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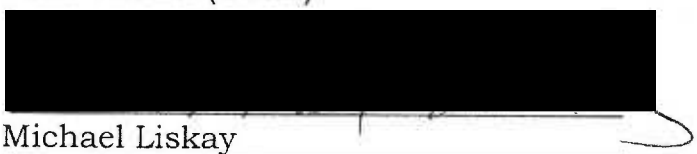

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Characterization of the SCF^{Skp2} Substrate, UBP43, an ISG15 Isopeptidase

by

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A DISSERTATION

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Abbreviations

3-AT	3-amino-1,2,4-triazole
IB	Immuno-blot
IFN	Interferon
IP	Immunoprecipitation
kb	Kilobase
Kd	Kilodalton
LPS	Lippopolysacharide
MIPL	Mammalian IP Lysis Buffer
PI	Proteasome Inhibitors
PhI	Phosphatase Inhibitors
TBS	Tris-Buffered Saline

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Abstract

The Skp2 oncoprotein belongs to the family of F-box proteins that function as substrate recognition factors for SCF (Skp1, cullin, F-box protein) E3 ubiquitin-ligase complexes. Skp2 promotes entry into S-phase by targeting various cell-cycle proteins for degradation. Using full length Skp2 as bait in a yeast two-hybrid screen, we have identified UBP43 as a novel substrate for Skp2. UBP43 was originally identified as an upregulated gene in a leukemia mouse model, as well as in melanoma cell lines treated with IFN-β. UBP43 belongs to the family of ubiquitin isopeptidases. It cleaves an IFN induced, ubiquitin-like molecule, ISG15. UBP43 knockout mice exhibit shortened life spans, hypersensitivity to IFN, and neuronal damage, suggesting that tight regulation of ISG15 conjugation is critical for normal cellular function. Here, we demonstrate that Skp2 binds to UBP43 and promotes its ubiquitination and degradation, resulting in higher levels of ISG15 conjugates. In contrast, in Skp2-null mouse cells, levels of UBP43 are consistently upregulated, while levels of ISG15 conjugates are reduced. In addition, Skp2 null MEFs demonstrate increased sensitivity to IFN. Our results demonstrate that SCF^{Skp2} is involved in controlling UBP43 protein levels and therefore plays an important role in modulating type 1 IFN signaling. Moreover, these results demonstrate a novel link between cell cycle control by Skp2, and the cellular response to stress.

Chapter I: Inroduction

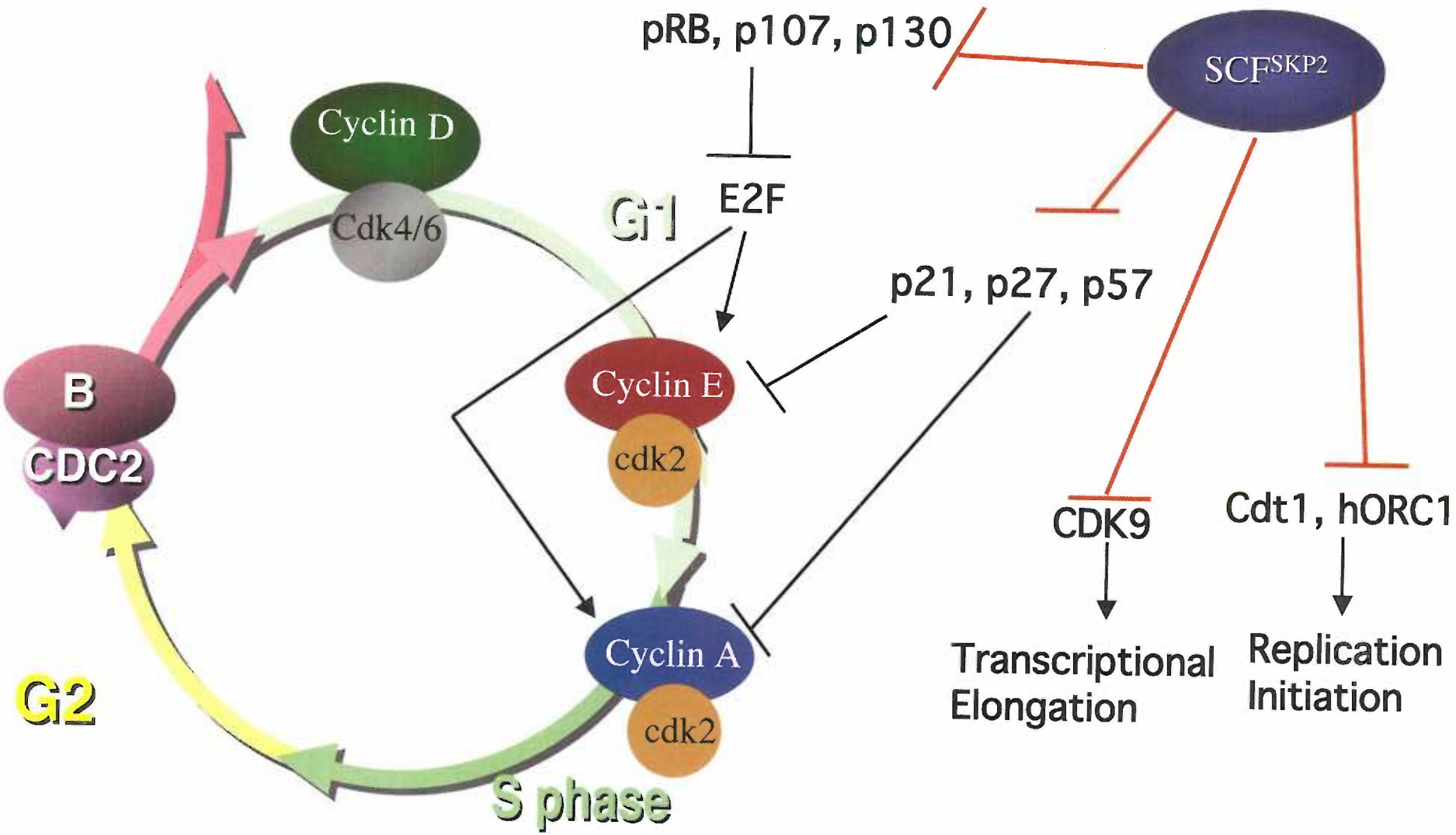
Introduction

Mutations in cell cycle regulatory and signaling proteins can allow cells to bypass normal cell cycle checkpoints, allowing unregulated cell growth and division that lead to tumorigenesis. One protein essential for normal regulation of the cell cycle is the F-box protein Skp2, which promotes cell-cycle progression via the ubiquitin-mediated degradation of many key players in the cell cycle (Figure 1) (Krek 1998). In proliferating cells, the cell cycle consists of four phases: Gap1 (G_1), S, Gap2 (G_2), and Mitosis (M). Gap 1 (G_1) is the interval between mitosis and DNA replication that is characterized by cell growth. The transition that occurs at the G_1/S checkpoint commits the cell to the proliferative cycle. If the conditions that signal this transition are not present, the cell exits the cell cycle and enters G_0, a nonproliferative phase during which growth, differentiation, and apoptosis occur. Replication of DNA occurs during the synthesis (S) phase; this phase is followed by a second gap phase (G_2) during which growth and preparation for cell division occurs. Mitosis and the production of two daughter cells occur in M phase. Skp2 is an important factor in pushing the cell cycle past the critical G_1/S cell-cycle checkpoint, as well as through S-phase.

Mutations that cause elevated levels of Skp2 result in aberrant cell proliferation and DNA replication. For example, over-expression of Skp2 in quiescent Rat1 cells induces DNA replication in low serum conditions (Sutterluty, Chatelain et al. 1999). Conversely, when Skp2 is inhibited with anti-sense oligonucleotides or antibodies, entry into S phase is dramatically reduced both in normal and in transformed human fibroblasts (Zhang, Kobayashi et al. 1995). In addition, mouse embryonic fibroblasts (MEFs) and T

Figure 1. A. Diagram of the mammalian cell cycle. B. Skp2 plays a major role in the progression of the cell cycle into S-phase. Skp2 promotes the induction of Cyclin E transcription, a major player in the G_1 to S transition, by stimulating E2F through the degradation of p130, which relieves the repression of E2F. Skp2 also relieves repression of CyclinE-cdk2 activity by down-regulating its repressors, CKIs p21, p57, and p27. In addition, Skp2 helps mediate transcription and replication initiation by regulating Cdt & hORC, which control the formation of pre-replication complexes.

Figure 1


pRB, p107, p130
SCF^SKP2
Cyclin D
Cdk4/6
G1
E2F
p21, p27, p57
B
CDC2
Cyclin E
cdk2
CDK9
Cdt1, hORC1
Transcriptional Elongation
Replication Initiation
Cyclin A
cdk2
G2
S phase

lymphocytes from Skp2-null mice demonstrate decreased proliferation compared to cells from wild type animals. The cell cycle profile for Skp2 -/- MEFs also shows a delayed entry into S-phase and prolonged S-phase compared to wild type MEFs (Nakayama, Nagahama et al. 2000). Such disruption in the proper timing of S-phase could account for the significant disruption in DNA content in the Skp2 -/- MEFs, which show increased polyploidy compared to wild type. This phenomenon was also observed in Skp2 null hepatocytes and lung and kidney epithelium MEFs (Nakayama, Nagahama et al. 2000). Normal Skp2 activity is therefore important for maintaining proper cell cycle timing, as well as genetic content in cells.

Unfortunately, these activities also make Skp2 a potent oncogene. Skp2 has been shown to contribute to tumorigenesis and malignant transformation both in animal models and in human cancers. Transgenic mice expressing high levels of Skp2 in T-cells develop T cell lymphoma when crossed with mice harboring an activated N-Ras, a potent oncogene (Latres, Chiarle et al. 2001). In addition, co-expression of Skp2 and another oncogene, activated H-Ras, in rat embryonic fibroblasts resulted in tumors in nude mice (Gstaiger, Jordan et al. 2001). Furthermore, prostate-targeted Skp2 causes dysplasia and low-grade carcinoma in mouse prostate (Shim, Zhang et al. 2003). Skp2 is also commonly elevated in human lymphomas, oral epithelial, and colorectal carcinomas and is associated with poor prognosis (Chiarle, Pagano et al. 2001; Kudo, Kitajima et al. 2001; Latres, Chiarle et al. 2001; Chiarle, Fan et al. 2002; Li, Wu et al. 2004).

This dissertation describes the identification and characterization of a novel Skp2 interacting protein, UBP43. I chose to search for novel Skp2-interacting proteins to help us better understand its role in oncogenesis. Through this pursuit, we identified a number

of novel interacting proteins. Among these, UBP43 was of interest because it was originally identified as a ubiquitin-specific protease, and this is important because Skp2 functions in ubiquitin modification of proteins. The discovery of this interaction deepens our knowledge of Skp2 activity and provides an expanding role for Skp2 in the cell cycle and tumorigenesis.

Cdk-Cyclin Complexes and Cdk Inhibitors (CKI) are Key Regulators of Cell Cycle Progression

Progression of the cell cycle is regulated by the sequential activation and de-activation of cyclins and cyclin-dependent kinases (cdks), as illustrated in Figure 1. Cdks phosphorylate downstream regulators of the cell cycle, and their activity is regulated by their interactions with Cyclins. There are three main groups of Cyclins: G_1, S-phase, and mitotic. Cyclin D1, a G_1 Cyclin, interacts with cdks 4 and 6. Mitogenic stimuli promote Cyclin D activation by inducing Cyclin D gene transcription and increasing Cyclin D stability at the protein level (Sherr and Roberts, 1999). The Cyclin D-cdk4/6 complex phosphorylates downstream regulators in the cell cycle, including the transcriptional repressors pRB, p130, and p107. This, in turn, releases repression of the transcription factor E2F and activates the expression of E2F-dependent genes, which include Cyclin E and A (Dahmann, Diffley et al. 1995).

Cyclins E and A, S-phase Cyclins, act in concert with cdk2 to promote progression of the cell cycle through the G_1 checkpoint and S phase (Gomez Lahoz, Liegeois et al. 1999). Cyclin E is believed to be a critical factor in the cell cycle progression through this restriction point. Cyclin E-cdk2 complexes also phosphorylate

pRB, increasing the de-repression of Cyclin E and Cyclin A transcription. Cyclin E-cdk2 also appears to activate downstream regulators involved in chromatin remodeling centrosome duplication, and origin of replication licensing, while Cyclin A-cdk2 major targets appear to be factors involved in DNA synthesis.

As cells progress into S-phase, Cyclin D-cdk4/6 activity and Cyclin E-cdk2 activity are shut off. In late S-phase, Cyclin A-cdk2 activity is attenuated, and the mitotic cyclin complex, Cyclin B-cdk1, takes over and aids in regulating factors necessary for completing mitosis. Finally, as mitosis is complete, Cyclin B-cdk1 activity is repressed. If mitogenic stimuli are still present, Cyclin D transcription will increase and begin the process again; otherwise, the cell enters G_0 and ceases proliferation.

One way a cell controls the temporal activity of Cyclin-cdk complexes is via cdk inhibitors (CKIs), which inhibit the activity of these complexes. CKIs comprise two families, the INK family and the WAP/CIP family. INK family CKIs (p15, p16, p18, and p19) inhibit Cyclin D-cdk4 and 6 complexes, while the WAP/CIP family members (p21, p27, and p57) inhibit Cyclin E-cdk2 and Cyclin A-cdk2 complexes. In early G_1, levels of the CKIs p27 and p21 are high and inhibit the activity of Cyclins E and A. As the cell progresses into mid to late G_1, levels of p21 and p27 are down-regulated via ubiquitin-mediated proteolysis; and Cyclin E-cdk2 and Cyclin A-cdk2 activity can increase, allowing the cell cycle to progress through S-phase.

Ubiquitin-Mediated Proteolysis Helps to Control Cell Function

In summary, then, the cell cycle is tightly controlled by sequential oscillations of cell cycle proteins through transcriptional regulation but also through modulation of

protein levels. One form of this modulation is ubiquitin-mediated proteolysis, which has been shown to be a critical regulatory process in the cell cycle (Hochstrasser, 2000). The classic function of protein ubiquitination is to target proteins for degradation via the 26S proteasome. Proteins are multi-ubiquitinated by a ubiquitin ligase complex and then targeted to the proteasome, where the protein is degraded into peptides.

The cell cycle transitions are rapidly and intricately timed and require the tight regulation of the level and activity of the cell cycle proteins. Ubiquitin-mediated degradation is a major mechanism with which the cell controls levels of Cyclins, cdks, and CKIs, and subsequently cell cycle progression. In late G_1, the CKIs p27, p21, and p57 are targeted for ubiquitin-mediated degradation, allowing the activity of S-phase cyclins A and E to increase (Kamura, Sato et al. 1998; Carrano and Pagano 2001; Bornstein, Bloom et al. 2003). In early S phase, Cyclin E is rapidly ubiquitinated and degraded, leading to progression through S phase (Clurman et al, 1996). Cyclins A and B are subsequently degraded, allowing entry in to G_2 and completion of mitosis.

Ubiquitination is also necessary for maintaining proper genetic stability. One example is the replication-licensing factor Cdt1. Cdt1 levels are high in early G_1, where replication is licensed, and Cdt1 is rapidly ubiquitinated and degraded at the G_1-to-S transition (Li, Zhao et al. 2003). The rapid down-regulation of Cdt1 helps to prevent re-replication and thus polyploidy. Likewise, human Orc1 is also a target for ubiquitin-mediated degradation (Mendez, Zou-Yang et al. 2002). hOrc1 is involved in the formation of pre-replication complexes as cells exit mitosis. Ubiquitin-mediated degradation of hOrc1 prevents a second round of pre-replication complexes from forming, thus helping to prevent polyploidy.

Ubiquitin-mediated proteolysis also plays a role in controlling DNA transcription. Proper cell division is dependent on the unidirectional progression through the cell cycle, and alterations in cell cycle progression can have significant effects on the cell, most notably causing genomic instability and uncontrolled proliferation. Ubiquitin-mediated degradation provides a mechanism by which the cell can rapidly and irreversibly regulate proteins levels in response to changes in cellular activity.

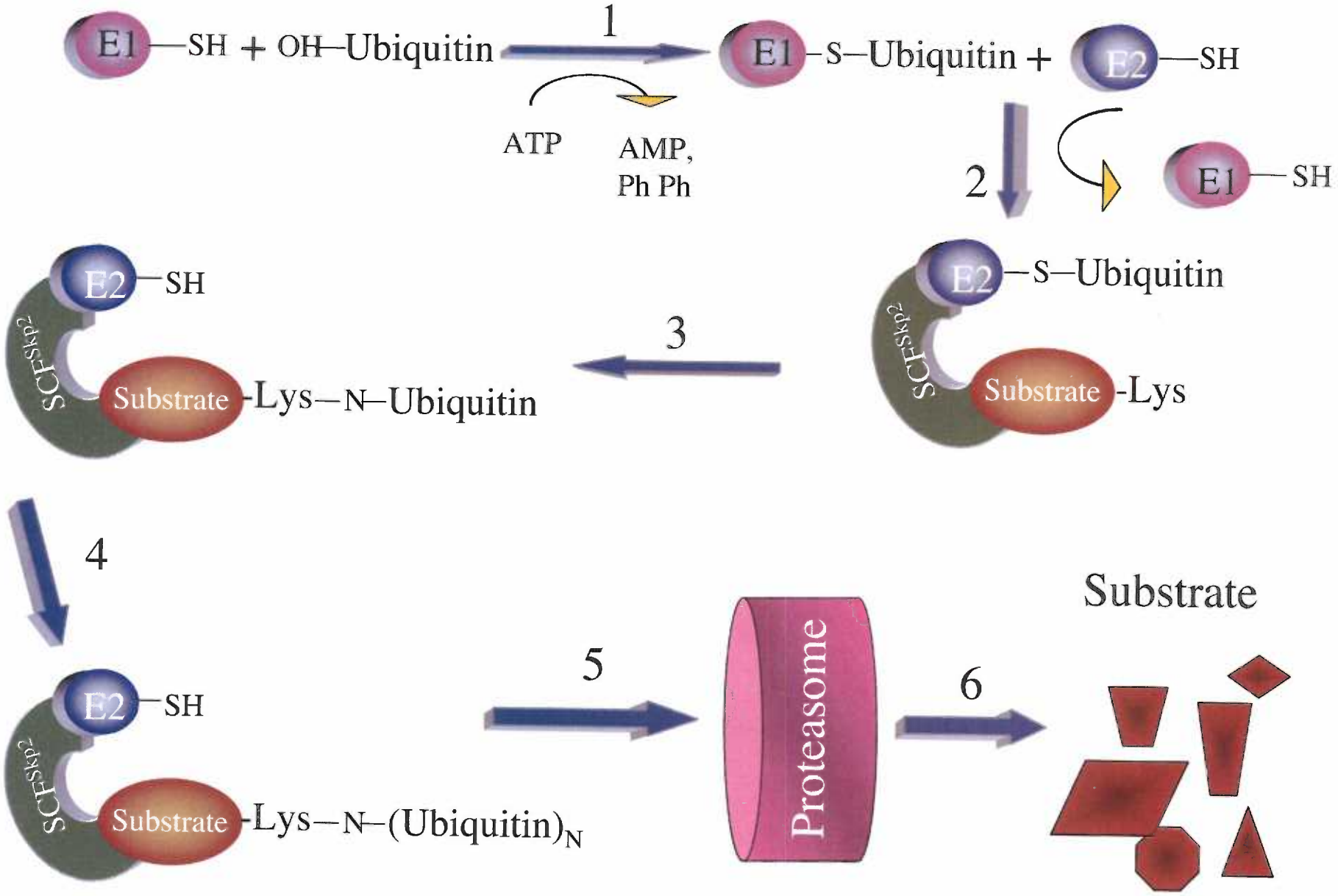
The Enzymatic Process of E3 Ligase-Mediated Ubiquitination

Ubiquitination is a process that involves three key enzymes, E1, E2, and E3. The ubiquitin-activating enzyme, E1, activates ubiquitin by using ATP to form a thioester bond between its active site cysteine and a ubiquitin molecule (Figure 2, step 1) (Scheffner, Nuber et al. 1995). This activated ubiquitin is then transferred to the cysteine of the ubiquitin conjugating enzyme, E2 (Figure 2, step 2). The ubiquitin-bound E2 binds the ubiquitin ligase, E3, and together the E2 and E3 enzymes promote the formation of isopeptide bonds between the C-terminal glycine of the ubiquitin molecule and the amino group of lysine residues on the substrate (Figure 2, step 3).

There are two families of E3 ligases, the HECT domain and the RING finger domain. HECT ubiquitin ligases pass the ubiquitin from the E2, to the E3, and then to the substrate. This is in contrast to RING-finger ubiquitin ligases that act as adaptor proteins between the E2 and substrate, with the ubiquitin moiety being passed directly from the E2 to the target (Jackson, Eldridge et al. 2000). Ubiquitin chains form to facilitate degradation, and ubiquitination chain elongation occurs when additional ubiquitin molecules are conjugated to internal lysine residues on the ubiquitin molecule

Figure 2. Diagram of the enzymatic process of ubiquitination. Ubiquitin molecules are activated by a thioester bond formation with the ubiquitin-activating enzyme, E1. Ubiquitin is then transferred to the conjugating enzyme E2, which forms a complex with the E3 ligase. The E3 ligase in turn promotes the ubiquitination of the bound substrate and ubiquitin chain elongation.

Figure 2


E1—SH + OH—Ubiquitin
1
E1—S—Ubiquitin +
E2—SH
ATP
AMP,
Ph Ph
2
E1—SH
E2—S—Ubiquitin
SCFSkp2
Substrate
-Lys
3
E2—SH
SCFSkp2
Substrate
-Lys—N—Ubiquitin
4
E2—SH
SCFSkp2
Substrate
-Lys—N—(Ubiquitin)N
5
Proteasome
6
Substrate

bound to the substrate. These multi-ubiquitinated proteins are then subject to degradation by the 26S proteasome (Figure 2, step 4-5) (Hershko, Ciechanover et al. 2000; Pickart 2001). It is unclear exactly how ubiquitin chain formation is regulated, but recent evidence suggests that an additional enzyme, E4, is responsible for promoting the formation of these chains (Querido, Blanchette et al. 2001; Hoppe, Cassata et al. 2004).

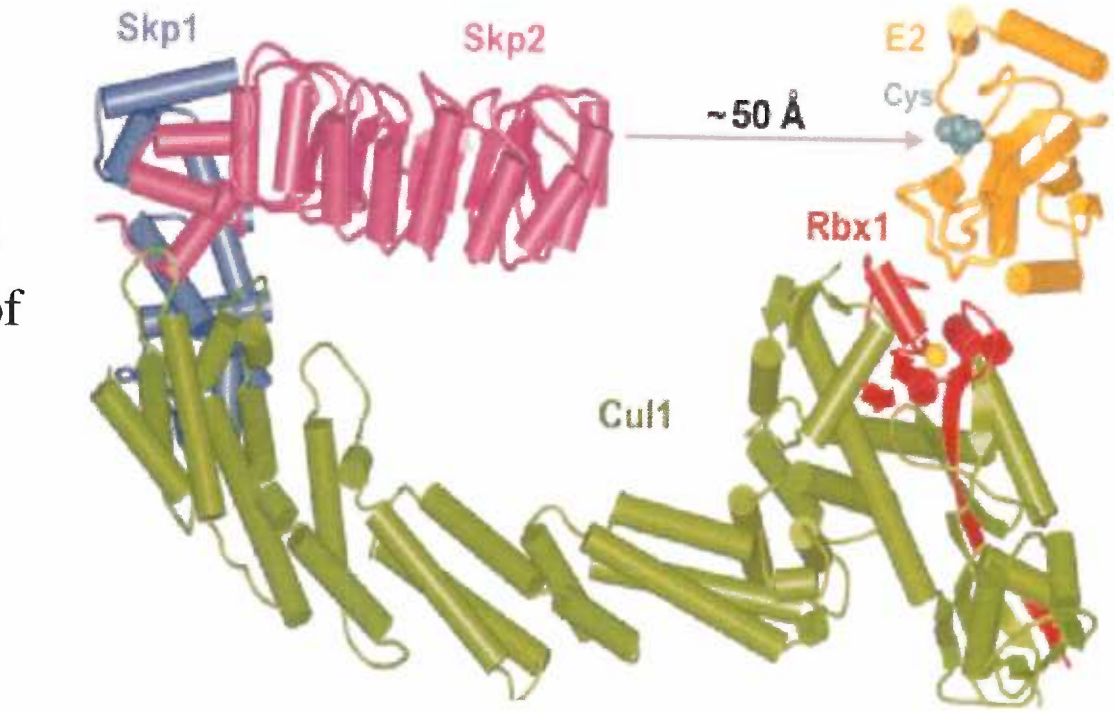
The SCF^{Skp2}, an E3 Ubiquitin Ligase

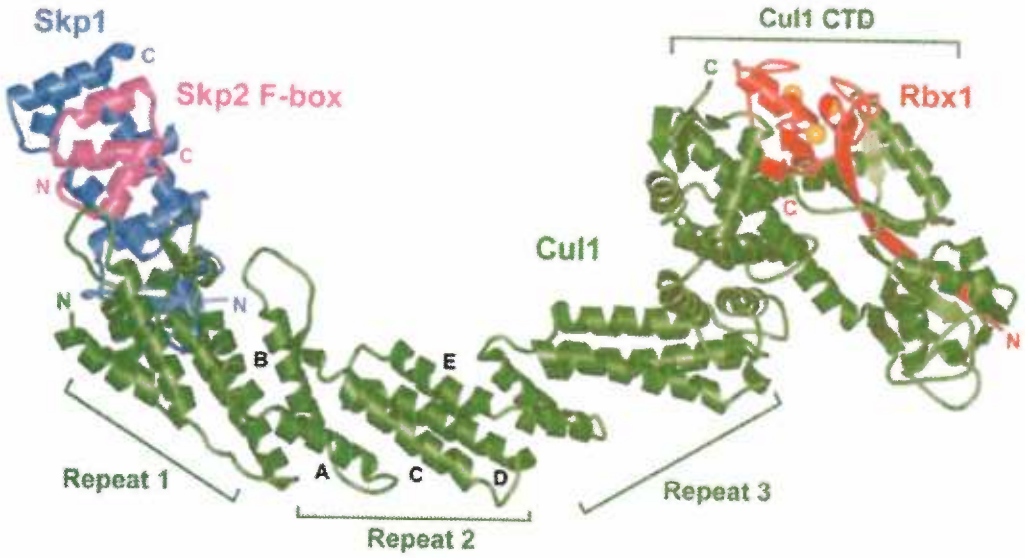
My thesis will focus on the RING finger E3 ubiquitin ligase, SCF^{Skp2}. The SCF complex consists of four subunits, Skp1, Cul1, Rbx/Roc1, and a variable F-box protein (Figure 3). The F-box proteins function as the substrate recognition factors for E3 ubiquitin ligases (Laney and Hochstrasser 1999). Figure 3 shows a visual interpretation of the crystal structure of SCF^{Skp2} complex. The F-box protein Skp2 interacts with Skp1 via its "F-box" domain (Kipreos and Pagano 2000). Skp1, in turn, interacts with Cullin, linking Skp2 to the rest of the E3 ligase components (Zheng, Schulman et al. 2002). Cullin consists of three repeats of a novel five-helix structural motif and two C-terminal domain repeats (CTD) (Figure 3). Cullin binds Skp1 through its N-terminal repeat and Rbx1 at the CTD. The domains of Cullin are arranged so that it forms a rigid bridge between the Skp1/F-box proteins and Rbx1. Finally, Rbx1 mediates the interaction between Cullin and the E2 conjugating protein. Together Skp1, Cul1, and Rbx1 create a horseshoe-shaped complex that effectively places the Skp2-bound substrate in proximity to the E2-bound ubiquitin. A non-rigid mutant of Cullin appeared to maintain binding to both the Rbx1 and the F-box-bound substrate; however, it was no longer able to ubiquitinate the substrate (Zheng and Ruderman 1993). The SCF complex most likely

Figure 3. The SCF complex consists of three main components. The SCF^{Skp2} structure reveals a multi-subunit complex composed of the F box element of Skp2, Skp1 (the adaptor between Skp2 and Cul1), Cul1 (the bridge), and Rbx1 (the RING finger subunit) as described by Zheng *et al.* 2002. Rbx1, Cul1, and Skp1 form a rigid structure that brings the ubiquitin molecule (attached to E2) in proximity with the substrate (bound to Skp2) facilitating ubiquitination.

Figure 3

- E3/ ubiquitin ligase
- Contains
  - Skp1 scaffolding protein
  - Cul1 (or other member of the cullin family)
  - Variable F-box protein
  - Rbx1/Roc1-ring finger protein





Pavletich lab
(Zheng *et al., Cell 102 (2000)*

serves no catalytic role in ubiquitination, but rather ensures proper assembly of the components.

The Role of Skp2 in Cell Cycle Progression

The F-box protein Skp2 is a critical factor in the regulation of the cell cycle. SCF^{Skp2} promotes progression through S-phase via ubiquitin-mediated degradation of many key players in the cell cycle (Figure 1) (Krek 1998). Skp2 links the cell cycle to gene expression through its-down regulation of the pRB pocket protein, p130 (Hofferer, Wirbelauer et al. 1999; Kiernan, Emiliani et al. 2001). Down-regulation of p130 alleviates repression of the transcription factor, E2F, which is responsible for transcription of S-phase-promoting genes, including Cyclin E and Cyclin A. Skp2 also regulates the cell cycle by targeting for degradation many regulatory proteins important in the G_1-S transition, including p27, p57, and p21 (Tedesco, Lukas et al. 2002; Bornstein, Bloom et al. 2003; Kamura, Hara et al. 2003). As described earlier, p27, p57, and p21 are Cyclin-cdk inhibitors that block the G_1 to S transition by binding and inactivating Cyclin-cdk complexes. SCF^{Skp2} down-regulates p21 and p27, preventing repression of G1 and S phase Cyclin-cdk complexes allowing the cell to enter S-phase.

Skp2 is also important in maintaining genetic content. SCF^{Skp2} is the F-box for both Cdt1 and hOrc1. As described previously, regulation of both of these factors is essential in preventing inappropriate DNA synthesis and excess rounds of replication that could result in polyploidy.

Skp2 itself is subject to ubiquitin-mediated degradation by another ubiquitin ligase, anaphase-promoting complex (APC/C^{CDH1}), in late M phase (Bashir, Dorrello et

al. 2004; Wei, Ayad et al. 2004). Skp2 can also undergo auto-ubiquitination in G_1/S (Wirbelauer, Sutterluty et al. 2000). It has been shown recently that this auto-ubiquitination may be induced by tumor suppressor proteins connexin43 and TGF-β (Zhang, Nakayama et al. 2003; Wang, Ungermannova et al. 2004). Proper temporal control of Skp2 is important in maintaining proper cellular function, and disruption of Skp2 regulation plays a role in oncogenesis

The role of Skp2 in oncogenesis often correlates to its degradation of the tumor suppressor cdk inhibitor p27, thereby releasing the cell cycle from p27-induced repression. High Skp2 levels in various carcinomas usually correlate with low p27 levels; however, this is not always the case. In addition, Skp2 has recently been identified as a possible transcriptional cofactor of the strong oncoprotein, c-Myc (von der Lehr, Johansson et al. 2003). Finding additional Skp2 targets may aid in further understanding its role in cellular function and consequently the development and progression of cancers.

The Skp2 Interacting Protein, UBP43, is an ISG15 Isopeptidase

This thesis investigates the interaction of Skp2 with a novel interacting protein UBP43. Using the yeast two-hybrid technique to isolate potential Skp2-interacting proteins, I identified five novel proteins, including UBP43, which will be described in this report.

UBP43 belongs to the family of ubiquitin isopeptidases, a class of ubiquitin and ubiquitin-like (Ubl) deconjugating enzymes (Schwer, Liu et al. 2000). Ubiquitin and ubiquitin-like isopeptidases have been linked to many cellular processes, including

development, intracellular transport, immunology, and cell signaling (Cope, Suh et al. 2002; Panse, Kuster et al. 2003).

UBP43 was originally identified as a strongly expressed mRNA in hematopoietic tissue from an acute myeloid leukemia (AML) mouse model (Liu, Ilaria et al. 1999). The mouse contained a knock-in of the leukemia-associated fusion protein, AML1-ETO, which represses the transcription of anti-proliferative, AML1 responsive genes. UBP43 mRNA levels were upregulated in MEFs from these knock-in mice compared to MEFs from wild type animals. Subsequently, UBP43 was identified as an interferon-induced gene in melanoma cells treated with IFN-ß. This treatment causes cells to irreversibly lose proliferative potential and begin progression toward terminal differentiation (Schwer, Liu et al. 2000).

UBP43 functions as an isopeptidase responsible for cleaving the ubiquitin-like protein, ISG15, from substrates (Malakhov, Malakhova et al. 2002). ISG15 is a 15 kD protein induced by type I interferons (IFN); lipopolysaccharide (LPS), which mimic bacterial infections; and viral infections (Loeb and Haas 1992). ISG15 is composed of two domains, each with about 30% homology to ubiquitin. It is synthesized as a 17kD protein that is then processed into the 15kD form, exposing the C-terminal di-glycine residues required for its conjugation to other proteins, similar to ubiquitin (Narasimhan, Potter et al. 1996; Potter, Narasimhan et al. 1999). An E1 and E2 enzyme have been identified for ISG15 conjugation. The E1, Ubiquitin activating enzyme 1-like protein (UBE1L), initiates conjugation by forming a thioester bond with ISG15, thus preparing it for transfer to the next series of enzymes (Krug, 2001). And recently, an E2 enzyme, Ubc8, has been shown to mediate ISG15 conjugation (Er-Zhang, 2004). No E3 ligases

have been found; however, Ubc8 is known to interact with the E3 ubiquitin ligases, E6AP and HHARI (P. M. Howley. 1997, C. H. Buys. 2000). Substrates of ISG15 conjugation have been shown to be poly-ISGylated; it is unknown whether this is the result of ISG15 chain formation or multiple ISGylated sites on a protein.

In this dissertation, I describe the discovery of UBP43 as a novel Skp2 interacting protein. I have characterized UBP43 as a substrate for SCF^{skp2}- mediated ubiquitination and degradation. Understanding the role of Skp2 in regulating IFN signaling and the cellular immune response may provide insight into its role in oncogenesis.

Chapter II: Methods and Materials

DNA Constructs.

Skp1 and Skp2 were cloned into MatchMaker® (CloneTech) yeast two-hybrid vectors pGADT7 and pGBKT, respectively by Mark Lui. For subsequent GATEWAY® cloning Skp1, Skp2 and UBP43 were PCR amplified using a human lymphocyte cDNA library (HL4006AE, ClonTech). Forward primers included the first 20-22 nucleic acids of the coding sequence and a 5' CACC for TOPO cloning (Table 2). Reverse primers contained the last 22-24 nucleic acids. All plasmids were created with the GATEWAY® cloning system using vectors from Invitrogen Inc. following manufacturer's protocols. Skp2 was subcloned into pDEST31 (Invitrogen) containing an N-terminal 6His epitope and UBP43 was subcloned into pDEST40 (Invitrogen) containing C-terminal V5 and 6His epitopes. The HA-ubiquitin and 6His-ubiquitin constructs were a gift from Dr. Rosalie Sears (OHSU, Portland, OR).

The UBP43-GFP adenovirus was made by Kevin Friedman (OHSU, Portland, OR), and the Flag-Skp2 and transactivating viruses were made by Dr. Catherine Berset (ESBATech AG, Zürich-Schlieren, Switzerland) .

cDNA Library

We used a human lymphocyte cDNA library derived from an EBV-transformed human peripheral blood lymphocyte cell line (CloneTech, HL4006AE). This library was estimated to contain 3.0 X 10^6 independent clones with an average insert size of 1.7 Kb. We titered the library by diluting 1μl of the library into 1ml of LB broth supplemented with 50μM ampicillin. Dilutions of 1:10^6 and 1:10^9 were plated on LBA plates and left at 37^0C for 20 hours. Colonies were counted and the titer was calculated to by 3 X 10^7

cfu/ml. The Library was amplified by plating 40,000 colony-forming units (cfu) per 150mm plate, 3 X 10^6 cfu was plated in total. The plates were incubated at 37^0C for 20 hours. Five milliliters of LB/25% glycerol was added to each plate and colonies were scraped into one flask. Aliquots of 50 milliliters were set aside for future use and 3 liters was used for immediate DNA plasmid isolation. Plasmid isolations were carried out using Wizard Plus Maxiprep kit from Promega.

Yeast Two-hybrid screen.

The MatchMaker® (CLONTECH) yeast two-hybrid system was used to screen for Skp2-interacting proteins. Yeast strain AH109 was transformed with control vectors included in the MatchMaker protocol , the plasmid pGBKT-Skp2 containing full-length Skp2 fused to the GAL4 DNA-binding domain (BD), and pGADT7-Skp1 containing Skp1 fused to the GAL4 activation domain (AD).(Table 1). Transformations were carried out according to the Gietz Protocol. The parent strain was grown overnight at (30^0C shaking at 250 rpm) and diluted to an OD_{600} of 5 X 10^6 (50 ml) the next morning. Cells were allowed to go through two divisions and then harvested by centrifugation at 3000X g. The pellets were washed once in sterile water, resuspended in 100mM lithium acetate to a volume of 1ml, and incubated at 30^0C for 10min. 100µl of the cell suspension was transferred to a microfuge tube and pelleted. After removing the supernatant the following were added: 240µl 50%PEG, 36µl 1M LiAc, 50µl ss-DNA (2mg/ml), 1µl (1µg) of plasmid DNA and 33µl water. Tubes were incubated at 30^0C for 30 minutes, heat shocked at 42^0C for 30 minutes, and pelleted. The cells were resuspended in 100µl of H_2O and plated on appropriate media.

Transformed cells were streaked on plates lacking either leucine or tryptophan or both to select for single clones. 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of yeast *HIS3* protein, is used to prevent leaky expression of the HIS3 nutritional marker. Appropriate concentrations of 3-AT were determined by testing different concentrations for growth of the pGBKT-Skp2/pGADT7 and pGBKT-Skp2/pGADT7-Skp1 strains. The concentration that allowed growth of Skp2/Skp1 but not Skp2/empty vector was chosen.

Table A. Yeast Two-Hybrid Strains

Plasmid Combinations	**Description**
pGBKT-Skp2	Parent strain for library transformation
pGBKT-Skp2/pGADT7	Control for Skp2 transactivation of GAL4-AD
pGBKT/pGADT7-Skp1	Control for Skp1 transactivation of GAL4-BD
pGBKT-Skp2/pGADT7-Skp1	Positive control
pGBKT-53/pGADT7-Large T	Positive control provided by MatchMaker
pGBKT-Lam/pGADT7-Large T	Negative control provided by MatchMaker

The parent strain containing Skp2 was then transformed with the human lymphoma cDNA library (HL4006AE, Invitrogen Inc.). Library transformation was carried out according to the Gietz protocol described above but scaled up 30X.

LacZ Expression Assays

Filter lifts and liquid β-galactosidase assays were done according to protocols in the Yeast Protocols Handbook from Clonetech. For filter lifts, Whatman #5 filters were soaked in Z buffer (50mM Na_2HPO_4-$7H_2O$, 40mM $Na_2H_2PO_4$-H_2O, 10mM KCL, 1mM $MgSO_4$-$7H_2O$, pH7.0) containing 2.7mg/ml X-gal and placed in a dry petri dish. A dry filter was then placed on a 100mm culture dish containing yeast colonies (2-3 days

growth). Colonies were lifted with the filter and subjected to two freeze thaw cycles using liquid nitrogen to lyse the cells. The filter containing the lysed yeast colonies was then placed on the X-gal filter and incubated at 30^0 C. Colonies were monitored for color change and ranked based on time of color change and intensity of color change. Plasmids were isolated from yeast colonies exhibiting the highest *LacZ* expression, and subjected to liquid β-galactosidase assays which are more sensitive

For liquid β-galactosidase assays, 2 mL of overnight cultures grown in restrictive media (lacking both leucine and tryptophan) were added to 8ml of YAPD (10g/L yeast extract, 20g/L peptone, 20g/L glucose) and allowed to grow until they reached OD_{600} .8-.9. 1.5 milliliters of the cultures were added to microfuge tubes and pelleted. Supernatant was removed and cells were washed once in Z buffer. 300μl of buffer was added and 100μl was transferred to a fresh tube. Samples were subjected to three freeze thaw cycles using N_2. (liq) and a 37^0C water bath. Protein concentration was determined using Bio-Rad protein assay. 100μg of protein was aliquoted to fresh tubes containing 700μl Z buffer with 50μM β-mercaptoethanol followed by 160μl of ONPG 4mg/ml. Samples were placed at 30^0C and 1M Na_2CO_3 was added once color developed. OD_{420} were taken and values determined using the OD_{420}/elapsed time.

Yeast Plasmid Isolation

The protocol for yeast plasmid isolation was published in *Biotechniques* Vol.14, no.4 (1993). Briefly, yeast were grown in yeast media lacking both leucine and tryptophan overnight and 5ml of culture at 1 OD_{600} was collected by centrifugation.

500μl of buffer (50mM tris-HCL, pH7.5, 20mM EDTA, 1% SDS) was added and cells were transferred to a 1.5ml microfuge tube and pelleted. 500μl fresh buffer was added and .5mm glass beads were added so that cell suspension was just above the bead line. Samples were vortexed for 1min and iced for 1 minute, repeated five times. Cell lysis was checked by microscopy. Samples were incubated at 70^0C for 10min and then incubated on ice for 20 minutes following the addition of 200μl 5M potassium acetate and 150μl 5M NaCl. Supernatants were harvested by centrifugation (15,000 rpm/20 minutes) and transferred to a new tube where 1/3 volume of 30% PEG_{6000} was added to precipitate plasmid DNA. After a ten-minute incubation on ice, pellets containing the yeast plasmid DNA were harvested by centrifugation and resuspended in 40μl of TE (10mM Tris-HCL, 1mM EDTA, pH7.5).

GST-Skp2 *In Vitro* Interaction Assays

Escherichia coli BL21® (Invitrogen) cells were used to express the vector, pDEST15, (Invitrogen) containing GST or a GST-Skp2 fusion protein. Overnight cultures (250 mL) grown in LB-ampicillin (50ug/ul) were diluted to an OD_{600} of 0.1 in 1 L. At OD_{600}, 0.5 each strain was induced with 500mM IPTG for 4 hours. Cells were pelleted, washed once in PBS, and re-suspended in the PBS/1% triton. Protein was harvested using a 3X freeze-thaw protocol with a dry ice and ethanol bath. Protein concentration was determined by OD_{280}.

Extracts were purified over Glutathione Sepharose® 4B matrix (Pharmacia) according to manufacturer's instructions. 1 mg of protein from bacteria expressing GST-Skp2 or 10μg from bacteria expressing GST alone was added to PBS 1% triton to a

volume of 4mls. We used ten times more GST-Skp2 than GST because the GST-Skp2 fusion was much more unstable in the bacteria and therefore accounted for a much lower portion of the total extract than in the lysates from GST alone. 100µl of matrix was added and samples were rocked at room temperature for 1 hour and washed three times in PBS/triton.

100µg of extracts from HEK 293 cells transfected with indicated plasmids was added to the matrix containing either the GST-Skp2 or GST alone and brought up to a total volume of 500ul with mammalian IP lysis buffer (50mM Tris-HCL pH 7.5, 150mM NaCl, .1% NP-40, 5mM EDTA pH 8.0, 1mM DTT, 1X phosphates inhibitors, 1X protease inhibitors). Samples were incubated at 4^0 C overnight, washed three times with mammalian IP lysis buffer, and finally boiled in SDS sample buffer. Bound fractions were analyzed by SDS-PAGE and Western blotting using the indicated antibodies.

Deletion Constructs

All UBP43 and two Skp2 deletion constructs were created via PCR amplification with indicated primers (Table 2). The forward primers contained a 5' CACC for the Gateway cloning system as well as an ATG followed by the indicated sequences contained in the cDNA. Reverse primers are indicated in Table 2. Skp2 forward primers contained the sequence for the Flag epitope between the 'CACC' ATG sequence and the beginning of the Skp2 sequence.

Table 2. Primers for UBP43 and Skp2 deletion constructs (nucleotides)

Construct	**Forward**	**Reverse**
UBP43-FL	CACCATG 1-21	1095-1116
$UBP43^{1-120}$	CACCATG 1-21	341-360

$UBP43^{121-373}$	CACCATG 361-379	1095-1116
$UBP43^{121-352}$	CACCATG 361-379	1031-1056
$UBP43^{121-285}$	CACCATG 361-379	797-825
$UBP43^{121-183}$	CACCATG 361-379	524-549
Skp2-FL	CACCATG Flag 1-24	1254-1272
$Skp2^{109-436}$	CACCATG Flag 298-320	1254-1272
$Skp2^{209-436}$	CACCATG Flag 625-646	1254-1272

PCR products were run on a 1% agrose gel to confirm the product and then gel isolated using QIAquick Gel Extraction kit from Qiagen. Purified oligos were cloned into pENTR,, a shuttling vector, according to manufacture's protocol. Once in pENTR the constructs can be shuttled to the destination vector of choice via a recombination event between sequences flanking the gene of interest in pENTR and sequences contained on the destination vectors. This was also done according to manufacture's protocols. UBP43 constructs were cloned into pDEST32 for analysis in yeast and pDEST40 (containing C-terminal V5 and 6HIS epitopes) for mammalian expression. Skp2 deletions were cloned into pDEST22 for analysis in yeast and pDEST31 (containing an N-terminal 6HIS epitope) for mammalian expression.

The F-box deletion construct of Skp2 was created by restriction digest. pENTR-Skp2-FL was cut with *XcmI* and *BsgI* to remove 139 nucleotides that contained the F-box motif. The vector fragment was gel isolated and subjected to further restriction digest to confirm that both cuts were made efficiently. The doubly digested fragment was cut with *HindIII* and yielded 5.2, 1.4, 0.5 and 0.140 Kb bands. This pattern would not be present if either restriction cut was not made. The gel-isolated product was then treated with Klenow fragment. The digest left two 3' overhangs. Klenow excised the overhangs and

left blunt ends for ligation while maintaining the reading frame. After Klenow treatment the plasmid was ligated using Quick-Stick ligation kit from Bioline, transformed into Gold competency cells from Bioline, and plated on LB-Amp (50ug/ml). The next day liquid colonies were grown from transformed colonies and incubated at 37^0 C and 225 rpm overnight. Plasmids were isolated using Wizard Plus Mini-Preps from Promega, and cut with *SpeI* for verification. Positive clones were sent for sequencing at the Vollum core facility (OHSU, Portland, OR) to ensure the reading frame was maintained. Clones containing the correct sequence were subcloned into pDEST31 using the Gateway cloning system as described above.

Generation of Primary and Transformed Mouse Embryonic Fibroblasts (MEFS)

Primary MEFs were made from 13.5 day embryos from crosses of Skp2 +/- and Skp2 -/- mice. After processing, one embryo was plated to a p100 tissue culture dish (20% FBS, DMEM, 1X pen/strep, 1X L-Glutamine) and incubated for two days. Each sample was then passaged to a p150 dish and harvested in freezing media (20% FBS, DMEM, 1X pen/strep, 1X L-Glutamine, 10% DMSO) after 3 days, resulting in passage 2 primary MEF stocks.

Transformed cell lines were established by transfecting primary MEFs with a plasmid containing a CMV driven SV40 Large T that causes cellular transformation through inhibition of p53. Cells were passaged every 2 days until cell growth arrested. Cells were then incubated and media was changed every two days. Colonies of immortalized cells began to appear after 10 days. Immortalized cells were passaged two more times into p150 dishes and then harvested in freezing media.

Construct Expression in Mammalian Cell Culture

The Gateway mammalian expression vectors that we used contained a CMV promoter to drive gene expression. For mammalian transfections, bacterial stocks were grown overnight and the plasmids were isolated using Wizard Plus Mini-Preps from Promega (Madison, WI). Care was taken to not contaminate the DNA, which should be sterile following the ethanol washes in the Wizard Prep protocol. Transfections were carried out using Effectene transfection reagent from Qiagen (Valencia, CA). Transfections were performed in 100mM plates at approximately 80% confluency. 2μg total DNA was used per plate. DNA was brought up to a volume of 300μl with the DNA Condensation (EC) buffer and 16μl of Enhancer buffer was added. This solution was vortexed lightly and incubated at room temperature for 10 minutes. We used a ratio of 1:30 DNA to Effectene. 60μl of Effectene was added, mixed, and incubated at room temperature for another 15 minutes. The transfection solutions were added to the plates in a drop wise manner. Total transfection time was 36 hours. Proteasome inhibitors, LPS, or IFN were typically added 5, 7, or 24 hours, respectively, prior to harvest. This protocol was used for all cell lines discussed, however it should be noted that for human transformed cells this yielded approximately 80% transfection efficiency, while in the Skp2 MEFs the efficiency was closer to 25-30%.

For viral infections indicated amounts of virus was diluted in 1ml of DMEM/20mM HEPES per 100mM plate. While the virus solution was equilibrating at room temperature, the media containing serum and antibiotics was removed and replaced with 8mls of basic media (F-12, 1X L-Glutamine). The virus was added and the plates

incubated the plates at 37^0 C for six hours, rocking every 20min for the first hour. The serum and antibiotics (10% FBS, F-12, 1X L-Glutamine, 1X pen/step) were added back and plates incubated for another 36 hours. For the Skp2 MEFs the above protocol was used, however, 2ul of Lipofectamine 2000 (Invitrogen) was added to the virus solution prior to equilibration. MEFs are more difficult to infect as they typically have low levels of mCAR, the main adenovirus receptor. Addition of Lipofectamine, allowed viral particle enter the cells by lipid membrane fusion similar to transfections.

All cells were harvested in 200μL/100mM plate mammalian IP lysis (MIPL) buffer (50mM Tris-HCL pH7.5, 150mM NaCl, 0.1% NP-40, 5mM EDTA pH8.0, 1mM DTT, and 1X phosphates and protease inhibitors). Cells were scraped into a microfuge tube and incubated on ice for 10 minutes. Cells were centrifuged at 15,000 rpm, 4^0 C for 10 minutes and supernatant transferred to a fresh tube.

Immunoprecipitation.

Cell lysates in mammalian IP lysis buffer were immunoprecipitated with antibodies indicated in the figure legends. One 100mM plates of cells was used per IP. Transfection efficiencies were assessed via expression of a GFP to ensure equal protein expression. GFP expression was from a co-transfected plasmid containing CMV-GFP (in the case of transfections) or from an internal ribosomal entry sequence (IRES) contained within the Ad-UBP43 (in the case of infections). For Skp2 MEFs, two plates of Skp2 -/- cells were used for every 1 plate of Skp2+/+ cells because the transfection efficiency was approximately 50% in Skp2 null cells compared to wild type.

For native V5 immunoprecipitations, 20μl of protein A/G beads (Invitrogen) were

added per sample. Total volume was brought up to 1ml with MIPL buffer and incubated rocking at room temp for 1 hour. 2 μl of V5 antibody was added to each sample and incubated for another hour. Beads were washed three times in MIPL and then boiled in SDS loading buffer.

A protocol from William P. Tansey (http://tanseylab.cshl.edu/protocols.html) (Cold Spring Harbor Laboratory, NY) was followed for denaturing V5 immunoprecipitations. Cells were harvested by scrapping into PBS, and centrifuged 10 min at 15,000 rpm. 100μl of the solution TDS (50mM Tris pH 7.5, 5mM DTT, 1% SDS) was added to the cell pellet and samples were boiled for 10 minutes. Lysates were centrifuged and supernatant transferred to a fresh tube. 1.2 mL of TNN (50mM Tris pH7.5, 250 mM NaCl, 5mM EDTA, .5% NP-40) was added to renature the proteins. The V5 immunoprecipitations were carried out as described for the native immunoprecipitations except the final washes were done with TNN.

For Flag immunoprecipitation, we used EZview™ Red ANTI-FLAG M2 beads (Sigma Santa Rosa, CA) that have the FLAG M2 antibody covalently attached. We choose this method because tagged Skp2 runs near the IgG heavy chain band in SDS-PAGE that is present when uncoupled beads are used. Cell harvesting was carried out as described previously in this chapter and 20μl of M2 resin was added to each IP and the total volume was brought up to 1ml with MIPL buffer. Samples were incubated overnight at 4^0C. Samples were washed times in MIPL buffer. Flag-Skp2 and associated proteins were eluted in 50μl of TBS containing 150ng/ul of Flag peptide (Sigma) at 4^0C for 1hour, mixing every 15 minutes. Samples were centrifuged and supernatant transferred to a fresh tube with SDS sample buffered and then boiled for 3 minutes prior

to loading gel. 1/4 of the IP was used for Skp2 detection while the other 3/4 was used to detect UBP43-V5.

Proteins were run on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and analyzed with indicated antibodies.

Interaction Assays Using Nickel Ni^{2+}-NTA Resin and a 6His Epitope

For denaturing His pull-downs, cell lysates in mammalian IP lysis buffer were brought to a total volume of 1ml in denaturing buffer (100mM NaH_2PO_4, 10mM Tris-Cl, and 8M urea). 20 ug of Nickel Ni^{2+}-N TA beads (Qiagen) were added to each sample and incubated rocking at room temp for 1 hour. Beads were washed times in denaturing buffer and then boiled in SDS loading buffer. Proteins were run on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and analyzed with indicated antibodies.

^{35}S Pulse/Chase.

REF52 and A549 cells were infected with UBP43-V5 adenovirus for 20 hours as described above. Cells were then labeled for 30 min with 7mCi of ^{35}S methionine, chased with media containing 5mM methionine and 3mM cysteine, washed in PBS, and collected at indicated time points. Proteasome inhibitors (PI, 5μM MG132 + 5μM lactacystin) were added 5 hours prior to the pulse and maintained throughout the pulse and chase phases. Samples were subjected to a denaturing V5 immunoprecipitation (described above) to reduce background signal. Immunoprecipitations were run on a 10% SDS-PAGE and the gel was then dried and placed on a phospho-imager for ^{35}S

detection.

IFN-ß Treatment.

Skp2 wild type and Skp2-/- MEFs were treated with 1000 U/ml mouse IFN-ß and human A549 samples were treated with 1000 U/ml of human IFN-α. Extracts were harvested 24 hours later. For cell growth measurements, 3.6 $x10^4$ immortalized MEF cells plated in 6-well plates, were either not treated or treated with 500 U/ml of mouse IFN-ß, and harvested in duplicate. For each time point, counting cells with a hemocytometer assessed total viable cell number. Trypan blue staining was used to identify dead cells. A minimum of 150 cells were counted per sample.

Chapter III: Identifying Skp2 Interacting Proteins

Introduction

The Oncoprotein Skp2 plays an important role in regulating the cell cycle by promoting progression through S-phase. However, its oncogenic potential does not appear to be limited to its pro-proliferative activity. For example, Skp2 levels also appear to positively correlate with increased metastasis and tissue invasiveness in lung cell carcinomas (Inazawa J., 2004). Skp2 likely serves a broader function than just that of cell cycle control. While several targets of the SCF^{Skp2} complex are known, elucidating more Skp2 interacting proteins will undoubtedly allow us to better understand Skp2's role in cellular physiology.

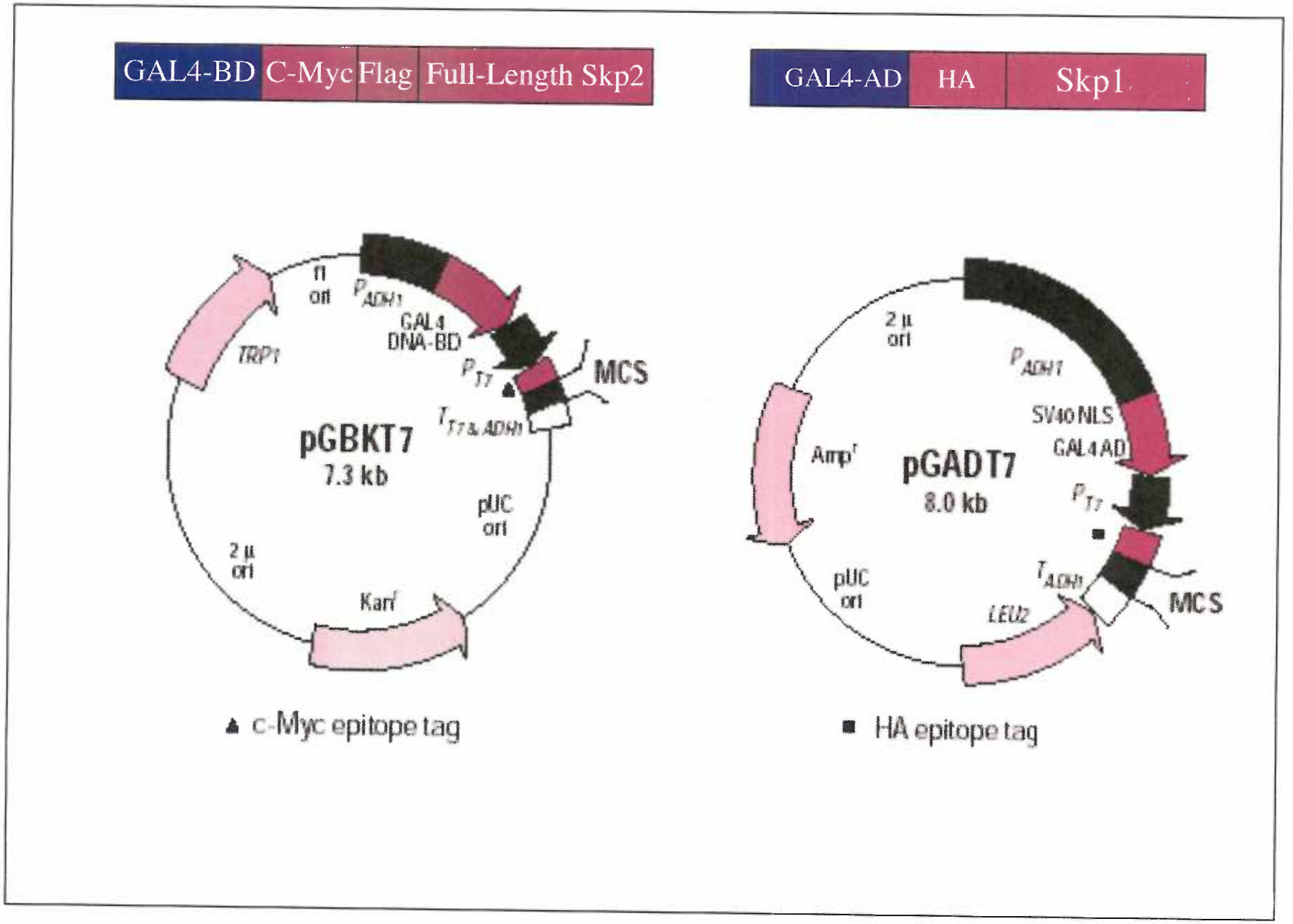
Results

A. Generating the Yeast Two-Hybrid

I used a GAL4-based MatchMaker yeast two-hybrid system from ClonTech® to assay for Skp2-interacting proteins. This system allows the screening of high numbers of cDNA transcripts, in this case 2.4 million, with relatively simple assays for interactions using yeast physiology. GAL4 is a transcription factor that requires both a DNA binding domain (BD) and an activation domain (AD) to drive transcription of reporter genes. Figure 4 shows the constructs created for use in this screen. In the MatchMaker yeast two-hybrid system, the BD and AD, shown in Figure 4 in blue, have been cloned into separate vectors. I cloned Skp2 into pGBKT7 to create a GAL4-BD-Skp2 fusion.

Figure 4. Yeast Two-Hybrid Constructs. Skp2 and Skp1 were cloned into the MCS of pGBKT and pGADT7 respectively. This resulted in a GAL4 binding domain (BD) fusion with Skp2 that also contained N-terminal c-myc and Flag epitopes (the Flag epitope was generated in the PCR and was not contained in the pGBKT vector). Skp1 was fused to the GAL4 activation domain (AD) and contained an N-terminal HA epitope. PGBKT7 and pGADT7 express the genes TRP1 and LEU2 respectively to allow for nutritional selection.

Figure 4


GAL4-BD
C-Myc
Flag
Full-Length Skp2
GAL4-AD
HA
Skp1
f1 ori
P_{ADH1}
GAL4 DNA-BD
P_{T7}
MCS
$T_{T7\ \&\ ADH1}$
TRP1
pGBKT7
7.3 kb
pUC ori
2 µ ori
Kan^r
2 µ ori
P_{ADH1}
SV40 NLS
GAL4 AD
Amp^r
pGADT7
8.0 kb
P_{T7}
pUC ori
T_{ADH1}
MCS
LEU2
▲ c-Myc epitope tag
■ HA epitope tag

In addition, we created a GAL4-AD-Skp1 fusion as a positive control. We used a pre-made cDNA library made from human, transformed B-lymphocytes fused to GAL4-AD for the screen.

GAL4 specific promoters in the yeast strain are used to drive transcription of reporter genes, typically nutritional markers and *LacZ*. As Figure 5A indicates, either the BD or AD fusion alone is not sufficient to drive transcription of the reporter genes. However, when the BD and AD domains are brought together through the interaction of the fused proteins, expression of the associated gene is initiated. The MatchMaker system utilizes three reporter constructs, HIS3, ADE2 and a *LacZ,* to reduce the occurrence of false positives. Each reporter gene is fused to a different promoter to allow for different stringency screens. Weaker promoters (MET1) require stronger interactions of the fusion proteins to drive transcription and therefore result in a high stringency screen (Figure 5B). Conversely, stronger promoters (GAL1) do not require a strong interaction between the fusion proteins and therefore result in a low stringency, but more inclusive, screen. I separated the screen into selecting for either, HIS3, or both HIS3 and ADE2, effectively giving two different stringency screens.

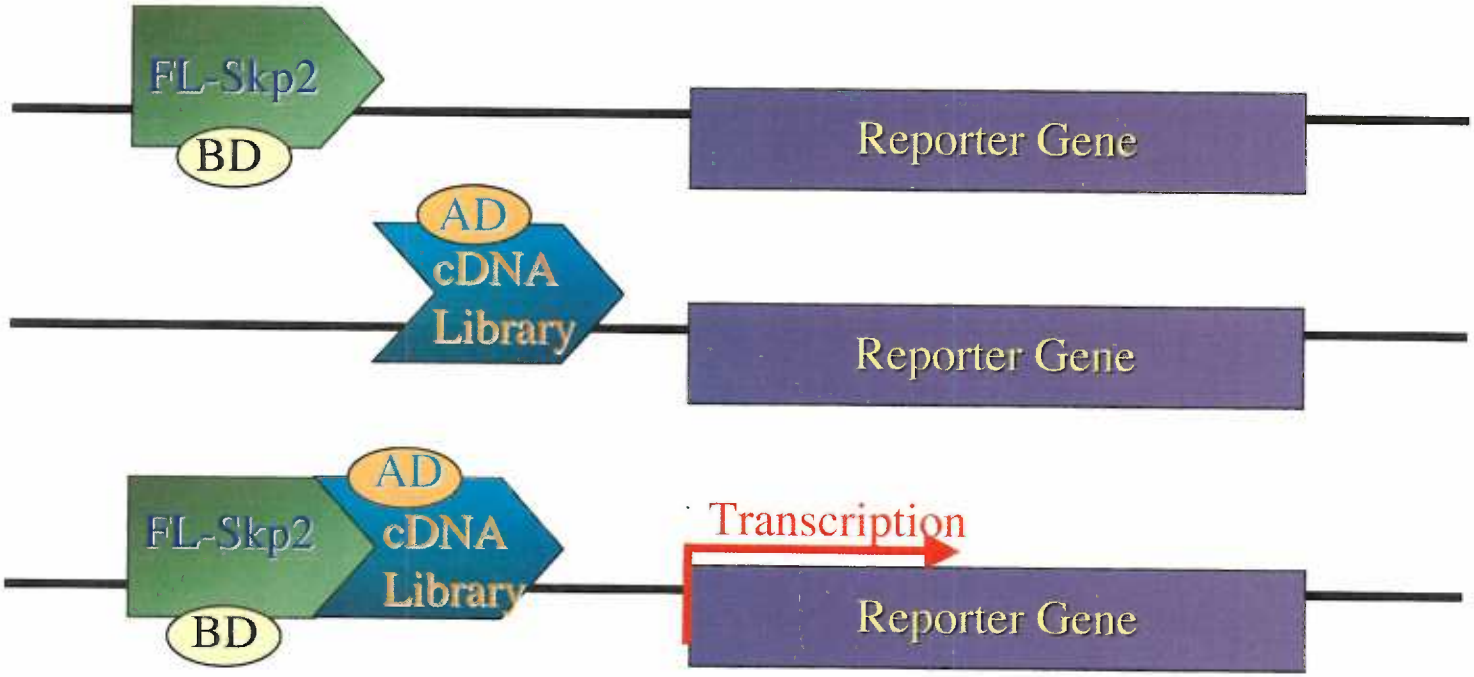
We screened 2 X 10^6 clones from the cDNA library on both low stringency (-his + 3-AT) and high stringency conditions (-ade, -his + 3-AT). 3-AT, a competitive inhibitor of the yeast HIS3 protein, is included in the plate to inhibit low levels of HIS3 resulting from leaky expression at this promoter. All plates were also lacking in leucine and tryptophan to select for vectors containing the bait and prey GAL4 domain fusions as described in Figure 4. The Library contained an estimated 3 X 10^6 independent clones, so we covered about 2/3 of the library in our screen.

Clones from both screens were streaked for single colonies and then tested for *LacZ* expression using the filter lift technique described in the Material and Methods section. *LacZ* is expressed from the weakest promoter (Figure 5B) and represented the most stringent assay. Approximately 60 clones remained potential interactors after the *LacZ* assay. We isolated the prey plasmids from the strongest interactors, as determined by the *LacZ* assay, and retransformed them into the strain containing GAL4-BD-Skp2.

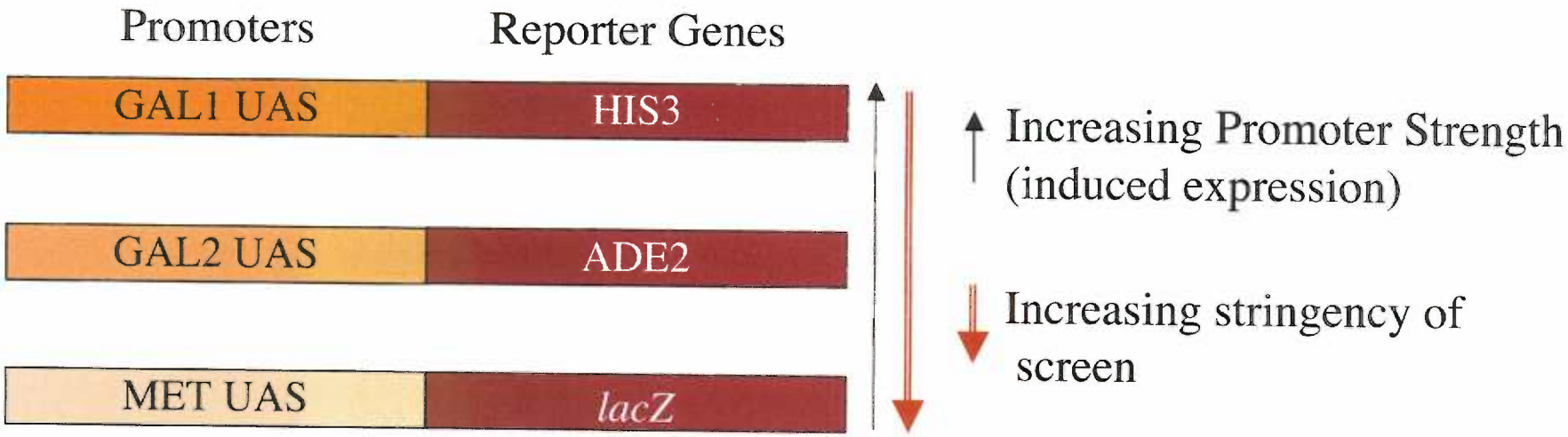
Figure 5. A. Diagram of the MatchMaker© Yeast Two-Hybrid System. A protein fused to either the GAL4 AD or BD will not promote transcription on its own. When the two GAL4 domains are brought in proximity due to the interaction of fused proteins, transcription of associated genes is initiated. B. Three different promoter strengths provide for the ability to conduct screens of varying stringencies; weaker promoters yield more stringent screens. The GAL1 promoter is the strongest followed by GAL2 and MET1.

Figure 5

A


FL-Skp2
BD
Reporter Gene
AD
cDNA
Library
Reporter Gene
AD
FL-Skp2
cDNA
Library
BD
Transcription
Reporter Gene

B


Promoters
Reporter Genes
GAL1 UAS
HIS3
GAL2 UAS
ADE2
MET UAS
lacZ
Increasing Promoter Strength
(induced expression)
Increasing stringency of
screen

Clones that were verified to grow on nutritional markers after retransformation were assayed for *LacZ* using the more sensitive liquid assay. Strong interactors were then sequenced to determine their coding sequence. Out of approximately 30 clones sequenced, only eight demonstrated valid orientation and open reading frames. The relative β-galactosidase activity for each of these clones is shown in Figure 6A.

B. Skp2 Interactors Identified in the Yeast Two-Hybrid

The eight putative Skp2 interacting clones, summarized in figure 6B, consisted of proteins with a wide variety of functions. Three clones, Cks1 and Skp1 (2 clones), were previously described as Skp2 interactors. Skp1 is a part of the SCF complex and Cks1 is an important adaptor protein required for the SCF^{Skp2} -mediated ubiquitination of p27 (Hershko, A 2001). Two proteins were identified that have roles in cell signaling and growth regulation; RAGA, a member of the family of Ras-related GTPase binding proteins, and PP1, a protein phosphatase (Shcurmann, 1995,Wadzinski BE, 1999). RNAPOLII subunit E and MMS19 play roles in RNA transcription and mismatch repair respectively (Berk, 2003, Hoeijmakers, 2002). Finally, UBP43, described as a ubiquitin specific protease, was identified.

I chose to focus on UBP43 due to its implied role in cleavage of ubiquitin molecules. I originally pulled out the C-terminal region of UBP43 in the yeast two-hybrid screen. To determine if full length UBP43 would also interact we PCR-amplified the full-length cDNA, as well as the C-terminal region, out of the lymphocyte library. These constructs were cloned into the Gateway vectors for the Invitrogen® Yeast Two-

Figure 6. A) Relative levels of *LacZ* (as assayed by β-galactosidase activity) for the putative Skp2 interacting clones identified in this screen. Skp2 interaction with Skp1 was the strongest interaction (++++) and all other values were compared to this. RNA Polymerase II subunit E and UBP43 show equally high *LacZ* expression, while Cks1 demonstrated the lowest expression. B) Table of putative Skp2 interacting proteins and their known functions.

Figure 6

A

	-Ade-His+3At	*LacZ*
RNA POLII E	+	++++
PP1	+	+++
RAGA	+	++
MMS19	+	N/A
UBP43	+	++++
cks1	+	+
Skp1 (2)	+	++++

B

Cellular Function	**Proteins**
SCF Mediated Ubiquitination	Skp1, Cks1
Signal Transduction/Growth Regulation	RAGA, PP1
Transcription/DNA Repair	RNAPOLII Subunit E, MMS19
Ubiguitin Modification	UBP43

Hybrid system ProQuest. I chose to do this to facilitate cloning into a variety of vectors including our bacterial and mammalian vectors to be used later. Interaction was again assessed on nutritional markers. Figure 7A shows the growth of yeast strains containing the indicated plasmids on selective media. Full length UBP43, as well as the C-terminal region from the original screen ($UBP43^{121-373}$), demonstrated robust growth, indicating an interaction between them and GAL-BD-Skp2. Expression of the various constructs in yeast was verified by western blot analysis as shown in figure 7B.

Discussion

Skp2 plays a pivotal role in controlling the cell cycle. Identifying additional interactors of Skp2 would help expand our knowledge of its function in the cell and how it contributes to oncogenesis. I show here that Skp2 can interact with the protein UBP43 in a yeast two-hybrid system. I pursued UBP43 because of its proposed role as a de-ubiquitinating enzyme. UBP43 was first identified as an upregulated mRNA in the mouse leukemia model AML1-ETO (Zhang, 2000). It contained structural motifs similar to those of other ubiquitin-specific proteases and was therefore classified as a de-ubiquitinating enzyme (DUB).

Ubiquitination is a highly dynamic process, and DUBs play key roles in regulating this process, as seen in Figure 8. DUBs affect ubiquitin recycling by facilitating ubiquitin chain cleavage after proteasomal degradation of the substrate (Amerik, Hochstrasser, 2004). They also play a role in modulating protein activity. For instance, mono-ubiquitination is known to cause a change in the activity or cellular localizations of some proteins. DUBs provide a means of rapid, reversible regulation of

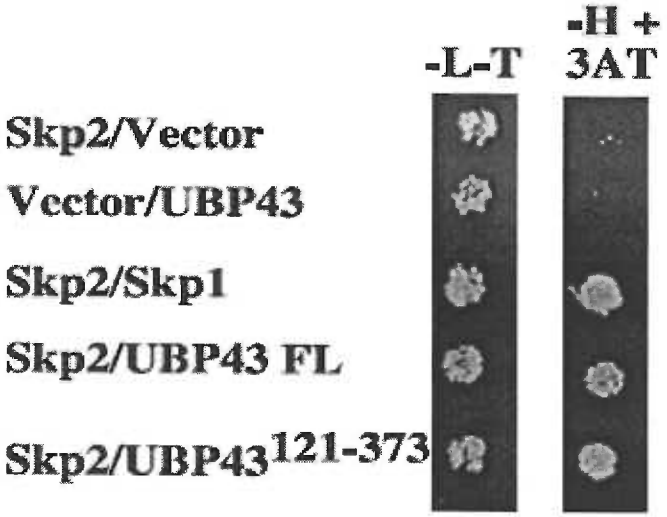
mono-ubiquitination. In addition, multi-ubiquitinated proteins can be "edited" to a mono-ubiquitinated form, thus altering their activity, levels, or cellular localization. This was of obvious interest to me, due to Skp2's role in ubiquitin conjugation.

Figure 7. A) Skp2/UBP43 interaction in the Yeast Two-hybrid. Indicated plasmids were expressed in yeast strain AH109 and plated on media lacking histidine to assay for interaction. Clones containing either Skp2 or UBP43 with empty vectors do not promote growth on –his media, indicating that there is no transactivation occurring. Skp2 and Skp1, as well as Skp2 and UBP43, support growth on the restrictive media, indicating the proteins interact. B) Protein was harvested from yeast and subjected to western blot analysis to confirm protein expression of the construct. The anti-HA blot shows expression of HA-tagged UBP43.

Figure 7

A Growth on Selective Media

LacZ assay



B Protein Expression in Yeast

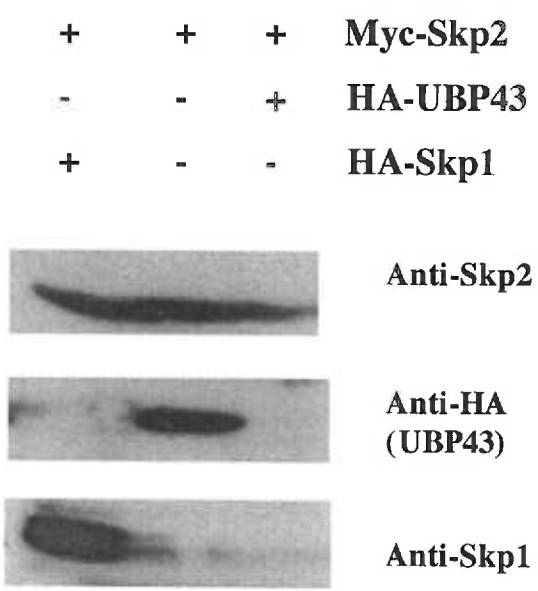
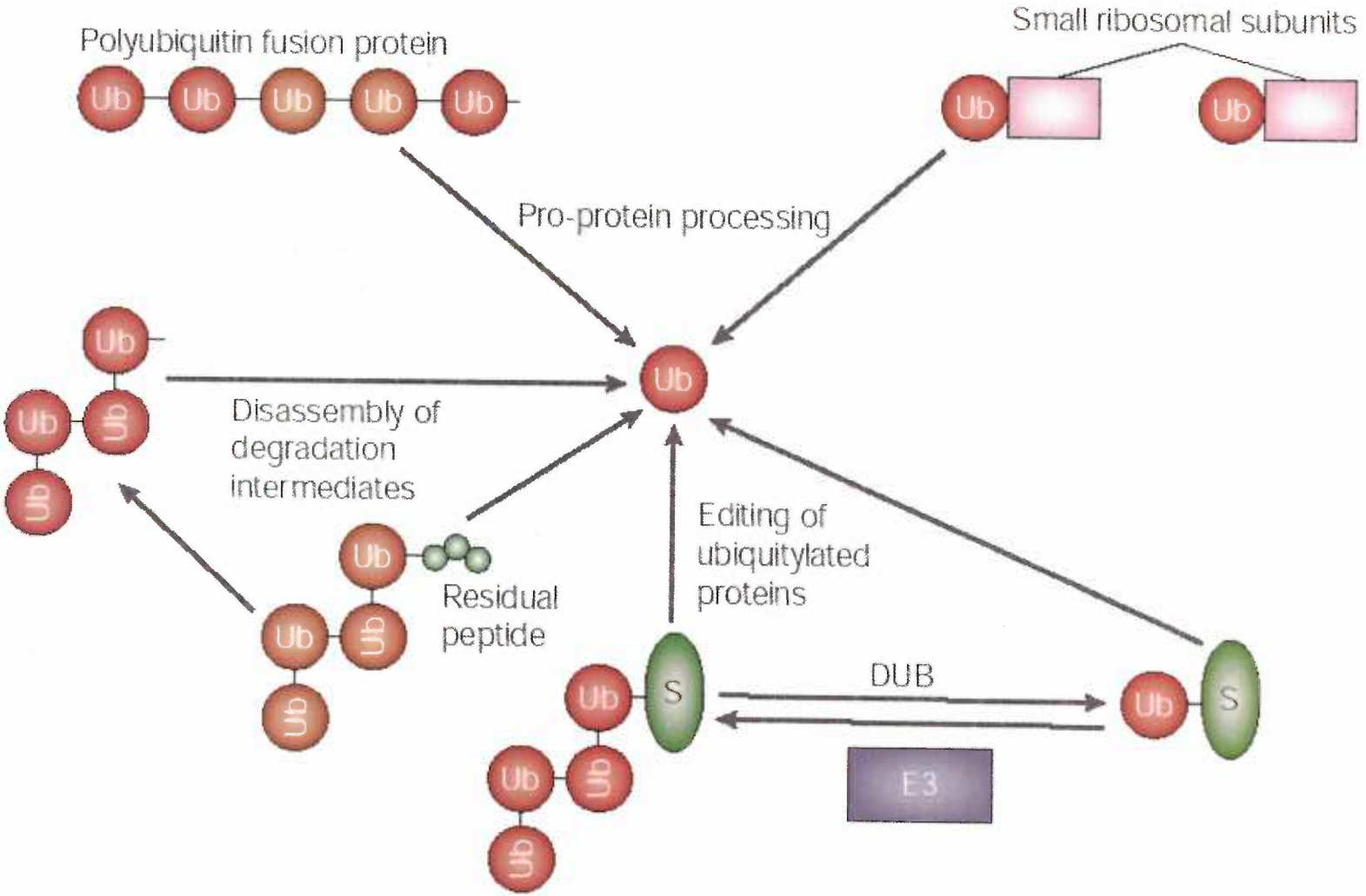


Figure 8. Diagram of the cellular functions of de-ubiquitinating enzymes. DUBs are critical factors in ubiquitin recycling, substrate modification, and modulation of substrate activity.

Figure 8

Deubiquitination Enzymes (DUBs)



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Chapter IV: Characterizing The Skp2/UBP43 Interaction

Introduction

In my Y2H screen, I identified UBP43 as a putative Skp2 interacting protein. I then verified this interaction in mammalian cells and characterized the binding regions. Skp2 has four main protein domains: an N-terminal region, an F-box motif, a leucine rich repeat (LRR), and a small C-term tail (Figure 9A). The F-box and LRR regions are the two main protein interaction regions of Skp2. Skp2 interacts with Skp1 through the F-box motif, connecting it to the rest of the SCF E3 ligase (Ilyin, Rialland et al. 1999). The LRR region has been implicated as the region responsible for Skp2 binding to substrates (Hsiung, Chang et al. 2001; Kobe and Kajava 2001). Therefore, we may suspect that Skp2 binds UBP43 via its LRR region.

Little is known about the domain structure of UBP43. The C-terminal 252 amino acids of UBP43 was originally identified as the Skp2 interacting region, shown in Figure 9B. However, within this region, no specific protein structures or domains are apparent (Zhang 2001). Further isolating the Skp2 binding region of UBP43 could define a regulatory domain within UBP43.

Results

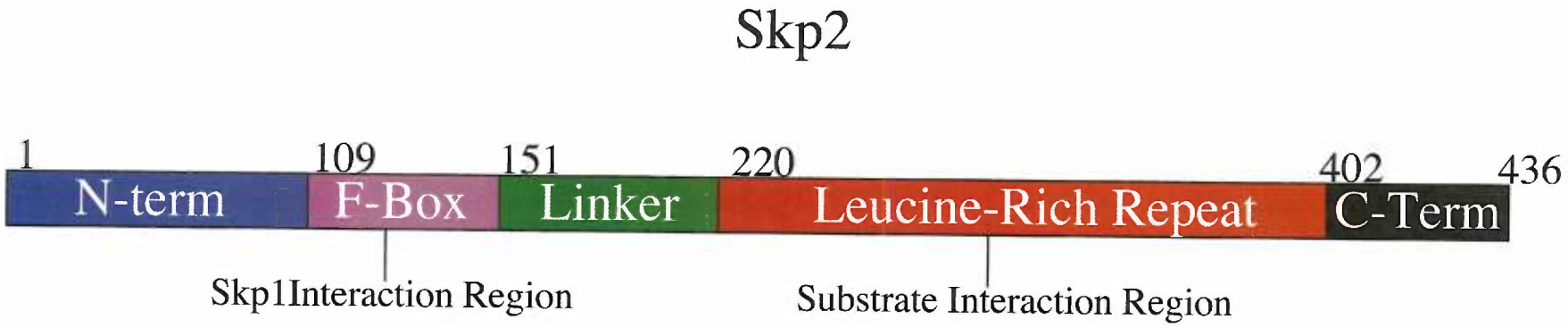
A. Skp2 and UBP43 Interact *In Vitro*

While the yeast two-hybrid system is an effective method for screening entire libraries, I next had to verify interaction outside of the yeast system. I began by making a GST-Skp2 fusion protein and expressing it in an IPTG inducible strain of *E. coli* .

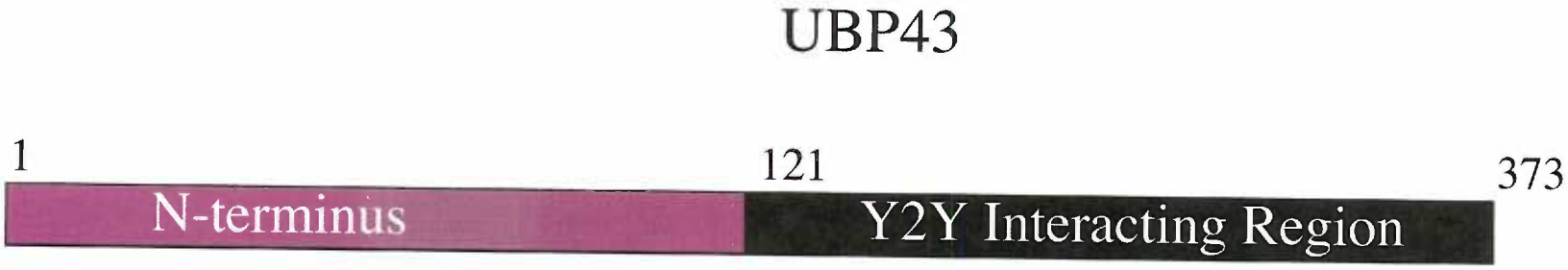
Figure 9. Illustration of the protein domains of Skp2 and UBP43. Skp2 has four main protein domains: N-terminus, F-box, Leucine-Rich Repeat and C-terminus. UBP43 has no described protein domains. UBP43 is a 373 amino acid protein. No domains have been described to date. We have termed the region between 121 and 373 the yeast two-hybrid interacting region.

Figure 9

A


Skp2
1
109
151
220
402
436
N-term
F-Box
Linker
Leucine-Rich Repeat
C-Term
Skp1Interaction Region
Substrate Interaction Region

B


UBP43
1
121
373
N-terminus
Y2Y Interacting Region

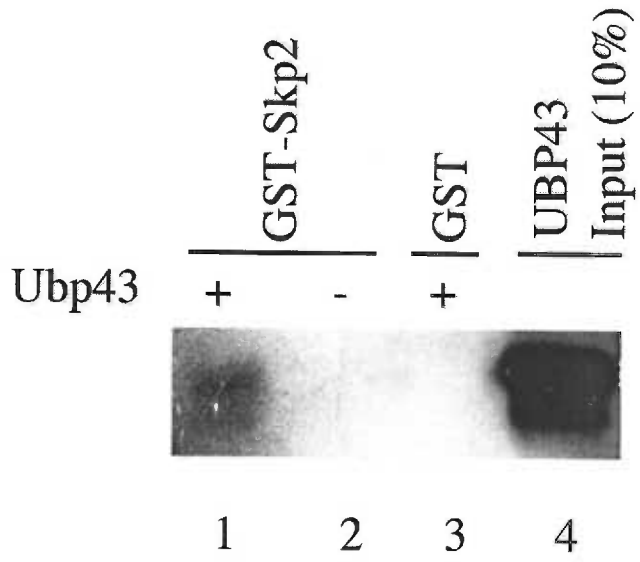
Substrate recognition by F-box proteins often depends on specific phosphorylation of the substrates (Carrano, Eytan et al. 1999; Tedesco, Lukas et al. 2002; Bornstein, Bloom et al. 2003; Kamura, Hara et al. 2003; Li, Zhao et al. 2003). Therefore, I expressed UBP43 in mammalian cells in order to maintain any post-translational modification of UBP43 required for its binding to Skp2. As shown in Figure 10A, UBP43 was retained in the bound fraction when extracts were passed over Glutithione beads with bound GST-Skp2 (Figure10A, lane 1) but not beads with bound GST alone (Figure10A, lane 3). We conclude that UBP43 is able to interact with a bacterially expressed Skp2 *in vitro*.

Because Skp2 and UBP43 are both mammalian proteins, we also needed to verify an *in vivo* interaction in mammalian cells. This was accomplished through Co-immunoprecipitation of Flag-Skp2 and UBP43-V5. There is no human UBP43 antibody, and Skp2 levels are usually low in cycling cells; therefore we examined *in vivo* binding using ectopically expressed UBP43 and Skp2. I infected cells with a CMV-driven Ad-V5-UBP43 and a tetracycline repressible Ad-Flag-Skp2 and performed immunoprecipitations on the extracts. Beads covalently linked to the anti-Flag epitope were used to pull down Flag-Skp2, and extracts were probed with the indicated antibodies. As shown in Figure 10B, UBP43 co-immunoprecipitated when Flag-Skp2 was present (lane 1) but not in the absence of Flag-Skp2 (lane 2). I confirmed the interaction by reversing the protocol, pulling down V5-UBP43 and looking for Skp2 in the bound fraction (data not shown). These data demonstrate that Skp2 and UBP43 indeed interact in mammalian cells.

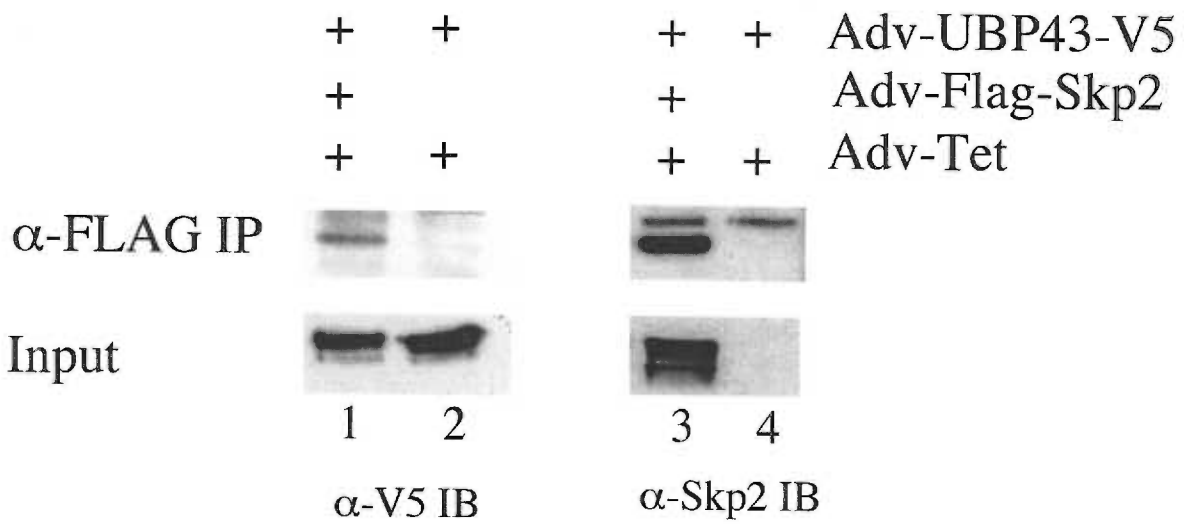
Figure 10. *In vitro* and *In vivo* binding of Skp2 and UBP43. A) GST-Skp2 binds UBP43 in vitro. GST-Skp2 (lanes 1 and 2) or GST (lanes 3) were expressed in E. coli and purified over glutathione beads. Beads were incubated with HEK293 cell extracts expressing UBP43 where indicated. 10% HEK293 extract input is shown in lane 4. B) Mammalian co-immunoprecipitation. Human lung carcinoma A549 cells were infected with adenovirus expressing UBP43-V5 and FLAG-Skp2. Cell extracts were immunoprecipitated with anti-FLAG beads, separated by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Immunoprecipitated fractions are shown in the top panel and 10% inputs are shown below. The Ad-Flag-Skp2 virus requires the addition of the tetracycline-controlled transactivator (Ad-Tet) to activate transcription of the gene of Skp2 from a silent promoter.

Figure 10

A


GST-Skp2
GST
UBP43
Input (10%)
Ubp43
+
-
+
1
2
3
4

B


+ + + + Adv-UBP43-V5
+ + Adv-Flag-Skp2
+ + + + Adv-Tet
α-FLAG IP
Input
1 2 3 4
α-V5 IB
α-Skp2 IB

B. The Leucine-Rich Region (LRR) of Skp2 is Required for Binding to UBP43

After confirming the interaction of both full-length proteins *in vivo* and *in vitro*, I investigated the binding of deletion mutants to further define interaction domains. I created deletion mutants of Skp2, illustrated in Figure 11A, lacking the N-terminus (Flag-Skp2$^{109-436}$), both the N-terminal and F-box regions (Flag-Skp2$^{209-436}$), and lacking the LRR and C-terminal regions (Flag-Skp2$^{1-232}$). These constructs, along with the plasmid expressing V5-UBP43, were transfected into human lung carcinoma A549 cells (as described in Methods) and protein extracts were subjected to a Flag IP (Figure 11B). Full-length Flag-Skp2, as well Flag-Skp2$^{209-436}$ and Flag-Skp2$^{109-436}$, was able to co-immunoprecipitate UBP43 (Figure 11B, lanes 2 and 4, upper panel). The construct lacking the LRR and C-terminal regions was unable to co-immunoprecipitate full-length UBP43 (Figure 11B, lane 5). The construct lacking the N-terminal region appeared to pull down less UBP43 (Figure 11B, lane 3). The involvement of the LRR region of Skp2 in UBP43 binding suggests that UBP43 is binding to Skp2 as a target for E3-mediated ubiquitination. To date, the C-terminus has not been implicated in direct substrate binding; however, UBP43 binding could be facilitated by an adaptor protein, such as Cks1, bound to the carboxy-terminus of Skp2.

C. A Central Region of UBP43 Mediates Interaction with Skp2

To determine which region of UBP43 interacts with Skp2, I created C-terminal truncations of the portion of UBP43 isolated in the two-hybrid screen (Figure 12A). We were unable to express the C-terminal truncations of UBP43 in mammalian cells, so we

Figure 11. Interaction of Skp2 Deletion Mutants with UBP43. A) Diagram of Skp2 deletion constructs used in the mammalian co-immunoprecipitation. B) Mammalian co-immunoprecipitation. Human lung carcinoma A549 cells transfected with constructs expressing UBP43-V5 and indicated Flag-Skp2 constructs. Cell extracts were immunoprecipitated with anti-Flag beads, separated by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Endogenous Skp2 is denoted by a white arrowhead and ectopic Skp2 by black arrowheads. The asterisk indicates a background band.

Figure 11

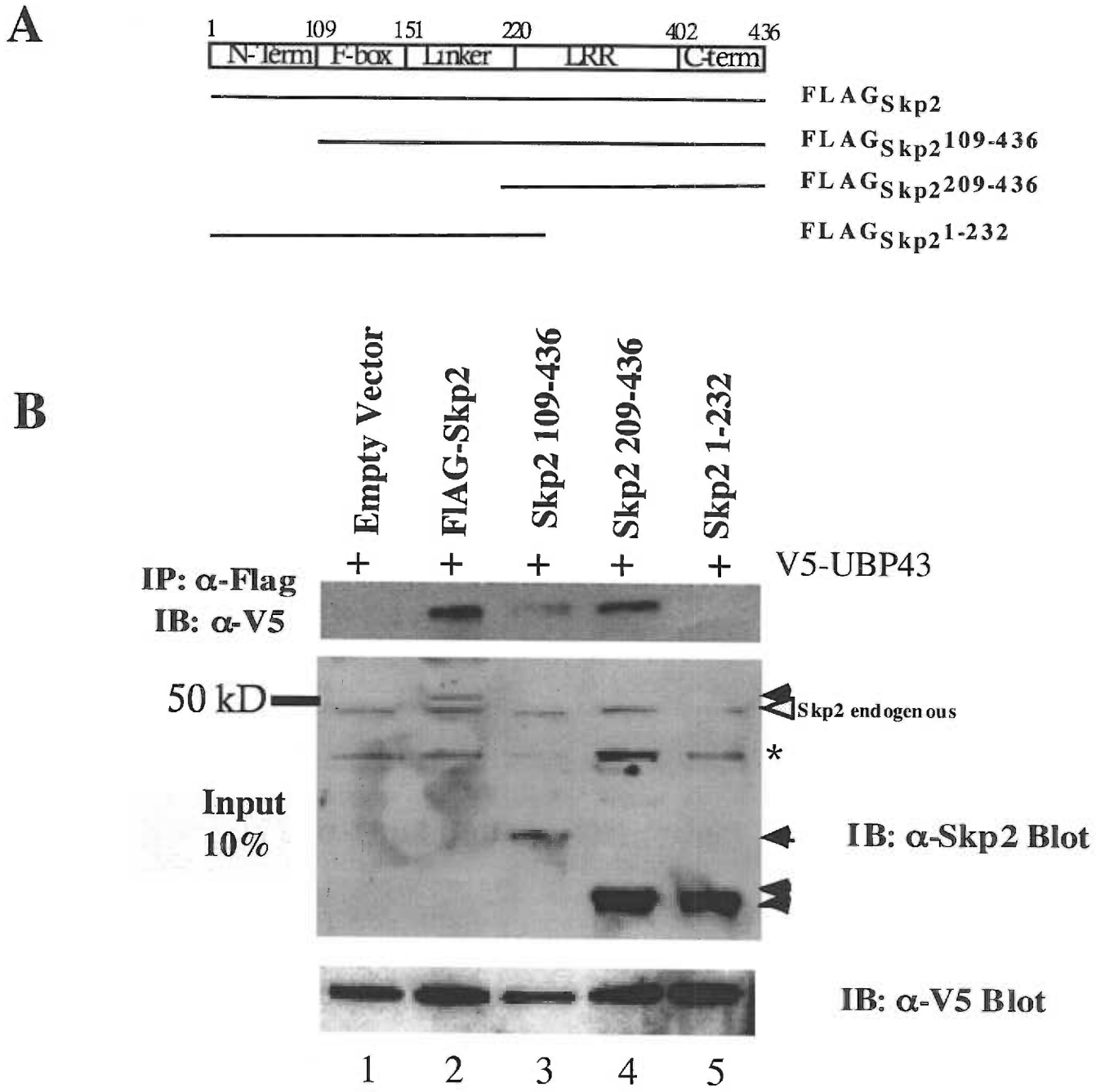
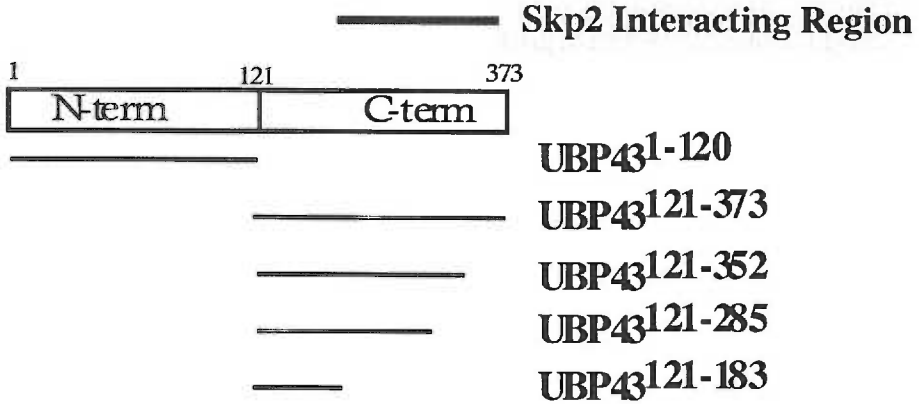

A
1
109
151
220
402
436
N-Term
F-box
Linker
LRR
C-term
FLAG Skp2
FLAG Skp2 109-436
FLAG Skp2 209-436
FLAG Skp2 1-232
B
Empty Vector
FlAG-Skp2
Skp2 109-436
Skp2 209-436
Skp2 1-232
+
+
+
+
+
V5-UBP43
IP: α-Flag
IB: α-V5
50 kD
Skp2 endogenous
*
Input
10%
IB: α-Skp2 Blot
IB: α-V5 Blot
1
2
3
4
5

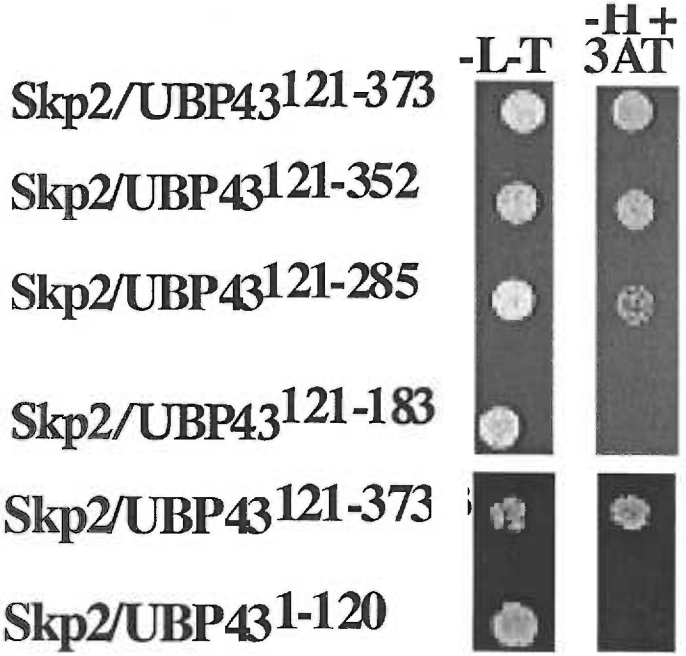
Figure 12. Interaction of UBP43 Deletion Mutants with Skp2. A) Diagram of UBP43 deletion constructs analyzed for Skp2 interaction in a yeast two-hybrid assay. B) Yeast two-hybrid interaction assay. Yeast strains expressing the indicated constructs were grown on restrictive media to assess protein interaction. Transformants were analyzed for transcriptional activation of *GAL4* driven HIS3 by assessing growth on media lacking histidine. *Left*, growth on –Leu-Trp plates (*-L-T*) *Right*, on -His plates containing 30 mM (3-AT) (*-H+3-AT*).

Figure 12

A


Skp2 Interacting Region
1
121
373
N-term
C-term
UBP43 1-120
UBP43 121-373
UBP43 121-352
UBP43 121-285
UBP43 121-183

B


-L-T
-H+ 3AT
Skp2/UBP43 121-373
Skp2/UBP43 121-352
Skp2/UBP43 121-285
Skp2/UBP43 121-183
Skp2/UBP43 121-373
Skp2/UBP43 1-120

cloned the constructs in yeast two-hybrid vectors and tested their ability to drive expression of nutritional markers when co-expressed with full-length Skp2 (Figure 12B). The portion of UBP43 containing amino acids 121 through 373 was originally pulled out in the yeast two-hybrid screen with full-length Skp2. As expected, this portion ($UBP43^{121-373}$), as well as one lacking the last 21 amino acids ($UBP43^{121-352}$), was able to grow on restrictive media. When the last 90 amino acids were deleted ($UBP43^{121-285}$), it appeared to reduce growth and when amino acids 184 to 373 were removed ($UBP43^{121-183}$), growth was abrogated completely. Moreover, the N-terminal 120 amino acids were also unable to interact and support growth. These data identify the Skp2-interacting domain of UBP43 as being between amino acids 183 and 352.

Discussion

I show here that both full length Skp2 and full length UBP43 are able to interact both *in vitro* and *in vivo*. In addition, I was able narrow down the putative interacting domains for each protein. Little is know about the domain and structure of UBP43. By determining UBP43's Skp2 binding region, we have identified a possible regulatory region for UBP43 contained within amino acids 183-373.

There is considerably more information known regarding the domains and structure of Skp2. Identifying the UBP43 binding region of Skp2 has given insight to the nature of their relationship. The LRR portion of F-box proteins has been implicated in substrate binding. For example, the 12 LRRs of Grr1 are essential for its binding to targets, Cln1 and Gic2 (Hsiung, Chang et al. 2001). In addition, the crystal structure of the SCF^{Skp2} shows that its 7 LRRs are positioned within 50 angstroms of the E2,

suggesting that this region binds the substrate (Zheng, Schulman et al. 2002). We show here that indeed the LRR region of Skp2 likely plays a role in binding to UBP43, suggesting that UBP43 may be a substrate of SCF^{Skp2} mediated ubiquitination.

Chapter V: Skp2 Down-Regulates UBP43 via Ubiquitin-Mediated Proteolysis

Introduction

Skp2 aids in the down-regulation of selected proteins by targeting them for ubiquitination and subsequent degradation. I previously determined that Skp2 likely binds UBP43 through a region typically involved in substrate binding, the LRR domain. Therefore I began by investigating the possibility that UBP43 is a substrate of SCF^{Skp2}-mediated ubiquitination and subsequent downregulation.

SCF-mediated ubiquitination classically causes rapid degradation by the 26S proteasome; however, ubiquitin modifications can also result in changes in protein function or activity. Substrate degradation is initiated by poly-ubiquitination, typically four or more ubiquitin molecules, while substrate activation/inactivation is usually achieved through mono-ubiquitination (Moren, Moustakas et al. 2003). As previously discussed, UBP43 mediates the cell stress signaling pathway initiated by interferon stimulation and viral or bacterial infection. UBP43 cleaves the Ubl, ISG15, from targets and attenuates the cell's response to these stressors (Ritchie, 2002; Malakhova, 2003). To date, Skp2 has not been directly implicated in the regulation of the cellular immune response. Determining what role Skp2 plays in the function and regulation of UBP43 will provide insight into Skp2's role in the cellular immune and stress responses.

Results

A. UBP43 is degraded by the 26S Proteasome

We began by looking at the effect of proteasome inhibition on steady-state UBP43 levels. Inhibition of the proteasome prevents 26S proteasome-dependent protein degradation and should increase levels of any protein subject to this form of regulation.

To determine whether UBP43 accumulates in cells that have been treated with a proteasome inhibitor, I transfected HEK293 cells with V5-UBP43 and treated the cells with the proteasome inhibitor (PI) MG132. Indeed, proteasome inhibition increased the steady state levels of UBP43 (Figure 13A, compare lanes 1 and 2). I also measured the half-life of UBP43 by pulse/chase analysis. For these experiments, rat fibroblasts (REF52) were infected with an adenovirus expressing V5-UBP43 (Ad-V5-UBP43) generated as described in Materials and Methods. As shown in figure 13B, proteasome inhibition with MG132 increased the stability of UBP43 (right panel) when compared to no inhibition (left panel).

B. Skp2 Expression Down-Regulates UBP43 Protein Levels

I then determined the effects of altered Skp2 expression on both the steady state levels and the half-life of UBP43. As a substrate of Skp2, both the steady state levels and half-life of UBP43 should decrease in the presence of Skp2. To assess the influence of Skp2 on steady state UBP43 levels, I expressed ectopic Skp2 and V5-UBP43 in HEK293 cells. UBP43 levels were strongly reduced when Skp2 was over-expressed (Figure 14A, lane 2), compared with UBP43 expressed alone (lane 1). (UBP43 is detected as a doublet as a result of a post-translational modification, discussed later.) To test whether levels of UBP43 are increased in cells that are devoid of Skp2, I performed immunoblotting of primary, low-passage MEF extracts derived from wild-type or Skp2 -/- mice. As shown in Figure 14B, UBP43 levels are significantly increased in extracts lacking Skp2 (lane 2) compared to wild type (lane 1). Note that

Figure 13. UBP43 is degraded by the 26S proteasome A. HEK293 cells were transfected with an expression construct for V5-UBP43 and treated with either the proteasome inhibitor MG132 (10 μM) or Me2SO (DMSO) seven hours before cell harvest. The *asterisk* indicates a loading control, cdk2. B. Pulse-chase analysis of Ad-V5-UBP43 infected REF52 cells. Proteasome Inhibitor (PI) MG132 was added seven hours prior to harvest, where indicated. Arrowhead indicates V5-UBP43 and the asterisk marks a non-specific band.

Figure 13

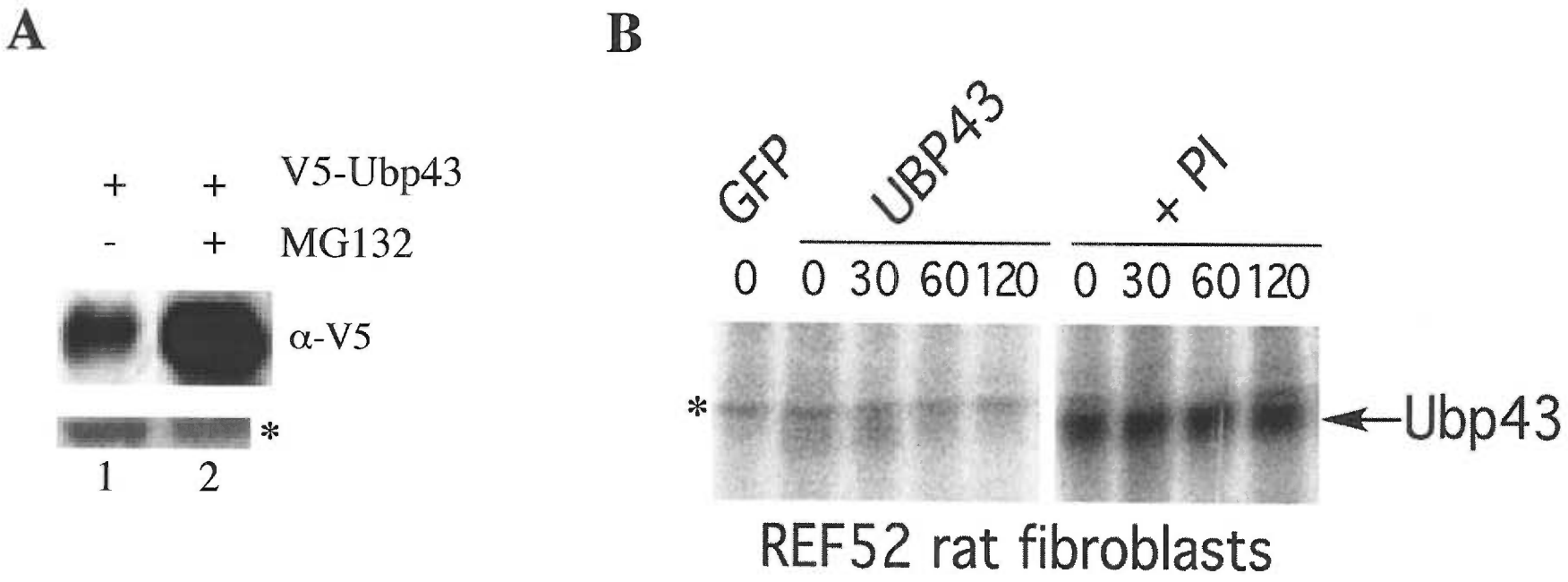
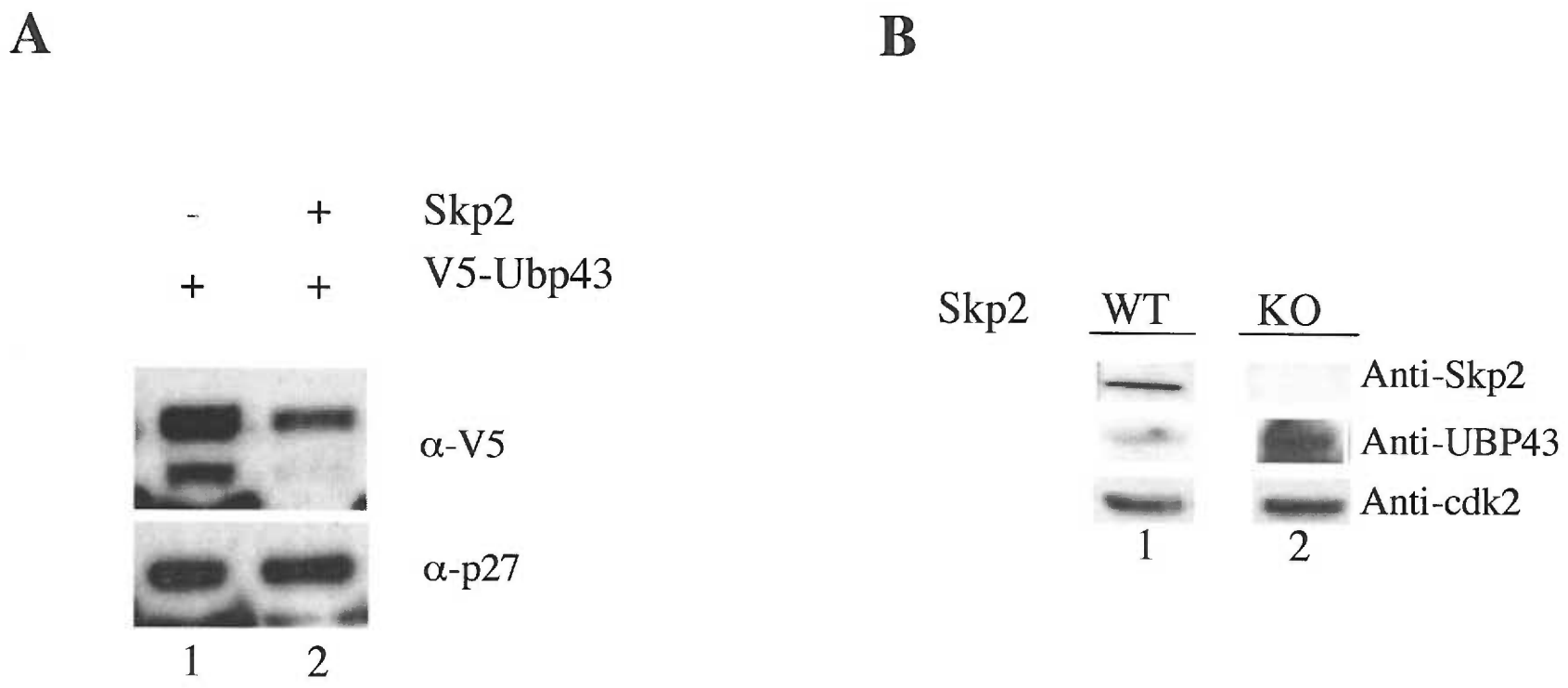

A
+ + V5-Ubp43
- + MG132
α-V5
*
1 2
B
GFP
UBP43
+ PI
0 0 30 60 120 0 30 60 120
*
Ubp43
REF52 rat fibroblasts

Figure 14. Skp2 Down-regulates UBP43 Protein Levels. A. UBP43 steady state levels are modulated by Skp2 levels. HEK293 cells were transfected with V5-UBP43 and either control vector (-) or Skp2. Extracts were subjected to Western blot analysis with indicated antibodies. p27 was used as a loading control. UBP43 is seen as a doublet here. B. The absence of Skp2 results in increased UBP43 levels. Extracts from asynchronous, low-passage, primary Skp2 wild-type and -/- MEFs were immunoblotted with antibodies specific to the indicated proteins. UBP43 antibody was a gift from Dr. Dong Er-Zhang. cdk2 is used as a loading control.

Figure 14


A
- + Skp2
+ + V5-Ubp43
α-V5
α-p27
1 2
B
Skp2 WT KO
Anti-Skp2
Anti-UBP43
Anti-cdk2
1 2

UBP43 levels are very low in un-stimulated wild-type cells and are hardly detectable with the available antibody. We conclude that Skp2 levels inversely affect UBP43 levels.

C. There is an Inverse Relationship Between Skp2 and UBP43 Expression Levels in Cycling cells

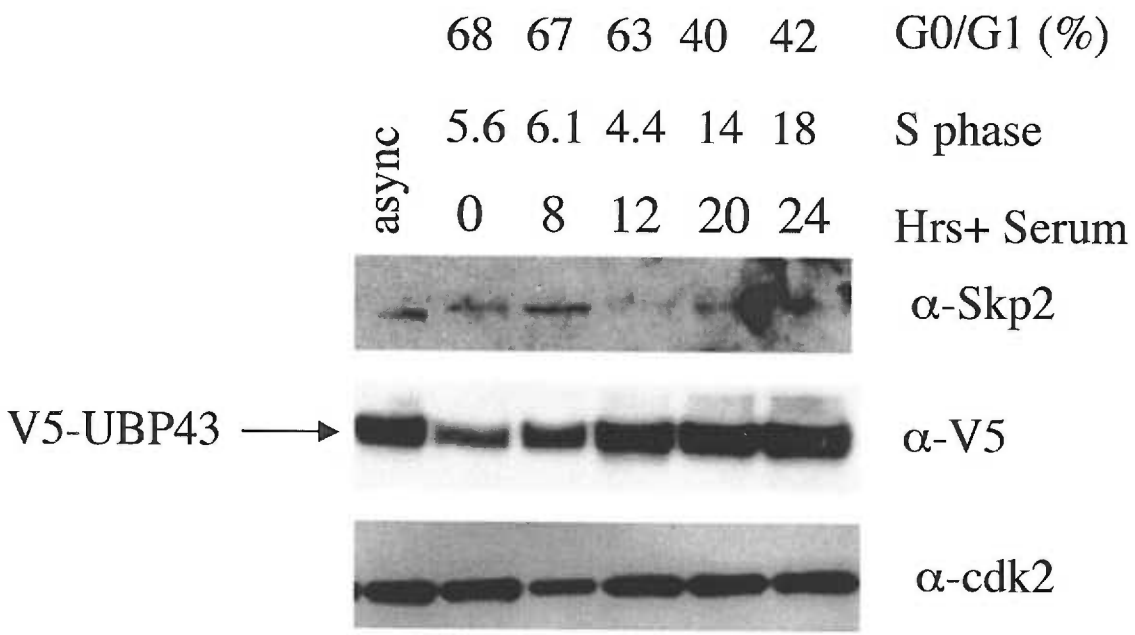
Skp2 protein levels fluctuate throughout the cell cycle. Levels are low in G_0 and early G_1, peak at the G_1/S transition, and then decline rapidly in late M phase (Carrano and Pagano, 2001). I would expect UBP43 levels to decrease as Skp2 levels rise in G_1 and S phase and inversely increase as Skp2 levels fall in mitosis.

For cell cycle analysis, I used A549 cells that had been synchronized at G_0. Cells were starved for 36 hours in low-serum media (.2% FBS,F12, 1X pen/strep, 1X L-glut) and then synchronously stimulated to enter the cell cycle with 15% serum stimulation. The percentage of cells in G_0/G_1 and S phase was determined by propidium iodide staining and FACS analysis. Propidium iodide intercalates the DNA helix and fluoresces upon excitation with 488nm light. Using this method, one can determine the relative DNA amount per cell and therefore assess the cell cycle position.

As shown in Figure 15, Skp2 expression was atypical in these cells, most likely because the cell lines were cancer-derived and therefore Skp2 levels were de-regulated. We observed that Skp2 levels were high in G_0 and early G_1, and decreased in late G_1 (Figure 15). However, we do find that when Skp2 levels are high UBP43 levels are relatively low as I would expect from my previous data showing that Skp2 protein levels negatively affect UBP43 levels.

Figure 15. Skp2 and UBP43 levels are inversely proportional in cycling cells. A549 cells were infected with Ad-V5-UBP43, synchronized and harvested at indicated time points after serum stimulation. Extracts were analyzed by both FACS analysis using propidium Iodide, to determine cell cycle phase, and by western blot with indicated antibodies. The percentage of cells in each cell cycle phase are indicated above. An asynchronous (async) sample was loaded for comparison. cdk2 levels remain fairly constant throughout the cell cycle and were used as a loading control.

Figure 15


68 67 63 40 42
G0/G1 (%)
5.6 6.1 4.4 14 18
S phase
async
0 8 12 20 24
Hrs+ Serum
α-Skp2
V5-UBP43
α-V5
α-cdk2

D. Skp2 Expression Decreases the Half-Life of UBP43

These results suggested that UBP43 levels are controlled by SCF^{Skp2}-mediated degradation via the ubiquitin-proteasome pathway. To measure UBP43 degradation rates *in vivo*, we performed pulse-chase experiments in Ad-V5-UBP43 infected Skp2 +/+ and -/- primary MEFs. The half-life of UBP43 was approximately 50 minutes in Skp2 wild-type cells (Figure 16A, lanes 1-5; 16B, squares) compared to 120 minutes in knockout cells (Figure 16A, lanes 7-11; 16B, triangles). When Skp2 was re-expressed in the Skp2 -/- cells, levels of UBP43 dropped dramatically at the 15 min time point, and then remained at low levels for the rest of the chase period (Figure 16A, lanes 12-16; 16B, inverted triangles). The GFP control lane (Figure 16A, lane 6) clearly demonstrates the specificity of the UBP43 band. In addition, cycloheximide treatment of cultures followed by analysis of UBP43 protein levels confirmed the pulse-chase results (data not shown). These data strongly support a model in which Skp2 reduces the half-life of UBP43, most likely by targeting it for ubiquitin-mediated proteolysis.

E. Skp2 Induces Poly-Ubiquitination of UBP43

Proteasomal degradation of proteins is triggered by multi-ubiquitination of targeted polypeptides. Ubiquitination is a covalent modification, and therefore ubiquitinated species of UBP43 should appear as higher molecular weight bands in SDS-PAGE. I infected REF52 cells with a constant amount of Ad-V5-UBP43 and increasing amounts of Ad-Flag-Skp2 (Figure 17). Under proteasomal inhibition with MG132 and lactacystin, covalently modified, high-molecular weight bands of UBP43 can be seen

Figure 16. UBP43 half-life increases in Skp2-null cells A. UBP43 has increased stability in Skp2-/- MEFs. Skp2+/+ and Skp2-/- MEFs (passage 4) were infected with Ad-V5-UBP43 adenovirus and, where indicated, with Flag-Skp2 adenovirus, followed by pulse-chase analysis. B. Graphical representation of A. Squares, dashed line, Skp2+/+ MEFs; triangles, solid line, Skp2-/- MEFs; inverted triangles, dotted line, Skp2-/- MEFs infected with Ad-Flag-Skp2 and its transactivator Ad-Tet.

Figure 16

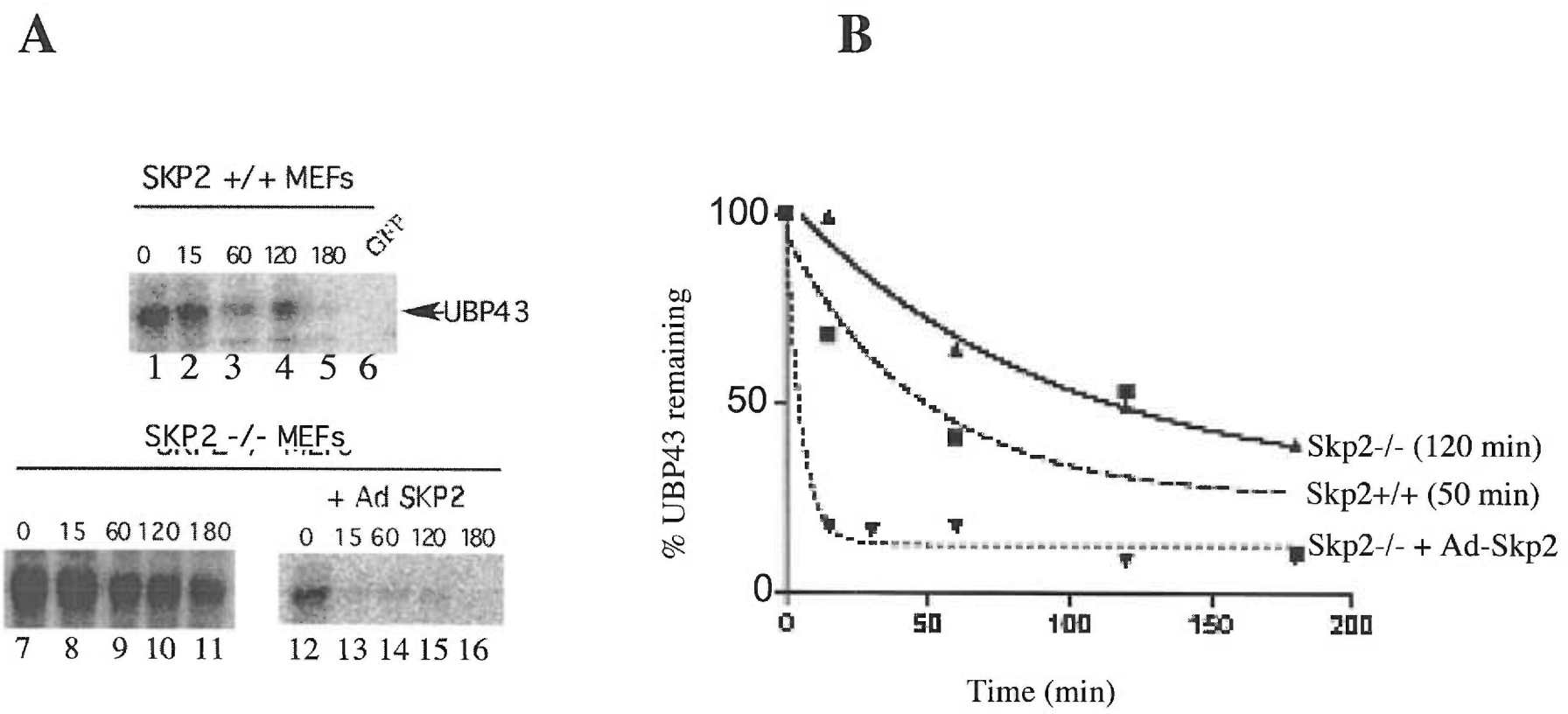
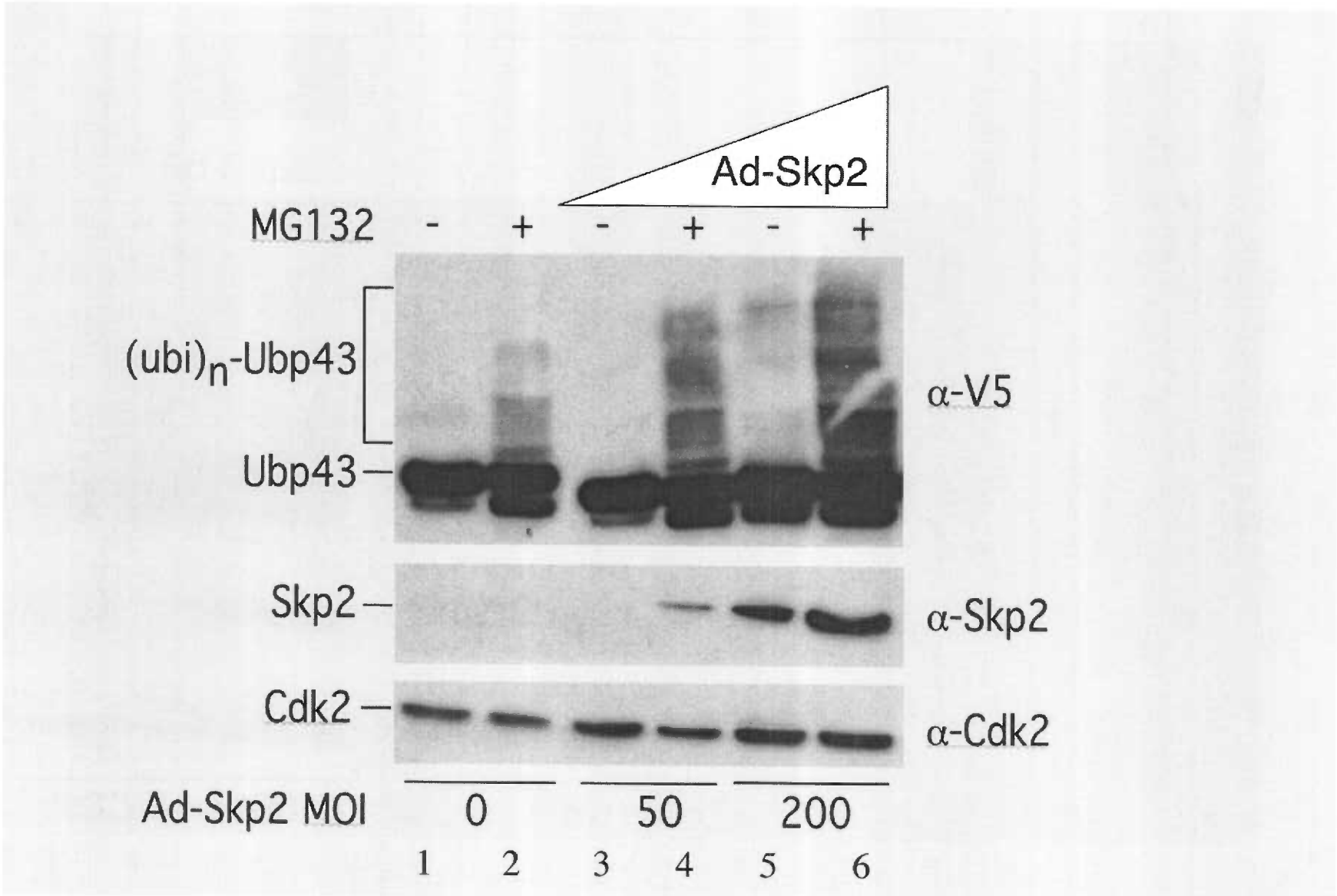

A
SKP2 +/+ MEFs
0 15 60 120 180 GFP
UBP43
1 2 3 4 5 6
SKP2 -/- MEFs
+ Ad SKP2
0 15 60 120 180
0 15 60 120 180
7 8 9 10 11
12 13 14 15 16
B
% UBP43 remaining
100
50
0
0 50 100 150 200
Time (min)
Skp2-/- (120 min)
Skp2+/+ (50 min)
Skp2-/- + Ad-Skp2

Figure 17. Skp2 expression increases high-molecular-weight conjugates of UBP43. A. REF52 cells were infected with constant amounts of adenovirus expressing V5-UBP43 or GFP and increasing amounts of adenovirus expressing Ad-Flag-Skp2 in the presence or absence of proteasome inhibitor, PI (5 μM MG132, 5 μM lactacystin). Lysates were subjected to Western blot analysis with indicated antibodies.

Figure 17

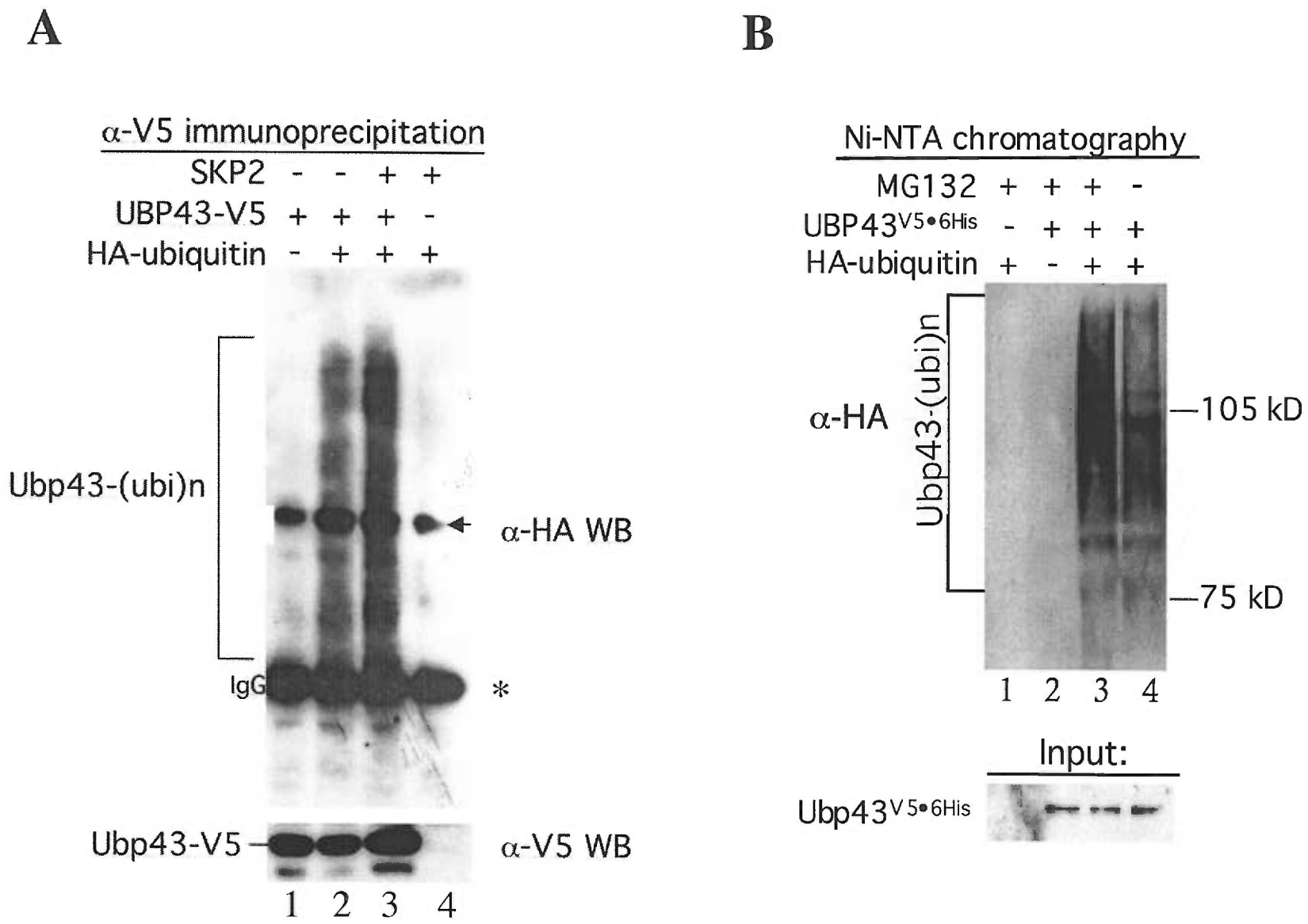

Ad-Skp2
MG132 - + - + - +
(ubi)n-Ubp43
Ubp43
α-V5
Skp2
α-Skp2
Cdk2
α-Cdk2
Ad-Skp2 MOI 0 50 200
1 2 3 4 5 6

(Figure 17, lanes 2, 4, and 6). These modified forms increase in both amount and molecular weight with increasing amounts of Skp2.

To determine if these modified forms of UBP43 were the result of ubiquitination, I transfected HEK293 cells with plasmids that expressed Skp2, V5- UBP43, and HA-ubiquitin. I immunoprecipitated for UBP43 using the V5 epitope and probed for HA to identify ubiquitinated species. UBP43 does appear to be ubiquitinated as seen by the high-molecular-weight smear in the presence of HA-ubiquitin (Figure 18A, lane 2). These high-molecular-weight bands were intensified upon overexpression of Skp2 (Figure 18A, lane 3). The specificity of this smear as ubiquitinated UBP43 is supported in the control lanes lacking either HA-ubiquitin or V5-UBP43 (lanes 1 and 4). In these experiments, the amount of transfected UBP43 is high compared to Skp2 levels, and therefore we did not observe a significant reduction in UBP43 steady-state levels upon Skp2 co-transfection. If these high-molecular-weight bands correspond to ubiquitinated UBP43 species, then inhibition of the proteasome should result in an increase in the intensity, and possibly a shift to higher molecular weights, of this smear. To test this, I transfected A549 cells with indicated vectors. His-V5-tagged UBP43 was captured on Ni-NTA beads under denaturing conditions, followed by immunoblotting against the HA tag on ubiquitin. As seen in the previous panel, ubiquitinated UBP43 is seen by the HA immuno-reactive smear in lanes 3 and 4 (Figure 18B) which is not present in the absence of either ectopic ubiquitin or UBP43 (lanes 1 and 2). Furthermore, proteasome treatment did result in both an overall increase in the amount as well as the molecular weight of multi-ubiquitinated UBP43 (compare lanes 3 and 4, Figure 18B). From these results, we conclude that UBP43 is ubiquitinated *in vivo* and that UBP43 ubiquitination is enhanced

Figure 18. UBP43 is ubiquitinated in a Skp2-dependent manner A. Extracts from HEK293 cells transfected with UBP43-V5 (lanes 1–3), Skp2 (lanes 3 and 4), and HA-ubiquitin (lanes 2–4) were immunoprecipitated with anti-V5 antibody, separated by SDS-PAGE, and analyzed with the indicated antibodies. The asterisk marks the heavy chain IgG band. The arrow indicates a non-specific band. B. A549 cells were transfected with UBP43-V5-6HIS (lanes 2–4) and HA-ubiquitin (lanes 1, 3, and 4). Proteasome inhibitor, 5 μM MG132, was added 5 hours before harvest. Extracts were purified using nickel-nitrilotriacetic acid (Ni-NTA) beads under denaturing conditions (8 M urea) to capture UBP43-V5-6HIS. Eluates were separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies.

Figure 18


A
α-V5 immunoprecipitation
SKP2 - - + +
UBP43-V5 + + + -
HA-ubiquitin - + + +
Ubp43-(ubi)n
α-HA WB
IgG
*
Ubp43-V5
α-V5 WB
1 2 3 4
B
Ni-NTA chromatography
MG132 + + + -
UBP43V5•6His - + + +
HA-ubiquitin + - + +
α-HA
Ubp43-(ubi)n
105 kD
75 kD
1 2 3 4
Input:
Ubp43V5•6His

by increasing levels of Skp2 (Figure 17 and 18A).

Because SCF substrates are often recognized by phosphorylation events, we examined the phosphorylation status of UBP43. V5-UBP43 was expressed in A549 cells and extracts were prepared with and without phosphatase inhibitors. Upon treatment with calf intestinal phosphatase (CIP) to remove any phosphorylation modifications, the lower band of the UBP43 doublet begins to disappear (Figure 19, compare lanes 1 & 2 and 4 & 5). However, if phosphatase inhibitors are included with the CIP treatment, the intensity of the lower band does not change (Figure 19, lanes 3 and 6). Typically, we would expect the higher-molecular-weight band to represent the phosphorylated form; however, phosphorylation can result in a more rapidly migrating band by affecting the ability of SDS to fully denature the protein. Alternatively, the upper band could represent a form that has additional modifications.

Discussion

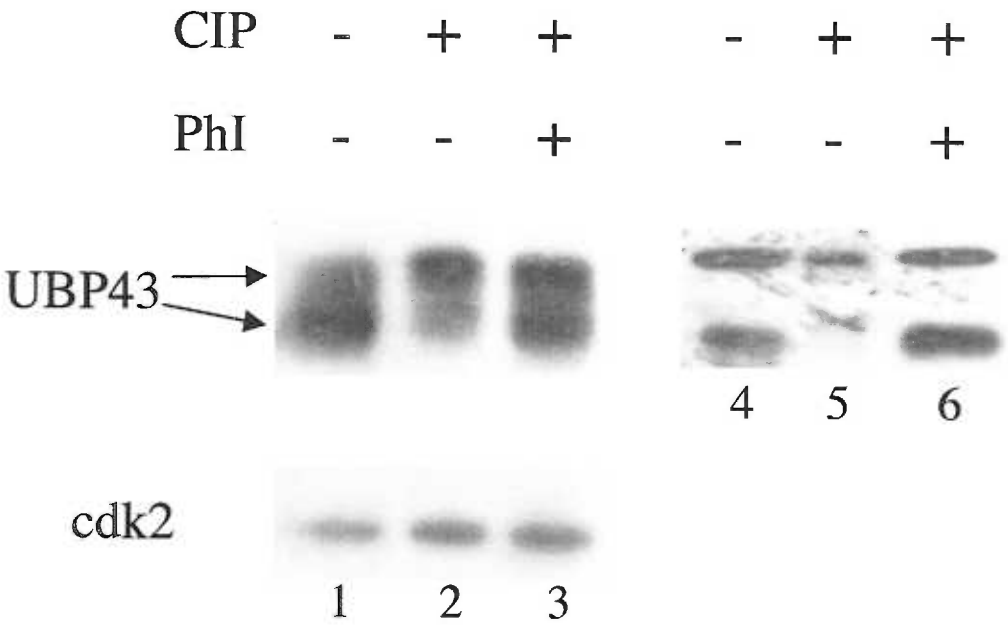
We show here, through analysis of protein degradation rates and ubiquitination assays, that UBP43 is a substrate of SCF^{Skp2}-mediated ubiquitination and subsequent degradation. In Skp2-/- cells UBP43 levels were up-regulated and over-expression of Skp2 decreased steady-state levels of ectopic UBP43. I also show here that ectopic UBP43 levels are inversely proportional to endogenous Skp2 in synchronized, cycling cells.

Skp2 expression also appears to negatively affect the half-life of UBP43. Skp2 null MEFs show an increased half-life for UBP43. When Skp2 is re-introduced and over-

expressed, we see UBP43 stability rapidly decrease suggesting that Skp2 is the major regulator of UBP43. However, we see that even in Skp2 null cells UBP43 is still degraded over time, suggesting that Skp2 is not the sole mechanism for regulating UBP43 protein levels.

Figure 19. UBP43 is modified by phosphorylation. Extracts from A549 cells transfected with V5-UBP43 were harvested in lysis buffer with or without phosphatase inhibitors. Samples were then treated with either calf intestinal phosphatase (CIP) alone or both CIP and phosphates inhibitors (PhI). Samples were run on an SDS-PAGE gel and probed with indicated antibodies. Arrows indicate UBP43 doublet.

Figure 19



Chapter VI: Physiological Role of Skp2-mediated Down Regulation of UBP43

Introduction

UBP43 cleaves the ubiquitin-like modification, ISG15 from proteins. ISG15 conjugation can be stimulated by treating cells with either LPS (mimicking bacterial infection) or IFN α/β (Liu, Reimschuessel et al. 2002; Malakhova, Malakhov et al. 2002). Type I IFNs are present at low levels in unstressed cells and are rapidly induced in response to cell stress including viral and bacterial infections. Free ISG15 levels rise at about eight hours post IFN induction, followed by an increase in ISG15 conjugates at about 18 hours (Loeb, Haas, 2001). Skp2 may help modulate this pathway by regulating UBP43 levels, and consequently ISG15 conjugation (Figure 20). Consequently, I would expect a change in ISG15 conjugation patterns and the cell's physiological response to interferon in the absence of Skp2.

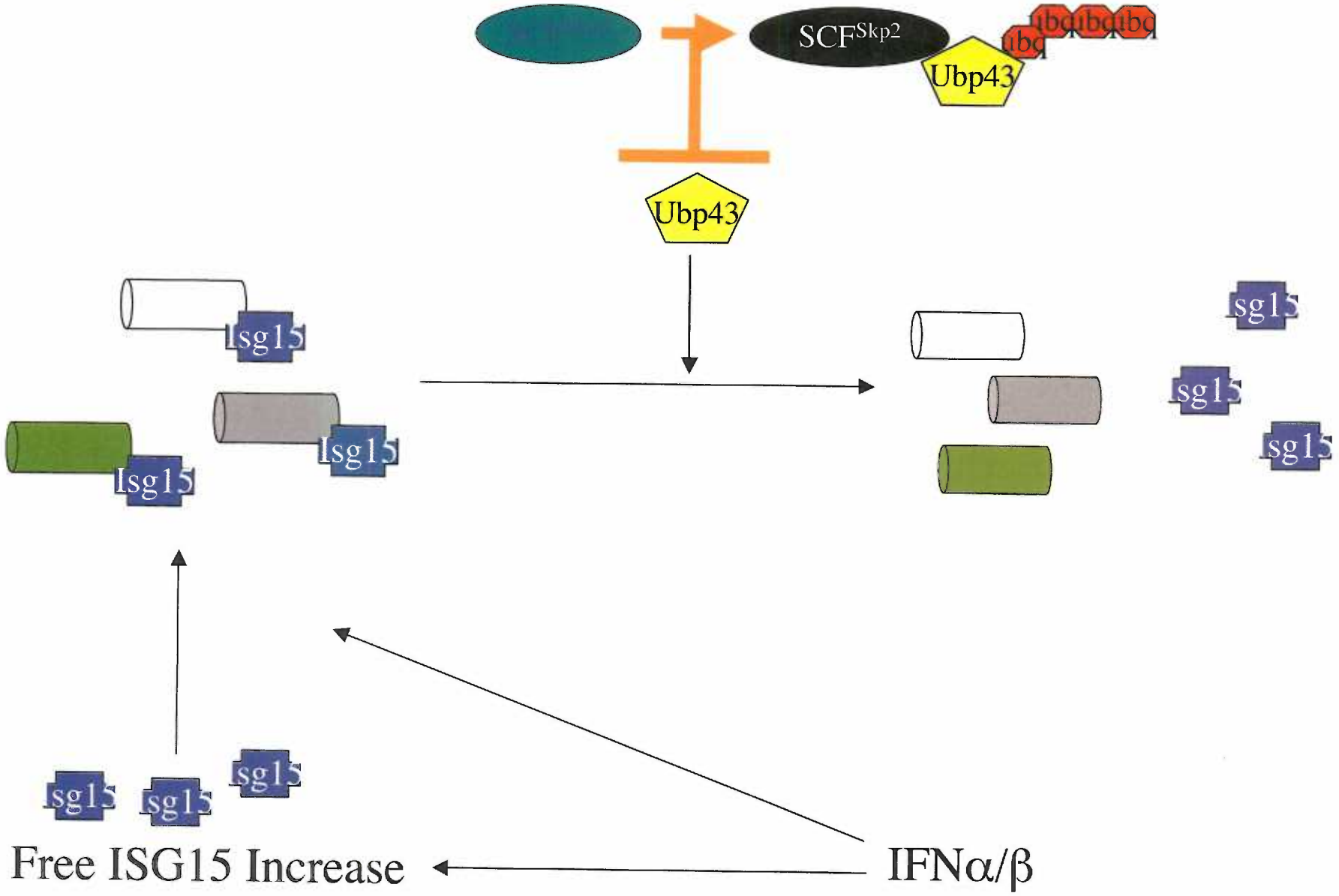
Results

A. Skp2 Expression Affects the Activity of UBP43 as Measured by ISG15 Conjugation

To assess the effects of Skp2 on UBP43 function, we looked at ISG15 conjugation. Using an antibody that specifically recognizes ISG15, we looked at conjugation in LPS/IFN-induced cells over-expressing Skp2 or null for Skp2. As shown in Figures 21 and 22, Skp2 did appear to positively influence ISG15 conjugation in stimulated cells. First, in LPS-treated Skp2 -/- cells, free ISG15 levels are remarkably increased compared to wild type (Figure 21; compare lanes 1 and 2 to 3 and 4) suggesting that these cells have impaired ISG15 conjugation. When looking at ISG15 conjugates,

Figure 20. Hypothetical model of Skp2 regulation of ISG15 conjugation. IFNα/β induces an increase first in free ISG15, and then in ISG15 conjugates; UBP43 modulates this response by cleaving ISG15 from conjugates, thus increasing free ISG15 and decreasing conjugates. Skp2 in turn regulates UBP43 activity by down-regulation. High Skp2 would result in increased ISG15 conjugates, while low levels would promote an increase in free ISG15

Figure 20


SCF^{Skp2}
Ubp43
Ubp43
Isg15
Isg15
Isg15
Isg15
sg15
sg15
sg15
sg15
sg15
Free ISG15 Increase
IFNα/β

levels are greatly reduced in Skp2 -/- cells due to increased UBP43 cleavage of ISG145 (Figure 22A). Conversely, overexpression of Skp2 had the opposite effects. When I overexpressed Skp2 in A549 cells that expressed ectopic V5-UBP43 and ISG15, I found greatly increased ISG15 conjugation due to UBP43 down-regulation (Figure 22B).

B. Skp2-Null Cells are Insensitive to IFN Treatment

I looked at a growth curve of IFN α/β treated cells with or without Skp2 to assess the role of Skp2 in the IFN α/β pathway. Interferon has strong anti-proliferative effects on cells and can also induce apoptosis (Stark, Schreiber, 1998). The proliferation of Skp2 MEFs (immortalized with SV40) was monitored in IFN-treated cells compared to untreated cells. Cell numbers of IFN treated cells at each time point were divided by the cell numbers in the untreated sample of the same time point. A ratio of one indicates that both samples are proliferating at equal rates, while a ratio of less than one indicates that IFN-treated samples have decreased proliferative potential compared to untreated samples. In Figure 23, we see that, as expected, in wild-type cells, IFN causes a reduction of proliferation, evident by a decrease in the growth ratio of IFN-treated cells compared to untreated cells. In contrast, Skp2 null cells show little difference in cell

Figure 21. Absence of Skp2 results in increased free ISG15 levels. LPS was added 7 hours prior to harvest. Extracts from asynchronous, low-passage primary Skp2 wild-type (+/+) or knockout (-/-) MEFs were immunoblotted with antibodies specific to the indicated proteins. Free ISG15 is strongly induced in Skp2 null cells (lane 4) compared to a relatively small induction in Skp2 wild type MEFS (lane 2). cdk2 is used as a loading control.

Figure 21

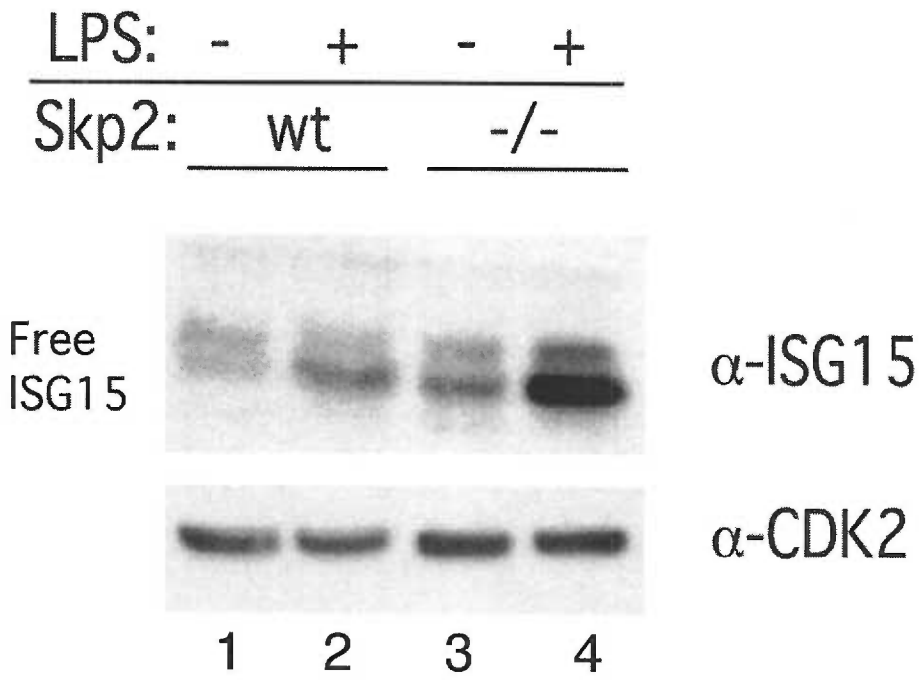
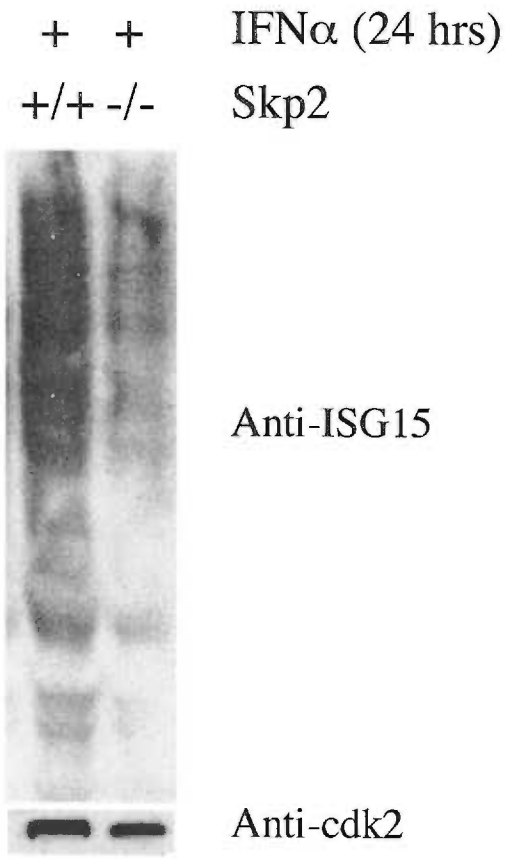


Figure 22. Skp2 Affects ISG15 Conjugation. A. Asynchronously growing wild type or Skp2-/- MEFs were treated with 1000 units/ml of mouse IFN-α for 24 hours, and extracts were analyzed for ISG15 conjugation using the anti-ISG15 antibody. B. Skp2 affects ISG15 conjugate levels. A549 cells were transfected with indicated vectors and stimulated with 500 units/ml of human IFN-β. Extracts were analyzed for ISG15 conjugation using an affinity-purified polyclonal anti-ISG15 antibody (gift from Arthur Haas).

Figure 22

A

SV40 Transformed MEFs



B

A549 Lung Cell Carcinomas

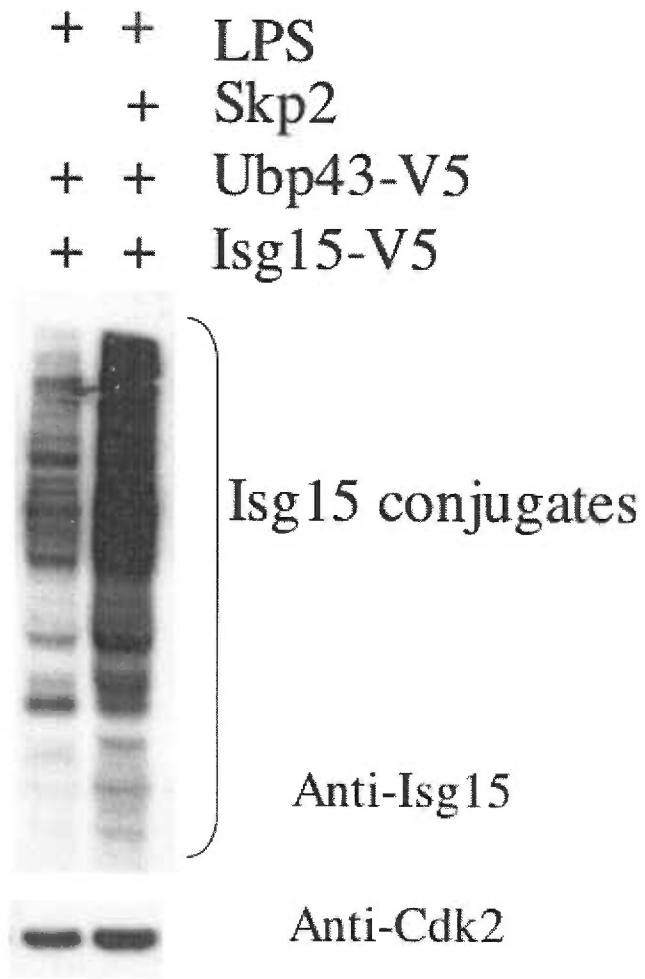
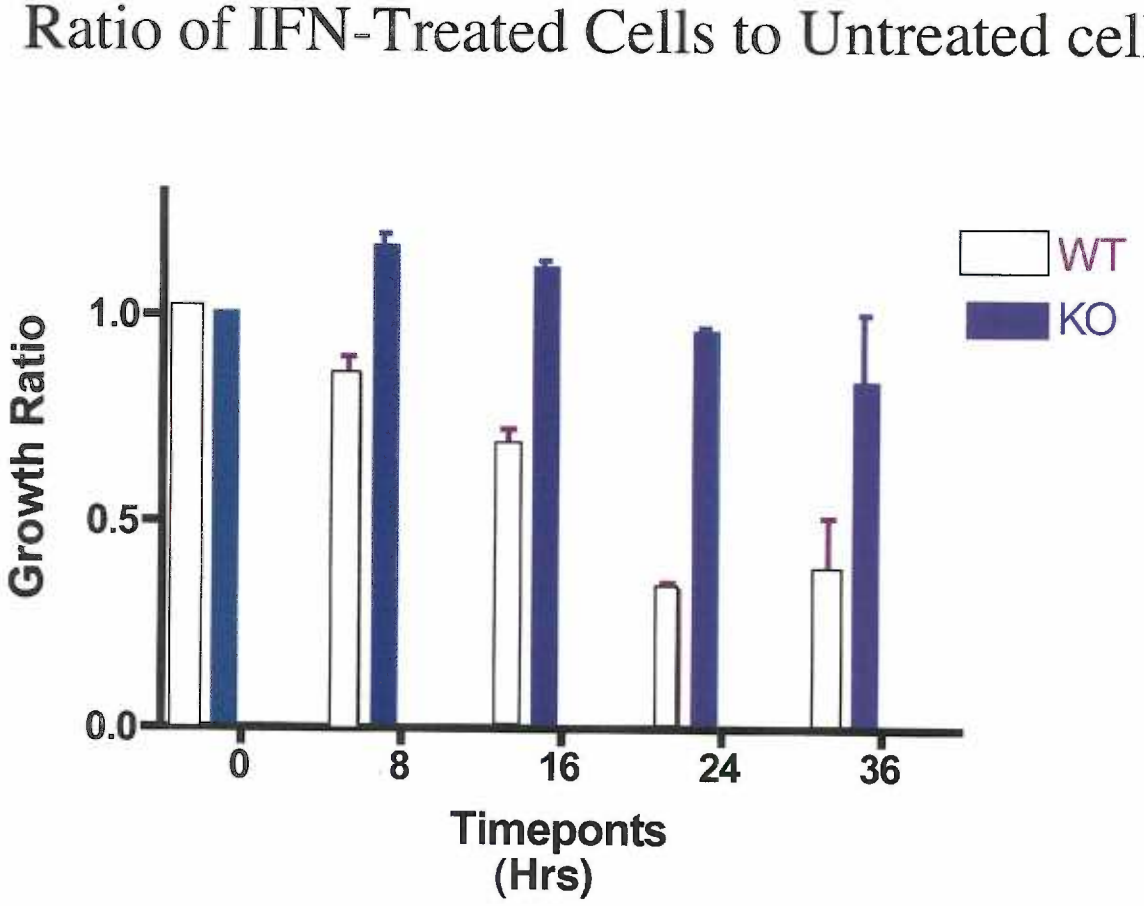


Figure 23. Skp2 null cells are insensitive to IFN-β treatment. Asynchronously growing wild-type or Skp2-/- MEFs were treated with 500 units/ml mouse IFN-β and viable cells were counted at the indicated time points. Growth ratio is defined as number of cells with interferon treatment divided by number of cells without interferon treatment.

Figure 23


Ratio of IFN-Treated Cells to Untreated cells
WT
KO
Growth Ratio
1.0
0.5
0.0
0
8
16
24
36
Timeponts
(Hrs)

proliferation, suggesting that Skp2 null cells are insensitive to the anti-proliferative effects of IFN treatment.

Discussion

UBP43 and ISG15 are both IFN regulated genes. The implication of Skp2 in the regulation of UBP43 suggests that the absence of Skp2 could affect ISG15 conjugation and the cell's response to IFN treatment.

Indeed, levels of free ISG15 are high in Skp2 -/- cells compared to wild-type cells. We hypothesized that this was due to a loss of regulation of UBP43 and subsequent increase in cleavage of ISG15 conjugates (Figure 20). This is supported by the fact that ISG15 conjugates are reduced in IFN-stimulated cells lacking Skp2. Likewise, over-expression of Skp2 caused a dramatic increase in ISG15 conjugation in response to IFN treatment, probably as a result of UBP43 degradation.
when Skp2 is over expressed. These data support Skp2 as being an F-box protein that down-regulates UBP43 protein levels and thus regulates UBP43 function.

I also show that Skp2 null cells demonstrate IFN insensitivity in relation to cell proliferation. ISG15 conjugation is known to prolong IFN signaling, and the apparent insensitivity of Skp2 -/- cells to IFN stimulation is probably due in part to increased ISG15 cleavage as a result of deregulation of UBP43. It should be noted that IFN treatment, in addition to thwarting cell proliferation, also increases cellular apoptosis. I did not notice a significant decrease in cell numbers in IFN treated cells with or without Skp2, nor did the cells demonstrate apoptotic phenotypes. I conclude therefore that the

majority of the effect is due to decreased proliferation.

Chapter VII: Conclusions and Discussion

A. Conclusions

In this dissertation, I describe a novel target of SCF^{Skp2}-mediated ubiquitination, UBP43. UBP43 was identified as a putative interactor in a yeast two-hybrid screen using full length Skp2 (Figure 7). Further investigation revealed that Skp2 also interacts with UBP43 *in vitro*, using a GST pull-down (Figure 10A), and *in vivo*, through a mammalian co-immunoprecipitation (Figure 10B). I show that Skp2 binding to UBP43 requires a region containing the leucine-rich repeat (LRR) (Figure 11B), a known substrate-binding region present in some F-box proteins. Furthermore, I show that the Skp2 interacting region of UBP43 is between amino acids 183 and 352 (Figure 12A), identifying a possible regulatory domain of UBP43.

In addition, I show that the SCF^{Skp2} ubiquitin ligase controls UBP43 protein levels by ubiquitin-mediated degradation via the 26S proteasomal pathway. My data show that Skp2 protein levels negatively affect UBP43 protein levels and half-life (Figure 14 A and B, Figure 15, and Figure 16). In addition, Skp2 promotes UBP43 poly-ubiquitination, resulting in UBP43 degradation via the 26S proteasome (Figure 18 A and B). Finally, I show evidence that UBP43 is phosphorylated, a common modification in SCF targets, which triggers their recognition by the F-box protein (Wirbelauer, Sutterluty et al. 2000; Ganoth, Bornstein et al. 2001; Hsiung, Chang et al. 2001; Spruck, Strohmaier et al. 2002). Whether this phosphorylation is important for binding to Skp2 will require further investigation.

UBP43 cleaves ISG15 from substrates, and consequently, reduced UBP43 results in increased ISG15 conjugation. I show that in MEFs lacking Skp2, levels of free ISG15

are high, and ISG15 conjugates are low, consistent with increased UBP43 levels (Figure 21). Conversely, high levels of Skp2 result in the down-regulation of UBP43 and in an increase in ISG15-conjugated proteins (Figure 22 A and B). Lastly, Skp2 did appear to play a role in the cellular response to IFN stimulation. Skp2-null cells appeared to be insensitive to the anti-proliferative effects of IFN treatment compared to wild-type cells (Figure 23). ISG15 conjugation plays a role in propagating the IFN response, and this was most likely disrupted in Skp2-null cells due to unregulated ISG15 cleavage.

B. The Importance of Skp2 Regulation of UBP43

In this dissertation, I describe a novel function for Skp2 that could possibly impact modulation of IFN signaling. Cellular levels of UBP43 are controlled at both the level of transcription, which is induced by LPS and IFN type 1 induction and, as described here, by post-translational ubiquitin modification that targets it for degradation (Kang, Jiang et al. 2001; Kang, Yi et al. 2001; Malakhov, Malakhova et al. 2002). Skp2 down-regulation of UBP43 thus affects ISG15 conjugation activity in the cell.

The biological function of ISG15 modification is not well understood, but it has been implicated in a wide variety of cellular functions, including cellular immunity, malignant transformation, and brain development. Free ISG15 acts as a cytokine that appears to play a role in the activation of immune cells. ISG15 stimulates the production of IFN-γ and other cytokines in $CD3^+$ lymphocytes. These cytokines in turn stimulate a number of other immune pathways, including the activation of monocyte cytotoxicity and upregulation of natural killer (NK) cell proliferation. In addition, free ISG15 added to the media of human melanoma cell lines induces upregulation of E-cadherin in co-cultured

dentritic cells (Padovan, Bolli et al, 2002). This could possibly impede immune surveillance of tumors by inhibiting dendritic cell migration.

ISG15 conjugation appears to play a role in regulating IFN signaling. Few targets of ISGylation are known; however, some, including Stat1 and Jak1, are known. Stat1 and Jak1 are both modulators of the IFN signaling pathway and conjugation of ISG15 to Stat1, an IFN-responsive transcription factor, is believed to prolong IFN signaling. UBP43 knockout mouse embryonic fibroblasts (MEFs), in which ISG15 cleavage is strongly reduced, demonstrate prolonged Stat1 signaling and increased activation of IFN-stimulated genes (Ritchie, Malakhova, et al. 2003). Consequently, UBP43 -/- MEFs demonstrate a hypersensitivity to PolyI/C treatment, a potent inducer of IFN production. Specifically, Malakhova et al. have shown that 90% of UBP43 null mice treated with PolyI/C die, while their wild-type littermates were resistant to the treatment (Malakhov, Kim et al. 2003). In these knock mice, ISG15 cleavage from Stat1 is inhibited, prolonging Stat1 phosphorylation and subsequent expression of interferon-stimulated genes. The inability of these cells to cleave ISG15 conjugates disrupts the ability of the cell to down-regulate IFN signaling, causing IFN hypersensitivity. Finally, a human leukemia cell line, K562, void of the E1 enzyme for ISG15, UBE1L, and therefore deficient in ISG15 conjugation, shows an increased in IFN-stimulated genes when UBE1L is restored (Malakhova, Kim et al. 2003).

In addition, UBP43, ISG15 and the E1 enzyme for ISG15, UBE1L, have all been shown to be deregulated in various malignant cell lines, suggesting that the lack of ISG15 conjugation contributes to malignant transformation (Malakhova, Kim et al, 2003)

Carefully controlled ISG15 conjugation and deconjugation to substrates is crucial

for the health of a cell and of an organism. Indeed, mice lacking UBP43 are short-lived, develop neuronal injury, exhibit hypersensitivity to IFN, and demonstrate increased apoptosis in hematopoietic tissues (Ritchie, Malakhov et al. 2002). In brain tissue from UBP43 -/- mice, Malakhova et al. observed an elevated level of ISG15 conjugation and cellular necrosis, resulting in brain malformation and severe neurological disorders. Ectopic expression of ISG15 in various cell types also appears to initiate apoptosis (Stefan Lanker, unpublished). Clearly, both free and conjugated ISG15 may have wide-reaching implications for cell stress response, cancer immunology, and embryonic development.

The coordinated induction of both the ISG15 conjugating (ISG15, UBE1L) and deconjugating (UBP43) pathways upon IFN stimulation suggests that ISG15 modification is a dynamic process that must be carefully controlled for normal cellular function and viability. In this context, Skp2-mediated degradation of UBP43 might play a fine-tuning role, helping to adjust the levels of free ISG15 and conjugated ISG15 according to the growth and stress conditions of a cell.

C. The Role of Skp2 in IFN Signaling

Type-I IFNs are present at low levels in unstressed cells and are rapidly induced in response to cell stress, including viral and bacterial infections. IFNα/β stimulation of cells induces transcription of IFNα/β target genes that have apoptotic and anti-proliferative functions essential to the cells' immune response. These properties of IFNα/β signaling make it an effective chemotherapy for cancer treatment. In one study, IFN treatment induced either G_0 or G_1 arrest in three malignant lymphoid cell lines

(Sangfelt, Erickson et al. 1999). Additionally, Talpaz et al. showed that 10-20% of chronic myelogenous leukemia (CML) cases in their study exhibited complete remission when treated with IFN in the chronic phase (Talpaz 2001).

IFN-induced apoptosis and cell cycle arrest is achieved through inducing a wide variety of cell cycle control proteins. IFNα/β treatment induces up-regulation of the cdk inhibitors p21, p15, and p27, which in turn cause down-regulation of the G_1 and S cyclin-cdk complexes cdk4/6-cyclin D and cdk2/cyclin E. This cascade of events impedes the cell's ability to transition from G_1 to S phase by suppressing the phosphorylation of pRb and the pocket proteins p130 and p107 (Sangfelt, Erickson et al. 1999). Thus, Skp2 may antagonize IFN signaling by down-regulating p21, p27, and p130.

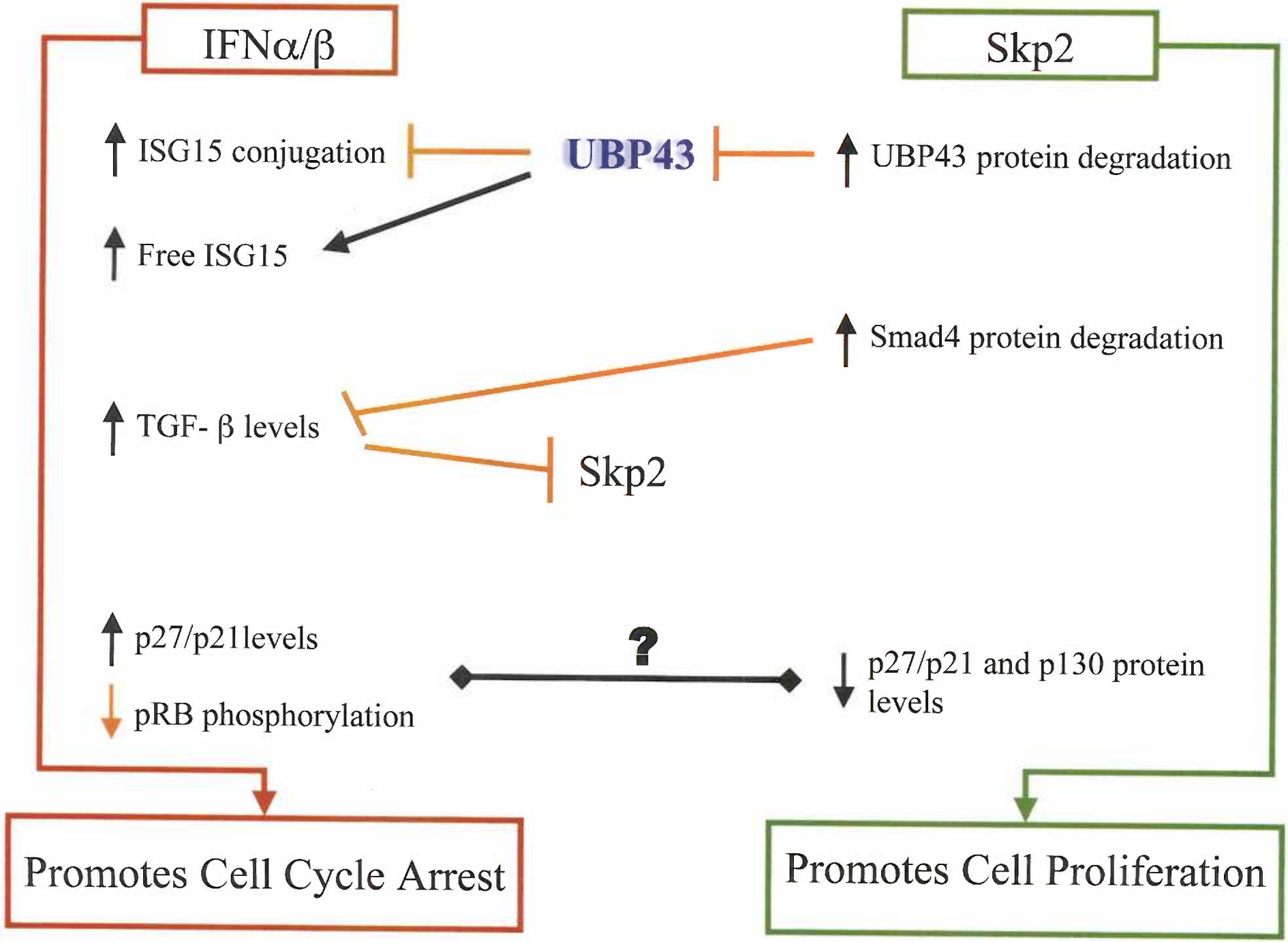
It is intriguing that Skp2 controls the levels of factors involved in cell stress because it suggest a deeper role in cell-cycle regulation by Skp2 than has previously been shown. Connections between cell cycle control and the stress response at the level of SCF-mediated degradation have been documented. For example, the yeast E3 ligase SCF^{Grr1} degrades yeast G1 cyclins and has an important role in the organism's response to glucose starvation (Lanker, Valdivieso et al. 1996; LaRue, unpublished). The yeast SCF^{Cdc4} complex controls the CDK inhibitors Sic1 and Far1, as well as the transcription factor Gcn4, which are involved in the yeast response to amino acid starvation, thus linking the cell cycle to the cellular environment.

Skp2 has been known as a cell cycle control protein; however, involvement of Skp2 in IFN signaling has been suggested by its negative regulation of the protein Smad4 (Liang, Liang et al. 2004). Smad4 cooperates with other transcription factors, including Smad3, to induce the expression of Transforming Growth Factor β (TGF-β)-responsive

genes. TGF-β is a growth factor that regulates cell proliferation and apoptosis, and it plays a vital role in a cell's immune response (Letterio and Roberts 1998). Recently, it was discovered that TGF-β plays a role in prolonged type I-IFN signaling. Qing *et al.* showed that Smad3 interacts with interferon regulatory factor IRF-7 and cooperates in the activation of the IFN-β promoter (Qing, Liu et al. 2004). Skp2-mediated destruction of Smad4 could interfere with TGF-β stimulation of the IFN pathway. Similarly, TGF-β is capable of down-regulation of Skp2. TGF-β treatment of lung epithelia cells showed a destabilization of Skp2 caused by the induction of Skp2 self-ubiquitination (Wang, Ungermannova et al. 2004). While this serves to stabilize p27 and prevent cell proliferation, we see that Skp2 down-regulation may also be necessary for proper Smad4 function and prolonged IFN signaling.

My research indicates that Skp2 may have a positive effect on IFN signaling as well as the negative effect that has previously been described. Skp2 appears to counteract the anti-proliferative effects of IFN treatment through its down-regulation of p21, p27, and p130, as well as Smad4. However, Skp2-mediated down-regulation of UBP43 and consequent up-regulation of ISG15 conjugation suggest that Skp2 also positively affects IFN signaling. Skp2's apparent dual roles in IFN signaling may reflect the dynamic and complex nature of this signaling pathway. Upon IFN induction, transcription of ISG15, UBE1L, and UBP43 are all rapidly upregulated (Malakhova, Malakhov et al. 2002). Free ISG15 appears approximately 8 hours after IFN induction in the human lung carcinoma cell line A549, but conjugates are not visible until 18 hours post IFN. Skp2-mediated down regulation of UBP43 may aid in regulating the balance between free ISG15 and ISG15 conjugates in early and late IFN signaling.

Figure 24. Skp2 Positive and Negative Regulation of IFN Signaling. The role of Skp2 in IFN signaling through UBP43, and subsequent ISG15 regulation, may serve both positive and negative effects based on cell type and the stage of IFN signaling. Skp2 has negative effects on IFN signaling through the down regulation of Smad4, a transcription factor necessary for TGF-β expression. Skp2 may also exert a negative effect on IFN signaling by antagonizing the cell cycle arrest phenotypes of increasing p27 and p21 and decreasing pRB phosphorylation.


IFNα/β
Skp2
ISG15 conjugation
UBP43
UBP43 protein degradation
Free ISG15
Smad4 protein degradation
TGF- β levels
Skp2
p27/p21levels
?
p27/p21 and p130 protein levels
pRB phosphorylation
Promotes Cell Cycle Arrest
Promotes Cell Proliferation

UBP43 and ISG15 conjugation, then, play an important role in many cellular functions. Levels of UBP43, as well as ISG15, need to be carefully controlled in cells to maintain normal physiology. Indeed, UBP43 protein levels are regulated at the level of transcription (Malakhov, Malakhova et al. 2002) and, as we show here, post-translationally via the SCF^{Skp2}-mediated ubiquitin degradation pathway.

It will be interesting to further understand the molecular mechanism that ties Skp2 to cellular stress, and the role of ISG15 conjugation in this process. Dissecting how Skp2 connects cell cycle control and cellular stress signaling will serve as a paradigm for similar pathways that integrate cell division and external signals.

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Appendix I: Journal of Biological Chemistry Publication 2004

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The ISG15 Isopeptidase UBP43 Is Regulated by Proteolysis via the SCF^{Skp2} Ubiquitin Ligase*

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The Skp2 oncoprotein belongs to the family of F-box proteins that function as substrate recognition factors for SCF (Skp1, cullin, F-box protein) E3 ubiquitin-ligase complexes. Binding of the substrate to the SCF^{Skp2} complex catalyzes the conjugation of ubiquitin molecules to the bound substrate, resulting in multi-ubiquitination and rapid degradation by the 26 S proteasome. Using Skp2 as bait in a yeast two-hybrid screen, we have identified UBP43 as a novel substrate for Skp2. UBP43 belongs to the family of ubiquitin isopeptidases and specifically cleaves ISG15, a ubiquitin-like molecule that is induced by cellular stresses, such as type 1 interferons (IFN), nephrotoxic damage, and bacterial infection. UBP43 was originally identified as an up-regulated gene in knock-in mice expressing an acute myelogenous leukemia fusion protein, AML1-ETO, as well as in melanoma cell lines treated with IFN-β. The phenotype of UBP43 knockout mice includes shortened life span, hypersensitivity to IFN, and neuronal damage, suggesting that tight regulation of ISG15 conjugation is critical for normal cellular function. In this study, we demonstrate that UBP43 is ubiquitinated *in vivo* and accumulates in cells treated with proteasome inhibitors. We also show that Skp2 promotes UBP43 ubiquitination and degradation, resulting in higher levels of ISG15 conjugates. In Skp2−/− mouse cells, levels of UBP43 are consistently up-regulated, whereas levels of ISG15 conjugates are reduced. Our results demonstrate that the SCF^{Skp2} is involved in controlling UBP43 protein levels and may therefore play an important role in modulating type 1 IFN signaling.

Modification of proteins by ubiquitin and ubiquitin-like (Ubl)[1] molecules, including SUMO, Nedd8, and ISG15, has emerged as a critical regulatory process in eukaryotes, controlling pathways such as the cell cycle, cellular stress response, intracellular signaling, development, and the immune response (1–5). Deregulation of ubiquitin or Ubl modification can cause autoimmune and neurodegenerative diseases, developmental abnormalities, and cancer.

Conjugation of ubiquitin and Ubls involves a three-step mechanism initially demonstrated for ubiquitin as follows. A single E1 ubiquitin-activating enzyme activates the Ubl molecule via formation of a thioester bond. Activated Ubl is then transferred to one of a large family of E2 ubiquitin-conjugating enzymes. In most cases, E2 enzymes are targeted to appropriate substrates by a class of substrate receptor complexes termed E3 ubiquitin ligases. Together, the E2 and E3 enzymes catalyze the formation of isopeptide bonds between ubiquitin and lysine residues on the target proteins. In the case of multiubiquitination, additional ubiquitin molecules are added to form ubiquitin chains. The multiubiquitinated proteins are then recognized and rapidly degraded into short peptides by the 26 S proteasome (3, 6). Modification by ubiquitin and Ubl molecules is a reversible process mediated by a large family of isopeptidases that exist to remove these molecules from their substrates. Although the function and regulation of these isopeptidases is poorly defined, the few that have been studied indicate an important role in growth and development (7, 8).

Specificity of the ubiquitin and Ubl pathways is conferred by the nature and activity of the E3 complexes. One of the best-studied E3 ligase complexes is the SCF complex. SCF complexes are composed of four subunits: the three core components Skp1, a Cullin family member, the RING finger protein Rbx1/Roc1, and one variable component, the F-box protein, that acts as the substrate recognition factor. A large number of F-box proteins have been identified in organisms ranging from yeast to humans. The mammalian F-box protein Skp2 may play a pivotal role in oncogenesis and has been implicated in degradation of several key regulators of cell proliferation including the tumor suppressor proteins p27^{Kip1}, p57^{Kip2}, (9) and p130 (10) and the replication factor, hORC1 (11).

We used the yeast two-hybrid technique to isolate potential Skp2-interacting proteins. We identified seven proteins, including UBP43, which will be described in this report. UBP43 belongs to the family of ubiquitin isopeptidases, one of the two classes of ubiquitin and Ubl deconjugating enzymes (12). UBP43 was identified as strongly expressed mRNA in hematopoietic tissue from an acute myeloid leukemia mouse model (13). Thereafter, UBP43 was identified as an up-regulated gene in melanoma cells treated with IFN-β and the protein kinase C activator MEZ. This treatment causes cells to irreversibly lose proliferation potential and begin progression toward terminal differentiation (14). UBP43 functions as an isopeptidase re-

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[1] The abbreviations used are: Ubl, ubiquitin-like; SCF, Skp1, cullin, F-box protein; LPS, lipopolysaccharide; IFN, interferon; MEF, mouse embryonic fibroblast; LRR, leucine-rich repeat; HEK, human embryonic kidney; HA, hemagglutinin; SCF^{Skp2}, Skp1-Cul1-F-box protein Skp2.

sponsible for cleaving the ubiquitin-like protein ISG15 from substrates (15). Upon treatment of cells with type I IFN or lipopolysaccharide (LPS), ISG15, UBP43, and the ISG15 E1 enzyme, UBE1L, are rapidly up-regulated (16). It seems that tight regulation of both the ISG15 conjugation and deconjugation pathways is required to ensure proper response to cellular stress. In UBP43 knockout mouse embryonic fibroblasts (MEFs), ISG15 cleavage from cellular substrates is strongly reduced, suggesting that UBP43 is the major ISG15 isopeptidase (17). UBP43−/− MEFs demonstrate prolonged STAT1 signaling and IFN hypersensitivity, which is in accordance with data showing that signal transducer and activator of transcription 1 is an ISG15-modified protein (18). These observations and the identification of UBP43 in the two tumor models suggest that UBP43 plays an important role in cellular proliferation and differentiation and that UBP43 levels need to be carefully controlled in cells. Indeed, UBP43 protein levels are regulated at the level of transcription (16) and post-translationally, as we show here, via the SCF^{Skp2}-mediated ubiquitin pathway.

MATERIALS AND METHODS

DNA Constructs—Skp1, Skp2, and UBP43 were PCR-amplified using a human lymphocyte cDNA library (HL4006AE; BD Biosciences Clontech). Skp1 and Skp2 were cloned into MatchMaker (BD Biosciences Clontech) yeast two-hybrid vectors pGADT7 and pGBKT, respectively. All other plasmids were created with the GATEWAY cloning system using vectors from Invitrogen following manufacturer's protocols. The C-terminally V5-tagged UBP43 adenovirus construct was made using the pADeasy system (Qbiochem). Amplified clones were verified by sequencing.

Antibodies—The Skp2 antibody used for immunoprecipitation was purchased from Zymed Laboratories Inc.. Skp2 (His-435) and Cdk2 (Glu-119) antibodies used in immunoblotting analysis were purchased from Santa Cruz. His, HA, and V5 antibodies were purchased from Amersham Biosciences, Babco, and Invitrogen, respectively. The affinity-purified polyclonal ISG15 antibody was kindly supplied by Dr. Arthur Haas and does not cross-react with ubiquitin (19).

Cell Extracts—Unless stated otherwise, cells were harvested in mammalian lysis buffer (150 mM NaCl, 0.1% Nonidet P-40, and 50 mM Tris-HCl, pH 7.5) by scraping, incubating on ice for 10 min, and then centrifuging at 14,000*g* (40° C) for 15 min; the supernatant was retained.

Yeast Two-hybrid Screen—The MatchMaker (BD Biosciences Clontech) yeast two-hybrid system was used to screen for Skp2-interacting proteins. Yeast strain AH109 was transformed with plasmid pGBKT-Skp2 containing full-length Skp2 fused to the GAL4 DNA-binding domain. Transformed cells were streaked on plates lacking tryptophan to select for single clones. This strain was then transformed with a human lymphoma cDNA library (HL4006AE; Invitrogen) cloned into the vector pACT1 that fuses cDNAs to the GAL4 activating domain. 2×10^6 clones were screened on plates lacking histidine (+10 mM 3AT) or adenine and histidine (+10 mM 3AT). Interaction of positive clones was verified by retransforming clones into the original Skp2 bait strain and assaying for interaction. Skp2 and UBP43 constructs were then cloned into yeast expression vectors compatible with the ProQuest two-hybrid system from Invitrogen. Constructs were transformed into AH109 and screened on plates lacking leucine, tryptophan, or histidine (+30 mM 3AT).

Production of Recombinant Proteins in Bacteria and GST Purification—*Escherichia coli* BL21 (Invitrogen) cells were used to express the vector, pDEST15 (Invitrogen) containing GST or a GST-Skp2 fusion protein. One liter of each strain was induced with 100 mM isopropyl β-D-thiogalactoside for 2 h. Protein was isolated using a 3× freeze-thaw protocol with a dry ice and ethanol bath. Extracts were purified over glutathione-Sepharose 4B matrix (Amersham Biosciences) according to manufacturer's instructions. Extracts from HEK 293 cells transfected with indicated plasmids were added to the matrix. Bound fractions were analyzed by Western blotting using the indicated antibodies.

Immunoprecipitation—80% confluent A549 cells on a 10-cm plate were infected with adenoviruses expressing Skp2 with an N-terminal FLAG tag, and UBP43 with a C-terminal V5 tag. Infection was confirmed with GFP. 36 h after infection, cells were harvested in mammalian lysis buffer. The lysate was subjected to a FLAG column (Sigma), and bound proteins were eluted with 1.5× FLAG peptide (Sigma) and subjected to Western blot analysis.

Ubiquitination—Skp2 was subcloned into pDEST31 (Invitrogen) containing an N-terminal His_6 epitope. UBP43 was subcloned into pDEST40 (Invitrogen) containing C-terminal V5 and His_6 epitopes. HA-ubiquitin and His_6-ubiquitin constructs were a gift from Dr. Rosalie Sears (Oregon Health and Sciences University, Portland, OR). A549 cells, transfected with indicated plasmids, were treated for 5 h with 10 μM MG132 (Calbiochem).

^{35}S Pulse-chase—REF52 and A549 cells were infected with UBP43-V5 adenovirus for 20 h. Skp2 wild-type (+/+) and Skp2 knockout (−/−) primary MEFs were infected in the presence of Effectene reagent. Cells were then labeled for 30 min with 7 mCi of [^{35}S]methionine, chased with media containing 5 mM methionine and 3 mM cysteine, washed, and collected at 0, 15, 30, 60, 120, and 180 min. Cell extracts were denatured and then renatured for V5 immunoprecipitation with anti-V5 antibody from Invitrogen. Proteins were separated by SDS-PAGE and visualized by autoradiography. Proteasome inhibitors (5 μM MG132 + 5 μM lactacystin) were added 5 h before the pulse and maintained throughout the pulse and chase phases.

IFN-β Treatment—Skp2 wild-type and Skp−/− MEFs were treated with 1000 units/ml mouse IFN-β, and extracts were harvested 24 h later. For cell growth measurements, 3.6×10^4 immortalized MEF cells were plated in six-well plates, treated with 500 units/ml of mouse IFN-β, and harvested in duplicate. For each time point, total viable cell number was assessed by counting with a hemacytometer. Trypan blue staining was used to identify dead cells. A minimum of 150 cells was counted per sample.

RESULTS

UBP43 Interacts with Skp2—To identify Skp2-interacting proteins, we performed a yeast two-hybrid screen and obtained eight clones coding for putative Skp2 interacting proteins (see "Materials and Methods"). Sequencing of the cDNA inserts revealed two previously identified and five novel Skp2 interactors. In validation of our screen, we isolated Skp1 (two clones) and Cks1. Skp1 is the scaffold protein that anchors Skp2 to the SCF complex via the Skp2 F-box domain (18). Cks1 is a protein recently identified to interact with Skp2 and facilitate substrate recognition of p27 (20, 21). Of the other five clones isolated, we focused our attention on a clone coding for a C-terminal portion of UBP43. Both the UBP43 C terminus (amino acids 121–373) that was isolated in the original screen and full-length UBP43, but not the N terminus (amino acids 1–121), were able to interact with Skp2, as assayed by production of β-galactosidase and growth on plates lacking histidine (Fig. 1*A*). To further isolate the Skp2-interacting region of UBP43, we made successive C-terminal truncations of the 121–373 fragment (Fig. 1*B*). We located a region between amino acids 183 and 352 of UBP43 that seems to be involved in its interaction with Skp2. The deletion construct containing the entire C-terminal region (121–373) as well as a construct lacking the last 21 amino acids, fragment 121–352, demonstrated robust interaction. However, a construct containing amino acids 121–285 showed markedly reduced interaction. When fragment 121–183 was expressed, interaction with Skp2 was completely abrogated. We therefore conclude that the region between amino acids 121 and 285 of UBP43 is required for the interaction between UBP43 and Skp2.

Skp2 Interaction with UBP43 Requires the Skp2 Leucine-rich Repeat (LRR) Domain—Next, we analyzed the interaction of UBP43 and Skp2 *in vivo*. Fig. 1*C* demonstrates that full-length Skp2 can co-immunoprecipitate UBP43 ectopically expressed in the human lung cancer cell line A549. A construct lacking the LRR and C terminus showed no binding to UBP43. However, constructs lacking the N terminus or both the N terminus and F-box regions but retaining the LRR and C terminus did associate with UBP43. The LRR region of Skp2 has been implicated in substrate binding. Our data thus suggest that UBP43 is a substrate of SCF^{Skp2}.

Skp2 and UBP43 Interact in Vitro—To verify the interaction of UBP43 with Skp2, we performed *in vitro* binding experiments. *Escherichia coli* that expressed GST-Skp2 or GST alone

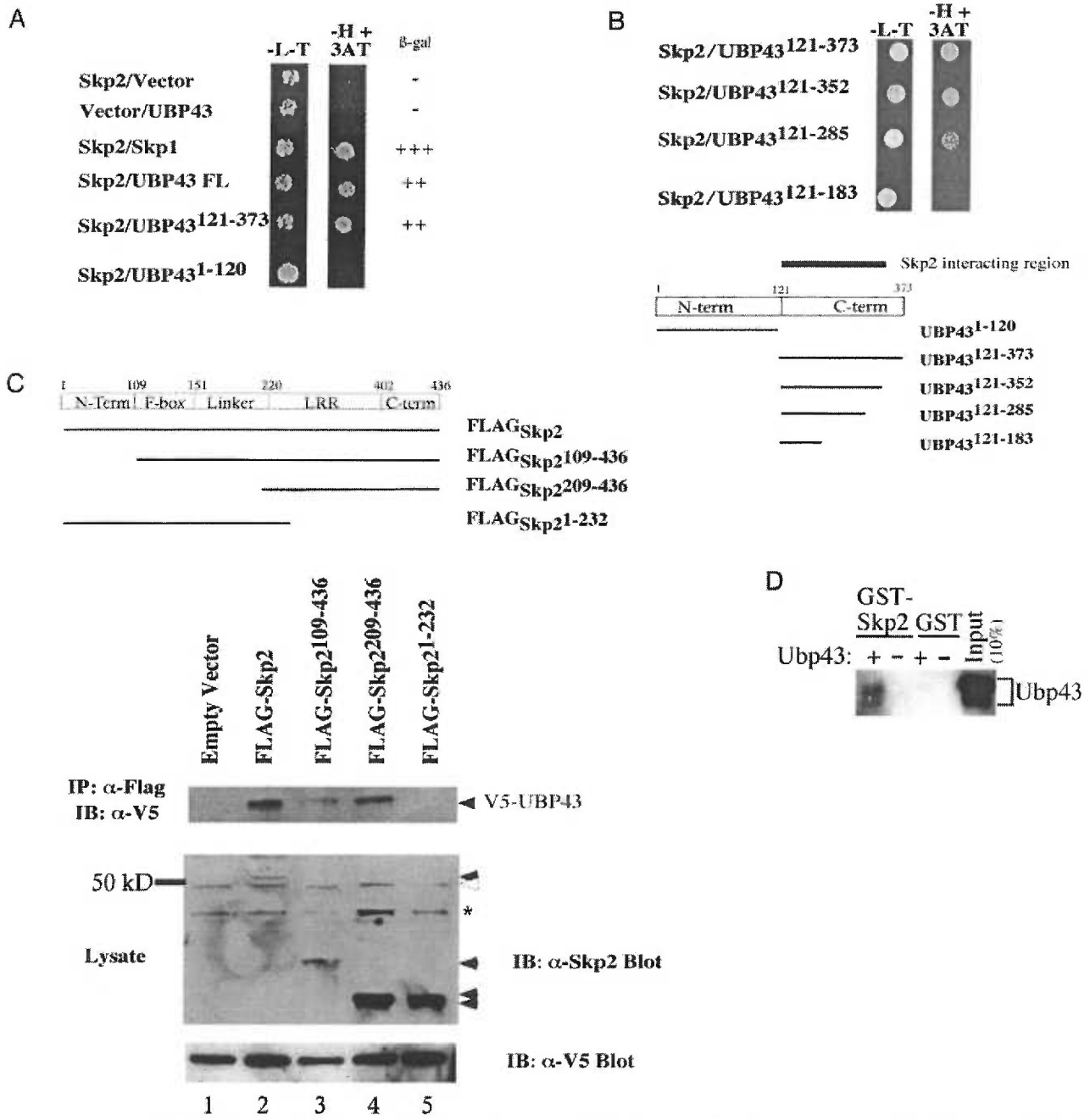

FIG. 1. **UBP43 and Skp2 interact *in vivo* and *in vitro*.** *A*, yeast two-hybrid screen. Transformants were analyzed for transcriptional activation of the *GAL4* promoter driven reporter constructs. *Left*, growth on −Leu−Trp plates (*-L-T*) or on −His plates containing 30 mM 3-amino-1,2,4-triazole (3-AT) (*-H+3-AT*) to test expression of the *HIS3* reporter gene (*-His+3-AT*). *Right*, β-galactosidase activity. *B*, yeast strains expressing the indicated constructs were grown on −H+3-AT plates to assess protein interaction as above. *C*, mammalian co-immunoprecipitation. Human lung carcinoma A549 cells were infected with adenovirus expressing UBP43-V5 and various FLAG-Skp2 constructs. Cell extracts were immunoprecipitated with anti-FLAG beads, separated by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. The *asterisk* indicates a background band. Endogenous Skp2 is denoted by a *white arrowhead*, ectopic Skp2 by *black arrowheads*. The proteasome inhibitor lactacystin was added to a final concentration of 10 μM 5 h before harvest. *IB*, immunoblot; *IP*, immunoprecipitation. *D*, GST-Skp2 binds UBP43 *in vitro*. GST-Skp2 (*lanes 1* and *2*) or GST (*lanes 3* and *4*) were expressed in *E. coli* and purified over glutathione beads. Beads were incubated with HEK293 cell extracts expressing UBP43 (*lanes 1* and *3*) or empty vector (*lanes 2* and *4*).

were bound to glutathione beads, followed by incubation with extract from human embryonic kidney (HEK) 293 cells that expressed ectopic UBP43. Fig. 1*D* shows that GST-Skp2, but not GST, binds to UBP43 *in vitro*.

Levels of UBP43 Are Modulated by Skp2 and by Proteasome Inhibitors—Our finding that UBP43 interacts with the LRR domain of Skp2 suggested that UBP43 is a substrate of SCF^{Skp2}. Therefore, UBP43 protein levels could be controlled by protein degradation. To address this question, we expressed Skp2 and UBP43 in HEK293 cells. UBP43 levels were strongly reduced when Skp2 was expressed (Fig. 2*A*, *lane 2*) compared with when UBP43 was expressed alone (*lane 1*). Because Skp2 functions in an E3 ligase complex that targets substrates for degradation by the 26 S proteasome, we next assayed whether UBP43 accumulates in cells that have been treated with the proteasome inhibitor MG132. Indeed, under these conditions,

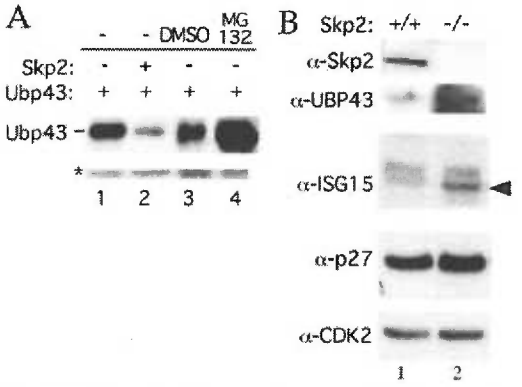

FIG. 2. **UBP43 steady state levels are modulated by Skp2 and by proteasome inhibitors.** *A*, HEK293 cells were transfected with UBP43 and either control vector (−) or Skp2 and subjected to Western blot analysis. Proteasome inhibitor MG132 (10 μM) or Me_2SO (*DMSO*) as control was added 7 h before cell harvest. The *asterisk* indicates a loading control. *B*, absence of Skp2 results in increased UBP43 levels. Extracts from asynchronous low passage primary Skp2 wild-type (+/+) or knockout (−/−) MEFs were immunoblotted with antibodies specific to the indicated proteins. The *arrowhead* denotes free ISG15.

robust UBP43 accumulation can be observed (Fig. 2*A*, *lane 4*). Together, these results suggest that UBP43 is degraded in the proteasome most likely by SCF^{Skp2}-mediated ubiquitination.

Levels of UBP43 and ISG15 Are Altered in Skp2−/− Cells—To test whether levels of UBP43 are increased in cells that are devoid of Skp2, we performed immunoblotting of primary, low passage MEF extracts derived from wild-type or Skp2−/− mice. As shown in Fig. 2*B*, UBP43 levels are significantly increased in Skp2−/− extracts (*lane 2*) compared with wild type (*lane 1*). Note that UBP43 levels are very low in unstimulated wild-type cells and are hardly detectable with the available antibody. We also observed a significant increase in steady-state UBP43 levels in Skp2−/− cells stimulated with LPS (data not shown). In addition to the increase in UBP43 levels in Skp2−/− cells, an increase in free ISG15 levels was observed (*lane 2*, *arrowhead*). This suggests that in the absence of Skp2, UBP43 levels are up-regulated, resulting in increased cleavage of ISG15 from substrates (see Fig. 5*B*) and a concomitant increase in free ISG15.

Skp2 Enhances UBP43 Ubiquitination in Vivo—Proteasomal degradation of proteins is triggered by multi-ubiquitination of targeted polypeptides. To determine whether UBP43 is ubiquitinated *in vivo*, we transfected HEK293 cells with plasmids encoding Skp2, V5-tagged UBP43, and HA-tagged ubiquitin. UBP43, in the presence of overexpressed Skp2, appeared in higher molecular mass forms consistent with ubiquitination (Fig. 3*A*). These high molecular mass bands were intensified upon addition of the proteasome inhibitor lactacystin. Next, we performed immunoprecipitation with anti-V5 antibodies to capture UBP43 protein. High molecular mass bands were observed that were immunoreactive against anti-HA antibodies, indicating that these bands represented ubiquitinated UBP43 (Fig. 3*B*, *lanes 2* and *3*). Ubiquitination was enhanced by ectopic Skp2 (*lane 3*) and was absent if either V5-UBP43 or HA-ubiquitin was omitted (*lanes 1* and *4*). If these high molecular mass bands correspond to ubiquitinated UBP43 species, then inhibition of the proteasome should result in an increase and possibly a shift to even higher molecular mass bands. This is indeed the case, as shown in Fig. 3*C*, *lane 3*. In this experiment, His-V5-tagged UBP43 was captured on nickel-nitrilotriacetic acid beads under denaturing conditions, followed by immunoblotting against the HA tag on ubiquitin. Because in these experiments, the amount of transfected UBP43 was high compared with Skp2, we did not observe a significant reduction in UBP43 steady-state levels upon Skp2 co-transfection. We conclude that UBP43 is ubiquitinated *in vivo*. To test whether Skp2 can increase the amount of ubiquitinated UBP43, we infected normal rat embryo fibroblast (REF52) cells with V5-tagged UBP43 expressed from an adenovirus together with increasing levels of adeno-Skp2 virus. Fig. 3*D* shows that under proteasome inhibition conditions (MG132, *lanes 3*, *5*, and *7*), UBP43 ubiquitination is enhanced by increasing levels of Skp2. We kept the level of Skp2 at or near endogenous levels (data not shown), whereas UBP43 levels were higher to allow detection of ubiquitinated species. In conclusion, these experiments demonstrate that UBP43 is ubiquitinated *in vivo* by Skp2 and is then degraded in a proteasome-dependent manner.

Skp2 Promotes Degradation of UBP43 in Vivo—Our results suggested that UBP43 levels were controlled by SCF^{Skp2}-mediated degradation via the ubiquitin-proteasome pathway. To measure UBP43 degradation rates *in vivo*, we performed pulse-chase experiments in adenovirus-UBP43 infected rat fibroblast (REF52) cells. UBP43 has a half-life of ~60 min under unstressed conditions (Fig. 4*A*, *lanes 2–5*). Again, UBP43 is very hard to detect under these conditions; however, the GFP control lane (*lane 1*) clearly demonstrates the specificity of the UBP43 band. When cells were treated with a proteasome inhibitor (Fig. 4*A*, *lanes 6–9*), UBP43 was stabilized significantly. We obtained similar results in human A549 cells (data not shown). In addition, cycloheximide treatment of cultures followed by analysis of UBP43 protein levels confirmed the pulse-chase results (data not shown). Ectopic Skp2 expression resulted in accelerated degradation of UBP43 (data not shown). We repeated the pulse-chase analysis in low passage Skp2+/+ and Skp−/− primary MEFs. The half-life of UBP43 was ~50 min in Skp2 wild-type cells compared with 120 min in knockout cells (Fig. 4, *B* and *C*). When Skp2 was re-expressed in the Skp2−/− cells, levels of UBP43 dropped dramatically at the 15-min time point and then remained at that low level for the rest of the chase period. We conclude that Skp2 can initiate rapid degradation of UBP43 via the ubiquitin-proteasome pathway.

Skp2 Activity Modulates the ISG15 Conjugation Pathway—ISG15 conjugation to substrates is induced upon IFN-β stimulation. To determine whether Skp2 has an effect on ISG15 conjugation by way of its regulation of UBP43, we overexpressed Skp2 in the A549 human lung carcinoma cell line. After treatment with LPS, cells overexpressing Skp2 showed a marked increase in ISG15 conjugation compared with cells transfected with empty vector (Fig. 5*A*). On the other hand, Skp2−/− MEFs induced with IFN-β displayed a reduction in ISG15 conjugation (Fig. 5*B*) and an increase in free ISG15 (Fig. 2*B*) compared with wild-type MEFs. We conclude that Skp2 can modulate the level of ISG15 conjugates, most likely via degradation of UBP43.

Cells from UBP43−/− mice exhibit increased levels of ISG15 conjugates; these animals are hypersensitive to induction of the type I IFN pathway (17). Therefore, we would expect that Skp2−/− cells, which have reduced levels of ISG15 conjugates, might be more resistant to the growth-inhibiting effects of IFN-β treatment. To test this, we analyzed the sensitivity of Skp2−/− cells toward IFN-β. Skp2 wild-type cells showed a marked reduction in cell growth 24 h after IFN-β reduction (Fig. 5*C*). In contrast, the growth rate of Skp2−/− cells was unaffected by IFN-β induction at 24 h. These data suggest that absence of Skp2 decreases or delays the response to IFN-β, most likely because of the higher levels of UBP43 observed (Fig. 2*B*).

DISCUSSION

The biological function of ISG15 modification is not well understood. It is clear, however, that carefully controlled

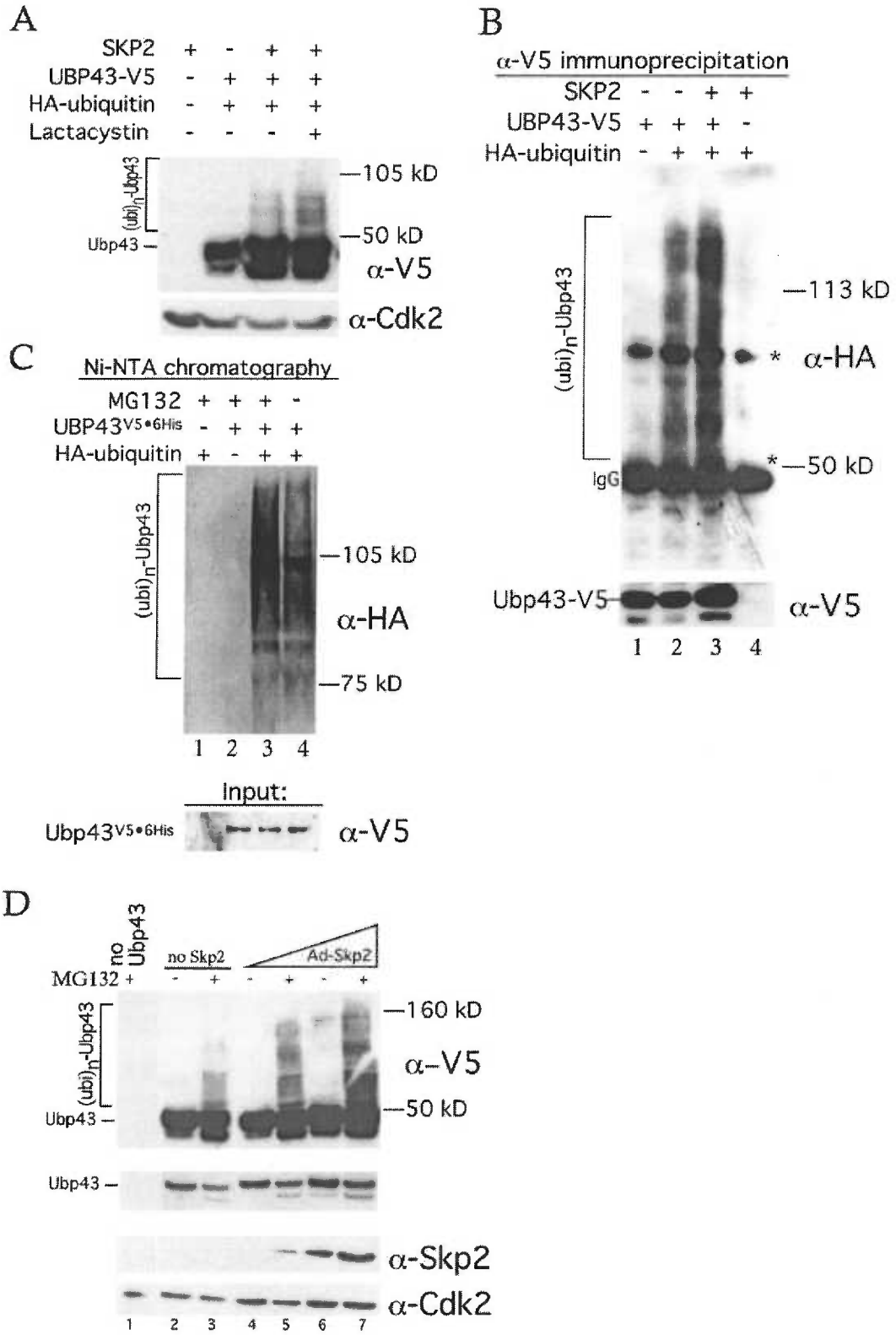

FIG. 3. **Skp2 increases UBP43 ubiquitination *in vivo*.** *A*, extracts from HEK293 cells were transfected with UBP43-V5 (*lanes 2–4*), Skp2 (*lanes 1, 3, 4*), and HA-ubiquitin (*lanes 2–4*), separated by SDS-PAGE, and analyzed with the indicated antibodies. Lactacystin (10 μM) was added 5 h before harvest. B, extracts from HEK293 cells transfected with UBP43-V5 (*lanes 1–3*), Skp2 (*lanes 3* and *4*), and HA-ubiquitin (*lanes 2–4*) were immunoprecipitated with anti-V5 antibody, separated by SDS-PAGE, and analyzed with the indicated antibodies. *Asterisks* mark heavy and light IgG bands. *C*, A549 cells were transfected with $UBP43^{V5\text{-}6HIS}$ (*lanes 2–4*) and HA-ubiquitin (*lanes 1, 3,* and *4*). Proteasome inhibitor, 5 μM MG132, and/or 5 μM lactacystin (*lanes 1–3*) was added 5 h before harvest. Extracts were purified using nickel-nitrilotriacetic acid (Ni-NTA) beads under denaturing conditions (8 M urea) to capture $UBP43^{V5\text{-}6HIS}$. Eluates, separated by SDS-PAGE, were analyzed by Western blotting with the indicated antibodies. *D*, REF52 cells were infected with constant amounts of adenovirus expressing UBP43-V5 or GFP and increasing amounts of adenovirus expressing Skp2 (*Ad-Skp2*) in the presence or absence of proteasome inhibitor (5 μM MG132, 5 μM lactacystin). Lysates were subjected to Western blot analysis.

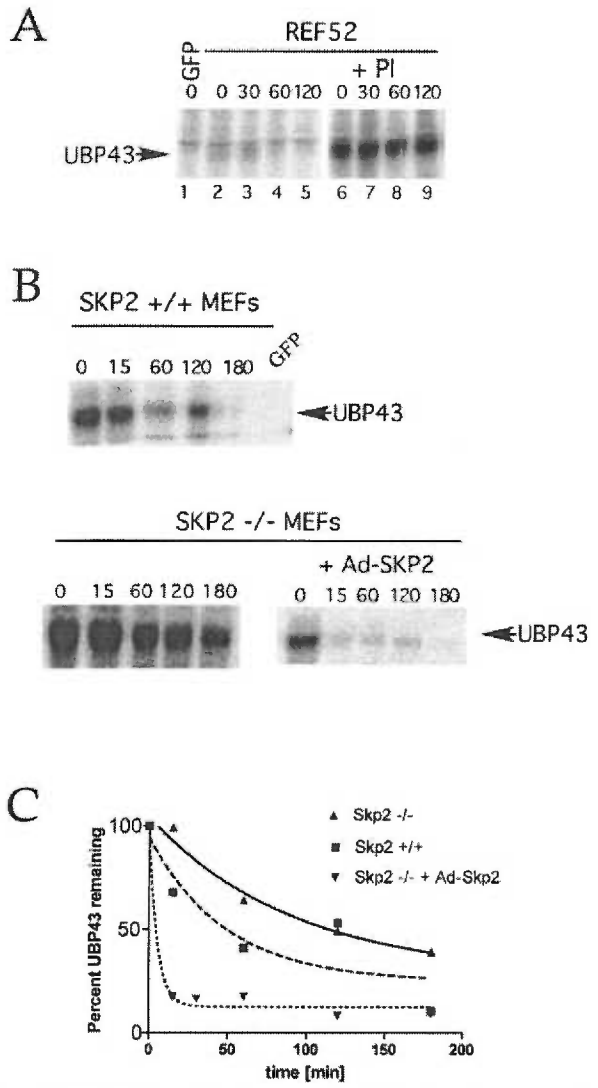

FIG. 4. **UBP43 stability.** *A*, proteasome inhibitors increase UBP43 protein stability. Normal rat embryo fibroblast REF52 cells were infected with UBP43-V5 adenovirus for 20 h and subjected to pulse-chase analysis in the presence or absence of proteasome inhibitors (PI, 5 μM MG132, 5 μM lactacystin). *GFP*, control infection with green fluorescent protein virus. *B*, UBP43 has increased stability in Skp2−/− MEFs. Skp2+/+ and Skp2−/− MEFs (passage 4) were infected with UBP43 adenovirus and, where indicated, with Skp2 adenovirus, followed by pulse-chase analysis. *C*, graphical representation of *B*. *Squares, dashed line*, Skp2+/+ MEFs; *triangles, solid line*, Skp2−/− MEFs; *inverted triangles, dotted line*, Skp2−/− MEFs infected with Adeno-Skp2.

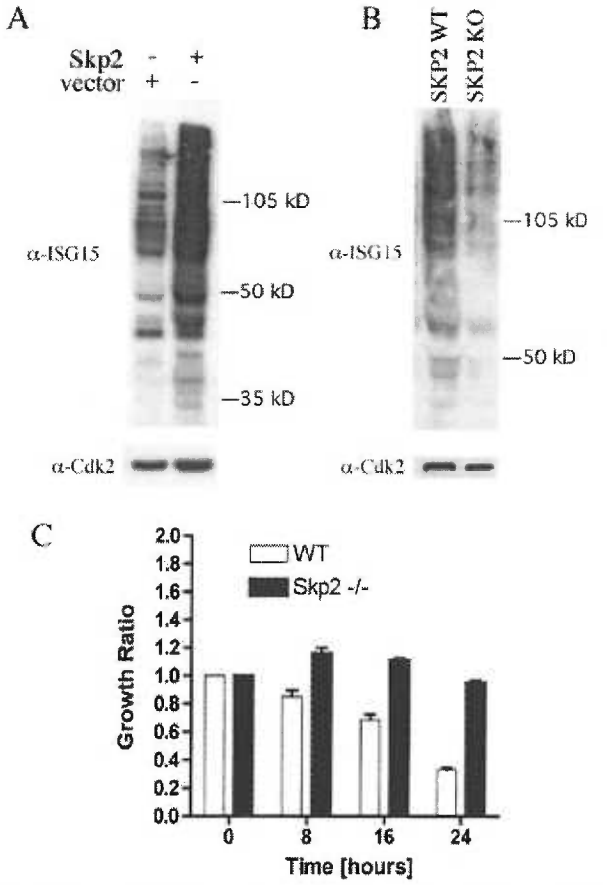

FIG. 5. **Skp2 interferes with IFN signaling.** *A*, Skp2 affects ISG15 conjugate levels. A549 cells were transfected with indicated vectors and extracts analyzed for ISG15 conjugation using an affinity-purified polyclonal α-ISG15 antibody (gift from Arthur Haas). *B*, asynchronously growing wild-type or Skp2−/− MEFs were treated with 1000 units/ml of mouse IFN-β for 24 h, and extracts were analyzed for ISG15 conjugation. *C*, Skp2−/− cells are resistant to IFN. MEFs were treated with 500 units/ml mouse IFN-β and viable cells were counted at the indicated time points. Growth ratio is defined as number of cells with interferon treatment divided by number of cells without interferon treatment.

ISG15 conjugation and deconjugation to substrates is crucial for the health of a cell and of an organism. This suggests that the level and activity of enzymes that control ISG15 modification, including UBE1L and UBP43, need to be tightly regulated. Indeed, mice lacking UBP43 are short-lived, develop neuronal injury, exhibit hypersensitivity to IFN, and demonstrate increased apoptosis in hematopoietic tissues (17). On the other hand, ectopic expression of UBP43 blocks monocyte differentiation in cell culture (13). In addition, the E1 enzyme for ISG15, UBE1L, is absent in all 14 lung cancers examined for UBE1L expression, suggesting that the lack of ISG15 conjugation contributes to malignant transformation. Cellular levels of UBP43 are controlled at the level of transcription by LPS and IFN type 1 induction (14, 16). We demonstrate here that the SCF^{Skp2} ubiquitin ligase controls the UBP43 protein level by ubiquitin-mediated degradation via the proteasomal pathway. Our data demonstrate that Skp2 binds to UBP43 and initiates its multi-ubiquitination, resulting in UBP43 degradation via the proteasome. In MEFs lacking Skp2, levels of free ISG15 are high, and ISG15 conjugates are low, consistent with increased UBP43 levels. On the other hand, high levels of Skp2 result in an increase in ISG15-conjugated proteins. It was interesting that upon LPS treatment, UBP43 protein was stabilized, an effect that was countered by high Skp2 levels (data not shown). The coordinated induction of both ISG15 conjugating and deconjugating pathways suggests that ISG15 modification is a dynamic process that needs to be carefully controlled for normal cellular function and viability. Indeed, ectopic expression of ISG15 in various cell types initiates apoptosis.[2] In this context, Skp2-mediated degradation might play a fine-tuning role to adjust the levels of UBP43 according to the growth and stress conditions of a cell. Skp2 itself is regulated at the level of transcription and protein degradation (22, 23). Skp2 protein is absent in G_0 and early G_1 cells, rises as cells enter S phase, and declines in mitosis. We have observed an inverse correlation between Skp2 and ectopic UBP43 levels in synchronized A549 cells (data not shown), consistent with the role of Skp2 in

[2] S. Lanker, unpublished observations.

degrading UBP43. We have not yet been able to follow endogenous UBP43 levels through the cell cycle, mainly because UBP43 levels are very low in cells not treated with IFN or LPS, and the available antibody does not detect endogenous UBP43. Our data do not exclude the possibility that more than one F-box protein participates in the degradation of UBP43; indeed, in Skp2−/− cells, UBP43 is still fairly unstable, with a half-life of about 2 h (Fig. 4*C*). However, UBP43 steady-state levels are greatly increased in Skp2−/− cells, and Skp2 reexpression reduces the levels back to normal, arguing that Skp2 does have a major effect on UBP43 protein levels.

The recognition of substrates by SCF complexes is often catalyzed by substrate phosphorylation at particular residues (24–31). This is also true for SCF^{Skp2} (9, 10, 22, 32, 33). In addition, the adapter protein Cks1 was shown to be required for efficient targeting of p27 and p130 (20, 21). We have preliminary evidence that UBP43 is phosphorylated, but whether phosphorylation is important for binding and whether Cks1 is needed for efficient interaction with Skp2 are under investigation.

ISG15 conjugation plays an important role in stress response, and is also implicated in controlling cell proliferation and differentiation. It is noteworthy that influenza virus proteins inhibit ISG15 expression and conjugation, suggesting that ISG15 mediates antiviral activity (34). It is intriguing that Skp2 controls the levels of cell cycle regulators and, as we show here, a factor in stress response. A connection between cell cycle control and stress response at the level of SCF-mediated degradation has been documented. For example, the yeast SCF^{Cdc4} complex controls the CDK inhibitors Sic1 and Far1 as well as the transcription factor Gcn4 involved in the response to amino acid starvation. SCF^{Grr1} degrades G_1 cyclins and has an important role in the cellular response to glucose starvation. It will be interesting to understand the molecular mechanism that ties Skp2 to cellular stress and the role of ISG15 conjugation in this process. Dissecting how Skp2 connects cell cycle control and cellular stress signaling will serve as a paradigm for similar pathways that integrate cell division and external signals.

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