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<sub>Skp2</sub> 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 a 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, 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, 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, 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, leukemia fusion protein, AML1-ETO, as well as in melanoma cell lines treated with IFN-β.
nisable 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 knock-out 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-translational, as we show here, via the SCP3/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 pGBKKT, respectively. All other plasmids were created with the GATEWAY cloning protocol with a dry ice and ethanol bath. Extracts were purified over glutathione-Sepharose 4B matrix (Amersham Biosciences) according to protocol with a dry ice and ethanol bath. Extracts were denatured and then reneutralized 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 lactacyclin) were added 5 h before the pulse and maintained throughout the pulse and chase phases.

IFN-β Treatment—Skp2 wild-type and Skp2–/– MEFs were treated with 1000 units/ml mouse IFN-β, and extracts were harvested 24 h later. For cell growth measurements, 3.6 × 10⁴ 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 (19). 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. 1A). To further isolate the Skp2-interacting region of UBP43, we made successive C-terminal truncations of the 121–373 fragment (Fig. 1B). 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 vitro. Fig. 1C 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 SCP2/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
were bound to glutathione beads, followed by incubation with extract from human embryonic kidney (HEK) 293 cells that expressed ectopic UBP43. Fig. 1D 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 SCFSkp2. 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. 2A, 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,
isopeptidase UBP43 is regulated by proteolysis

By proteasome inhibitors. The asterisk indicates a loading control. The arrowhead denotes free ISG15.

Robust UBP43 accumulation can be observed (Fig. 2A, lane 4). Together, these results suggest that UBP43 is degraded in the proteasome most likely by SCFSkp2-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 knockout (−/−) MEFs. We conclude that UBP43 is degraded in the proteasome most likely by SCFSkp2-mediated ubiquitination.

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. 3A). 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. 3B, 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. 3C, lane 3. In this experiment, His-V5-tagged UBP43 was captured on nickel-nitriotriacetic 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 expressing from an adenovirus together with increasing levels of adenovirus-Skp2 virus. Fig. 3D shows that under proteasome inhibition conditions (MG132, lanes 3, 5, and 7), UBP43 ubiquitination is enhanced by increasing levels of Skp2.

To test whether levels of UBP43 are increased in cells transfected with empty vector (Fig. 5A), 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. 5B). 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. 4A, 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 Skp2−/− primary MEFs. The half-life of UBP43 was ~50 min in Skp2 wild-type cells compared with 120 min in knockout cells (Fig. 4B). 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. 5A). On the other hand, Skp2−/− MEFs induced with IFN-β displayed a reduction in ISG15 conjugation (Fig. 5B) and an increase in free ISG15 (Fig. 2B) 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. 5C). 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. 2B).

**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*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*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.
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 SCFSkp2 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 G0 and early G1 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

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2 S. Lanker, unpublished observations.
Isopeptidase UBP43 Is Regulated by Proteolysis

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. 4C). However, UBP43 steady-state levels are greatly increased in Skp2−/− cells, and Skp2 re-expression 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 SCFSkp2 (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^{Cdc4} degrades G1 cyclins and has an important role in the cellular response to glucose starvation. It will be interesting to understand 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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The ISG15 Isopeptidase UBP43 Is Regulated by Proteolysis via the SCF$^{\text{Skp2}}$ Ubiquitin Ligase

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