeast two hybrid screening was performed to isolate novel ARF associated proteins, which later led to identification of COMMD1 as a novel ARF binding candidate. Co-immunoprecipitation and immunofluorescence were used to identify the physical interaction and localization of the two proteins. Furthermore the functions of their interaction were also studied.

3.2 Materials and methods

3.2.1 Yeast two hybrid screening

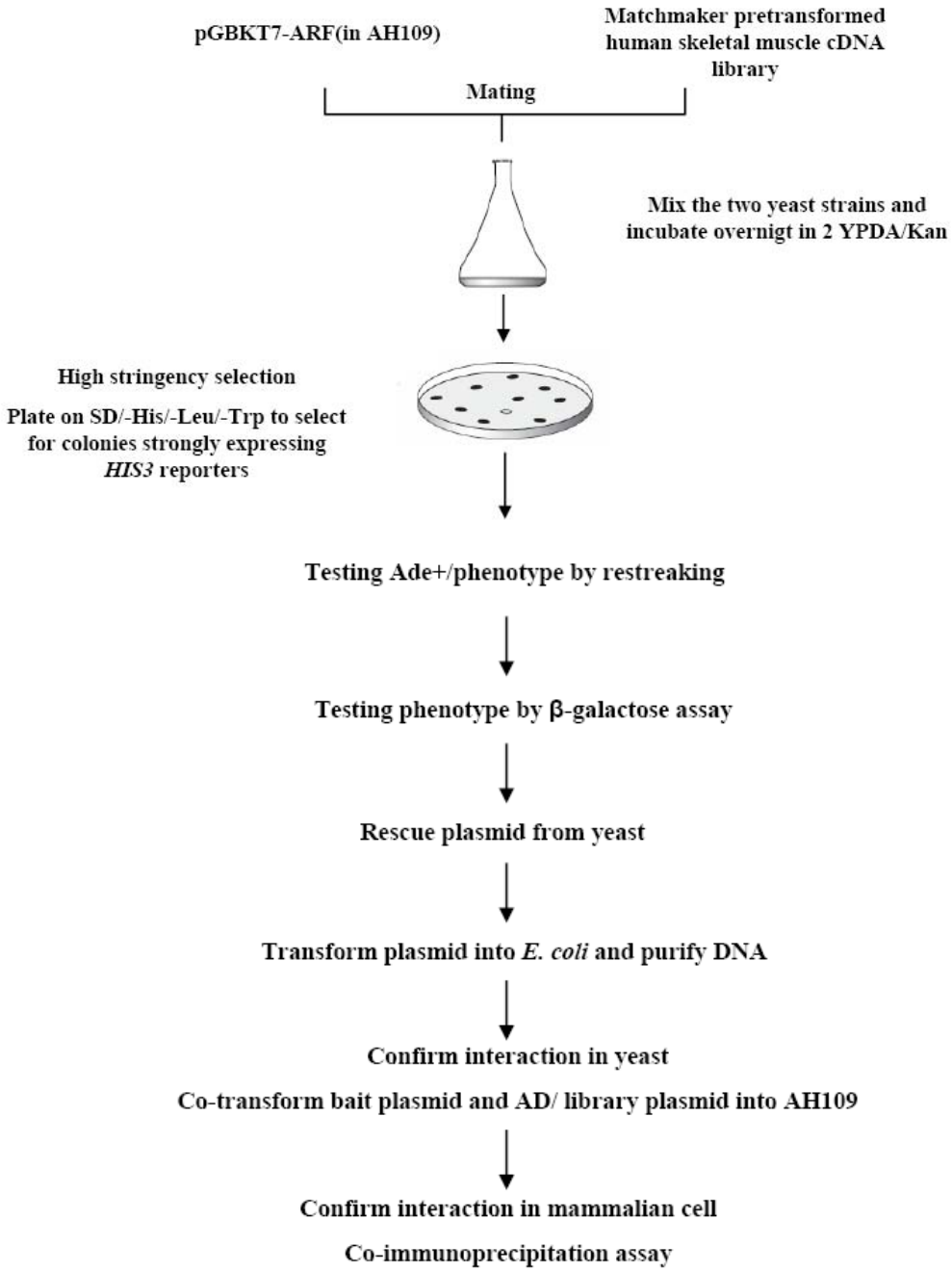


Figure 3.2.1 Strategy for yeast two hybrid screening

3.2.1.1 Yeast mating

Materials

Yeast strains

Table 3.2.1 Yeast host strain genotypes

Strain	Genotype	Reporters	Transformation Markers
AH109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80ΔlYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3,GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2,URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-LacZ MEL1*	*HIS3, ADE2, MEL1,LacZ*	*Trp1,leu2*
Y187	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met–,URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-LacZMEL1*	*MEL1,LacZ*	*Trp1,leu2*

Media for yeast

- Rich media (for routine culturing of untransformed yeast)

  YPDA medium

- Selective Media

  Minimal SD base medium + dropout supplement (-Trp, -Leu, -Leu/-Trp, -His/-Leu/-Trp)

- 1M 3-AT stock solution

  3-AT (3-amino-1, 2, 4-triazole), a competitive inhibitor of the yeast *HIS$_3$* protein (His$_3$p), is used to inhibit low levels of His$_3$p expressed in a leaky manner in some reporter strains

cDNA library

Clontech Matchmaker pretransformed human skeletal muscle cDNA library: Human skeletal muscle cDNA was ligated into the pACT2 vector which carries a DNA activation domain at upstream and transformed into the Y187 yeast stain.

Plasmids

- pGBKT7 (Provided by Dr. Wu Mian)
- pGBKT7-p14ARF (Constructed by Dr. Wu Mian's lab in China)

*The self-activation and toxicity of bait plasmid $pGBKT_7$-ARF have been tested and proved to be suitable for the screening experiment.

Method

Inoculate a fresh colony carrying bait plasmid into 50ml of SD/-Trp liquid medium and incubate until the OD_{600} reaches 0.8. Centrifuge cells down and combine the pellet with the library strain Y187 in 50ml of 2xYPDA liquid medium containing 50μg/ml kanamycin. Incubate cell cultures at 30rpm, 30°C for 24 hours. 24 hours later, Spin down cells and resuspend them in 10ml of 0.5xYPDA/Kan liquid medium. Plate the cultures 200μl per 150mm on SD/-His/-Leu/-Trp agar plate and incubate at 30°C for 3–8 days. Score the number of screened clones by counting the colonies from the SD/-Leu/-Trp plates after 3–5 days.

3.2.1.2 Testing Ade^+/ phenotypes by restreaking

Material

Selective media: SD/-Ade/-His/-Leu/-Trp agar plate

Method

Restreaked the positive colonies on SD/-Ade/-His/-Leu/-Trp plates. Positive colonies will grow to single colonies and appear white after 2–4 days. False positive colonies which fail to activate *ADE* reporter gene will not grow and turn to pink color. Colonies that turned to pink should be discarded.

3.2.1.3 β-galactosidase assay

Materials

- Z buffer: 16.1g/L $Na_2HPO_4 \cdot 7H_2O$, $NaH_2PO_4 \cdot H_2O$ 5.50g/L, KCl 0.75 g/L, $MgSO_4 \cdot 7H_2O$ 0.246 g/L. Adjusted to pH 7.0 and autoclaved, stored at RT.
- Z buffer/X-gal solution: 100ml Z buffer, 0.27ml β-mercaptoethanol, 1.67ml X-gal stock solution. Prepared before use.
- X-gal stock solution: Dissolve X-gal in DMF at a concentration of 20mg/ml. Stored in the dark at –20°C.
- Whatman No. 5, 0.75mm paper filters
- Liquid nitrogen

Method

Restreak colonies on the SD/-Ade/-His/-Leu/-Trp plates and incubate at 30°C. Let colonies grown to 1–3mm in diameter. Presoak a No.5 filter paper by

placing it in 2.5 ml of Z buffer/X-gal solution. Place a filter over the surface of the plate of colonies to be assayed. Lift the filter off the agar plate and transfer it to a pool of liquid nitrogen and submerge the filters for 10 sec. Remove the filter from the liquid nitrogen and allow it to thaw at room temperature. Place the filter, colony side up, on the presoaked filter and incubate at room temperature and check for the appearance of blue colonies.

3.2.1.4 Rescue of the prey plasmids from yeast

Materials

- Lyticase solution
- S buffer (1ml): 996µl lyticase solution + 4µl mercaptoethanol
- Lysis buffer: 0.25M Tris-HCl, pH7.5, 25mM EDTA, 2.5%SDS
- 3M potassium acetate

Method

Get yeast cells from one day liquid culture (4ml) and resuspend in 50µl S-buffer. Incubate at 37^{o}C for 1hr. Add 50µl lysing solution. Vortex to mix and incubate at 65^{o}C for 30 min. Add 88µl of 3M potassium acetate and chill on ice for 10min. Spin in centrifuge for 10 min. Pour supernatant in a new tube containing 1ml ethanol. Chill on ice for 10 min, spin 10min and then discard the supernatant. Wash the pellet with 70% ethanol twice and dry pellet by concentrator. Resuspend pellet in 20µl TE buffer.

3.2.1.5 Electroporation

Materials

- Bio-Rad 0.2cm electroporation Cuvette
- Electrocompetent *DH5α* cells
- Bio-Rad electroporator

Method

Thaw electrocompetent *E. coli* cells on ice. Add 1µl of yeast plasmid solution to 100µl of electrocompetent cells on ice. Transfer samples to a prechilled cuvette. Perform the electroporation according to the manufacturer's instructions. Plate cells on supplemented LB/amp agar medium. Incubate plates at 37°C for 12-18 hours.

3.2.1.6 Preparation of competent yeast cells

Materials

- 10×TE: 0.1M Tris-HCl, 10mM EDTA, pH7.5, autoclaved.
- 10×LiAc: 1M LiAc, adjust to pH7.5 with diluted acetic acid and autoclave.

Method

Transfer 5µl of fresh yeast culture to 50ml of YPDA. Incubate shaking until the OD_{600} reaches ~0.3. Centrifuge the cells and resuspend the pellet in 100ml of fresh YPDA. Incubate at 30°C until the OD_{600} reaches ~0.5. Centrifuge the cells and resuspend each pellet in 30ml sterile H_2O. Centrifuge the cells, and resuspend each pellet in 1.5ml of 1.1xTE/LiAc. Transfer the cell suspensions to

two respective tubes; centrifuge at 12000g for 15 sec. Discard the supernatant and resuspend each pellet in 600µl of 1.1xTE/LiAc. The cells are now ready to be transformed with plasmid DNA.

3.2.1.7 Yeast transformation

Materials

- 50% PEG3350
- PEG/LiAc solution: 40% PEG4000 + 1×TE + 1×LiAc
- Herring testes carrier DNA(10mg/ml)

Method

Combine 100ng plasmid DNA and 5µl Herring Testes Carrier DNA in a tube; Add 50µl competent cells and gently mix. Add 0.5ml PEG/LiAc and gently mix. Incubate at 30°C for 30 min. Mix gently every 10 min. Add 20µl DMSO and gently mix. Incubate in a 42°C water bath for 15 min and mix cell every 5 min. Centrifuge to pellet yeast cells, discard supernatant. Resuspend cells in 1ml YPD medium and incubate shaking for 90 min. Spin down the pellet and resuspend cell pellet in 1ml of 0.9% NaCl. Spread 100µl of 1/100 dilution onto a 100mm plate containing SD/-Leu/-Trp medium. Incubate plates at 30°C until colonies appear. Calculate transformation efficiency.

3.2.2 Cloning of genes into mammalian expression vectors

Materials

Vectors

- pCDNA3-Flag (From Dr. Wu Mian)
- pCMV5-HA (From Dr. Peter Cheung)
- pCMV5-Myc-ub (From Dr. Peter Cheung)

Enzymes

- Restriction enzymes (NEB, MA, USA; Roche, Switzerland)
- Calf intestine phosphytase (NEB, MA, USA)
- T4 ligase (NEB, MA, USA)
- *Pfu* (Promega, WI, USA)
- *Tag* (Promega, WI, USA)

*Kits including Gel extraction kit, PCR purification kit, Plasmid mini-prep and midi-prep kit are purchased from same companies as previously described.

Method

pCDNA3-Flag-COMMD1

Primers:

P1 5'CGGGATCCAGATGGCGGCGGGCGAGC 3'

P2 5'AGATCCCGCTCGAGTCAGTTAGGCTGGCTG 3'

cDNA encoding full length COMMD1 was amplified by reverse transcription and PCR using RNA template from HeLa cells with primers P1/P2. The PCR product and pCDNA3-Flag vector were individually digested by restriction

enzymes BamH1/Xho1 for 4 hours. Digested PCR product was then purified and inserted into the pCNDA3-Flag vector. The plasmid was verified by DNA sequencing.

pCMV5-HA-ARF and ARF deletion mutants

Primers:

P3 5'ACTATAGG G GTA CCG ATG GTG CGC AGG TTC TTG3'

P4 5'ATACCGCGGATCCTCAGCCAGGTCCACG3'

P5 5'CATACCGCGGATCCTCATGGTCTTCTAGGAAGCG3'

P6 5'ACATAGGGGTACCGGGTCATGATGATGGGC3'

P7 5'TATAGGGGTACCGTGTGGCCCGCCGCGAGTGA3'

P8 5'ACTATAGGGGTACCACGGCTCACGGGGGAGT3'

P9 5'ACTATAGGGGTACCGGTGCTGATGCTACTGAGG3'

P10 5'CCT CAG TAG CAT CAG CAC CCG CGG GAT GTG AAC3'

P11 5'GTGCTGATGCTACTGAGGAGCCAGCGTCTA3'

P12 5'ACTATAGGGGTACCGATGGTGCGCAGGTTCTTGGTGACCCTCCGGATTC GGCGCGCGGTGCTGATGCTACTGAGG3'

Full length ARF gene was amplified by PCR using pGBKT$_7$-ARF template with primer P3/P4. PCR product was then sequentially digested by restriction enzymes BamH1 and Kpn1 and subcloned into pCMV-HA backbone. The plasmid pCMV5-HA-ARF was verified by DNA sequencing.

DNA fragments encoding ARFΔN14, ΔN29, ΔN45 and 65-132 mutants were amplified by using forward primer P7, P8, P9 or P10 with reverse primer P4. The ARF1-64 fragment was amplified by using primer P3/P5. Assembly PCR was utilized to generate the ARFΔN30-45 DNA fragment. As shown in Figure

3.2.2, the 1-29 and 45-132 fragments were first amplified by PCR with primers P3/P10, P11/P4 respectively. ARFΔN30-45 was then generated by second step PCR using 1-29 and 45-132 as templates with primers P3/P4. For mutant ARFΔN15-45, the DNA fragment encoding the first 14 amino acids were added to the 5' end of primer P12 and the DNA fragment encoding ARFΔN15-45 was amplified by PCR with primers P12/P4. DNA fragments encoding various ARF mutants were subcloned into the BamH1/XhoI sites of pCMV5-HA backbone. Expression vectors were then verified by DNA sequencing.

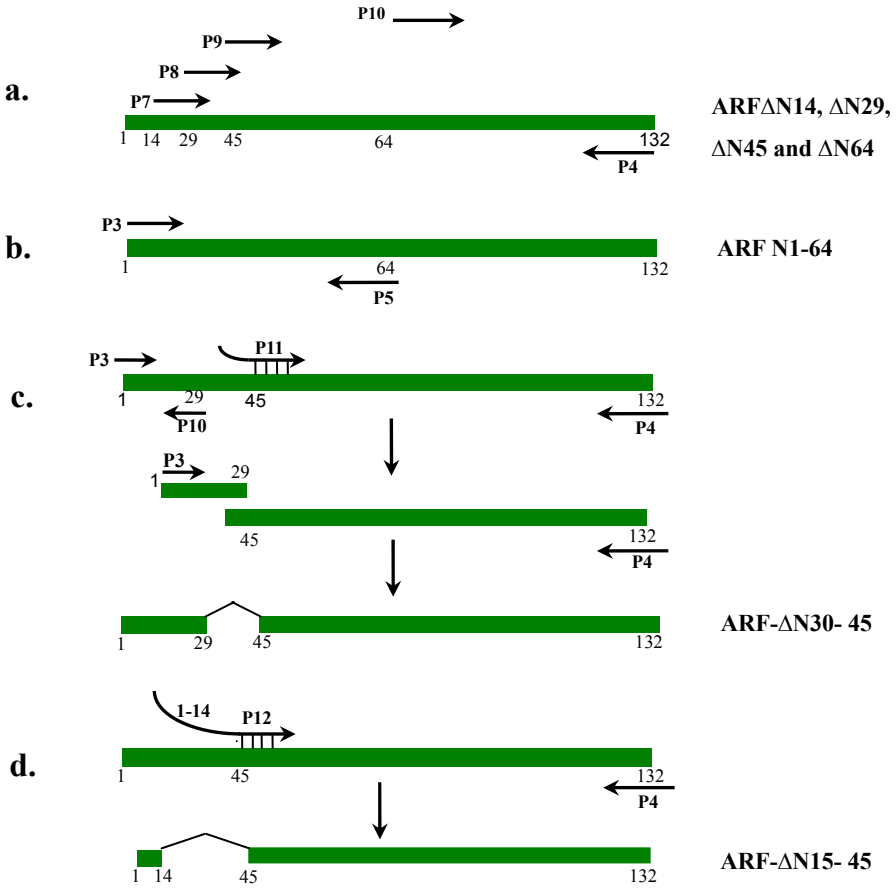


Figure 3.2.2 Strategy for generating various ARF deletion mutants DNA fragments.

PCMV5-Myc-ub-K63R

Primers:

P16 5'ATATACCGGAATTCGCCACCATGGAA3'

P17 5'ATATACGCGGATCCTCAGCCACCTCGCAGGCGCAACACCAGGTGCAGG GTGGACTCTCTCTGGATGTTGTAGTCTGAGAGA3'

The mutation site at K63 was introduced to the reverse primer P17. DNA fragment encoding ub-K63R was generated by PCR using primer P16/P17 and subsequently subcloned into EcoR1/BamH1 site of pCMV5 backbone.

3.2.3 Mammalian cell culture

Materials

Cell lines

- NCI-H1299 (From Dr. Yoon Ho Sup's lab)
- A549 (From Dr. Peter Droger's lab)

Media

- RPMI (Roswell Park Memorial Institute) 1640 containing 25mM HEPES buffer, 10% FBS, 100mg/ml penicillin and 100mg/ml streptomycin, 2.5g/L sodium bicarbonate, 1×sodium pyruvate.
- DMEM containing 10% FBS, 100mg/ml penicillin and 100mg/ml streptomycin, 3g/L sodium bicarbonate, 1×sodium pyruvate.

Method

H1299 and A549 cell line were cultured in complete RPMI and DMEM medium respectively. Cell cultures were incubated in humidified atmosphere

containing 5% CO_2 at 37^{o}C and subcultured 2-3 times a week.

Transfection of cells with various mammalian expression vectors by Lipofectamine 2000 was according to the method described by the manufacture's instruction.

3.2.4 Western blotting and immunoprecipitation

Materials

Primary antibodies

- anti-Flag M2 mAb (Sigma, St. Louis, MO, USA)
- anti-Flag pAb (Sigma, St. Louis, MO, USA)
- anti-HA pAb (Santa Cruz biotechnology, CA, USA)
- anti-p14ARF pAb (Lab vision, CA, USA)
- anti-COMMD1 pAb (Abnova, Taiwan)
- anti-Actin pAb (Santa Cruz biotechnology, CA, USA)
- anti-α-Tubulin mAb (Molecular Probes, USA)
- anti-GCN5 mAb (Cell Signaling Technology, USA)

Secondary antibodies

- Anti-mouse, rabbit or goat HRP conjugated secondary antibodies are purchased from Dako, Denmark.

Membranes

- Nitrocellulose, 0.45 μm (Bio-Rad, CA, USA)
- PVDF, 0.2μm (Bio-Rad, CA, USA)

*PVDF membrane with pore size 0.2μm was used for transferring ARF deletion mutant proteins with MW less than 10KD.

Beads

- Protein G sepharose fast flow 4B (Amersham, UK)
- Flag-M2 affinity gel (Sigma, St. Louis, MO, USA)

Buffers

- M-PER Lysis buffer (Pierce Biotechnology, IL, USA)
- Wash buffer for IP: 20mM Tris-HCl, pH7.6, 500mM NaCl
- SDS sample buffer (Bio-Rad, CA, USA)

Other buffers are same as previously described in chapter 2.

ECL detection kit

- ECL Western Blotting Substrate (Pierce, IL, USA)
- ECL advanced western Blotting detection kit (Amersham, UK)

Method

Protocols for western blotting and immunoprecipitation are same as described in chapter 2.

3.2.5 Immunostaining and microscopy

Materials

Antibodies

- Anti-Flag M2 mAb (Sigma, St. Louis, MO, USA)
- Anti-HA pAb (Santa Cruz biotechnology, CA, USA)

- Anti-p14ARF pAb (Lab vision, CA, USA)
- Anti-COMMD1 pAb (Abnova, Taiwan)

Secondary antibodies

- Alexa Fluor 488, 594 anti-rabbit and anti-mouse antibodies are purchased from Molecular Probes.

Mounting medium

- Prolong Gold antifade reagent with DAPI (Molecular Probes)

Microscope

Carl Zeiss LSM510 confocal microscope

Method

H1299 cells were seeded on the glass coverslip at 60-70% confluence. Day 2, cells were transfected with indicated plasmid. Day 3, cells were washed with PBS twice, fixed with 4% paraformaaldehyde in PBS for 10min. Wash cells with PBS twice. Permeabilize cells with 0.1% NP-40 in PBS for 10min. Wash cells twice with PBS. After permeabilization, cells are first blocked with 4% BSA for 30min and incubated with primary antibody for 2 hours. Wash cells three times with TBST, add secondary antibody and incubate for 1 hour. Repeat wash step for 3 times. Incubate cells with the other primary antibody for 2 hours followed by corresponding secondary antibody for 1hour. Add mounting medium to the coverslip and put coverslip onto the glassslide. Examine the fluorescence with microscope.

3.2.6 Protein stability assay

Materials

- Cycloheximide (Sigma, St. Louis, MO, USA)
- MG132 (Sigma, St. Louis, MO, USA)

Method

Method for protein stability assay is similar as previously described in chapter 2.

3.3 Results

3.3.1 Isolation of novel ARF binding proteins

For better understanding the function of ARF, we performed a yeast two hybrid screening to isolate novel ARF interacting proteins. Full length p14ARF fused with Gal-4 DNA binding domain was used to screen a human skeletal muscle library fused with DNA activation domain. The screening led to identification of totally 10 proteins as the ARF binding candidates. Among them, YY1 (Yin-Yang 1) transcription factor, E4F1 and Tat binding protein 1 (TBP1) have been reported as ARF associating proteins. Previously it was demonstrated that NF-κB regulates apoptosis process in response to a wide range of stimuli and COMMD1 could potently inhibit NF-κB mediated transcription at both basal level or in response to cytokine stimulation [104, 169]. Therefore it is possible that association with ARF might regulate COMMD1's inhibition effect on NF-κB. We then specifically chose candidate COMMD1 for our study.

Table 3.3.1 ARF binding candidates identified by yeast two hybrid screening.

Positive clones on QDO	β-galacsidase -assay	PCR (kb)	Co-transformation		Sequencing result
			pGBKT7	pGBKT7 -ARF	
1	+	0.8	−	+	Triadin
2	+	1.5	−	+	COMMD1
3	+	2	−	+	Zinc finger 95
4	+	1.5	−	+	Myomesin
5	+	0.4	−	+	ZNF 3115
6	+	2	−	+	ZNF408
7	+	0.6	−	+	YY1
8	+	2	−	+	Tat binding protein 1
9	+	2.5	−	+	E4F1
10	+	0.9	−	+	ZNF408

3.3.2 ARF interacts with COMMD1 *in vivo*

The interaction between ARF and COMMD1 in the mammalian cells was verified by co-immunoprecipitation (co-IP) assay. As shown in Figure 3.3.1 a, HA-ARF can be detected in the Flag-COMMD1 immunoprecipitates but not Flag mock (Figure 3.3.1a). In a reciprocal co-IP experiment when HA tagged proteins were precipitated from cell lysates (Figure 3.3.1b), Flag-COMMD1 could only be pulled down together with HA-ARF but not HA, demonstrating that ARF interacts with COMMD1 specifically in mammalian cells.

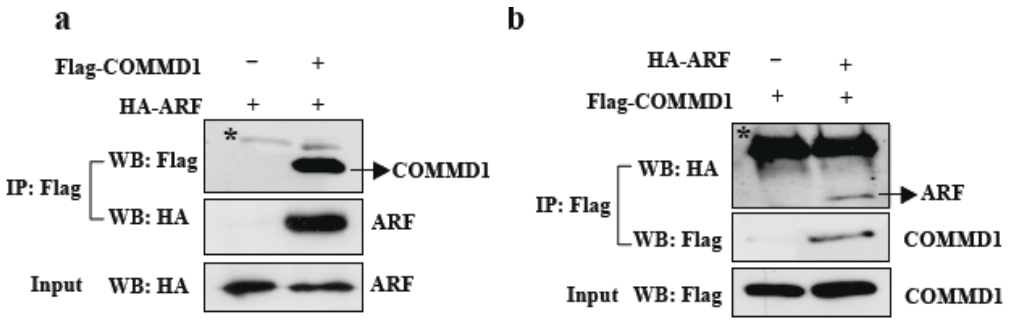


Figure 3.3.1 ARF interacts with COMMD1 *in vivo*. H1299 cells were co-transfected with Flag-COMMD1 and HA-ARF. 24 hours after transfection, cells were lysed in the Pierce M-PER lysis buffer. Immunoprecipitation was performed by using antibody as indicated and then immnobloted with anti-Flag or HA antibody. *: IgG light chain.

To delineate the region of ARF involved in the interaction with COMMD1, full length ARF and various deletion mutants were constructed and tested their ability to bind COMMD1. The N-terminal domain (amino acid 1-64) of ARF encoded by exon1 β was found to be sufficient for the interaction with COMMD1 (Figure 3.3.2, lane 3), whereas exon2 encoded C-terminal domain (amino acid 65-132) did not show any binding to COMMD1 (Figure 3.3.2, lane

4). To better define the interacting domain, ARF mutants with more discrete deletion in the N-terminus were generated and employed for the mapping assay. Deletion of the first 14 residues of ARF (ARFΔN14) had no discernable effect on the interaction with COMMD1, while deletion of 1-29 residues (ARFΔN29) still showed some binding to COMMD1. Deletion of the 1-45 residues of ARF (ARFΔ45) totally abolished the interaction with COMMD1, suggesting that the binding site may lie in the region 30-45. However, ARFΔN30-45 mutant still retained some capacity to bind COMMD1, whereas deletion of 15-45 completely abrogated the association, implicating that N-terminal amino acids 15-45 contribute to the interaction of ARF with COMMD1.

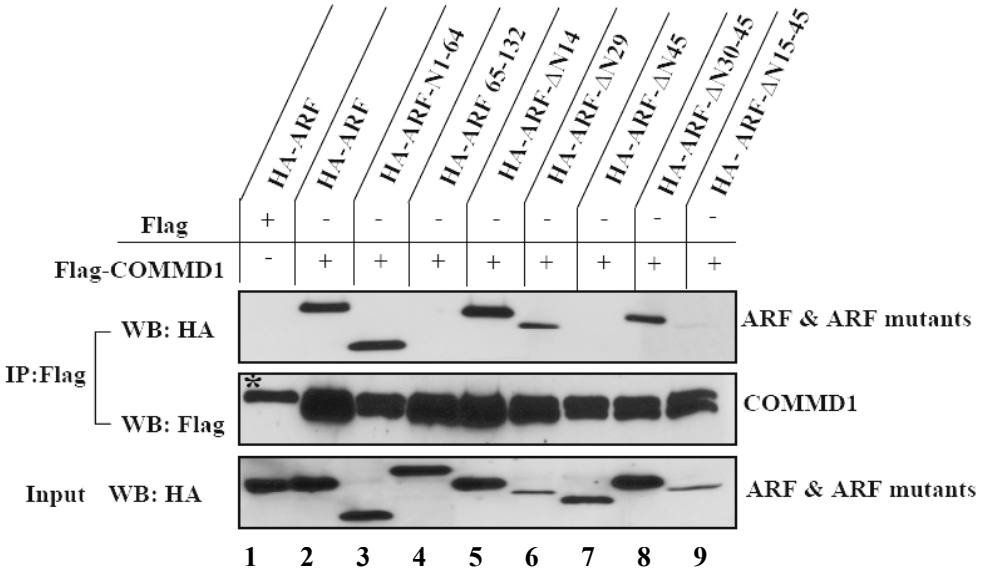


Figure 3.3.2 Deletion mapping: H1299 cells were cotransfected with Flag-COMMD1 and HA-ARF full length or various truncated mutants as indicated. Cell lysates were immunoprecipitated with anti-Flag M2 affinity gel. The pulled down materials were separated by SDS-PAGE and immunoblotted with anti-HA or Flag antibody. *: IgG light chain.

ARF normally localizes in the nucleolus in cancer cell lines and is capable of co-localizing its associated proteins such as MDM2, E2F1, and HIF-α into the nucleolus [88, 149, 152, 172]. Therefore, we examined the localization of COMMD1 upon simultaneous expression with ARF. After transient transfection with HA-ARF and Flag-COMMD1 or corresponding empty vector, H1299 cells were fixed and stained with Flag antibody for COMMD1 and HA antibody for ARF. As shown in the Figure 3.3.3, when ARF was coexpressed together with empty vector, it localized in the nucleolus (as arrows indicates). In contrast, COMMD1 exhibited a mainly cytoplasmic localization pattern with accumulation of the protein in some discrete perinuclear regions. COMMD1 could also be detected in the nucleus, but they were excluded from the nucleolus. However, when COMMD1 was coexpressed along with ARF, COMMD1 was found in the nucleus and colocalized with ARF in the nucleoplasm. Previous studies have shown that although ARF is capable of sequestering its binding partner into the nucleolus, the NoLS of the binding partners may also be required for the nucleolus co-localization [133, 152] . We found that COMMD1 did not localize to the nucleolus when it was co-expressed with HA-ARF, instead they both localized in the nucleoplasm. It could be due to the lack of NoLS in COMMD1. Nevertheless, this result indicates that formation of COMMD1-ARF complex alone is not sufficient for ARF to sequester protein to the nucleolus and the functional consequence of ARF-COMMD1 interaction may partially rely on their co-localization in

nucleoplasm.

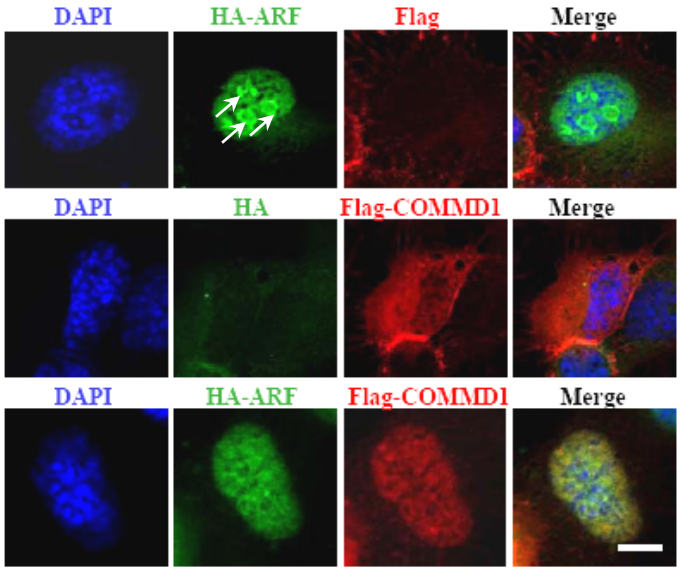


Figure 3.3.3 ARF co-localizes with COMMD1 in the nucleoplasm. H1299 cells plated on the slides were transfected with HA-ARF and Flag vector, Flag-COMMD1 and HA empty vector or HA-ARF and Flag-COMMD1. 24 hours after transfection, cells were fixed with 4% paraformaldehyde in PBS. Fixed cells were first stained with anti-Flag M2 antibody, followed by Rhodamine conjugated secondary antibody, and then stained with anti-HA antibody, followed by FITC conjugated secondary antibody. Nuclei were visualized with DAPI. Scale bar indicates 10μm. White arrows indicate the nucleolus.

3.3.3 DNA damage induces endogenous ARF/COMMD1 interaction

Previously it was reported that DNA damage reagent actinomycin D can induce nucleolus disruption and rapid redistribution of endogenous ARF into nucleoplasm [129]. It is possible that ARF/COMMD1 interaction in the nucleoplasm could be enhanced after DNA damage stimulation. To test this idea, immunostaining and co-IP assay were performed after treatment of H1299 cells with actinomycin D for 8 hours. As shown in Figure 3.3.4 lower panel,

actinomycin D induced a complete redistribution of ARF in the nucleus and subsequently led to more ARF co-localize with COMMD1 in the nucleoplasm. H1299 cells are deficient of *p53*, so the redistribution of ARF in response to actinomycin D treatment is independent to p53. Consistent with this observation, the complex of endogenous ARF-COMMD1 could only be detected in H1299 cells with actinomycin D stimulation but not in untreated cells (Figure 3.3.5). Thus the interaction of ARF and COMMD1 under physiological condition appeared depend, at least partially, on their co-localization in the nucleoplasm.

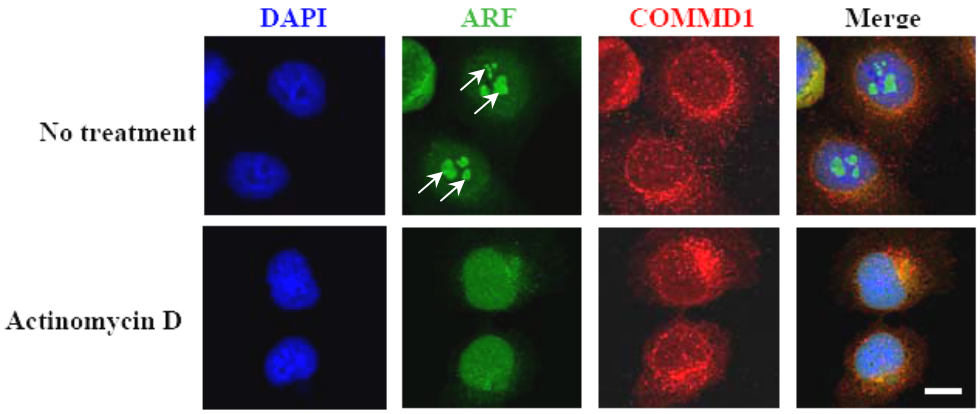


Figure 3.3.4 Actinomycin D induces nuclear redistribution of ARF. H1299 cells were treated with 0.01μg/ml actinomycin D for 8 hours and first stained with anti-ARF antibody for detection of endogenous ARF and then stained with anti-COMMD1 antibody for detection of COMMD1. Scale bar indicates 10μm. White arrows indicate the localization of the nucleolus.

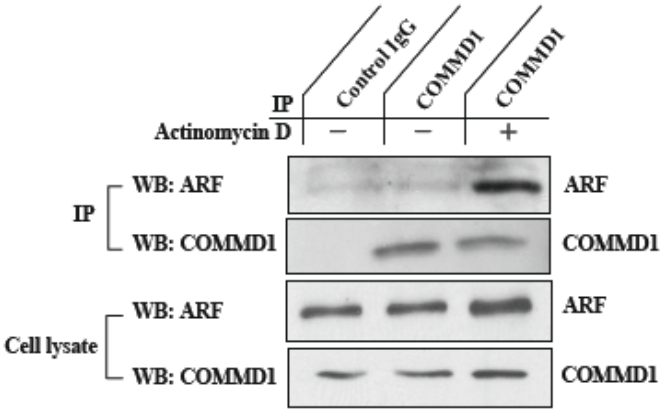


Figure 3.3.5 ARF interacts with COMMD1 in response to actinomycin D stimulation. H1299 cells were treated with actinomycin D or left untreated for 8 hours. Cells were then harvested and COMMD1 was immunoprecipitated with mouse anti-COMMD1 conjugated protein G agarose for 6 hours. Endogenous ARF co-precipitated with COMMD1 was detected by rabbit anti-ARF antibody. Immunoprecipitated COMMD1 was analyzed by western blotting with mouse anti-COMMD1 antibody followed by HRP conjugated protein G to avoid detection of IgG light and heavy chain.

3.3.4 ARF stabilizes COMMD1 by regulating its turnover

We noticed that the level of COMMD1 appeared to be higher in cells transfected with ARF than in cells transfected with empty vector (Figure 3.3.6). The accumulation of COMMD1 was observed in both A549 (*p53*+/+) and H1299 (*p53*-/-) cells， indicating that it is p53 and MDM2 independent. Conversely, we found that knockdown of endogenous ARF by RNAi led to decrease of COMMD1 protein level (Figure 3.3.7).

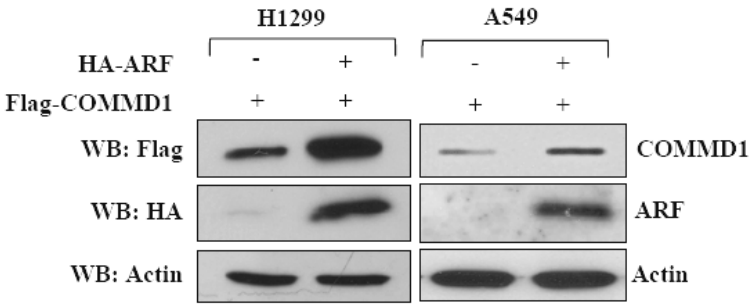


Figure 3.3.6 COMMD1 is stabilized in the presence of ectopically expressed ARF. H1299 cells were co-transfected with 1μg Flag-COMMD1 along with 1μg HA vector or HA-ARF. 24 hours after transfection, cells were lysed and subjected to western blotting by using anti-Flag antibody. Endogenous Actin levels were used as the loading controls.

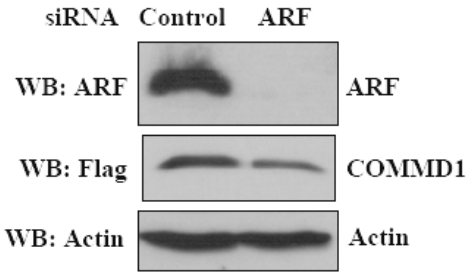


Figure 3.3.7 Knockdown of ARF destabilizes COMMD1. ARF in H1299 cells were knocked down by ARF siRNA and then transfected with Flag-COMMD1. The protein level of COMMD1 was analyzed by western blotting with anti-Flag antibody.

We next examined whether elevation of COMMD1 by ARF occurs at transcriptional or post-translational level. Cycloheximide was used to block protein synthesis in cells. H1299 cells transfected with HA-ARF or empty vector were incubated with cycloheximide and harvested at the time indicated. The cells were lysed and cells lysate were then analyzed by western blot. As shown in Figure 3.3.8, in the presence of cycloheximide, COMMD1 appeared to be quite unstable, with half life about 1 hr as previously described [170].

However, in cells ectopically expressing HA-ARF, COMMD1 decayed at much lower rate with half life of 4 hours. This result suggests that COMMD1 is stabilized in the presence of ARF, which occurs at post-translational state.

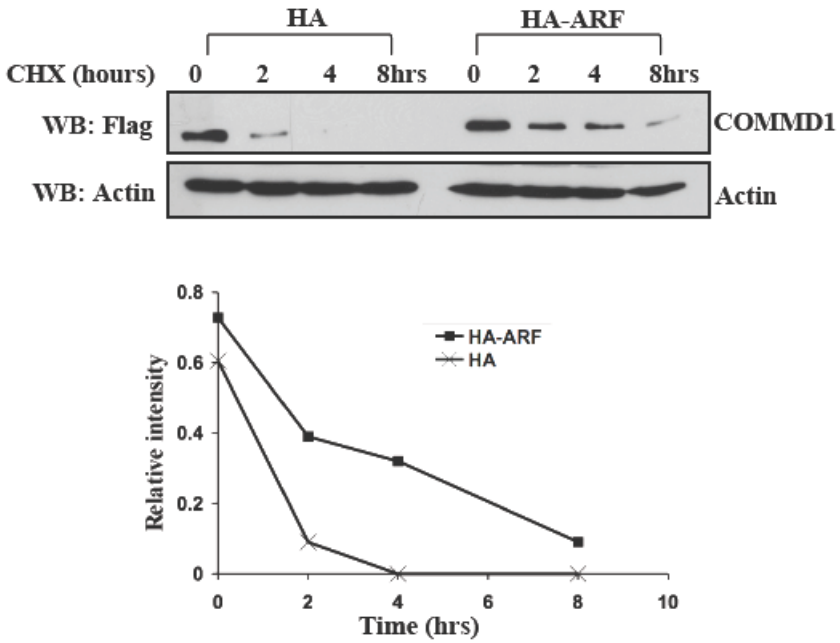


Figure 3.3.8 Stabilization of COMMD1 by ARF occurs at posttranslational state. H1299 Cells were cotransfected with 1μg Flag-COMMD1 together with 1μg HA empty vector or HA-ARF. 24 hours post transfection, cells were treated with 20μg/mL cycloheximide for the time indicated and protein levels were detected by using anti-Flag antibody. Relative densities of COMMD1/Actin are analyzed by Photoshop software.

COMMD1 is a substrate of proteasome and ubiquitination is essential for its specific degradation [170]. Therefore we reasoned that ARF may affect COMMD1 expression by regulating the ubiquitination process. We then tested whether upregulation of COMMD1 by ARF is sensitive to the treatment with MG132, a potent proteasome inhibitor. COMMD1 level increased dramatically

after 2 hours proteasome inhibition (Figure 3.3.9, lane 2), indicating that degradation of COMMD1 is mediated by the 26s proteasome complex. However, no obvious accumulation of COMMD1 was detected after MG132 treatment in the presence of ectopically expressed ARF, suggesting that ARF may stabilize COMMD1 by either downregulating the level of polyubiquitinated COMMD1 that is targeted for proteasomal degradation or acting similarly as proteasome inhibitor to enhance and sustain COMMD1.

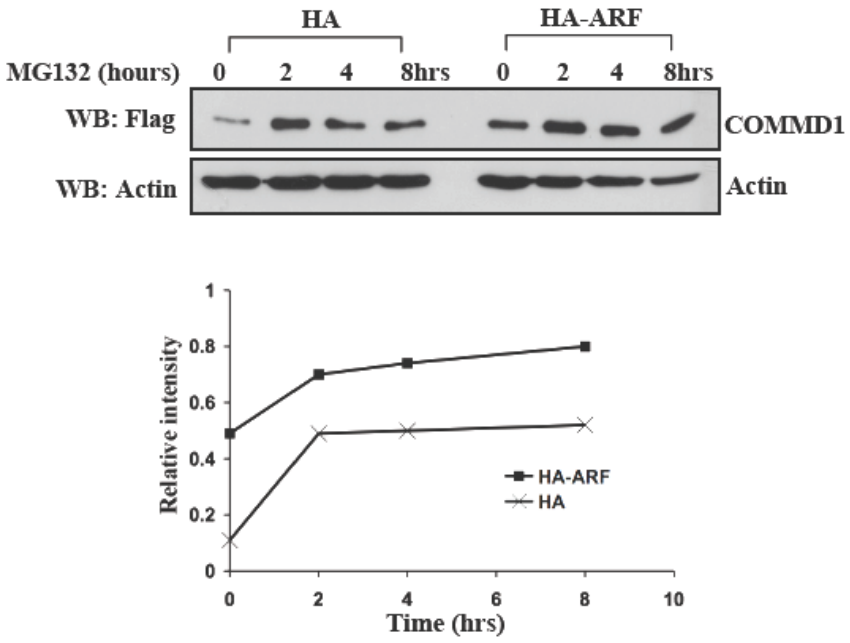


Figure 3.3.9 COMMD1 stabilization induced by ARF is inert to the proteasome blockade. H1299 cells were co-transfected with 1μg Flag-COMMD1 together with 1μg HA empty vector or HA-ARF. One day after transfection, cells were treated with 2μM MG132 for the period of time indicated. COMMD1 levels were analyzed by western blotting with anti-Flag antibodies. Relative densities of COMMD1/Actin are analyzed by Photoshop software.

3.3.5 ARF promotes proteasomal independent K63 linked ubiquitination of COMMD1

Next we examined the effect of ARF expression on the level of ubiquitinated COMMD1 by performing *in vivo* ubiquitination assay. H1299 cells were transfected with Myc-ub along with Flag-COMMD1 and HA-ARF or corresponding empty vector. 24 hours after transfection cells were treated with 2μM MG132 or left untreated for another 8 hours. Flag-COMMD1 was immunoprecipitated from cell extracts and detected by anti-Flag antibody. Consistent with previous finding by [170], polyubiquitination of COMMD1 could be detected in the presence of MG132 (Figure 3.3.10). Surprisingly, we found that in the sample co-expressing Flag-COMMD1, Myc-ub and HA-ARF without MG132 treatment, the level of ub conjugated COMMD1 was increased significantly, demonstrating that ARF promotes ubiquitination of COMMD1. When MG132 was added to allow accumulation of polyubiquitinated COMMD1 that is ultimately degraded by the proteasome, a high molecular weight smear characteristic of polyubiquitination was detected in cells transfected with HA vector and HA-ARF. However, transfection with ARF led to greater recovery of ubiquitinated COMMD1 (Figure 3.3.10, upper right panel). The ubiquitination of COMMD1 in the absence or presence of MG132 has also been confirmed by using anti-Myc antibody to probe the precipitated COMMD1-ub conjugates. We noted that compared with untreated sample, the amount of COMMD1-ub conjugates induced by ARF was not elevated after

proteasome blockade. In many cases, ubiquitinated form of proteasome substrate can only be detected in the presence of proteasome inhibitors. Therefore, the possible explanation for this result is that ARF facilitated ubiquitination of COMMD1 is proteasome independent and it does not target the protein for degradation.

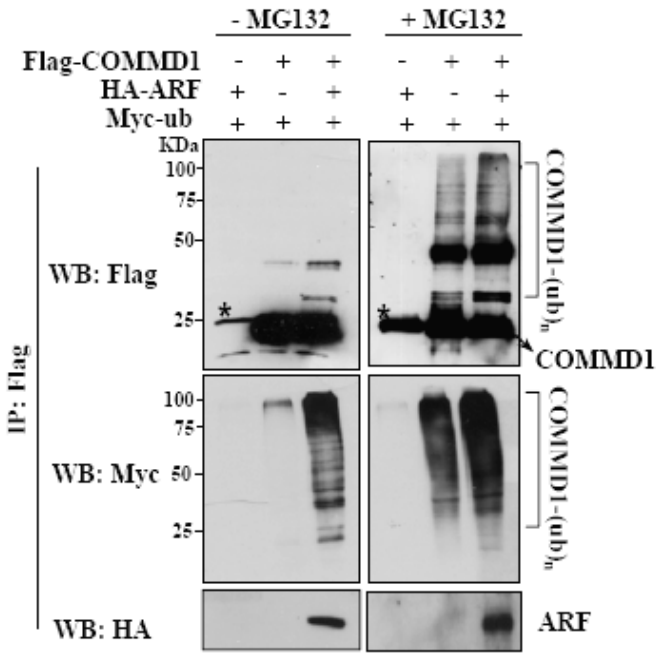


Figure 3.3.10 ARF promotes ubiquitination of COMMD1. H1299 cells were co-transfected with myc-ub, Flag-COMMD1 and HA-ARF or empty vector as indicated. One day later, cells were treated with 2μM MG132 or left untreated for another 8 hours. Cell lysates were prepared and immunoprecipitated with anti-Flag M2 affinity gel. The immunoprecipitated materials were then analyzed by western blotting with rabbit anti-Myc, anti-Flag or anti-HA antibody. *: IgG light chain.

To further confirm that ARF induced COMMD1 ubiquitination is proteasome independent, a K48R point mutant of ubiquitin that can not form K48

conjugated polyubiquitin chain was employed to carry out the *in vivo* ubiquitination assay. As shown in Figure 3.3.11 left panels, ARF induced COMMD1 ubiquitination was not impaired by introducing the K48R mutant. Compared with cells transfected with empty vector, more ubK48R conjugated COMMD1 could be detected in cells transfected with ARF, demonstrating that ARF induced proteasomal independent non-K48 ubiquitin chain conjugation to COMMD1. Together, we found that ARF promotes non-K48 mediated ubiquitination. As a result, COMMD1 would be protected from undergoing proteasomal dependent ubiquitination and degradation. In addition to K48 mediated ubiquitination, ubiquitination could also occur at K63 of ubiquitin. It has been reported K63 linked polyubiquitination does not involve proteolysis. Therefore we sought to investigate whether the apparent stability of ubiquitinated COMMD1 induced by ARF is due to the assembly of polyubiquitin chain involves K63 but not K48 on ubiquitin. As shown in Figure 3.3.11 right panels, when coexpressed with ubK63R mutant, anti-Flag immunoactivities associated with polyubiquitination of COMMD1 can be observed both in the absence and presence of ARF. However no enforced polyubiquitination was detected in sample transfected with ARF compared with the control transfection. ARF induced COMMD1 ubiquitination was totally abrogated when K63 in ubiquitin was mutated, demonstrating that ARF promoted COMMD1 ubiquitination is mediated through the K63-G76 linkage.

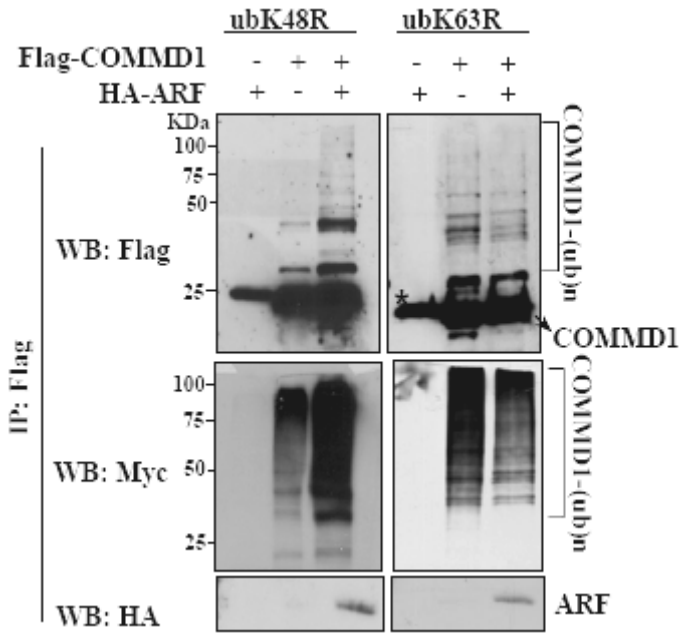


Figure 3.3.11 ARF induces non-K48 but K63 mediated polyubiquitination of COMMD1. H1299 cells were cotransfected with either K48R or K63R mutant of ubiquitin together with Flag-COMMD1, HA-ARF or empty vector control. One day later, cells were lysed and used for immunoprecipitation assay with anti-Flag affinity gel and then immunoblotted with anti-Flag or anti-HA antibody. *: IgG light chain.

Previously, it was reported that RNAi of XIAP, an E3 ligase to ubiquitinate and degrade COMMD1 through K48 linkage could enhance non-K48 ubiquitination of COMMD1 [170]. This result suggests that XIAP mediated K48 ubiquitin chains may be assembled at same lysine residues with that of ubiquitination occurs via non-K48 linkage. Hence reduction in K48 ubiquitination could result in greater ubiquitination through non-K48 linkage. In our study, we have shown that ARF facilitated K63 ubiquitination stabilizes COMMD1; COMMD1 is destabilized in cells deficient of ARF. It is possible that the K63 ubiquitination induced by ARF may compete with K48 ubiquitination and thus protect COMMD1 from proteasomal degradation. Therefore we sought to determine

COMMD1 ubiquitination pattern in cells depleted of ARF. As shown in Figure 3.3.12 left panels, knockdown of endogenous ARF by RNAi led to an obvious reduction in the total amount of COMMD1 ubiquitination, demonstrating that ARF mediated ubiquitination constitutes a large part of COMMD1 ubiquitination. Reduction of ubiquitinated COMMD1 was also observed in cells cotransfected with ubK48R but not in cells cotransfected with K63R mutant (Figure 3.3.12 right panels), indicating that K63 linked ubiquitination is mediated at least partially by endogenous ARF. We noticed that in contrast to the effect of XIAP RNAi, knockdown of ARF did not result in greater K48 ubiquitination of COMMD1. Consistent with this, the destabilization of COMMD1 caused by ARF RNAi was also not sensitive to the proteasome blockade (Figure 3.3.13). Together, these results suggest that ARF induced K63 ubiquitin chains are assembled at some specific lysine residues that are not responsible for K48 ubiquitination, hence decrease in K63 ubiquitination would not enhance K48 mediated ubiquitination and degradation. The possible explanation for the destabilization of COMMD1 in ARF deficient cells is that ARF induced K63 ubiquitination could protect COMMD1 from degradation in some proteasome independent manner, and thus COMMD1 would be degraded as a result of loss of protection from K63 ubiquitin chains.

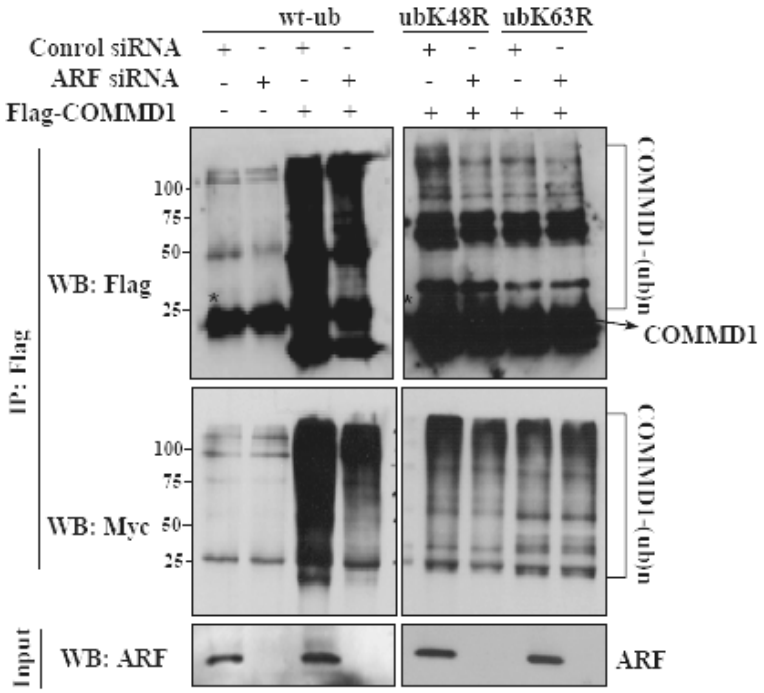


Figure 3.3.12 RNAi of ARF results in decrease of K63 linked ubiquitination of COMMD1. H1299 cells were transfected with ARF or control siRNA, Flag-COMMD1 along with wt-ub, ubK48R or ubK63R for 48 hours. Cells co-transfected with wt-ub were treated with MG132 for further 8 hours. Cells were then lysed and immunoprecipitated with mouse anti-Flag M2 affinity gel. The immunoprecipitates were immunoblotted with rabbit anti-Flag or anti-Myc antibody. *: IgG light chain.

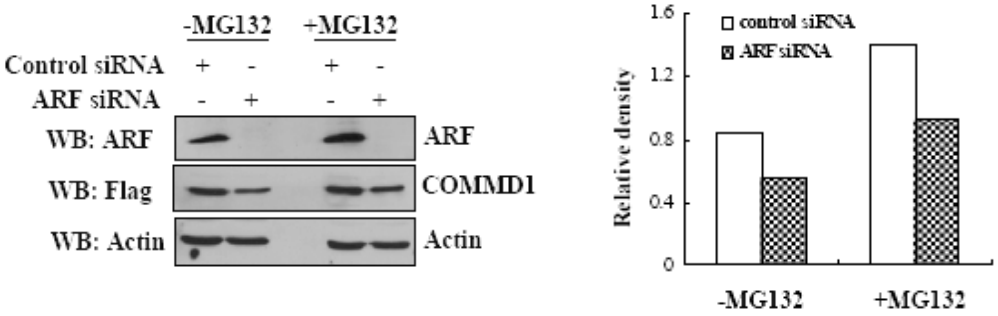


Figure 3.3.13 H1299 cells were transfected with ARF or control siRNA together with Flag-COMMD1 for 2 days. Cells were then split into two parts. Each part were treated with MG132 or left untreated for 8 hours. COMMD1 protein levels were detected by western blotting with anti-Flag antibody. Endogenous actin levels were used as loading controls. The relative density of COMMD1/Actin was analyzed by Photoshop software.

3.3.6 ARF interacts with COMMD1 under normal state but subjected to rapid degradation

The above data also implicate that interaction of ARF and COMMD1 may already exist under normal state and thus endogenous ARF could induce polyubiquitination of COMMD1 without any stimulation. Recently it was found that the non-nucleolar form of ARF mutant was inherently unstable compared with the nucleolar ARF and redirecting this ARF mutant to the nucleolus could dramatically stabilize the fusion protein [173]. Therefore we reasoned that proteasome inhibition treatment would result in more recovery of ARF in the nucleoplasm under physiological situation. To test this idea, H1299 cells were treated with 2μM MG132 for 8 hours and endogenous ARF were visualized by immunostaining with anti-ARF antibody. Under non-stimulated situation, most endogenous ARF was localized in the nucleolus (Figure 3.3.14 upper panel). Treatment with MG132 did not have obvious effect on the nucleolar ARF, but it led to great recovery of ARF in the nucleoplasm (Figure 3.3.14 lower panel), indicating that nucleoplasmic ARF is less stable than the nucleolar ARF. As it has been shown in Figure 3.3.4, under normal state we could not detect any endogenous ARF/COMMD1 interaction which could be due to the rapid degradation of ARF in the nucleoplasm. Next we examined whether accumulation of ARF in the nucleoplasm following proteasome inhibition could lead to detection of association between endogenous ARF with COMMD1. After transient transfection with Flag-COMMD1, cells were treated with

MG132 for 8 hours and endogenous ARF co-precipitated with Flag-COMMD1 were detected by anti-ARF antibody. As shown in Figure 3.3.15, in the absence of MG132, no endogenous ARF could be coprecipitated with Flag-COMMD1. However after MG132 treatment, ARF was readily be detected in the Flag-COMMD1 precipitates. Taken together, these data suggest that ARF interacts with COMMD1 under normal state but subjected to rapid degradation; endogenous ARF induces K63 polyubiquitination of COMMD1 in a proteasome independent way.

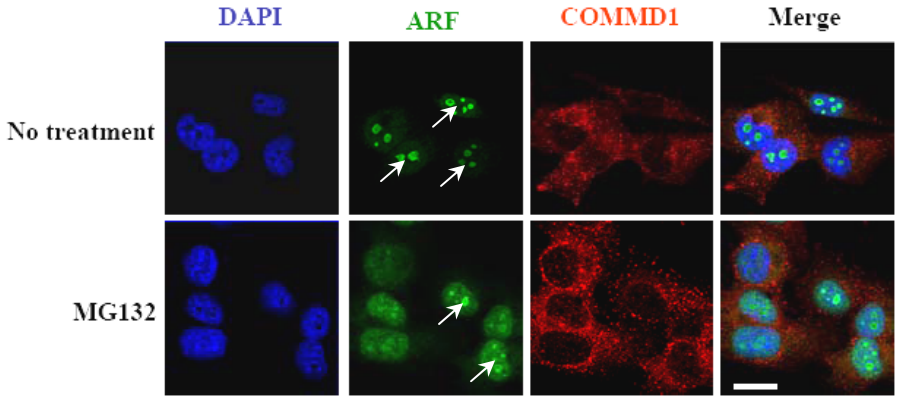


Figure 3.3.14 Proteasome inhibition leads to recovery of endogenous ARF in the nucleoplasm. H1299 cells were treated with 2μM MG132 for 8 hours and first stained with anti-ARF antibody followed by staining with anti-COMMD1 antibody. Scale bar indicates 20μm. White arrows indicate the localization of the nucleolus.

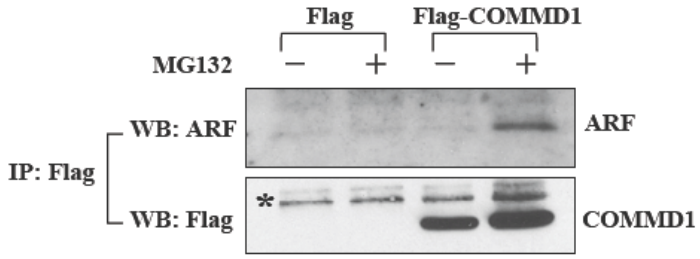


Figure 3.3.15 COMMD1 coimmunoprecipitates ARF after proteasome inhibition. After transient transfection with Flag-COMMD1, H1299 cells were treated with MG132 or left untreated for further 8 hours. Cells were then harvested and Flag-COMMD1 was immunoprecipitated with Flag-M2 affinity gel for 6 hours. Endogenous ARF co-precipitated with Flag-COMMD1 was detected by anti-ARF antibody. *: IgG light chain.

3.3.7 ARF specifically induces nuclear COMMD1 ubiquitination

Because ARF associates with COMMD1 in the nucleus, we also determined whether ARF specifically induces ubiquitination of COMMD1 in the nucleus. As shown in Figure 3.3.16, compared with cytoplasmic fraction, nuclear COMMD1 protein level was dramatically downregulated in ARF depleted cells. Consistent with this, the decreased COMMD1 polyubiquitination in nuclear fraction was also observed in cells deficient of ARF. Hence, these results suggest that ARF specifically induces COMMD1 polyubiquitination in the nucleus.

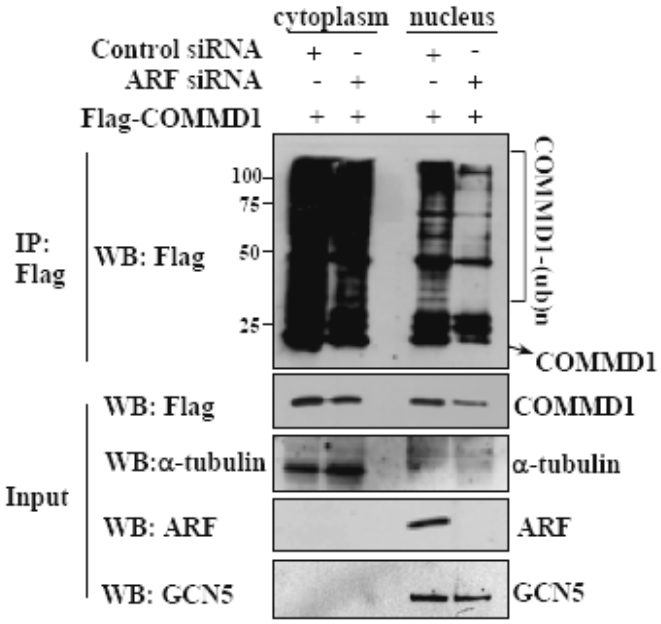


Figure 3.3.16 ARF knockdown leads to decrease of COMMD1 ubiquitination in the nucleus. H1299 cells were transfected with ARF or control siRNA and Flag-COMMD1 for 2 days and treated with MG132 for further 8 hours. The cytoplasmic and nuclear fractions were separated by Pierce cytoplasmic and nuclear extraction kit, followed by immunoprecipitation with anti-Flag M2 antibody. Immunoprecipitated COMMD1 in each fraction was analyzed by anti-Flag antibody. α-tubulin and GCN5 protein levels were used as loading controls for cytoplasmic and nuclear fractions respectively. *: IgG light chain.

3.3.8 ARF mutants are defective in promoting ubiquitination of COMMD1

In order to determine whether physical interaction of ARF with COMMD1 is a prerequisite condition for ARF to induce COMMD1 ubiquitination, we tested the effect of two ARF deletion mutants, ARF ΔN15-45 which is defective in binding COMMD1 and ARFN1-64 which binds to COMMD1 efficiently but lack the C-terminal domain encoded by exon2. As shown in Figure 3.3.17, full length ARF promoted COMMD1 ubiquitination as previously observed,

whereas expression of ARFΔN15-45 did not result in any ubiquitination of COMMD1, indicating that ARF facilitated ubiquitin conjugation of COMMD1 depends on the physical association of them. Previous study has established that the functional domain of ARF is encoded by the exon1β (amino acid 1-64) and ARF interacts with most of its binding partners through the N-terminal region [87, 108, 174]. In contrast, C-terminus contributes little to the function of ARF except nucleolus localization [133]. However our observation showed that although ARFN1-64 mutant strongly associates with COMMD1, it was also incapable of inducing ubiquitination of COMMD1. The possible explanation may be that deletion of the C-terminus impairs the association of ARF with some other unknown factor that also participates in the ubiquitination process. So in addition to nucleolus localization, the C-terminal region may also contribute to some other functions of ARF.

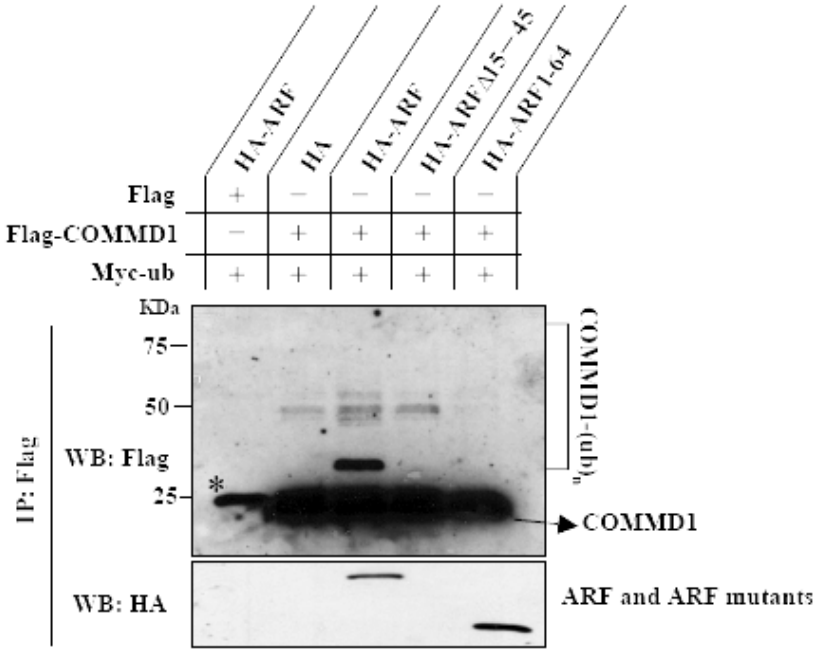


Figure 3.3.17 ARF mutants are defective in promoting COMMD1 ubiquitination. H1299 cells were transfected Myc-ub, Flag-COMMD1 with vectors encoding full length ARF or ARF deletion mutants. 24 hours post transfection, Flag-COMMD1 were precipitated from cell lysates and analyzed by western blotting. *: IgG light chain.

3.4 Discussion

Overexpression of ARF was reported to arrest proliferation of *p53* null or *p53/MDM2* null cells [136]. Mice deficient of *ARF*, *p53* and *MDM2* developed tumor at a greater frequency compared with mice lacking both *p53* and *MDM2* or *p53* alone [135]. These observations suggested ARF also possess p53 independent tumor suppressor functions. In this study we have characterized a novel pathway for ARF to control the stability of COMMD1 through promoting K63 mediated polyubiquitination of COMMD1 in a p53 independent manner.

3.4.1 Interaction of ARF and COMMD1 in the nucleoplasm

ARF localizes in the nucleolus and it was proposed that physical sequestration of MDM2 by ARF can stabilize p53 by separating it from MDM2 mediated degradation [88]. Recent data however demonstrated that nucleolar relocalization of MDM2 is not required for p53 activation and that redistribution of ARF into nucleoplasm enhances its association with MDM2 and p53 dependent growth suppressive activity [87, 88]. Together these evidences support the concept that ARF normally functions in the nucleoplasm where it can interact with MDM2 via multiple domains. Here, our results showed that under physiological state, the endogenous nucleoplasmic ARF is less stable than the nucleolar ARF and proteasome blockade induced greater recovery of ARF in the nucleoplasm, which is consistent with the observation that non-nucleolar ARF mutants is subject to rapid turnover through the ubiquitin-proteasome pathway [173]. When ectopically expressed together,

ARF colocalizes with COMMD1 in the nucleoplasm, but endogenous ARF/COMMD1 complex was difficult to detect unless ARF degradation is inhibited by the proteasome inhibitor, implicating that ARF interact with COMMD1 in the nucleoplasm but is subject to rapid proteasomal degradation consequently. Therefore, our observations with ARF/COMMD1 interaction support the model that ARF functions in the nucleoplasm where it meets and complexes with the binding partner and regulate the function of the protein.

3.4.2 ARF induced K63 linked ubiquitination of COMMD1

Overexpression of ARF was reported to induce degradation of certain E2F family members through ubiquitin-proteasome pathway. It was suggested that ARF may act conversely to trim down the level of E2F and thus protect cells from tumorgenesis upon oncogene activation [149]. Previous studies have shown that ARF promotes proteasomal degradation of nucleolar protein B23 and therefore inhibits B23 mediated ribosome biogenesis [141]. However, our result showed that instead of facilitating ubiquitination which leads to degradation of its binding partners, ARF promotes K63 linked polyubiquitination of COMMD1. K63 ubiquitination is known as non-classic ubiquitination as it does not target proteins for degradation. Instead, it has distinct roles in regulating cellular functions such as protein kinase activation, DNA repair and vesicle trafficking [165, 175, 176]. We found that ARF stabilizes COMMD1 through its ability to promote K63 mediated ubiquitination of COMMD1. Because the non-proteasomal proteolysis is also utilized for

COMMD1 degradation, it is possible that K63 ubiquitin chains may be capable of regulating and stabilizing structure of the protein or protecting it being recognized by some unknown protease. K63 linked ubiquitination could also regulate protein-protein interactions [176, 177], which may have additional functions, such as affecting COMMD1's binding affinitiy to other factors. COMMD1 has been demonstrated as a ubiquitously expressed inhibitor for NF-κB [169, 171]. It was reported that COMMD1 interacts with and accelerates degradation of Rel-A in the nucleus, which terminates the association of Rel-A with chromatins [171]. We found ARF specifically promotes nuclear COMMD1 ubiquitination through K63 linkage. Therefore it is possible that ARF induced K63 polyubiquitination may regulate the inhibition effect of COMMD1 on κB mediated transcription in the nucleus.

3.4.3 Is ARF an E3 ligase?

Does ARF act as an E3 ligase for the ubiquitination process? Overexpression of ARF was reported to induce sumoylation of its binding partners such as MDM2, E2F1, B23 and WRN. ARF also binds to UBC9, the only E2 found to be responsible for sumoylation, which suggest that ARF may serves as an E3 ligase to bridge the E2 complex to the its binding partners. ARF has been showed to promote ubiquitination of its binding partners such as E2Fs and B23, but it is still remained to be determined whether this is a direct effect or indirect effect that subsequently induce the ubiquitination. ARF does not possess typical E3 ligase structural feature or known catalytic activity for ubiquitination. We

found that physical association of ARF and COMMD1 is necessary for the ubiquitination and some unknown factor bind to the C-terminus of ARF may also contribute to the ubiquitination process. This factor may be some unknown E3 ligases for COMMD1 as ARF has been reported to indirectly activate p53 through its association and inhibition on MDM2. However, it is also possible that ARF is capable of binding to E2 in addition to UBC9 and serve directly as an E3 ligase for COMMD1 ubiquitination. Strikingly, comparison of monoubiquitination with sumoylation in the nucleus reveals some difference in functions but similarity in the mechanisms. Therefore, our study may contribute to the understanding of ARF's divergent function through its ability to induce posttranslational ubiquitin and sumo modification.

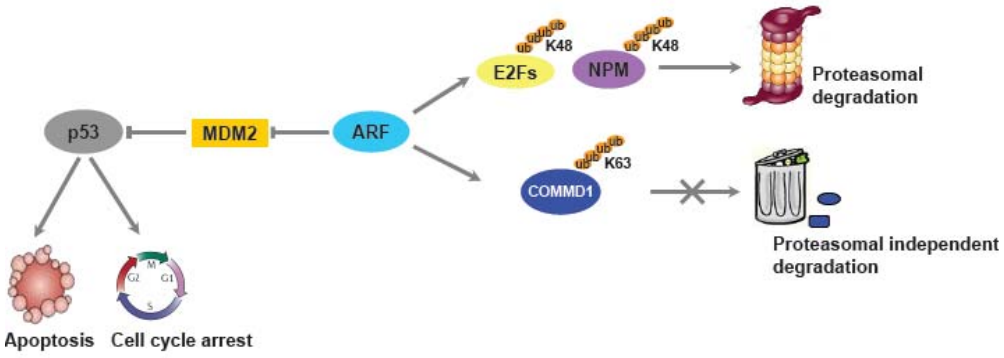


Figure 3.4.1 ARF has both p53 dependent and p53 independent anti-proliferative functions. Upon oncogenic stimulation, ARF inhibits E3 ligase MDM2 and activates p53 mediated cell cycle arrest or apoptosis. In addition to its p53 dependent tumor suppressor function, ARF can also regulate the turnover and function of its binding partners such as E2Fs, NPM and COMMD1 through affecting the ubiquitination pathway.

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Livin promotes Smac/DIABLO degradation by ubiquitin–proteasome pathway

L Ma[1,4], Y Huang[2,4], Z Song[1], S Feng[1], X Tian[1], W Du[1], X Qiu[3], K Heese[2] and M Wu*[,1]

[1] Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, People's Republic of China
[2] School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Republic of Singapore
[3] The National Laboratory of Medical Molecular Biology, Department of Biochemistry and Molecular Biology, The Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, 5 DongdanSantiao, Beijing 100005, People's Republic of China
[4] These authors contributed equally to this work.
* Corresponding author: M Wu, Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, People's Republic of China. Tel: +86 551 3606264; Fax: +86 551 3606264; E-mail: wumian@ustc.edu.cn

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Abstract

Livin, a member of the inhibitor of apoptosis protein (IAP) family, encodes a protein containing a single baculoviral IAP repeat (BIR) domain and a COOH-terminal RING finger domain. It has been reported that Livin directly interacts with caspase-3 and -7 *in vitro* and caspase-9 *in vivo* via its BIR domain and is negatively regulated by Smac/DIABLO. Nonetheless, the detailed mechanism underlying its antiapoptotic function has not yet been fully characterized. In this report, we provide, for the first time, the evidence that Livin can act as an E3 ubiquitin ligase for targeting the degradation of Smac/DIABLO. Both BIR domain and RING finger domain of Livin are required for this degradation *in vitro* and *in vivo*. We also demonstrate that Livin is an unstable protein with a half-life of less than 4 h in living cells. The RING domain of Livin promotes its auto-ubiquitination, whereas the BIR domain is likely to display degradation-inhibitory activity. Mutation in the Livin BIR domain greatly enhances its instability and nullifies its binding to Smac/DIABLO, resulting in a reduced antiapoptosis inhibition. Our findings provide a novel function of Livin: it exhibits E3 ubiquitin ligase activity to degrade the pivotal apoptotic regulator Smac/DIABLO through the ubiquitin–proteasome pathway.
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Abbreviations: IAP, inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; GST, glutathione *S*-transferase; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; CHX, cycloheximide; PARP, poly(ADP-ribose) polymerase; FADD, Fas-associated protein with death domain; RIP, receptor-interacting protein; RIP3, receptor-interacting protein 3; DR6, death receptor 6; XIAP, X-linked IAP; TNFR1, tumor necrosis factor receptor 1; Smac/DIABLO, second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI

Introduction

In recent years, an increasing amount of attention has been given to a class of antiapoptotic proteins, the IAPs (inhibitor of apoptosis proteins), owing to their highly specialized roles in the process of apoptosis. IAPs constitute a family of highly conserved apoptotic suppressor proteins and have been found in a wide variety of species ranging from insects to human beings throughout evolution.[1,2] Members of the IAP family are characterized by 1–3 tandem baculovirus IAP repeat (BIR) motifs and some of them also possess a RING finger motif at their carboxyl-termini. IAP proteins prevent cell death by binding to and inhibiting active caspases. They are negatively regulated by IAP-binding proteins, such as mitochondrial protein Smac/DIABLO, which alleviates the IAP–caspase interaction, thus freeing the caspase for its downstream apoptotic activity.[3,4] Mature Smac/DIABLO was reported to interact with XIAP, cIAP-1, cIAP-2, Survivin or Livin to inhibit their antiapoptotic activity.[5–9] Although RING domains have been identified in various proteins with different functions such as DNA and protein interactions,[10] the most prominent role the RING domain in XIAP, cIAP-1 or cIAP-2 plays has been attributed to its E3 ubiquitin ligase activity: promoting the degradation of both targeted proteins and themselves through ubiquitination. Although XIAP and cIAP-1 have been shown to mediate their self-degradation during apoptosis of thymocytes, XIAP, cIAP-1 and cIAP-2 have also been attested as ubiquitin-protein ligase E3 for Smac/DIABLO.[11–13] Additionally, whereas the cIAP-2 RING domain was shown to promote monoubiquitination of caspase-7 and caspase-3 *in vitro*,[14] XIAP regulates proteasomal degradation of caspase-3 through polyubiquitination thereby enhancing its antiapoptotic effect on Fas-induced cell death.[15] It has not yet been demonstrated, however, if this is generally true for all RING domain-containing IAP family members. Livin, also called KIAP and ML-IAP,[16–18] encodes a protein with a single BIR domain and a carboxy-terminal RING domain. Recent reports have demonstrated that Livin can interact via its BIR domain with caspase-3 and -7 *in vitro* and caspase-9 and Smac/DIABLO *in vivo*.[9,16] Disruption of interaction between Livin and Smac/DIABLO abrogated the ability of Livin to inhibit apoptosis. Yang and Du[19] reported that although Smac/DIABLO can efficiently promote the auto-ubiquitination of Livin, it was unable to target Livin for rapid degradation in HeLa cells. Although Livin was reported to be a poor caspase inhibitor, it can nonetheless protect cells from apoptosis

induced by multiple death stimuli such as FADD, Bax, RIP, RIP3 and DR6.[16] This gives rise to the possibility that Livin may exert its antiapoptotic properties by as yet uncharacterized caspase-independent mechanisms.

In this report, we provide the first evidence that Livin can act as a ubiquitin ligase E3 for targeting the degradation of Smac/DIABLO during the prevention of etoposide-induced cell death. The half-life of short-lived Livin was determined to be less than 4 h in the presence of cycloheximide (CHX), a potent eucaryotic translation inhibitor. We demonstrate that Livin-mediated polyubiquitination of Smac/DIABLO depends on the presence of both its functional RING domain and its intact BIR domain through which Smac/DIABLO interacts. Binding of Livin to Smac/DIABLO is the prerequisite for targeted ubiquitination of Smac/DIABLO. Moreover, Livin's RING domain is shown to promote its auto-ubiquitination, as RING-deleted Livin shows little, if any auto-ubiquitination. In contrast, mutations in the BIR domain of Livin do not greatly affect its self-ubiquitination *in vivo*, but rather significantly increase its instability. Finally, mutations in Livin's BIR domain abrogate its capability to bind to Smac/DIABLO for degradation *in vivo*, resulting in the reduction of its antiapoptotic inhibition. In conclusion, in addition to inhibiting caspase activity, Livin plays an alternative role in targeting the degradation of the death-promoting factor Smac/DIABLO through the ubiquitin–proteasome pathway.

Results

Livin is a short-lived protein and is degraded by the ubiquitin–proteasome pathway

Livin, a member of the IAP family, was reported to be an unstable protein.[19] To determine the half-life of this antiapoptotic factor, CHX (20 μg/ml) was added to HeLa cells and incubation was continued for indicated periods of time before cell extracts were collected and analyzed by Western blot. As shown in Figure 1a-1, endogenous Livin protein levels were dramatically decreased in the presence of CHX and approximately half of the Livin was shown to be degraded after 4 h CHX treatment. This conclusion was further confirmed by scanning densitometry and the plot is shown in Figure 1a-2. Nearly no Livin was detectable after 8 h of CHX treatment, indicating that in the absence of *de novo* protein synthesis, endogenous Livin degrades rapidly and its half-life ($t_{1/2}$) is approximately 4 h. The faint band just above Livin marked as Livin* is expected to be its modified form, although this awaits further characterization. Next, we examined whether Livin was subjected to ubiquitin-mediated proteasomal degradation. As shown in Figure 1b-1, degradation of Livin can be blocked by proteasome inhibitor MG132 or ALLN, suggesting that the inhibition of degradation of Livin is specific for MG132 and ALLN and hence Livin is a target for proteasomal degradation. The respective densitometry data are shown in Figure 1b-2. A baseline experiment showing accumulation of Livin with proteasome inhibitor MG132 treatment in the absence of CHX was also performed. As shown in Figure 1c-1, the protein level of Livin increases gradually over the indicated period of MG132 treatment. The densitometry data are shown in Figure 1c-2. These data further support the conclusion that Livin is subjected to ubiquitin-mediated proteasome degradation.

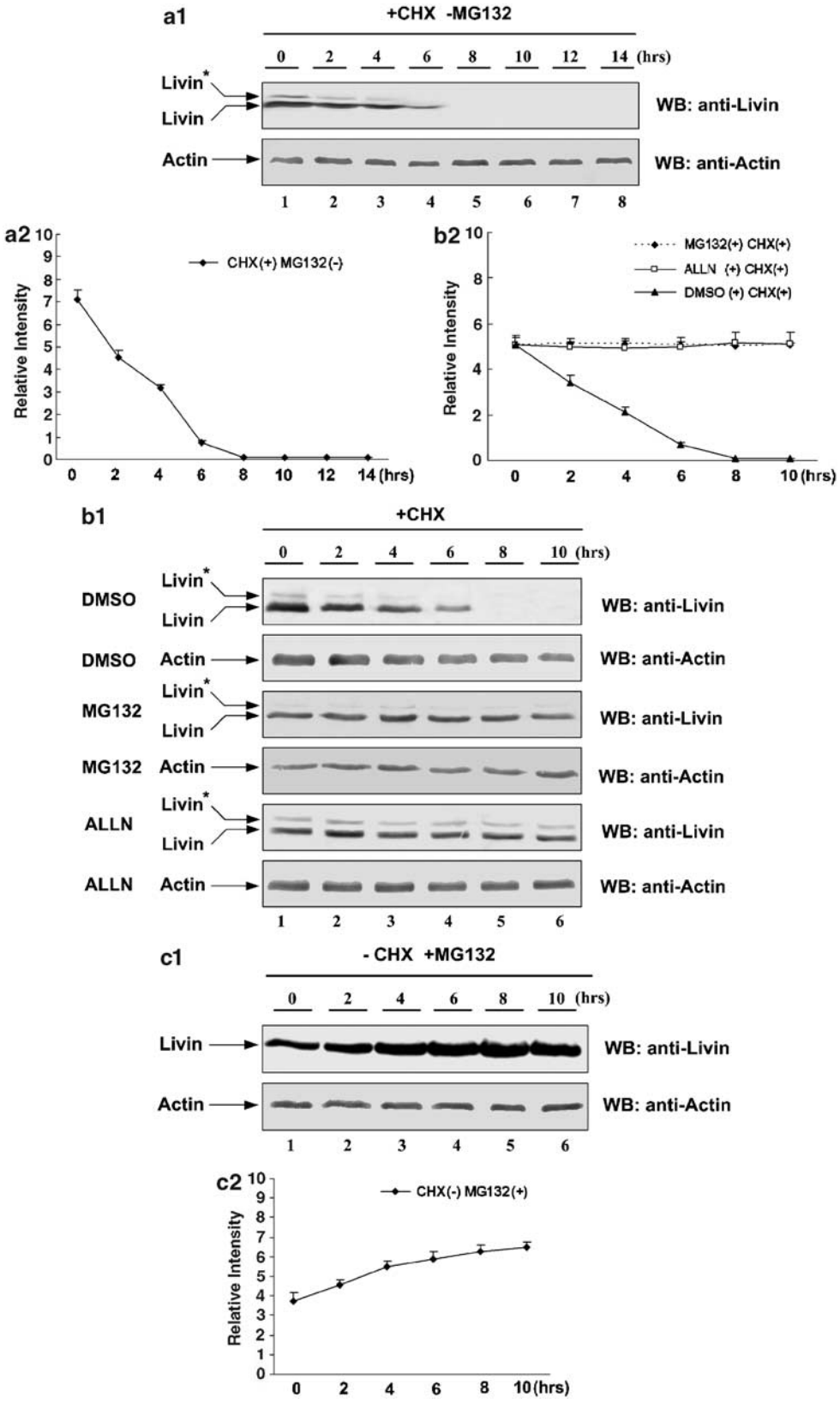
Auto-ubiquitination of Livin *in vivo*

To determine the involvement of self-ubiquitination of endogenous Livin *in vivo*, HeLa cells were treated with or without proteasome inhibitors MG132 or ALLN for 12 h before cells were collected. As shown in Figure 2a, high-molecular-weight Livin products characteristic of polyubiquitinated products were accumulated only in cells treated with MG132 or ALLN, but not in cells left untreated, indicating that Livin is subject to polyubiquitination modification under *in vivo* condition (Figure 2a).

To further examine the effects of BIR domain and RING domain of Livin on its self-ubiquitination, four Flag-tagged Livin fusion plasmids were constructed as shown in Figure 2b. HeLa cells expressing Flag-control, Flag-Livin, Flag-Livin-C124A or Flag-LivinΔRING, respectively, were treated with MG132 for 12 h before they were collected. Cell lysates were further used for immunoprecipitation and Western analysis. As shown in Figure 2c (lanes 1–4), a high-molecular-weight smear characteristic of polyubiquitylated products was detected in cells expressing Flag-Livin and BIR mutant Flag-Livin-C124A, but not in cells expressing RING domain deletion mutant Flag-LivinΔRING, indicating that the RING domain, but not the BIR domain, of Livin is involved in its polyubiquitylation *in vivo*. Immunoprecipitates used for detecting polyubiquitination of Livin by anti-Flag antibody were also examined by anti-Livin antibody, and a similar result was obtained (data not shown).

We notice from Figure 2c that the level of Livin BIR mutant Flag-Livin-C124A is much lower than that of wild-type (wt)-Livin or RING deletion mutant Flag-LivinΔRING. Therefore, we examined whether the BIR domain affects the stability of Livin. HeLa cells were transfected with pcDNA3/Flag-Livin, pcDNA3/Flag-LivinΔRING, pcDNA3/Flag-Livin-C124A or pcDNA3/Flag-Livin-C252A. The reason for additionally choosing Cys252 as a mutation residue, which is underlined

Figure 1 Determining the half-life and its degradation pathway for Livin. (**a**-1) HeLa cells cultured in 24-well plates were treated with CHX (20 μg/ml) for 0, 2, 4, 6, 8, 10, 12 and 14 h individually before soluble extracts were collected. The relative protein levels of Livin were analyzed by Western blot with anti-Livin antibody and endogenous Actin was used as loading control. Star denotes the modified form of Livin. **(b**-1**)** Overnight-cultured HeLa cells were pretreated with DMSO (5 μl/ml), MG132 (20 μM) and ALLN (100 μM) for 1 h before the treated cells were further incubated with CHX (20 μg/ml) for the indicated periods of time. Cell lysates were then subjected to SDS-PAGE and immunoblotting with anti-Livin antibody. Endogenous Actin was used as loading control. (**c**-1) HeLa cells were first cultured overnight and then treated with MG132 (20 μM) for 0, 2, 4, 6, 8 and 10 h individually before soluble extracts were collected in the absence of CHX. The relative protein levels of Livin were analyzed by Western blot with anti-Livin antibody and endogenous Actin was used as loading control. (**a**-2, **b**-2, **c**-2) Scanning densitometry was performed for each Western blot using commercial software provided by Eaglesight (Invitrogen). Values represent means $\pm$ S.D. from three independent experiments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WB: anti-Actin
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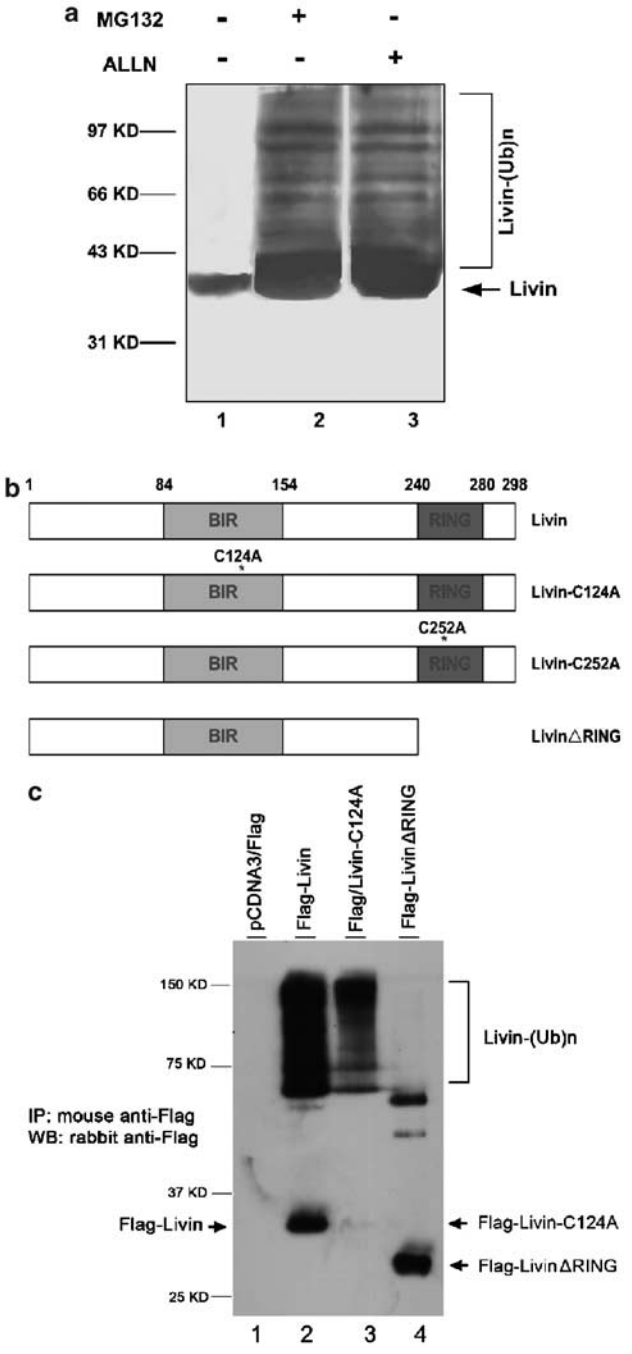


Figure 2 Auto-ubiquitination of Livin *in vivo*. (**a**) Overnight-cultured HeLa cells were treated with and without MG132 (20 μM) or ALLN (100 μM) for another 24 h. Cell lysates were then prepared and subjected to immunoblotting with anti-Livin antibody. Polyubiquitinated Livin is indicated as Livin-Ub(n) at the right side of the figure. (**b**) Schematic diagrams of pcDNA3/Flag-Livin fusion constructs for pcDNA3/Flag-Livin, pcDNA3/Flag-Livin-C124A, pcDNA3/Flag-Livin-C252A and pcDNA3/Flag-LivinΔRING. BIR domain is indicated by box in light gray and RING domain in dark gray. Numbers indicate the positions of amino-acid residues in Livin. (**c**) HeLa cells were transfected with pcDNA3/Flag, pcDNA3/Flag-Livin, pcDNA3/Flag-LivinΔRING and pcDNA3/Flag-Livin-C124A individually (Lanes 1–4). Twenty-four hours after transfection, cells were treated with MG132 (20 μM) for another 12 h. Cell lysates were prepared and incubated with mouse anti-Flag antibody bound to Protein A/G-Sepharose. After 6 h incubation at 4°C, the immunoprecipitates were washed five times in lysis buffer, and proteins were recovered by boiling beads in SDS sample buffer and analyzed by Western blotting using rabbit anti-Flag antibody

in the Livin RING domain $\underline{C}X_2CX_{9-39}CX_{1-3}HX_{2-3}C/HX_2$-C-$X_{4-48}CX_2C$, is the point that mutation at Cys252 (zinc-binding residues) was reported to abolish the capability of ubiquitination of Livin.[19,20] Protein stability was compared among wt-Livin, LivinΔRING, Livin-C124A and Livin-C252A with and without MG132 treatment. As shown in Figure 3, although the steady-state level of wt-Livin or Livin-C124A was considerably higher in the presence (lanes 2, 6) than in the absence of MG132 (lanes 1, 5) (densitometry data not shown), both RING deletion mutant LivinΔRING and RING point mutant Livin-C252A were expressed at comparable levels before and after MG132 treatment (lanes 3, 4, 7, 8), indicating that the RING domain, but not BIR domain, is responsible for Livin's ubiquitin-mediated proteasome degradation. We noticed that BIR mutant Livin-C124A always expressed at a lower level than wt-Livin, and moreover, MG132 treatment could not bring the level of BIR mutant back to a level similar to that of wt-Livin. Based on these observations, we explain it in more detail below: there might be two possibilities for explaining the accelerated instability of Livin BIR mutant: one, Livin BIR mutants may undergo an additional mechanism independent of proteasomal degradation (if only proteasomal degradation is involved, MG132 is expected to bring back the level of BIR mutant to the wt-Livin level, provided that BIR mutant expression level or structural stability is similar to wt-Livin); the alternative possibility is that Livin BIR mutants *per se* may have less expression level as a result of C124A point mutation.

Mutation in Livin BIR domain enhances its degradation and results in reduced antiapoptotic inhibition

To explore the role of Livin BIR domain in mediating protein stability, we constructed two additional Livin point mutants, namely Livin-W134A and Livin-H144A. Trp134 of Livin is highly conserved in IAP family and His144 of Livin corresponds to His220 of XIAP and His77 of Survivin.[21,22] Based

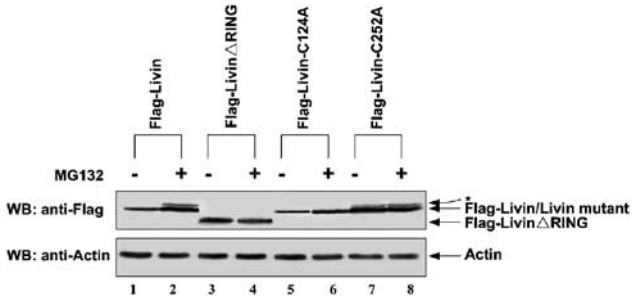


Figure 3 Livin RING domain affects its auto-ubiquitination and protein stability. HeLa cells were cotransfected with 0.5 μg of pEGFP-C1 (transfection efficiency index) plus either pcDNA3/Flag-Livin (0.5 μg), pcDNA3/Flag-LivinΔRING (0.5 μg), pcDNA3/Flag-Livin-C124A (0.5 μg) or pcDNA3/Flag-Livin-C252A (0.5 μg). Twenty-four hours after transfection, cells in each well were split into two equal parts and one part was treated with MG132 (20 μM) and the other part was left untreated for another 12 h. Expression levels of four examined Flag/Livin fusion proteins were compared by densitometry technique in the presence and absence of MG132 (upper panel, data from densitometry scanning not shown). Beta-actin was used as loading control (lower panel)

on this analogy, amino-acid residues Cys124, Trp134 and His144 of Livin are predicted to be critical for stabilizing the overall folding of the Livin molecule. We further compared the stability of Livin BIR domain mutants with that of wt-Livin in cells expressing Livin-C124A, Livin-W134A, Livin-H144A and wt-Livin. As shown in Figure 4a-1, the steady-state level of each BIR domain mutant protein in transfected cells was much lower than that of wt-Livin in the absence of MG132 (lanes 1, 3, 5, 7, densitometry data not shown), implying that BIR mutants are less stable than wt-Livin. Nevertheless, upon treatment with MG132, their levels were much increased (lanes 2, 4, 6, 8, densitometry data not shown). However, in the presence of MG132, levels of each BIR mutant (lanes 2, 4, 6) were still lower than that of wt-Livin (lane 8, densitometry data not shown).

If the notion that mutations in the BIR domain enhance Livin's degradation was true, we would expect that cells expressing Livin BIR mutant would be more prone to apoptosis than cells expressing wt-Livin. Kasof and Gomes[16] reported that Livin was able to prevent HeLa cells from apoptosis induced by RIP3; we therefore compared the antiapoptotic ability of wt-Livin and Livin BIR mutants in preventing RIP3-induced apoptosis. As shown in Figures 3, 4a-1, the BIR mutants expression level is lower than that of the wt-Livin. In order to have a better comparison of their apoptotic inhibition, we adjusted the amount of three BIR mutant plasmids to the same expression level as wt-Livin (Figure 4a-3, second panel). HeLa cells were cotransfected with pEGFP/C1-RIP3 plus either pcDNA3/Flag-Livin (0.5 μg), pcDNA3/Flag-Livin-C124A (1.5 μg), -W134A (1 μg) or -H144A (1.5 μg) individually. Transfected cells were stained with Hoechst 33342 and the viability of the cells was scored and compared (Figure 4a-2). As expected, wt-Livin (31.53%) was shown to significantly reduce RIP3-induced apoptosis compared with vector control (71.72%). In contrast, three BIR domain mutants, namely Livin-C124 (57.41%), -W134 (49.42%) and -H144 (48.48%), showed diminished antiapoptotic function ($P<0.05$, *t*-test), indicating that an intact BIR domain is essential for Livin's protective role. The cleavage of pro-caspase-9 and PARP representing exertion of apoptosis was detected in RIP3-induced apoptosis (Figure 4a-3), and the cleavage degree of procaspase-9 and PARP is in accordance with that of apoptosis shown in Figure 4a-2.

To explicate whether the reduced antiapoptotic function of Livin BIR mutants could be attributed to the loss of their ability to bind Smac/DIABLO, we first verified that Livin interacts with Smac/DIABLO in living cells. Immunoblotting results revealed that anti-Smac antibody could only co-immunoprecipitate (Co-IP) with endogenous Livin in the cells treated with drugs, but not in those cells left untreated (Figure 4b–1). We then examined whether Smac/DIABLO can interact with Livin mutants. As shown in Figure 4b-2, in the presence of Taxol treatment, anti-Smac antibody could only Co-IP wt-Livin and LivinΔRING, but not any of the Livin BIR mutants (Figure 4b–2, the left-hand side of the top panel). As a control, neither wt-Livin nor mutant Livin proteins could be co-immunoprecipitated with Smac/DIABLO by anti-Smac antibody without Taxol treatment (Figure 4b-2, the right-hand side of the top panel), suggesting that the association of Smac/DIABLO and Livin is dependent on Taxol or etoposide treatment. In conclusion, the Livin BIR domain is crucial for its binding to Smac/DIABLO and mutation in the BIR domain nullifies this interaction, and hence reduces Livin's antiapoptotic function.

Smac/DIABLO is a ubiquitination substrate for Livin

We have demonstrated that Livin physically interacts with Smac/DIABLO through its BIR domain. To further investigate whether Livin is able to target Smac/DIABLO for degradation and hence decreases the level of Smac/DIABLO in favor of cell survival, a Western analysis was performed. As shown in Figure 5a, in the absence of MG132, the protein level of cytosolic Smac/DIABLO was significantly decreased in cells expressing Flag-Livin (lane 3) when compared with cells expressing Flag control (lane 1), Flag-LivinΔRING (lane 5) or Flag-Livin-C124A (lane 7). In the presence of MG132, however, levels of Smac/DIABLO become comparable in all four transfected cells, implying that (i) Smac/DIABLO appeared to be targeted for degradation by Livin, but not by Livin-RING domain or -BIR domain mutant, and (ii) this Livin-targeted degradation is proteasome-mediated.

A reduced level of Smac/DIABLO is expected to decrease its apoptotic potential. Apoptosis induced by etoposide was measured in four above-mentioned transfected cells and the corresponding results are plotted in Figure 5b. In the absence of MG132, apoptosis of transfectants induced by etoposide ordered increasing is as follows: cells expressing Flag-Livin (37.17%), Flag-LivinΔRING (49.43%) and Flag-Livin-C124A (56.27%) ($P<0.05$, *t*-test). This result is in agreement with the hypothesis that reduced degradation of Smac/DIABLO by Livin mutant could result in increased apoptosis.

Next, we examined whether Livin truly ubiquitinates Smac/DIABLO for degradation *in vivo*. An immunoprecipitation experiment was performed. As shown in Figure 5c, Smac/DIABLO became heavily polyubiquitinated in those cells expressing wt-Livin, whereas cells expressing either Livin BIR mutant (Livin-C124A) or RING mutant (LivinΔRING) showed little, if any background ubiquitination of Smac/DIABLO. Immunoprecipitates used for detecting polyubiquitination by anti-ubiquitin antibody were also examined for the presence of Smac/DIABLO by anti-Smac antibody (Figure 5c, middle panel). In conclusion, Livin-mediated ubiquitination of Smac/DIABLO *in vivo* depends on the presence of both an intact Livin RING finger domain and its functional BIR domain.

Lastly, to provide more convincing evidence that Livin by itself can ubiquitinate Smac/DIABLO, an *in vitro* experiment was performed. As shown in Figure 5d, Smac/DIABLO was found to be ubiquitinated by GST-Livin (lane 8), but not GST-LivinRING or GST-Livin-C124A (lanes 6, 7), indicating that BIR domain and RING domain are essential for Livin's E3 ligase activity under *in vitro* conditions. However, if LivinRING is in excess, some degree of polyubiquination of Smac/DIABLO by LivinRING can be observed (data not shown). It is well known that excessive E2 can substitute for the

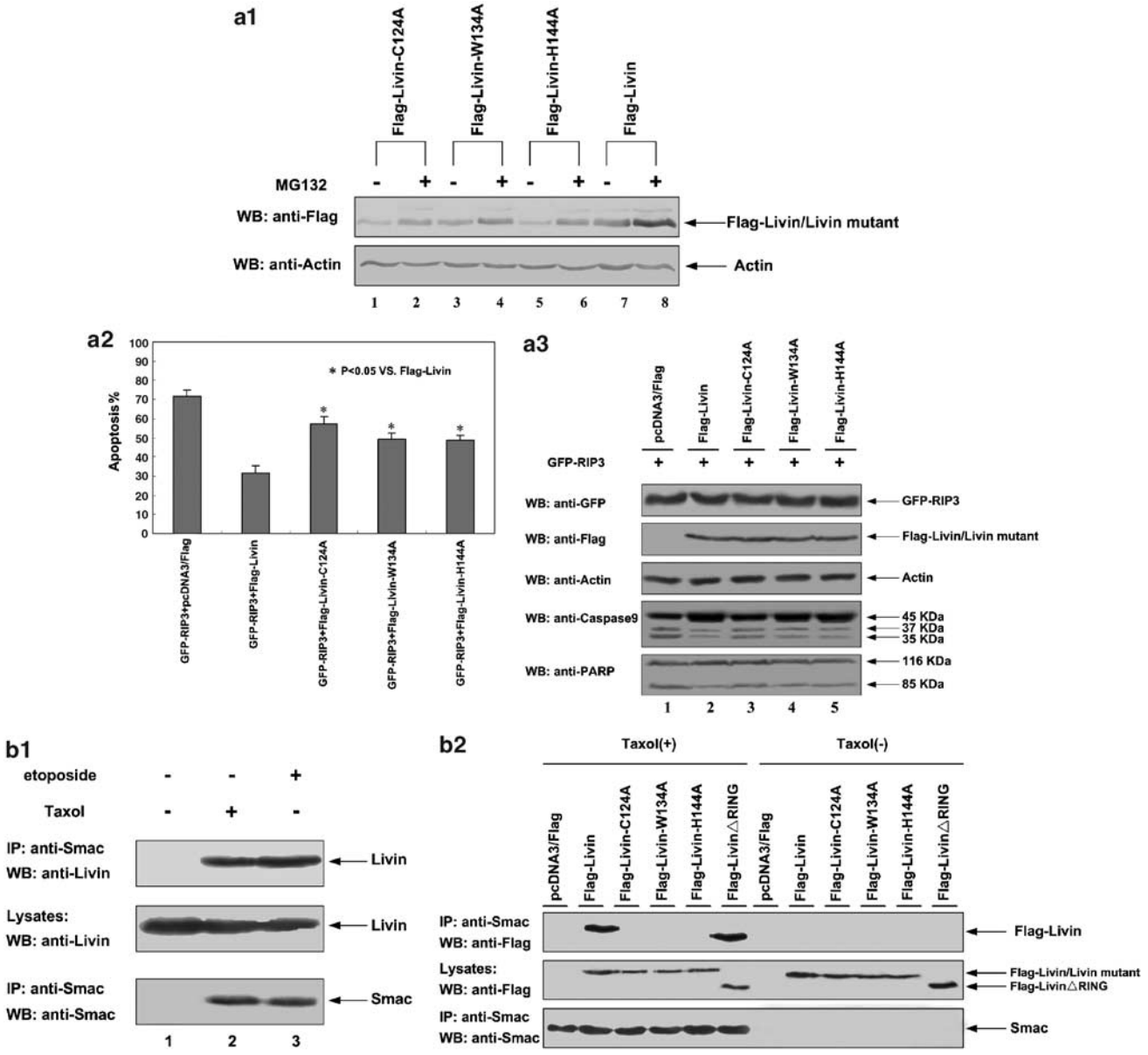


Figure 4 Livin BIR domain mutant fails to interact with Smac/DIABLO, resulting in a reduction of its antiapoptosis and an increase of its degradation. (**a**-1) HeLa cells were cotransfected with 0.5 μg of pEGFP-C1 (transfection efficiency index) plus either pcDNA3/Flag-Livin (0.5 μg), pcDNA3/Flag-Livin-C124A (0.5 μg), pcDNA3/Flag-Livin-W134A (0.5 μg) or pcDNA3/Flag-Livin-H144A (0.5 μg). Twenty-four hours after transfection, cells were split into two equal parts and one part was treated with MG132 (20 μM) and the other part was left untreated for another 12 h. Cells were then collected and analyzed by Western blotting using anti-Flag antibody (upper panel). Whole-cell lysates were used for detection of endogenous Actin to ensure equal sample loading per lane (lower panel). (**a**-2) To keep the expression level of BIR mutant and wt-Livin the same, different plasmid concentrations were used. HeLa cells were cotransfected with pEGFP/C1-RIP3 (0.5 μg) and either pcDNA3/Flag (0.5 μg), pcDNA3/Flag-Livin (0.5 μg), pcDNA3/Flag-Livin-C124A (1.5 μg), pcDNA3/Flag-Livin-W134A (1 μg) or pcDNA3/Flag-Livin-H144A (1.5 μg), respectively for 24 h and were then stained with Hoechst 33342 (5 μg/ml) for 10 min. The viability of the cells was determined by counting both the living and the dead GFP-positive cells. Five different microscopic fields containing 100–150 cells from each well were chosen at random for counting samples. Values are mean $\pm$ S.D. from three independent experiments. Statistical analysis was by *t*-test with $^*P < 0.05$. (**a**-3) Cell lysates were used for detection the expression of transfected Flag-Livin and Livin mutants with anti-Flag antibody and the expression of GFP-RIP3 with anti-GFP antibody. The cleavage of pro-caspase-9 was detected by anti-caspase-9 antibody and the cleaved products were indicated as 35 and 37 kDa, respectively. The cleavage of PARP was detected by anti-PARP antibody and the cleaved product was indicated as 85 kDa. Actin was used as loading control. (**b**-1) HeLa cells were cultured overnight and then treated with and without Taxol (200 nM) or etoposide (100 μg/ml) for 24 h. Cytosolic fraction prepared from each sample was immunoprecipitated by anti-Smac antibody and the immunoprecipitates were analyzed by Western blotting using anti-Livin antibody. Cell lysates were also used for detection of endogenous Livin by anti-Livin antibody. (**b**-2) HeLa cells were transfected with pcDNA3/Flag (0.5 μg), pcDNA3/Flag-Livin (0.5 μg), pcDNA3/Flag-Livin-C124A (0.5 μg), pcDNA3/Flag-Livin-W134A (0.5 μg), pcDNA3/Flag-Livin-H144A (0.5 μg) or pcDNA3/Flag-LivinΔRING (0.5 μg) for 24 h before each transfectant was split into two equal parts. One part was treated with Taxol (200 nM) and the other part was left untreated. Twenty-four hours later, cytosolic fractions prepared from each transfectant were then incubated with anti-Smac antibody-loaded Protein A/G-Sepharose for 6 h at 4°C, and the immunoprecipitates were analyzed by Western blotting using anti-Flag antibody (upper panel). Cell lysates were also used for detection of the expression of respective transfected Flag-Livin fusions by anti-Flag antibody (middle panel). The presence of Smac/DIABLO in each immunoprecipitate precipitated by anti-Smac antibody was confirmed by Western analysis (bottom panel)

requirement of an E3 in an *in vitro* assay; we therefore titrated down the concentration of E2 (UbcH5b) from 400 nM (concentration used in *in vitro* assay) down to 200 nM and 100 nM and found that ubiquitination of Smac/DIABLO is, at least *in vitro*, independent of E2 concentration (data not shown). We concluded that Smac/DIABLO ubiquitylation *in vitro* by Livin requires the RING domain. This conclusion is in accordance with the results *in vivo*.

Discussion

Among many mechanisms that regulate the activity of IAP, post-translational modifications, particularly ubiquitination, have received increasing attention. To investigate whether IAP protein Livin, like other RING finger proteins, can regulate itself through auto-ubiquitination,[11,23,24] we compared the endogenous Livin in the presence or absence of proteasome inhibitor MG132 or ALLN. We found that Livin is regulated by self-ubiquitination, and as a result, Livin undergoes proteasome-mediated degradation. A previous report demonstrated that although processed mature Smac/DIABLO is able to promote auto-ubiquitination of Livin, it has little, if any, effect on its degradation.[19] It is therefore reasonable to assume that Livin RING domain could be the candidate determinant of its self-ubiquitination. Data from our experiment proved that the RING domain of Livin plays a crucial role in its auto-ubiquitination, as Livin RING deletion mutant demolishes all its auto-ubiquitination activity (Figure 2c). However, we cannot completely exclude the possibility that Livin auto-ubiquitination is partially enhanced by processed mature Smac/DIABLO owing to longer MG132 or ALLN treatment.

We demonstrated that Livin BIR mutant Livin-C124A showed much less stability compared with wt-Livin (Figure 2c), implying that the BIR domain *per se* may normally possess suppressing function for protein degradation or, alternatively, some yet uncharacterized degradation-inhibitory factor regulates its stability through the BIR domain, and mutation in the BIR domain thereby occurs, resulting in its decreased stability. However, we found that the BIR mutant exhibits less auto-ubiquitination than wt-Livin, which may be explained by the possibility that Livin BIR mutant is degraded by an as yet uncharacterized mechanism that is independent of the auto-ubiquitination process. However, the possibility that BIR mutant has a lower expression level than wt-Livin cannot be excluded at the present time. Nevertheless, we have characterized some critical amino-acid residues within the BIR domain, which greatly sensitize Livin to ubiquitin–proteasome degradation. Vucic *et al*.[18] reported that Livin-C124A could no longer block Fas- and TNFR1-induced apoptosis, as C124 together with H144 is predicted to chelate zinc ions to stabilize the overall structure of Livin.[18] In the present study, we were able to demonstrate that Livin BIR domain point mutant Livin-C124A, Livin-W134A or Livin-H144A degrades more rapidly than wt-Livin. Accordingly, all three Livin BIR domain mutants showed less effectiveness in inhibiting RIP3-induced apoptosis, supporting the conclusion that Livin's antiapoptotic activity is dependent on its intact BIR domain.

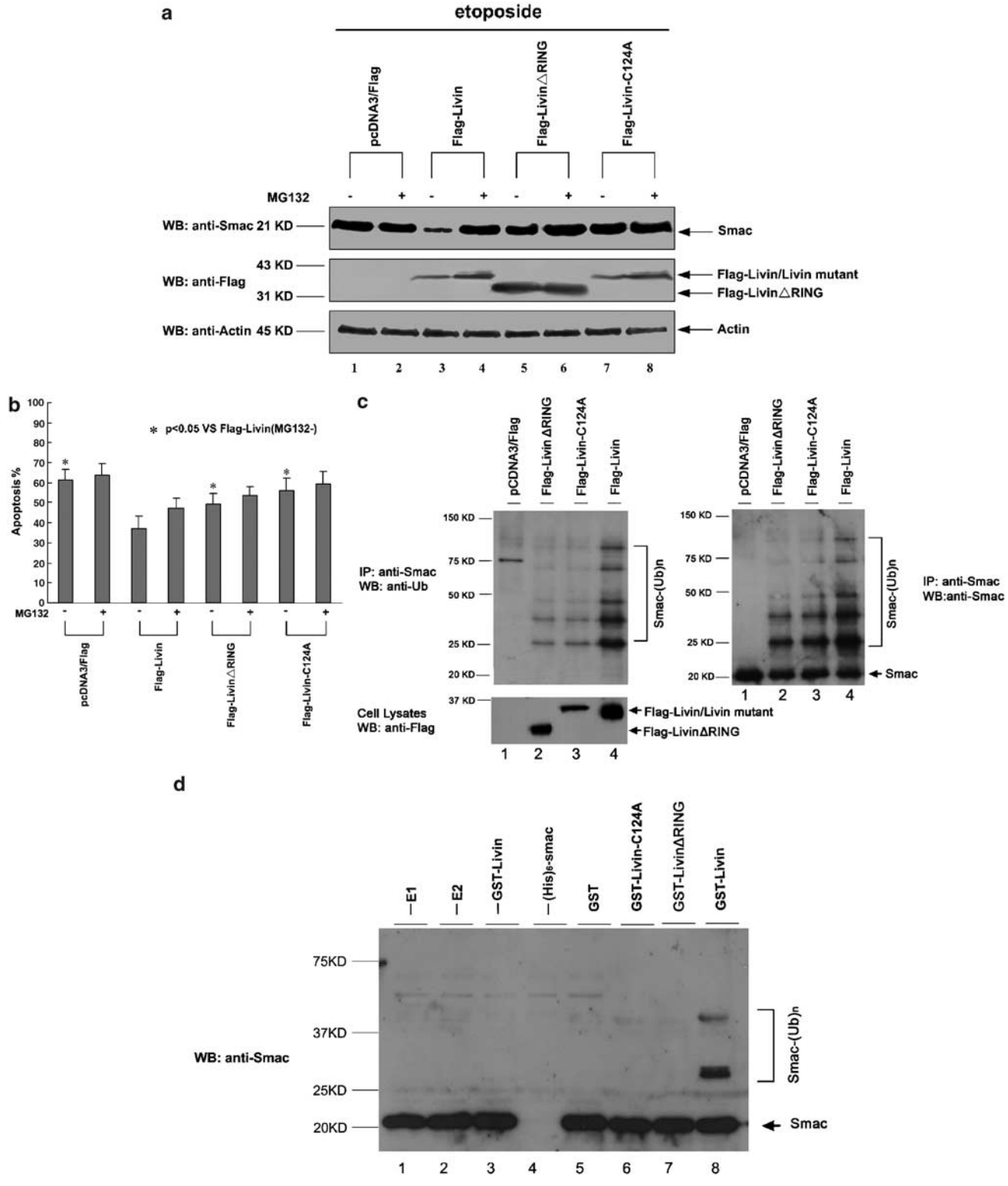
Thus far, no obvious role has been attributed to W67 in Survivin or W210 in XIAP for their antiapoptotic activity. We firstly showed that W134 of Livin (which corresponds to W67 in Survivin and W210 in XIAP) is functionally important, and that Livin BIR domain point mutant Livin-W134A not only decreases its ability to protect cells from RIP3-induced apoptosis, but also blocks interaction with mitochondria death inducer Smac/DIABLO. This suggests that W134, like C124 and H144, is a critical residue for Livin to bind Smac/DIABLO (Figure 4b-2). From these discussions, we conclude that integrity of BIR domain stabilizes Livin and enables it to exert its antiapoptotic activity.

Recent studies have shown that many RING finger-containing IAPs possess E3 activity, which appends additional biological activities to IAPs. In *Drosophila melanogaster*, the DIAP1 RING finger was demonstrated to mediate ubiquitination of Dronc and is indispensable for regulating apoptosis.[25] In addition, XIAP and DIAP1 were reported to serve as ubiquitin ligases for Reaper and this regulation has a significant impact on the ability of Reaper to initiate apoptosis.[26] Apollon was reported to function as an E2-Ubc to ubiquitinate both Smac/DIABLO and caspase-9 and thus displays an essential cytoprotection function in preventing Smac/DIABLO-induced apoptosis.[27] The interacting partners of Livin include caspase-3, -7 and -9 and proteins containing an IAP-interacting motif, which currently comprise the mitochondrial protein Smac/DIABLO and Omi/HtrA2. Smac/DIABLO is a negative regulator of Livin and is therefore a proapoptotic protein. Wilkinson *et al*.[28] reported that cytoprotective IAPs could inhibit apoptosis through the neutralization of IAP antagonists, such as Smac/DIABLO, rather than by directly inhibiting caspases. Regulation of apoptosis by Livin is believed to sequester Smac/DIABLO and prevents it from antagonizing XIAP-mediated inhibition of caspases.[29] Whether Livin directs the ubiquitination of interacting proteins, such as Smac/DIABLO through its RING domain, has not yet been characterized.

We demonstrate that ectopic expression of wt-Livin, but not of Livin RING domain or Livin BIR domain mutant, promotes Smac/DIABLO ubiquitination. Wt-Livin protects cells from etoposide-induced cell death more effectively than RING deletion mutant or BIR domain point mutant does. Ubiquitination of Smac/DIABLO by Livin requires both functional BIR and RING domain *in vivo* and *in vitro.* Livin binds to Smac/DIABLO through its BIR domain and their interaction is the prerequisite for targeting the degradation of Smac/DIABLO. BIR domain point mutant Livin-C124A, which was unable to interact with Smac/DIABLO, had abrogated its ability for ubiquitinating Smac/DIABLO. Equally important, Livin RING finger domain is also indispensable for ubiquitinating Smac/DIABLO *in vivo* (Figure 5c). However, it should be noted that the smeared ubiquitin-modified Smac/DIABLO bands in the anti-ubiquitin or anti-Smac blots were still detected in the presence of Livin-C124A or LivinΔRING (Figure 5c, lanes 2, 3). This is not unexpected as Smac/DIABLO is also subject to polyubiquitination by other IAP factors, such as Apollon/Bruce, cIAP-1 and cIAP-2.[14,27]

The struggle between cell survival and death is ubiquitous and this is maintained in balance under various intricate regulations including the mechanism of proteasome ubiquiti-

nation. On the one hand, by catalyzing its own ubiquitination, Livin lowers the threshold for inducing apoptosis and makes cells more perceptible for cell death. On the other hand, Livin targets death inducer Smac/DIABLO for degradation through the proteasome ubiquitination pathway, promoting cell survival. Understanding the detailed mechanisms underlying the dual functions of Livin in regulating cellular apoptosis and survival requires more extensive future research.



Materials and Methods

Oligonucleotides

The sequences of the oligonucleotides used in this study are listed as follows (all primers are read from 5′ to 3′):

P1: CGGAATTCCATGGGACCTAAAGACAGTGCCAAG;
P2: CGGTCGACCTAGGACAGGAAGGTGCGCACGCG;
P3: CAGGACAAGGTGAGGGCCTTCTTCTGCTATGGGGGC;
P4: AGCAGAAGAAGGCCCTCACCTTGTCCTGATG;
P5: GCAGAGCGCGAAGCGCGGGGACGACC;
P6: CCCCGCGCTTCGCGCTCTGCAGGCCCCCATA;
P7: GGACGGAGGCTGCCAAGTGGTTCCCCAG;
P8: TGGCAGCCTCCGTCCAGGGGTCG;
P9: GCAGGAGGAGAGGACGGCCAAGGTGTGCCTGGACCGC;
P10: CCAGGCACACCTTGGCCGTCCTCTCCTCCTGCAGCCGCCG;
P11: CGGTCGACCTACACATCCCTGGCTCCTGGGGGCTC.

Reagents and antibodies

The following antibodies were used in this study: polyclonal antibodies: anti-Actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pAb-ubiquitin (Calbiochem, La Jolla, CA, USA), pAb-anti-Flag (Sigma, St. Louis, MO, USA), pAb-anti-PARP (Upstate, Lake Placid, NY, USA). Monoclonal antibodies: mAb-Flag (Sigma), mAb-GFP (MBL, Nagoya, Japan), mAb-Smac/DIABLO (Cell Signaling Technology), mAb-caspase-9 (Immunotech, France) and mAb-Livin (IMGENEX, San Diego, CA, USA). Proteasome inhibitor MG132 (N-CBZ-leu-leu-leucinal) was purchased from Calbiochem (La Jolla, CA, USA) and ALLN (*N*-acetyl-leu-leu-nor leucinal, or calpain inhibitor I) was purchased from Sigma. CHX, Hoechst 33342 and etoposide were purchased from Sigma. Taxol used in our experiments was labeled as GCP (Good Clinical Practice) quality standard. Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). Medium compounds were obtained from Oxid (Basingstroke Hampshire, UK). Rabbit ubiquitin-activating enzyme E1 and recombinant His-tagged ubiquitin were purchased from Calbiochem.

Cell culture and transfection

HeLa cells were maintained in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 1 × nonessential amino acids, 1 × MEM sodium pyruvate, 100 μg/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cultured cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Transfection of cells with various mammalian expression vectors by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was according to the methods provided by manufacturer's specification.

PCR-mediated mutagenesis

PCR-mediated mutagenesis method described previously[8] was used to generate Livin point mutants including Livin-C124A, -W134A, -H144A, and -C252A. The primer pairs P1/P4 and P3/P2 were used for generating Livin-C124A; P1/P6 and P5/P2 for -W134A; P1/P8 and P7/P2 for -H144A; and P1/P10 and P9/P2 for -C252A. Mutations created were further verified by DNA sequencing.

Plasmid construction

Full-length Livin gene fragment was amplified from HeLa cDNA library by PCR using the primer pair P1/P2. The amplified fragment was subcloned into *Eco*RI/*Sal*I sites of pEGFP-C1 (for the preparation of GFP/Livin) or *Eco*RI/*Xho*I sites of pCDNA3/Flag (for the preparation of Flag/Livin). A cDNA fragment coding for LivinΔRING (residues1–239) generated by PCR reaction using primers P1/P11 was digested with restriction enzymes *Eco*RI/*Sal*I or *Eco*RI/*Xho*I to subclone into pEGFP-C1 and pcDNA3/Flag, respectively. PET22b-Smac (Δ55) was constructed by the method described previously.[8]

CHX inhibition

Protein stability was measured by using CHX as described previously.[30] Briefly, HeLa cells were cultured in 24-well plates overnight at 37°C before proteasome inhibitors MG132 or ALLN were added. After incubation with MG132 or ALLN for 1 h, cells were further treated with 20 μg/ml CHX for indicated periods of time and were then harvested and analyzed by Western blotting.

Cell death assay

The ability of Livin and Livin mutants to affect cell viability was assayed by transfecting HeLa cells in 24-well plates (2×10^4 cells/well) with various Livin constructs. In order to compare the protein level and to count the dead cells more accurately, same concentration of plasmids under the same conditions used for transfection was strictly employed. Twenty-four hours after transfection, cell incubation with the drug was continued for the desired period of time and the viability of the cells was measured by

Figure 5 Smac/DIABLO is a ubiquitination substrate for Livin *in vivo* and *in vitro*. (**a**) HeLa cells were transiently cotransfected with 0.5 μg of pEGFP-C1 (transfection efficiency index) plus either pcDNA3/Flag (0.5 μg), pcDNA3/Flag-Livin (0.5 μg), pcDNA3/ Flag-LivinΔRING (0.5 μg) or pcDNA3/Flag-Livin-C124A (0.5 μg). Twenty-four hours after transfection, cells in each well were split into two equal parts and cultured for a short period of time. All the samples that were split were first treated with etoposide (100 μg/ml) for 24 h, followed by treatment with or without MG132 (20 μM) for an additional 12 h. Cytosolic fractions were prepared for detection of the steady-state level of cellular Smac/DIABLO by Western blotting using anti-Smac antibody (top panel). The successful expression of transfected Flag-Livin fusions was verified by anti-Flag antibody (middle panel). Detection of endogenous beta-actin was used as loading control (bottom panel). (**b**) Cells were stained with Hoechst 33342 and both live and dying/dead cells were scored and the data were plotted as percent apoptosis. Each bar represents the mean ± S.D. from three independent experiments. Statistical analysis was by *t*-test with $*P < 0.05$. (**c**) HeLa cells were transiently transfected with pcDNA3/Flag, pcDNA3/Flag-LivinΔRING, pcDNA3/Flag-Livin-C124A or pcDNA3/Flag-Livin (lanes 1–4). Twenty-four hours after transfection, cell were treated with etoposide (20 μM) for another 12 h followed by treating with or without 10 μM MG132 for an additional 12 h. Cytosolic fractions were immnoprecipitated by anti-Smac antibody (mouse monoclonal antibody from Cell Signaling Technology) and the immunoprecipitates were further analyzed by Western blotting using anti-ubiquitin antibody (rabbit polyclonal antibody from Calbiochem) and donkey anti-rabbit IgG (from Amersham) (left upper panel) and anti-Smac antibodies (right panel). The same cytosolic fractions were used for detection of expression of various transfected Flag-Livin and Flag-Livin mutants (left lower panel). (**d**) Assays were performed in the presence of all the other assay components, but in the absence of purified recombinant His-Smac, E1, E2, GST-Livin for 120 min (lanes 1–4). The effect of incubating for 2 h at 30°C in the presence of purified GST, GST-Livin, GST-Livin-C124A or GST-LivinΔRING individually is shown in lanes 5–8. Assay reaction buffer utilized contained 50 mM Tris-HCl (pH 7.6), 100 nM rabbit E1 and 400 nM E2 UbcH5b (in E2 titration experiment, 200 nM and 100 nM E2 were also used, data not shown), 20 μM ubiquitin and 2 mM Mg-ATP. When the assay was completed, SDS was added to stop the reaction followed by Western blotting using anti-Smac antibody. High-molecular-weight polyubiquitinated Smac/DIABLO is marked as Smac-Ub(n) on the right side of the figure

counting round GFP-positive cells characterized with aberrant nuclei stained by Hoechst 33342. Data are expressed as percentages of control and are means of three independent experiments.

Western blot analysis and immunoprecipitation

Western blot analysis was performed according to the procedures described by Song *et al.*[8] For immunoprecipitation, cells were first lysed in a Triton X-100-based lysis buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, protease inhibitor cocktail) for 1 h, and the nuclear and cellular debris was cleared by centrifugation. Cytosolic lysate was then incubated with first monoclonal antibody-bound Protein A/G-Sepharose. After 1 h incubation at 4°C, the immunoprecipitates were washed five times in lysis buffer, and proteins were recovered by boiling beads in SDS sample buffer and analyzed by Western blot.

In vitro ubiquitination assay

GST and GST-fusion proteins were expressed in *Escherichia coli* strains DH5-alpha or BL21/DE3. The GST-fusion proteins were purified through the Glutathione Sepharose 4B beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Assay reaction buffer utilized for *in vitro* ubiquitination of Smac/DIABLO by Livin and Livin mutants contained 50 mM Tris-HCl (pH 7.6), 100 nM rabbit E1 and 400 nM E2 UbcH5b, 20 μM ubiquitin and 2 mM Mg-ATP. When the assay was completed, SDS was added to stop the reaction, followed by Western blotting using anti-Smac antibody.

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Tumor Suppressor ARF Promotes Non-classic Proteasome-independent Polyubiquitination of COMMD1*[S]

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Yafen Huang[‡], Mian Wu[§], and Hoi-Yeung Li[‡1]

From the [‡]Division of Molecular and Cell Biology, School of Biological Sciences, College of Science, Nanyang Technological University, Singapore 637551 and the [§]School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China

Although the tumor suppressor ARF is generally accepted for its essential role in activating the p53 pathway, its p53-independent function has also been proposed. Here, we report that ARF associates with COMMD1 and promotes Lys^{63}-mediated polyubiquitination of COMMD1 in a p53-independent manner. We found that ARF interacts with COMMD1 *in vivo*. Deletion analysis of ARF suggested that the N-terminal amino acids 15–45 are important for its interaction with COMMD1. In addition, we found that endogenous ARF redistributes from the nucleolus to the nucleoplasm and interacts with COMMD1 when DNA is damaged by actinomycin D. Interestingly, we found that ARF promotes the polyubiquitination of COMMD1 through Lys^{63} of ubiquitin but not the polyubiquitination of Lys^{48}, which does not target COMMD1 for proteasome-dependent proteolysis. Moreover, ARF mutants lacking the domain interacting with COMMD1 did not promote COMMD1 polyubiquitination, indicating that physical association is a prerequisite condition for the polyubiquitination process. Together, these data suggest that the ability to promote Lys^{63}-mediated polyubiquitination of COMMD1 is a novel property of ARF independent of p53.

The *INK4a/ARF* locus encodes two potent and distinct tumor suppressors, p16^{INK4a} and ARF (p14ARF in human and p19ARF in mouse) (1–3). p16^{INK4a} acts as an inhibitor of cyclin-dependent kinases to increase the growth-suppressive activity of pRb protein, whereas ARF activates the p53 pathway through its inhibitory effect on MDM2 (4). In response to oncogene activation, ARF binds to MDM2 and inhibits its E3[2] ligase activity for p53. Hence it protects p53 from degradation through a ubiquitin-proteasome pathway. In some conditions, sequestering MDM2 in the nucleolus by ARF may also contribute to stabilization and activation of p53 (5), but it has been demonstrated not to be essential for the inhibitory effect of ARF on MDM2 (6).

Apart from its major function in activating the p53 pathway, ARF does regulate other cellular activities such as ribosomal RNA processing and gene expression (7, 8). Although it does not contain any lysine residue, ARF undergoes proteasome-dependent degradation (9). Moreover, ARF also regulates the turnover of its binding partners by affecting the ubiquitination process. ARF inhibits the function of B23, a nucleolar endoribonuclease involved in 28 S RNA maturation, through promoting B23 polyubiquitination and proteasomal degradation (10). In some circumstances, overexpression of ARF destabilizes MDM2, E2F1, and DP1 by facilitating the polyubiquitination of the proteins (11–14). Strikingly, in addition to promoting ubiquitin conjugation, ARF also targets a number of its binding partners including MDM2, NPM (B23), Werner helicase, HIF-1α, and E2F1 for small ubiquitin-like modifier modification (11, 15, 16). The extensive effect of protein sumoylation induced by ARF may include control of protein stability, formation of subnuclear structure, and regulation of transcriptional activities in a p53- and MDM2-independent manner (17, 18).

COMMD1 (copper metabolism gene MURR1 domain-containing protein 1, previously known as MURR1) is a multifunctional factor that was identified to be involved in copper metabolism (18). Some of its functions are demonstrated related to the factors involved in apoptosis. XIAP, a potent inhibitor of apoptosis, can regulate cellular copper levels by promoting polyubiquitination and degradation of COMMD1 (19). In addition, COMMD1 blocks NF-κB activation by either inhibiting the proteasome-dependent degradation of IκB or interfering with the interaction between the NF-κB component RelA and chromatin (20, 21).

In this study, we have characterized COMMD1 as a novel binding partner of ARF. ARF colocalizes with COMMD1 in the nucleoplasm and promotes Lys^{63}-mediated, proteasome-independent polyubiquitination of COMMD1.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The following antibodies were used in this study: mouse anti-FLAG M2 monoclonal Ab (Sigma), rabbit anti-FLAG polyclonal Ab (Sigma), rabbit anti-HA polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Myc monoclonal Ab (Cell Signaling Technology), goat anti-actin polyclonal Ab (Santa Cruz Biotechnology), rabbit anti-ARF polyclonal Ab (Neomarkers), mouse anti-α-

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[1] To whom correspondence should be addressed. Tel.: 65-6316-2931; Fax: 65-6791-3856; E-mail: hyli@ntu.edu.sg.

[2] The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; E2, ubiquitin carrier protein; Ab, antibody; HA, hemagglutinin; ub, ubiquitin; PBS, phosphate-buffered saline; co-IP, co-immunoprecipitation; RNAi, RNA interference.

tubulin monoclonal Ab (Molecular Probes), rabbit anti-GCN5 Ab (Cell Signaling Technology), and mouse anti-COMMD1 Ab (Abnova). Rhodamine- and fluorescein isothiocyanate-conjugated secondary antibodies were purchased from Molecular Probes. Horseradish peroxidase-conjugated protein G was purchased from Bio-Rad. MG132 and cycloheximide were purchased from Sigma.

Yeast Two-hybrid Screening—A Gal4-based system (Matchmaker, Clontech) was used according to the manufacturer's instructions. Full-length human p14ARF was cloned into pGBKT7, which carries a DNA-binding domain, to create a bait protein. This plasmid was transformed into yeast strain AH109, and no spontaneous transactivation was detected. The transformed AH109 cells were then mated with yeast strain Y187 cells pretransformed with a Clontech Matchmaker human skeletal muscle cDNA library fused with a DNA activation domain in the pACT2 plasmid. After screening, plasmids were extracted from positive colonies, propagated in *Escherichia coli*, and analyzed by automated sequencing.

Plasmid Construction—To create the mammalian expression plasmids, full-length ARF was amplified using pGBKT7-ARF as the template and subcloned into the pcMV5-HA vector. COMMD1 cDNA was amplified using reverse transcription-PCR from HeLa total RNA extracts and subcloned into the pcDNA3-FLAG vector. pCMV5-Myc-ub and pCMV5-Myc-ubK48R were kindly provided by Dr. Peter Cheung. The K63R mutant of ubiquitin was generated by introducing the mutation site into the reverse primer and then subcloning it into the pCMV5 backbone.

Cell Culture and Transfection—Human NCI-H1299 cells were grown in RPMI 1640 medium with 2 mM L-glutamine, 2 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Transfection of cells with mammalian expression constructs by Lipofectamine 2000 (Invitrogen) was according to the methods recommended by the manufacturer.

Protein Stability Assay—H1299 cells grown on 6-well plates were transfected with the plasmid as indicated. 24 h after transfection, cells were treated with 25 μg/ml cycloheximide or 2 μM MG132 for the indicated period of time. Protein levels were analyzed by Western blotting.

Western Blotting and Immunoprecipitation—Cells were washed twice with PBS and lysed in Pierce M-PER lysis buffer supplemented with protease inhibitor mixture (Roche Applied Science). The protein samples were boiled in SDS sample buffer, resolved on 12% SDS-polyacrylamide gel, and transferred to nitrocellulose membrane (Bio-Rad). The membranes were blocked with 10% nonfat milk in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature. After blocking, membranes were incubated with antibodies as indicated for 3 h at room temperature or overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) or horseradish peroxidase-conjugated protein G (Bio-Rad) for 1 h at room temperature. Antibody detections were performed with a Pierce ECL detection kit or Amersham Biosciences ECL advanced Western blotting system according to the manufacturer's instructions.

For FLAG-tagged protein immunoprecipitation, FLAG M2 affinity gels were added to the cell lysates and incubated overnight at 4 °C. For HA-tagged ARF or endogenous COMMD1, cell lysates were first incubated with anti-HA or anti-COMMD1 polyclonal antibody for 6 h, followed by protein G-Sepharose bead incubation for another 3 h at 4 °C. The beads were then washed five times with wash buffer (20 mM Tris-HCl, pH 7.6, and 500 mM NaCl), boiled in SDS sample buffer, and subjected to Western blot analysis.

Indirect Immunofluorescence Staining and Microscopy—H1299 cells grown on a glass coverslip were transfected with plasmids as indicated. 1 day after transfection, cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Nonidet P-40 in PBS for 10 min, and blocked with 4% bovine serum albumin for 30 min. Fixed cells were first incubated with anti-HA antibody for 2 h, followed by incubation with fluorescein isothiocyanate-conjugated secondary antibody for another 1 h, incubation with anti-FLAG M2 antibody for 2 h, and incubation with rhodamine-conjugated secondary antibody for another 1 h. To detect localization of endogenous proteins, H1299 cells were treated with specific drugs or left untreated for the indicated period of time. After stimulation, cells were fixed and stained first with anti-ARF Ab for detection of ARF and then with anti-COMMD1 Ab for detection of COMMD1. Fluorescence signals were visualized using a Carl Zeiss LSM 510 confocal microscope.

RESULTS

ARF Interacts with COMMD1 in Vivo—We performed a yeast two-hybrid screening to identify novel ARF-interacting proteins. Full-length p14ARF fused with the Gal4 DNA-binding domain was used to screen a human skeletal muscle library fused with the DNA activation domain. This screen led to identification of a protein named COMMD1 as an ARF-binding partner candidate. The interaction between ARF and COMMD1 in the mammalian cells was verified by co-immunoprecipitation (co-IP) assay. As shown in Fig. 1*A* (*panel a*), HA-ARF could be detected in the FLAG-COMMD1 immunoprecipitate but not in FLAG mock. In a reciprocal co-IP experiment when HA-ARF was precipitated from cell lysates (Fig. 1*A*, *panel b*), FLAG-COMMD1 could be pulled down together only with HA-ARF but not with HA, demonstrating that ARF interacts with COMMD1 specifically in mammalian cells.

To delineate the region of ARF involved in the interaction with COMMD1, full-length ARF and various deletion mutants were constructed (Fig. 1*B*). The N-terminal domain (amino acids 1–64) of ARF encoded by exon 1β was found to be sufficient for the interaction with COMMD1 (Fig. 1*C*, *lane 3*), whereas the exon 2-encoded C-terminal domain (amino acids 65–132) did not show any binding to COMMD1 (*lane 4*). To better define the interacting domain, ARF mutants with more discrete deletions in the N terminus were generated. Deletion of the first 14 residues of ARF (ARFΔN14) had no discernible effect on the interaction with COMMD1, whereas deletion of residues 1–29 (ARFΔN29) still resulted in binding to COMMD1. Deletion of residues

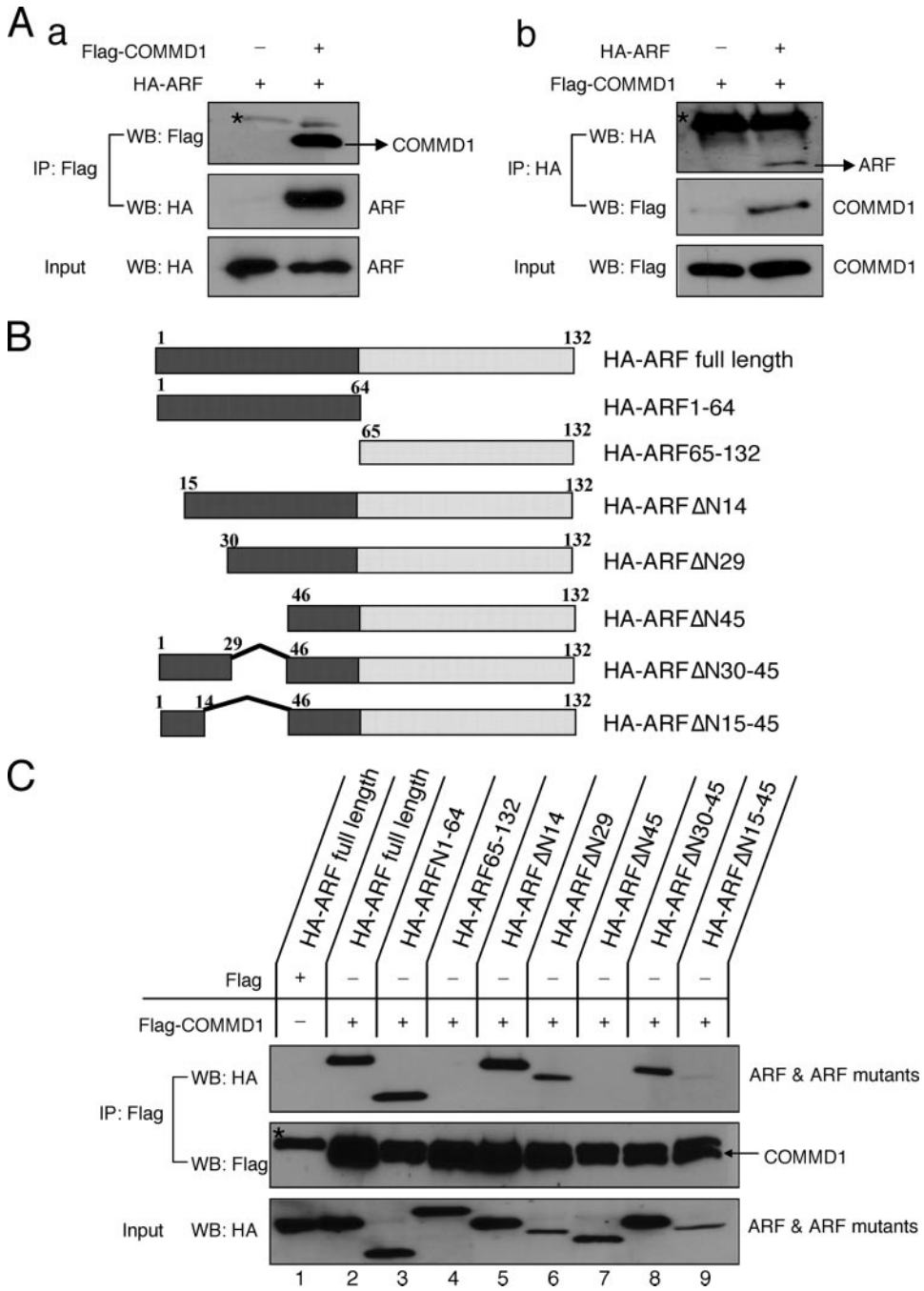

FIGURE 1. **ARF interacts with COMMD1 *in vivo*.** *A*, reciprocal co-IP assay. H1299 cells were cotransfected with FLAG-COMMD1 and HA-ARF. 24 h after transfection, cells were lysed in Pierce M-PER lysis buffer. Immunoprecipitation was performed using mouse anti-FLAG M2 or rabbit anti-HA antibody as indicated, and then cells were immunoblotted with rabbit anti-FLAG or anti-HA antibody. To test the input of different proteins, 2% cell lysates were loaded and probed with antibodies for specific proteins. *WB*, Western blotting. *B*, schematic representation of ARF and ARF truncated mutants used in the deletion mapping experiment. *C*, deletion mapping. H1299 cells were cotransfected with FLAG-COMMD1 and HA-ARF (full-length or various truncated mutants as indicated). Cell lysates were immunoprecipitated with anti-FLAG M2 affinity gel. The pulled down materials were separated by SDS-PAGE and immunoblotted with anti-HA or anti-FLAG antibody. 2% cell lysates were loaded and probed with specific antibodies for detection of the inputs of different proteins. *, IgG light chain.

1–45 of ARF (ARFΔ45) totally abolished the interaction with COMMD1, suggesting that the binding site may lie between amino acids 30 and 45. However, the ARFΔN30–45 mutant still retained some capacity to bind COMMD1, whereas deletion of residues 15–45 completely abrogated the association. These results suggest that amino acids 15–45 contribute to the interaction of ARF with COMMD1 (Fig. 1*C*).

COMMD1 Colocalizes with ARF in the Nucleoplasm—ARF normally localizes in the nucleolus in cancer cell lines and colocalizes with its associated proteins such as MDM2, E2F1, and HIF-1α in the nucleolus (5, 13, 22, 23). Therefore, we examined the localization of COMMD1 upon simultaneous expression with ARF. pCMV5-HA-ARF and pcDNA3-FLAG-COMMD1 or the corresponding empty vector were transfected into H1299 cells, followed by immunostaining using anti-FLAG antibody for FLAG-COMMD1 and anti-HA antibody for HA-ARF. As shown in Fig. 2*A*, HA-ARF localized in the nucleolus when it was coexpressed together with the empty vector. In contrast, FLAG-COMMD1 exhibited a mainly cytoplasmic localization pattern with accumulation of the protein in some discrete perinuclear regions. FLAG-COMMD1 could also be detected in the nucleus, but it was excluded from the nucleolus. However, when FLAG-COMMD1 was coexpressed along with HA-ARF, FLAG-COMMD1 was found in the nucleus and colocalized with HA-ARF in the nucleoplasm. Previous studies have shown that although ARF is capable of sequestering its binding partners into the nucleolus, the nucleolar localization signal of the binding partners may also be required for the nucleolus colocalization (23, 24). We found that FLAG-COMMD1 did not localize to the nucleolus when it was coexpressed with HA-ARF; instead, they both localized in the nucleoplasm. It could be due to the lack of the nucleolar localization signal in COMMD1. Nevertheless, this result indicates that formation of the ARF-COMMD1 complex alone is not sufficient for ARF to sequester protein to the nucleolus.

Previously it was reported that the DNA damage reagent actinomycin D can induce nucleolus disruption and rapid redistribution of endogenous ARF into the nucleoplasm (25). It is possible that ARF would colocalize and interact with

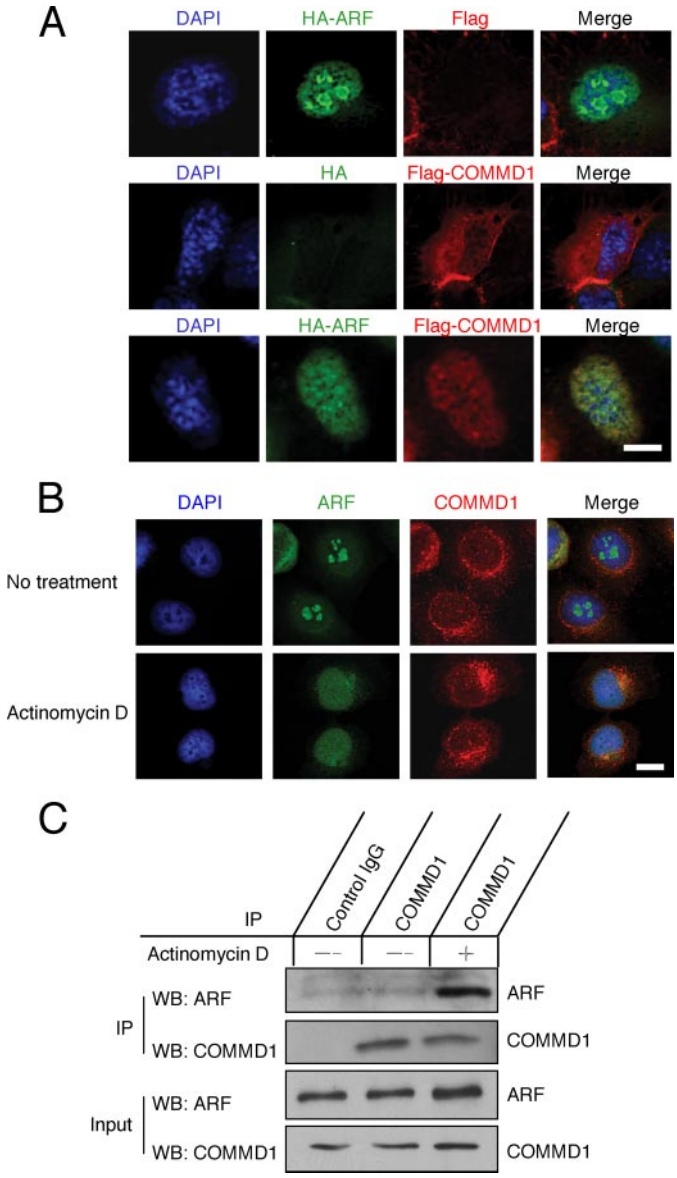

FIGURE 2. **ARF colocalizes with COMMD1 in the nucleoplasm.** *A*, H1299 cells plated on the slides were transfected with the HA-ARF and FLAG vector, FLAG-COMMD1 and HA empty vector, or HA-ARF and FLAG-COMMD1. 24 h after transfection, cells were fixed with 4% paraformaldehyde in PBS. Fixed cells were first stained with anti-FLAG M2 antibody, followed by rhodamine-conjugated secondary antibody, and then stained with anti-HA antibody, followed by fluorescein isothiocyanate-conjugated secondary antibody. Nuclei were visualized with 4′,6-diamidino-2-phenylindole (*DAPI*). *Scale bar* = 10 μm. *B*, H1299 cells were treated with 0.01 μg/ml actinomycin D for 8 h and first stained with anti-ARF antibody and then with anti-COMMD1 antibody. *Scale bar* = 10 μm. *C*, H1299 cells were treated with actinomycin D or left untreated for 8 h. Cells were then harvested, and COMMD1 was immunoprecipitated with mouse anti-COMMD1-conjugated protein G-agarose for 6 h. Endogenous ARF coprecipitated with COMMD1 was detected by rabbit anti-ARF antibody. Immunoprecipitated COMMD1 was analyzed by Western blotting (*WB*) with mouse anti-COMMD1 antibody, followed by horseradish peroxidase-conjugated protein G to avoid detection of IgG light and heavy chains.

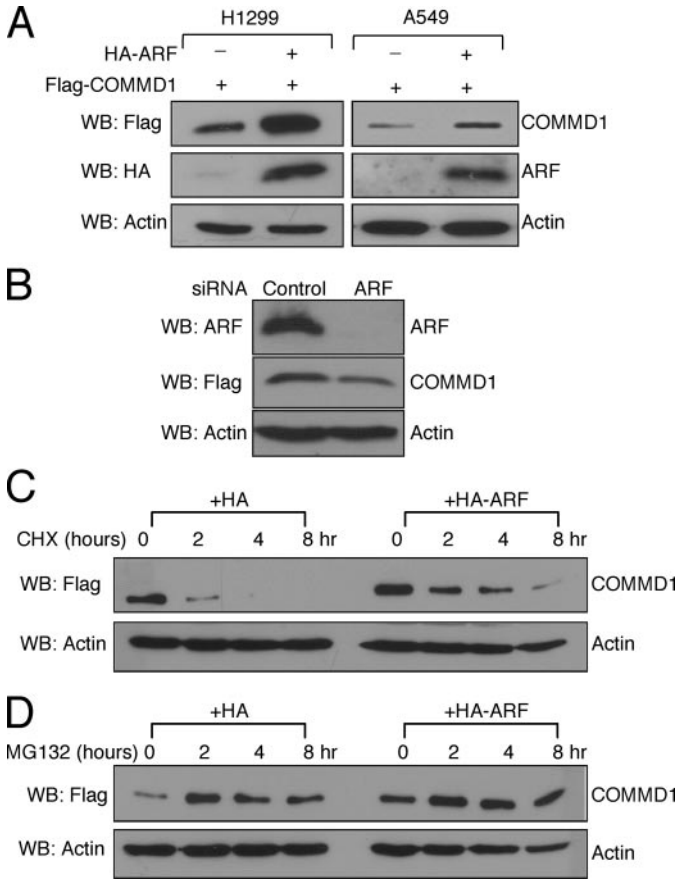

FIGURE 3. **ARF stabilizes COMMD1 by regulating its turnover.** *A*, H1299 or A549 cells were cotransfected with 1 μg of FLAG-COMMD1 along with 1 μg of the HA vector or HA-ARF. 24 h after transfection, cells were lysed and subjected to Western blotting (*WB*) using anti-FLAG M2 antibody. Endogenous actin levels were used as loading controls. *B*, H1299 cells were transfected with ARF or control small interfering RNA (*siRNA*) along with FLAG-COMMD1. COMMD1 levels were analyzed by Western blotting with anti-FLAG antibody 48 h post-transfection. *C*, H1299 cells were cotransfected with 1 μg of FLAG-COMMD1 together with 1 μg of the HA empty vector or HA-ARF. 24 h post-transfection, cells were treated with 20 μg/ml cycloheximide (*CHX*) for the time indicated, and protein levels were detected using anti-FLAG antibody. *D*, H1299 cells were cotransfected with 1 μg of FLAG-COMMD1 together with 1 μg of the HA empty vector or HA-ARF. 1 day after transfection, cells were treated with 2 μM MG132 for the period of time indicated. COMMD1 levels were analyzed by Western blotting with anti-FLAG antibody.

COMMD1 in the nucleoplasm after DNA damage stimulation. To test this idea, immunostaining and co-IP assay were performed after treatment of H1299 cells with actinomycin D for 8 h. As shown in Fig. 2*B* (*lower panelS*), actinomycin D induced a complete redistribution of ARF in the nucleus and subsequently led to more ARF colocalizing with COMMD1 in the nucleoplasm. H1299 cells are deficient of *p53*, so the redistribution of ARF in response to actinomycin D treatment is independent of p53. Consistent with this observation, the complex of endogenous ARF-COMMD1 could be detected only in H1299 cells with actinomycin D stimulation but not in untreated cells (Fig. 2*C*). Thus, the interaction of ARF and COMMD1 under physiological conditions appears to be dependent, at least partially, on their colocalization in the nucleoplasm.

ARF Stabilizes COMMD1 by Regulating Its Turnover—We noticed that the level of FLAG-COMMD1 appeared to be higher in cells transfected with HA-ARF than in cells transfected with the empty vector (Fig. 3*A*). The accumulation of FLAG-COMMD1 was observed in both A549 ($p53^{+/+}$) and H1299 ($p53^{-/-}$) cells, indicating that it is p53- and MDM2-independent. Conversely, in Fig. 3*B*, knockdown of endogenous ARF by RNAi led to a decrease in COMMD1 protein levels. We

next examined whether elevation of FLAG-COMMD1 by ARF occurs at the transcriptional or post-translational level. Cycloheximide was used to block protein synthesis in cells. H1299 cells transfected with HA-ARF or the empty vector were incubated with cycloheximide and harvested at the time indicated (Fig. 3*C*). The cells were lysed, and the cells lysates were then analyzed by Western blotting. As shown in Fig. 3*B*, in the presence of cycloheximide, FLAG-COMMD1 appeared to be quite unstable, with a half-life of ~1 h as described previously (19). However, in cells ectopically expressing HA-ARF, FLAG-COMMD1 decayed at much slower rate, with a half-life of 4 h. This result suggests that COMMD1 is stabilized in the presence of ARF, which occurs at the post-translational state.

COMMD1 is a proteasome substrate, and ubiquitination is essential for its specific degradation (19). Therefore, we reasoned that ARF might affect COMMD1 expression by regulating the ubiquitination process. We tested whether up-regulation of COMMD1 by ARF is sensitive to the treatment with MG132, a potent proteasome inhibitor. FLAG-COMMD1 levels increased dramatically after a 2-h proteasome inhibition (Fig. 3*D*, *second lane*), indicating that degradation of COMMD1 is mediated by the 26 S proteasome complex. However, no obvious accumulation of FLAG-COMMD1 was detected after MG132 treatment in the presence of ectopically expressed HA-ARF, suggesting that ARF may stabilize COMMD1 by either down-regulating the level of polyubiquitinated COMMD1 that is targeted for proteasomal degradation or acting similarly as a proteasome inhibitor to enhance and sustain COMMD1.

ARF Promotes Proteasome-independent Polyubiquitination of COMMD1—Next we examined the effect of ARF expression on the level of ubiquitinated COMMD1 by performing *in vivo* ubiquitination assay. H1299 cells were transfected with Myc-ub along with FLAG-COMMD1 and HA-ARF or the corresponding empty vector. Transfected cells were treated with 2 μM MG132 or buffer as a control for another 8 h. FLAG-COMMD1 was then immunoprecipitated from cell extracts, followed by Western blot analysis. Consistent with previous findings (19), COMMD1 was ubiquitinated in the presence of MG132 (Fig. 4*A*, *upper right panel*). Surprisingly, we found that in the sample coexpressing FLAG-COMMD1, Myc-ub, and HA-ARF (Fig. 4*A*, *upper left panel*) without MG132 treatment, the level of ub-conjugated COMMD1 was increased significantly, demonstrating that ARF promotes polyubiquitination of COMMD1. When MG132 was added to allow accumulation of polyubiquitinated COMMD1 that ultimately was degraded by the proteasome, a high molecular mass smear that is characteristic of polyubiquitination was detected in cells transfected with both the HA vector and HA-ARF, but transfection with HA-ARF led to greater recovery of ubiquitinated COMMD1 (Fig. 4*A*, *upper right panel*). The ubiquitination of COMMD1 in the absence or presence of MG132 was also confirmed using anti-Myc antibody to probe the precipitated COMMD1-ub conjugates (Fig. 4*A*, *middle panels*). We noted that the amount of COMMD1-ub conjugates induced by ARF was not elevated after proteasome blockade. In many cases, the ubiquitinated form of proteasome substrate can be detected only in the presence of proteasome inhibitors. Therefore, the possible explanation for this result is that ARF-facilitated ubiquitination of COMMD1 is proteasome-independent, and it does not target the protein for degradation.

To further confirm that ARF-induced COMMD1 ubiquitination is proteasome-independent, the K48R point mutant of ubiquitin, which cannot form Lys^{48}-conjugated polyubiquitin chains, was used for the *in vivo* ubiquitination assay. As shown in Fig. 4*B* (*left panels*), the ubK48R mutant did not inhibit ARF-induced COMMD1 ubiquitination. Interestingly, we found that overexpression of HA-ARF still enhanced the ubiquitination of COMMD1 in the presence of the Myc-ubK48R mutant, demonstrating that ARF induces proteasome-independent non-Lys^{48} ubiquitin chain conjugation to COMMD1. In addition to Lys^{48}-mediated ubiquitination, ubiquitination could also occur at Lys^{63} of ubiquitin. It has been reported that Lys^{63}-linked polyubiquitination does not involve proteolysis. Therefore we sought to investigate whether the apparent stability of ubiquitinated COMMD1 induced by ARF is due to the assembly of the polyubiquitin chain involving Lys^{63} but not Lys^{48} of ubiquitin. As shown in Fig. 4*B* (*right panels*), overexpression of HA-ARF did not enhance polyubiquitination of FLAG-COMMD1 as compared with control transfection in the presence of Myc-ubK63R. This result suggests that Lys^{63} of ubiquitin is important for ARF-induced COMMD1 polyubiquitination. We found that ARF promotes non-Lys^{48} ubiquitination of COMMD1, which is mediated through the Lys^{63}–Gly^{76} linkage.

Previously it was reported that the RNAi of XIAP, an E3 ligase that ubiquitinates and degrades COMMD1 through Lys^{48} linkage, could enhance non-Lys^{48} ubiquitination of COMMD1 (19). This result suggests that XIAP-mediated Lys^{48} ubiquitin chains may be assembled at the same lysine residues as those when ubiquitination occurs via non-Lys^{48} linkage. Hence, reduction in Lys^{48} ubiquitination could result in greater ubiquitination through non-Lys^{48} linkage. In our study, we have shown that ARF-facilitated Lys^{63} ubiquitination stabilizes COMMD1; COMMD1 is destabilized in cells deficient in ARF. It is possible that the Lys^{63} ubiquitination induced by ARF may compete with Lys^{48} ubiquitination and thus protect COMMD1 from proteasomal degradation. Therefore, we sought to determine COMMD1 ubiquitination pattern in cells depleted of ARF. As shown in Fig. 4*C* (*left panels*), knockdown of endogenous ARF by RNAi led to an obvious reduction in the total amount of COMMD1 ubiquitination, demonstrating that ARF-mediated ubiquitination constitutes a large part of COMMD1 ubiquitination. A decrease of ubiquitinated COMMD1 was also observed in cells cotransfected with ubK48R but not in cells cotransfected with the K63R mutant (Fig. 4*C*, *right panels*), indicating that Lys^{63}-linked ubiquitination is mediated at least partially by endogenous ARF. We noticed that in contrast to the effect of XIAP RNAi, knockdown of ARF did not result in greater Lys^{48} ubiquitination of COMMD1. Consistent with this, the destabilization of COMMD1 caused by ARF RNAi was also not sensitive to the proteasome blockade (Fig. 4*D*). Together, these results suggest that ARF-induced Lys^{63} ubiquitin chains are assembled at some specific lysine residues that are not responsible for Lys^{48} ubiquitination; hence, a decrease in Lys^{63} ubiquitination would not enhance Lys^{48}-mediated ubiquitination and degradation. The possible explanation for

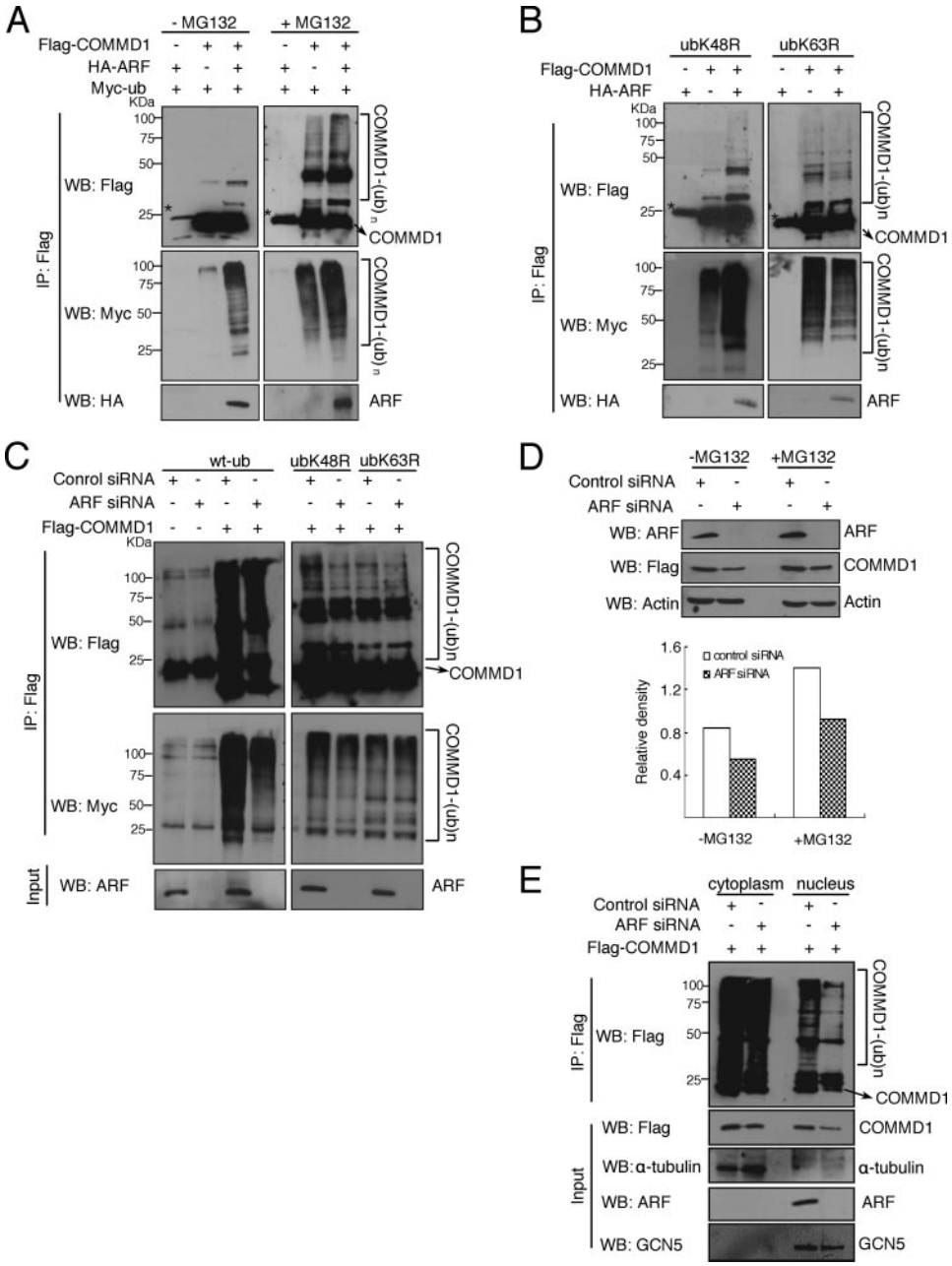

FIGURE 4. **ARF promotes Lys^{63}-mediated, proteasome-independent polyubiquitination of COMMD1.** *A*, H1299 cells were cotransfected with Myc-ub, FLAG-COMMD1, and HA-ARF or the empty vector as indicated. 24 h later, cells were treated with 2 μM MG132 or left untreated for another 8 h. Cell lysates were prepared and immunoprecipitated with mouse anti-FLAG M2 affinity gel. The immunoprecipitated materials were then analyzed by Western blotting (*WB*) with rabbit anti-Myc, anti-FLAG, or anti-HA antibody. *B*, H1299 cells were cotransfected with either the K48R or K63R mutant of ubiquitin together with FLAG-COMMD1, HA-ARF, or the empty vector control. Cells were lysed 24 h post-transfection and used for immunoprecipitation assay with anti-FLAG affinity gel and then immunoblotted with anti-FLAG or anti-HA antibody. *C*, H1299 cells were transfected with ARF, control small interfering RNA (*siRNA*), or FLAG-COMMD1 along with wild-type ub (*wt-ub*), ubK48R, or ubK63R for 48 h. Cells cotransfected with wild-type ub were treated with MG132 for another 8 h. Cells were then lysed and immunoprecipitated with mouse anti-FLAG M2 affinity gel. The immunoprecipitates were immunoblotted with rabbit anti-FLAG or anti-Myc antibody. *, IgG light chain. *D*, H1299 cells were transfected with ARF or control small interfering RNA together with FLAG-COMMD1 for 2 days. Cells were then split into two parts. Each part was treated with MG132 or left untreated for 8 h. COMMD1 protein levels were detected by Western blotting with anti-FLAG antibody. Endogenous actin levels were used as loading controls. The relative densities of COMMD1/actin were analyzed by Photoshop software. *E*, H1299 cells were transfected with ARF or control small interfering RNA and FLAG-COMMD1 for 2 days and treated with MG132 for an additional 8 h. The cytoplasmic and nuclear fractions were separated using a Pierce cytoplasmic and nuclear extraction kit, followed by immunoprecipitation with anti-FLAG M2 antibody. Immunoprecipitated COMMD1 in each fraction was analyzed by anti-FLAG antibody. α-Tubulin and GCN5 protein levels were used as loading controls for the cytoplasmic and nuclear fractions, respectively.

the destabilization of COMMD1 in ARF-deficient cells is that ARF-induced Lys^{63} ubiquitination could protect COMMD1 from degradation in some proteasome-independent manners; thus, COMMD1 would be degraded as a result of the loss of protection from Lys^{63} ubiquitin chains.

Meanwhile, the above data also imply that the interaction of ARF and COMMD1 may already exist in the normal state, and thus, endogenous ARF could induce polyubiquitination of COMMD1 without any stimulation. Recently, it was found that the non-nucleolar form of the ARF mutant is inherently unstable compared with nucleolar ARF (26). Like other proteins, ARF also undergoes proteasomal proteolysis (9). We reasoned that proteasome inhibition would result in recovery of the ARF-COMMD1 complex in the nucleoplasm in the normal state. As shown in supplemental Fig. 1*A*, treatment with MG132 led to great recovery of ARF in the nucleoplasm, which was not observed in non-stimulated cells. This indicates that nucleoplasmic ARF is less stable than nucleolar ARF. Similarly, in the co-IP experiment (supplemental Fig. 1*B*), no endogenous ARF could be coprecipitated with FLAG-COMMD1 in the absence of proteasome inhibition. However, after MG132 treatment, ARF could be readily detected in the FLAG-COMMD1 precipitates. Taken together, these data demonstrate that ARF specifically interacts with COMMD1 *in vivo* but is subjected to rapid degradation; endogenous ARF could induce Lys^{63} polyubiquitination of COMMD1 in the normal state.

Finally, as ARF associates with COMMD1 in the nucleus, we determined whether ARF specifically induces ubiquitination of COMMD1 in the nucleus. As shown in Fig. 4*E*, compared with the cytoplasmic fraction, the nuclear COMMD1 protein level was dramatically down-regulated in ARF-depleted cells. Consistent with this, the decreased COMMD1 polyubiquiti-

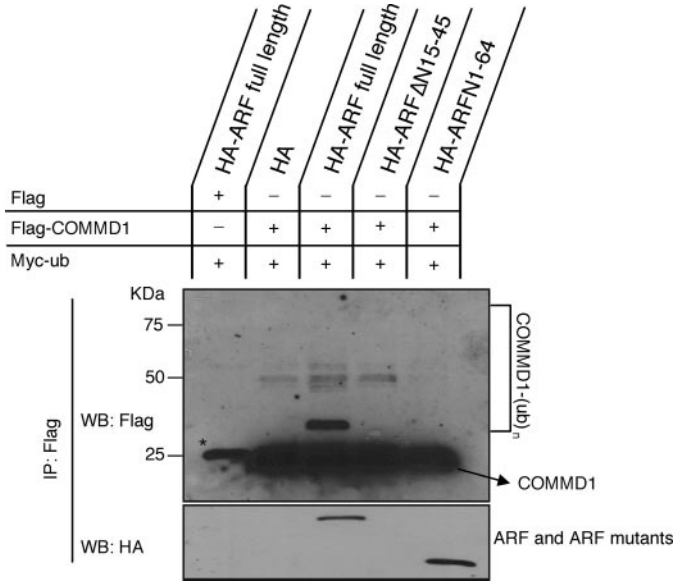

FIGURE 5. **ARF mutants are defective in promoting COMMD1 ubiquitination.** H1299 cells were cotransfected with Myc-ub and FLAG-COMMD1 with vectors encoding full-length ARF or ARF deletion mutants. 24 h post-transfection, FLAG-COMMD1 was precipitated from cell lysates and analyzed by Western blotting (*WB*). *, IgG light chain.

nation in the nuclear fraction was also observed in cells depleted of ARF. Hence, these results suggest that ARF specifically induces COMMD1 polyubiquitination in the nucleus.

ARF Mutants Are Defective in Promoting COMMD1 Polyubiquitination—To determine whether the physical interaction of ARF with COMMD1 is a prerequisite condition for COMMD1 ubiquitination, we tested the effect of two ARF deletion mutants: ARFΔN15–45, which is defective in binding COMMD1, and ARF-(1–64), which binds to COMMD1 efficiently but lacks the C-terminal domain encoded by exon 2. As shown in Fig. 5, full-length ARF promoted COMMD1 ubiquitination as observed previously, whereas expression of ARFΔN15–45 did not result in any ubiquitination of COMMD1, indicating that ARF-facilitated ubiquitin conjugation of COMMD1 depends on their physical association. Previous study has established that the functional domain of ARF is encoded by exon 1β (amino acids 1–64), and ARF interacts with most of its binding partners through the N-terminal region (6, 27, 28). In contrast, the C terminus contributes little to the function of ARF except nucleolus localization (24). However, our observation showed that although the ARF-(1–64) mutant strongly associated with COMMD1, it could not promote ubiquitination of COMMD1. We believe that deletion of the C terminus impairs the association of ARF with some other unknown factor that also participates in the ubiquitination process.

DISCUSSION

It was reported that overexpression of ARF arrests proliferation of *p53*-null or *p53*/*Mdm2*-null cells (29). Mice deficient in *Arf*, *p53*, and *Mdm2* develop tumors at a greater frequency compared with mice lacking both *p53* and *Mdm2* or *p53* alone (8). These observations suggest that ARF also possesses tumor suppressor functions that are independent of p53. In this study, we have characterized a novel p53-independent interaction between the tumor suppressor ARF and COMMD1. The physical association of the two proteins has been confirmed by co-IP assay and found to depend on their colocalization in the nucleoplasm. ARF normally localizes in the nucleolus, and it was proposed that physical sequestration of MDM2 by ARF can stabilize p53 by separating it from MDM2-mediated degradation (5). However, recent data demonstrated that nucleolar relocalization of MDM2 is not required for p53 activation and that redistribution of ARF into the nucleoplasm induced by DNA damage enhances its association with MDM2- and p53-dependent growth-suppressive activity (5, 6). Here, our results showed that ectopically expressed ARF relocalizes COMMD1 into the nucleoplasm without nucleolar sequestration. In non-stressed cells, endogenous ARF and COMMD1 have differential subcellular localization. However, the association of these two proteins could be observed when ARF was redistributed into the nucleoplasm in response to actinomycin D treatment. These observations suggest that under certain circumstances such as DNA damage stimulation, ARF would be released from the nucleolus into the nucleoplasm, and therefore, it can perform its function by interacting with its binding partners such as MDM2 and COMMD1.

Overexpression of ARF was reported to induce degradation of certain E2F family members through a ubiquitin-proteasome pathway. It was suggested that ARF may act conversely to trim down the level of E2F and thus protect cells from tumorigenesis upon oncogene activation (13). Previous studies have shown that ARF promotes proteasomal degradation of nucleolar protein B23 and therefore inhibits B23-mediated ribosome biogenesis (10). However, our results showed that instead of facilitating ubiquitination, which leads to degradation of its binding partners, ARF promotes Lys^{63}-linked polyubiquitination of COMMD1. Lys^{63} ubiquitination is known as non-classic ubiquitination, as it does not target proteins for degradation. Instead, it has distinct roles in regulating cellular functions such as protein kinase activation, DNA repair, and vesicle trafficking (17, 30, 31). We found that ARF stabilizes COMMD1 through its ability to promote Lys^{63}-mediated ubiquitination of COMMD1. Because non-proteasomal proteolysis is also utilized for COMMD1 degradation, it is possible that Lys^{63} ubiquitin chains may be capable of regulating and stabilizing the structure of the protein or protecting it from being recognized by some unknown protease. Lys^{63}-linked ubiquitination could also regulate protein-protein interactions (31, 32), which may have additional functions, such as affecting the binding affinity of COMMD1 for other factors. COMMD1 has been demonstrated as a ubiquitously expressed inhibitor for NF-κB (20, 33). It was reported that COMMD1 interacts with and accelerates degradation of RelA in the nucleus, which terminates the association of RelA with chromatins (33). We found that ARF specifically promotes nuclear COMMD1 ubiquitination through Lys^{63} linkage. Therefore, it is possible that ARF-induced Lys^{63} polyubiquitination may regulate the inhibitory effect of COMMD1 on NF-κB-mediated transcription in the nucleus.

Does ARF act as an E3 ligase for the ubiquitination process? Previous studies demonstrated that overexpression of ARF induces sumoylation of its binding partners such as MDM2,

E2F1, B23, and Werner helicase (11, 15, 16). ARF also binds to UBC9, the only E2 found to be responsible for sumoylation (16), which suggests that ARF may serve as an E3 ligase to bridge the E2 complex to its binding partners. In addition, overexpression of ARF promotes ubiquitination of its binding partners such as E2F and B23. Although ARF does not possess the typical E3 ligase structural feature or known catalytic activity for ubiquitination, it is possible that ARF mediates ubiquitination of these proteins indirectly by bringing different proteins such as E3 ligase together. We found that physical association of ARF and COMMD1 is necessary for the ubiquitination; therefore, we do not exclude the possibilities that the C terminus of ARF interacts with other factors, such as E3 ligase for COMMD1 ubiquitination, or that ARF itself serves as an E3 ligase for COMMD1 ubiquitination.

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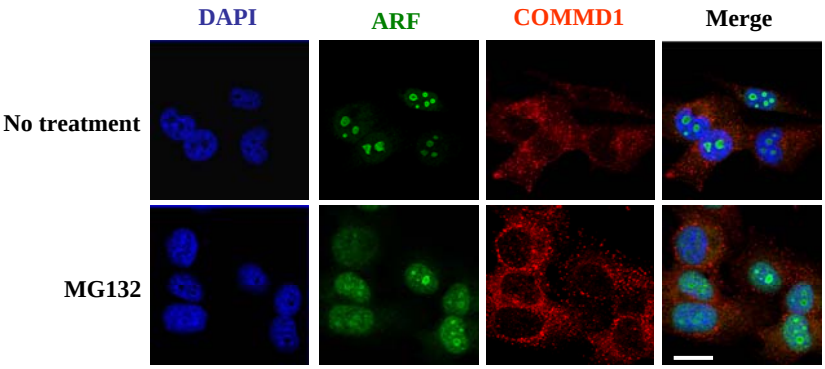
SUPPLEMENT FIGURE LEGENDS

Supplement Figure 1. (A) H1299 cells were treated with 2μM MG132 or left untreated for 8 hours. Cells were fixed, first stained with anti-ARF antibody for detection of endogenous ARF, and then stained with anti-COMMD1 for detection of COMMD1. Scale bar indicates 20 μm. (B) H1299 cells were transfected with Flag-COMMD1 for 24 hours and further treated with 2μM MG132 or left untreated for 8 hours. Cells were then lysed and immunoprecipitated with anti-Flag M2 antibody. Endogenous ARF in the precipitates were detected by anti-ARF antibody.

Supplement Figure 1

A



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