The p53 tumor suppressor plays a central role in integrating cellular responses to various stresses. Tight regulation of p53 is thus essential for the maintenance of genome integrity and normal cell proliferation. Previously, we reported that JFK, the only Kelch domain-containing F-box protein in human, promotes ubiquitination and degradation of p53 and that unlike the other E3 ligases for p53, all of which possess an intrinsic ubiquitin ligase activity, JFK destabilizes p53 through the assembly of a Skp1-Cul1-F-box complex. Here, we report that the substrate recognition by JFK requires phosphorylation of p53 in its central core region by CSN (COP9 signalosome)-associated kinase. Significantly, inhibition of CSN-associated kinase activity or knockdown of CSN5 impairs JFK-promoted p53 degradation, enhances p53-dependent transcription, and promotes cell growth suppression, G1 arrest, and apoptosis. Moreover, we showed that JFK is transcriptionally regulated by p53 and forms an auto-regulatory negative feedback loop with p53. These data may shed new light on the functional connection between CSN, Skp1-Cul1-F-box ubiquitin ligase, and p53 and provide a molecular mechanism for the regulation of JFK-promoted p53 degradation.

The p53 protein is considered to be “the guardian of the genome” for its crucial role in coordinating cellular responses to various stresses, and it is believed to be at the epicenter of the regulatory circuits that monitor signaling pathways from diverse sources, including DNA damage responses, abnormal oncogenic events, and aberrant cellular processes (1–4). In effect, p53 acts to prevent cells from entering or progressing through the cell cycle under conditions that could generate or perpetuate DNA damages. Mechanistically, in response to genotoxic insults and other stresses, p53 rapidly accumulates and functions as a sequence-specific transcription factor to regulate the expression of an array of downstream genes (1, 5). The antiproliferative effects of p53 thus are mediated by its target gene products and are imparted through a variety of mechanisms, including cell cycle arrest, apoptosis, and cellular senescence (2, 6). As inactivation or activation of p53 sets up life or death decisions, an exquisite mechanism has evolved to control its erroneous activation at the same time as initiating prompt stress responses. Central to this mechanism are the opposing actions by the essential p53 negative regulators and transcription co-activators.

Under normal cell growth conditions, the level of p53 protein is kept low through regulation of its protein stability by a number of negative regulators. Although earlier studies suggested that MDM2 is the primary factor regulating p53 turnover through mono- or poly-ubiquitination of p53 (1, 7), additional cellular factors have since been identified that facilitate p53 degradation through ubiquitin-proteasome-dependent mechanisms, indicating that the regulation of p53 stability is more complex than originally thought. Indeed, several other proteins, including Pirh2 (8), COP1 (9), and ARF-BP1 (10), have been reported to also promote p53 turnover. All these proteins possess an intrinsic ubiquitin ligase activity, and interestingly, MDM2, Pirh2, and COP1 each form an autoregulatory negative feedback loop with p53. Recently, we reported the identification of the first, and apparently only, human Kelch domain-containing F-box protein, JFK (11). We showed that JFK promotes ubiquitination and degradation of p53. But unlike MDM2, Pirh2, COP1, and ARF-BP1, all of which possess an intrinsic ubiquitin ligase activity, JFK destabilizes p53 through the assembly of a Skp1-Cul1-F-box (SCF)3 complex.

The SCF complex is the best characterized mammalian multi-subunit RING finger type of ubiquitin ligase. Each of the SCF complexes is composed of the following four subunits: Skp1, Cul1/Cdc53, Roc1/Reb1/Hrt1, and an F-box protein (12). F-box proteins constitute a large family of eukaryotic proteins that feature an ~40-amino acid F-box motif (13, 14). Although they may potentially influence a variety of cellular processes, F-box proteins were first described in the SCF complex (15) and have since been characterized as an integral subunit of the SCF ligase complexes responsible for substrate specification (12).

In the majority of the model organisms, substrate phosphorylation is one of the common prerequisites for target recognition by F-box proteins in the functioning of SCF complexes; it is crucial that one or more residues of the so-called substrate phosphodegron, a sequence with which F-box proteins specifically interact, are phosphorylated prior to the F-

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3 The abbreviations used are: SCF, Skp1-Cul1-F-box; MEF, mouse embryo fibroblast; λPPase, λ protein phosphatase.
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box protein-substrate interaction. Indeed, several well characterized F-box proteins such as β-TrCP, Skp2, and Fbw7 recognize their cognate substrates in a phosphorylation-dependent manner (9, 16–19).

CSN (COP9 signalosome) is an eight-subunit assembly and is highly homologous to the lid of the 26 S proteasome regulatory particle (20–22). It is required for cell cycle progression in yeast and is essential for development in plants and Drosophila. In mammals, CSN is believed to function at the interface between signal transduction and ubiquitin-dependent proteolysis by virtue of its associated two enzymatic activities, a protein kinase and a de neddylase. CSN has been shown to directly interact with and be required for the proper function of SCF ubiquitin ligases (20–22), and several studies have linked the CSN-associated kinase or CSN complex to p53 destabilization (23, 24). However, the molecular mechanism underlying the association of CSN with SCF ubiquitin ligases and p53 degradation is not fully understood. We report here that the substrate recognition by JFK requires phosphorylation of p53 in its central core region by CSN-associated kinase. We also showed that JFK is transcriptionally regulated by p53 and forms an auto-regulatory negative feedback loop with p53.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Reagents—The cDNAs for wild-type CSN5 was amplified by PCR and ligated into XbaI/EcoRI sites of a pcDNA3.1 vector that contains three copies of FLAG. CSN5m was produced through synonymous mutation of wild-type CSN5 (the sequence of the CSN5 cDNA at 64–81 bp, GCTCAGAGTATCGATGAA, which was targeted by CSN5-specific siRNA, was changed to GCAAGAGCAT-TGACGAG). Wild-type JFK, p53-AVV, or p53-DEE was constructed as described previously (11, 24–26). All clones were confirmed by DNA sequencing. pCMV-(HA-Ub)4 was from Dr. Yue Xiong (University of North Carolina). p53(R175H) was from Dr. Wei Gu (Columbia University). pG13-Luc was from Dr. Guillermina Lozano (University of Texas M. D. Anderson Cancer Center). Cells were maintained in Dulbecco’s modiﬁed Eagle’s medium (DMEM) (Hyclone) supplemented with 10% fetal bovine serum. All transfections were carried out in culture media for 14 days supplemented with 1 mg/ml G418 and were then stained with crystal violet.

RESULTS

Phosphorylation of p53 by CSN-associated Kinase Is Required for Substrate Recognition by JFK—Previously, we showed that JFK promotes p53 degradation through the formation of an SCF complex in which JFK serves as a substrate receptor (11). As mentioned above, substrate phosphorylation is one of the common prerequisites for target recognition by F-box proteins in the functioning of SCF complexes. To further strengthen the receptor-substrate relationship and to support the functional connection between JFK and p53, we tested whether phosphorylation of p53 is required for its interaction with JFK. To this end, p53-null H1299 cells were co-transfected with FLAG-tagged JFK (FLAG-JFK) and Myc-tagged p53 (Myc-p53) for 24 h followed by treatment with 10 μM proteasome inhibitor MG132. The cellular lysates were then collected in the presence or absence of phosphatase inhibitors and further challenged with λ protein phosphatase (APPase) prior to co-immunoprecipitation assays. As shown in Fig. 1A, a speciﬁc interaction between p53 and JFK was observed in the presence but not in the absence of the phosphatase inhibitors, suggesting that phosphorylation of p53 is required for its interaction with JFK. GST pulldown experiments were then performed to further conﬁrm the phosphorylation of p53 by JFK.
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A. phosphorylation of p53 is required for its interaction with JFK in vivo. H1299 cells were co-transfected with FLAG-JFK and Myc-p53, and treated with 10 μM MG132. Cellular lysates were collected in the presence or absence of phosphatase inhibitors and further challenged with λAPase followed by immunoprecipitation with anti-Myc and immunoblotting with anti-JFK. B. phosphorylation of p53 is required for its interaction with JFK in vitro. GST pull-down experiments were performed with GST or GST-JFK and in vitro mammalian reticulocyte extracts (MRE), or wheat germ extracts (WGE), transcribed/translated p53 in the presence or absence of λAPase (right panel). The phosphorylated p53 (pp53) was examined by immunoprecipitation of the extracts with anti-phosphoserine followed by SDS-PAGE analysis of the immunoprecipitates (left panel). C. inhibition of CSN-associated kinase or disruption of CSN complex prevents the interaction of JFK with p53. U2OS cells were treated with curcumin or curcumin and ATM inhibitors, followed by immunoblotting with anti-JFK and anti-ATM. D. phosphorylation of Ser-149, Thr-150, and Thr-155 residues of p53 in its binding to JFK were assayed by co-immunoprecipitation in the presence of λAPase. The phosphorylated p53 (pp53) was examined by immunoprecipitation of the extracts with anti-phosphoserine followed by SDS-PAGE analysis of the immunoprecipitates. E. phosphorylation of Ser-149, Thr-150, and Thr-155 of p53 in its binding to JFK were assayed by co-immunoprecipitation in the presence of λAPase. The phosphorylated p53 (pp53) was examined by immunoprecipitation of the extracts with anti-phosphoserine followed by SDS-PAGE analysis of the immunoprecipitates. F. phosphorylation of p53 is required for JFK-promoted ubiquitination and degradation of p53. In summary, we demonstrate that CSN-associated kinase is critical for JFK-promoted ubiquitination and degradation of p53.

In general, phosphorylation and acetylation of p53 result in its stabilization and accumulation in the nucleus and its subsequent activation. However, several kinases, including CSN-associated kinase, AURKA, JNK, and TAF, phosphorylate p53 and promote its ubiquitination and degradation (29). A total of 17 phosphorylation/dedephosphorylation sites in p53 have been identified in human cells, most of which are located in the N-terminal region (29, 30). Our previous data indicate that the p53 central core region is responsible for its binding to JFK (11). This region contains three phosphorylation sites, Ser-149, Thr-150, and Thr-155 (29, 30), all of which are potential targets for CSN-associated kinase (29, 30). CSN is a conserved protein complex consisting of eight subunits designated as CSN1–CSN8 (20–22). Studies with null mutants from Aspergillus nidulans, plants, and vertebrate animals indicate that the loss of one subunit leads to loss of the function of the entire complex (22). To test a possible role of CSN-associated kinase in the physical interaction of p53 with JFK, co-immunoprecipitation was performed under treatment of the cells with curcumin, a pharmacological inhibitor of CSN-associated kinase (20, 21), or under knockdown of CSN5, the catalytic subunit of CSN-associated kinase, by RNAi in these cells. The interaction between endogenous JFK and p53 was detected in U2OS cells by co-immunoprecipitation with anti-JFK, and co-immunoprecipitation was performed under treatment of the cells with curcumin, a pharmacological inhibitor of CSN-associated kinase (20, 21), or under knockdown of CSN5, the catalytic subunit of CSN-associated kinase, by RNAi in these cells. The interaction between endogenous JFK and p53 was detected in U2OS cells by co-immunoprecipitation with anti-JFK. The results revealed that either inhibition of CSN-associated kinase or knockdown of CSN5 thus disruption of CSN complex prevented the interaction of p53 with JFK (Fig. 1C). The result suggests that the CSN-associated kinase function is required for p53 recognition by JFK and further supports a phosphorylation-dependent interaction of p53 with JFK.

The next potential role of the phosphorylation of Ser-149, Thr-150, and Thr-155 of p53 was investigated in U2OS cells by co-immunoprecipitation with anti-JFK. The results showed that JFK was not co-immunoprecipitated with p53-AVB, but it was strongly co-immunoprecipitated with p53-DEE (Fig. 1D), supporting a model in which JFK recognizes p53 that is phosphorylated in the central core region by CSN-associated kinase.

p53 Phosphorylation by CSN-associated Kinase Is Required for JFK-promoted Ubiquitination and Degradation of p53—To investigate the effect of CSN-associated kinase in JFK-promoted p53 degradation, U2OS cells with CSN5 knockdown were transfected with JFK mammalian expression vector or JFK and the mammalian reticulocyte extract-synthesized p53, which was inhibited by λAPase (Fig. 1B). No interaction was detected between GST-JFK and the wheat germ extract-derived p53, regardless of the presence or absence of λAPase. Collectively, these experiments indicate that p53 recognition by JFK requires p53 phosphorylation.
treated with JFK siRNA. Cellular lysates were prepared, and the level of p53 protein was measured by Western blotting. The results of these experiments revealed that knockdown of CSN5 in U2OS cells resulted in a diminished effect on JFK-promoted p53 degradation (Fig. 2A), suggesting that CSN-associated kinase is required in JFK-mediated p53 degradation.

To further support this argument and to exclude the possibility that the effect of CSN5 knockdown on JFK-promoted p53 degradation is a result of perturbation of the MDM2 pathway, we employed a p53 mutant, p53W23S, which retains the transcriptional activity of p53 and the ability to interact with CSN5 but loses its ability to bind to MDM2 (31, 32). p53−/−/MDM2−/− MEFs cells with or without CSN5 knockdown were co-transfected with Myc-p53W23S and FLAG-JFK and analyzed for p53W23S expression by Western blotting. The results of these experiments indicated that JFK promoted p53W23S degradation in these cells (Fig. 2B). However, when CSN5 expression was knocked down, p53W23S degradation was severely impeded.

Next, we tested the effect of the phosphorylation of Ser-149, Thr-150, and Thr-155 of p53 on JFK-mediated p53 ubiquitination; p53−/−/MDM2−/− MEFs were co-transfected with HA-Ub, FLAG-JFK, and wild-type p53, p53-AVV, or p53-DEE. Immunoprecipitation of the cellular lysates with anti-HA and immunoblotting with anti-p53 demonstrated that p53 ubiquitination was enhanced in p53-DEE-transfected cells and inhibited in p53-AVV-transfected cells compared with wild-type p53-transfected cells (Fig. 2C). These data further support the phosphorylation-dependent nature and the role of CSN-associated kinase in JFK-promoted p53 degradation.

We then tested the effect of the phosphorylation of Ser-149, Thr-150, and Thr-155 of p53 on JFK-mediated p53 turnover; p53−/−/MDM2−/− MEFs were co-transfected with JFK and wild-type p53 or the p53 mutants. Cellular lysates were prepared, and the level of p53 protein was measured by Western blotting. There were no evident changes in p53 protein levels when the p53-AVV, which is unable to be phosphorylated at corresponding sites, was overexpressed, whereas in wild-type p53- or the phosphomimetic p53-DEE-transfected cells, p53 protein levels were greatly reduced (Fig. 2D). Collectively, the above data support the proposition that phosphorylation of p53 by CSN-associated kinase regulates the physical interaction between p53 and JFK and is required for JFK-promoted p53 degradation.

To further support the physiological significance of the relationship between JFK and p53, we examined the effect of genotoxic stresses on the physical association between JFK and p53. For this purpose, U2OS cells were treated with 5 μM of camptothecin for 2 h and then transferred to fresh medium for another 3 h. The cellular lysates were immunoprecipitated with anti-p53 followed by immunoblotting with anti-JFK. The results showed that a specific interaction between p53 and JFK was observed only under the normal conditions but not under camptothecin treatment (Fig. 2E). In addition, overexpression of JFK in U2OS cells under the treatment of camptothecin resulted in an impaired p53 destruction by JFK (Fig. 2F).

**FIGURE 2. Phosphorylation of p53 by CSN-associated kinase is required for JFK-promoted ubiquitination and degradation of p53.** A, effect of CSN-associated kinase on JFK-promoted p53 degradation. U2OS cells with CSN5 knockdown were transfected with JFK or treated with JFK siRNA. Cellular lysates were analyzed for protein expression by Western blotting with antibodies against the indicated proteins. B, effect of genotoxic stress on JFK-promoted p53 degradation. U2OS cells were treated with camptothecin. Cellular lysates were analyzed for protein expression by Western blotting with antibodies against the indicated proteins. C, effect of the phosphorylation of Ser-149, Thr-150, and Thr-155 of p53 on JFK-mediated p53 ubiquitination. p53−/−/MDM2−/− MEFs were co-transfected with the indicated plasmids. Cellular lysates were immunoprecipitated (IP) with anti-HA and immunoblotted (IB) with anti-p53. The expression of JFK and p53 was examined by Western blotting (WB) with anti-FLAG or anti-p53. D, phosphorylation of Ser-149, Thr-150, and Thr-155 residues of p53 is essential for JFK-mediated p53 turnover. p53−/−/MDM2−/− MEFs were co-transfected with FLAG-JFK and Myc-tagged wild-type (wt) p53 or p53 mutants. Cellular lysates were analyzed for protein expression by Western blotting with antibodies against the indicated proteins.
Collectively, these results suggest that JFK functions to maintain a low steady-state level p53 with the action of CSN5 under an unstressed condition.

**CSN-associated Kinase Is Specifically Involved in JFK-inhibited p53 Transactivation Activity**—To investigate the biological significance of CSN-associated kinase in JFK-promoted p53 degradation, we first assessed the effect of CSN-associated kinase on the suppression of p53 transcription activity by JFK. In these experiments, p53−/−/MDM2−/− MEFs cells with CSN5 knockdown were co-transfected with wild-type p53, p53-AVV, or p53-DEE plus pG13-Luc together with JFK or COP1. Cellular lysates were prepared, and luciferase activity was measured. Each bar represents the mean ± S.D. for triplicate experiments. Knockdown of CSN5 resulted in a diminished effect of JFK on the negative regulation of the mRNA expression of p21 and Bax. U2OS cells with CSN5 knockdown were transfected with JFK expression construct or treated with JFK siRNA. Total RNAs were prepared for real time RT-PCR analysis of mRNA expression of p21 and Bax. Each bar represents the mean ± S.D. for triplicate experiments. Knockdown of CSN5 resulted in a diminished effect of JFK on the negative regulation of the protein expression of p21 and Bax. U2OS cells with CSN5 knockdown were transfected with JFK expression construct or treated with JFK siRNA. Cellular lysates were prepared for Western blotting using antibodies against the indicated proteins.

2F). Collectively, these results suggest that JFK functions to maintain a low steady-state level p53 with the action of CSN5 under an unstressed condition.

**FIGURE 3. CSN-associated kinase is specifically involved in JFK-inhibited p53 transactivation activity.** A, CSN5 knockdown resulted in a diminished negative effect of JFK, but not COP1, on p53 transactivation activity. p53−/−/MDM2−/− MEFs cells with CSN5 knockdown were transfected with wild-type p53, p53-AVV, or p53-DEE plus pG13-Luc together with JFK or COP1. Cellular lysates were prepared, and luciferase activity was measured. Each bar represents the mean ± S.D. for triplicate experiments. B, knockdown of CSN5 resulted in a diminished effect of JFK on the negative regulation of the mRNA expression of p21 and Bax. U2OS cells with CSN5 knockdown were transfected with JFK expression construct or treated with JFK siRNA. Total RNAs were prepared for real time RT-PCR analysis of mRNA expression of p21 and Bax. Each bar represents the mean ± S.D. for triplicate experiments. C, knockdown of CSN5 resulted in a diminished effect of JFK on the negative regulation of the protein expression of p21 and Bax. U2OS cells with CSN5 knockdown were transfected with JFK expression construct or treated with JFK siRNA. Cellular lysates were prepared for Western blotting using antibodies against the indicated proteins.

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Real time RT-PCR measurement of the mRNA expression of these two genes in U2OS cells that were transfected with the JFK expression vector indicated that JFK overexpression resulted in a decreased mRNA expression of p21 and Bax. However, when the expression of CSN5 was knocked down, the negative effect of JFK on the expression of these two p53 target genes was abrogated (Fig. 3B, left panel). Consistent with this, overexpression of JFK was associated with a marked decrease in endogenous p21 and Bax proteins, whereas knockdown of CSN5 resulted in a diminished effect of JFK on the negative regulation of the expression of p21 and Bax proteins (Fig. 3C, left panel). We also measured the mRNA and protein levels of p21 and Bax in U2OS cells treated with CSN5 and/or JFK siRNA. The results indicate that knockdown of JFK or CSN5 abolished the negative effect of JFK on the transcription regulation of these genes by p53 (Fig. 3, B and C, right panels). These data clearly indicate that CSN-associated kinase is necessary for the inhibition of p53 transactivation activity by JFK.

Biological Significance of CSN-associated Kinase in JFK-promoted p53 Turnover—We next investigated the effect of CSN-associated kinase on the regulation of cell proliferation by JFK. For this purpose, U2OS cells were stably transfected with JFK shRNA and/or CSN5 shRNA plasmids that carried the gene for neomycin resistance. Cells were maintained in culture medium supplemented with 1 mg/ml G418 for 14 days and stained with crystal violet for colony counting. Colony formation assays demonstrated that depletion of JFK was associated with a significant inhibition in cell proliferation, which could be attenuated by simultaneous knockdown of CSN5 (Fig. 4A). These data suggest that JFK-regulated cell growth is dependent on CSN-associated kinase.

Given that JFK is a negative regulator of p53 and that the function of JFK is associated with the CSN-associated kinase, we wished to determine whether the CSN-associated kinase could affect the cell cycle. Mammary carcinoma MCF-7 cells with CSN5 knockdown were transfected with JFK. The cell cycle profile was analyzed by propidium iodide staining and flow cytometry. Compared with vector-transfected cells, JFK overexpression was associated with a decreased cell population in G0/G1 and an increased cell population in the S phase (Fig. 4B). This was reflected by a decrease in the G0/G1 ratio from 3.5 to 1.6. However, JFK-overexpressing MCF-7 cells exposed to CSN5 siRNA exhibited a similar cell cycle profile compared with control cells. Meanwhile, the effect of CSN5 knockdown on the regulation of the cell cycle by JFK could be rescued by a CSN5 mutant, CSN5m (Fig. 4B), which was generated by synonymous mutation of the CSN5 cDNA at 64–81 bp.

We then examined the effect of CSN-associated kinase on the regulation of p53-induced apoptosis by JFK. For this purpose, H1299 cells with CSN5 knockdown were co-transfected with p53 and JFK. The cells were then double-stained with annexin V and propidium iodide and subjected to flow cytometry. The results revealed that the forced expression of p53 caused ∼43% of H1299 cells to undergo apoptosis. However, overexpression of JFK resulted in a decrease in apoptotic cells by ∼16%. Meanwhile, overexpression of JFK but knockdown of CSN5 restored the number of apoptotic cells to ∼45% (Fig. 4C). Collectively, the above experiments support the association of JFK and the CSN-associated kinase in the regulation of p53-dependent transcriptional activation, cell growth suppression, G0 arrest, and apoptosis.

JFK Is a p53-inducible Gene—A striking feature of the p53 negative regulators, including MDM2, Pirh2, and COP1, is the existence of an autoregulatory feedback loop between each of these proteins and p53. Therefore, it is plausible to hypothesize that such a loop also exists for JFK and p53. We thus investigated whether JFK is a transcriptional target for p53. The p53 transcription factor recognizes a cognate sequence RRRGWY(0–13)RRRCWGWY (R = G or A; W = A or T; Y = C or T; N = any nucleotide). Scanning the promoter region of the JFK gene found no putative p53 consensus binding site. However, bioinformatics analysis with rVista2.0 and PROMO software (based on version 8.3 of TRANSFAC) of the whole genome of JFK found a putative p53 binding sequence (AGGCATGTCG(N = 0)CAGCAGCAG) in the +32,217 to +32,236 positions downstream of the transcription start site in its gigantic (∼40 kb) intron 1 (Fig. 5A). We then cloned, upstream of a luciferase reporter gene, a 1223-bp fragment (+31,257 to +32,480) from JFK intron 1 containing the sequence AGGCATG-
The reporter assays revealed that although the p53(R175H) mutant had little effect on either JFK-Luc or JFKmut-Luc activity, wild-type p53 significantly increased the luciferase activity of JFK-Luc but not that of the JFKmut-Luc (Fig. 5A). Furthermore, chromatin immunoprecipitation (ChIP) (34, 35) confirmed the recruitment of p53 in the region of the JFK intron 1 containing the identified sequence but not in the region ~2 kb upstream of this sequence in H1299 cells transfected with wild-type p53. In p53(R175H)-transfected H1299 cells, p53 recruitment was not detected in either of the two regions (Fig. 5A). Moreover, ectopic expression of wild-type p53 but not p53(R175H) was associated with an elevated expression of JFK at both the mRNA and protein level in H1299 cells (Fig. 5B, upper panel), and knockdown of p53 in U2OS cells led to a significant reduction in JFK expression (Fig. 5B, lower panel). Significantly, increased JFK protein levels were also detected in U2OS cells (with wild-type p53) but not in H1299 cells (p53-null) and MDA-MB-231 cells (p53 mutated, R280K) under the treatment of ionizing radiation (IR), which stabilizes p53 and activates p53-dependent transcription, as measured by Western blotting (Fig. 5C). Taken together, these data suggest that JFK is a p53-inducible gene that forms a negative feedback loop with p53.

**DISCUSSION**

The amount of p53 protein in unstressed cells is kept low, and this is accomplished by control of its degradation rather than its translation from mRNA. Current literature indicates that p53 degradation is executed by several ubiquitin ligases, including MDM2, Pirh2, COP1, and ARF-BP1, and is ensured by auto-regulatory negative feedback loops between p53 and each of MDM2, Pirh2, and COP1. Although Pirh2, COP1, and ARF-BP1 interact with p53 and promote its ubiquitination, MDM2 is believed to regulate p53 by masking the access of p53 to transcriptional machinery and by ubiquitinating and targeting it for proteasomal degradation (8, 9). MDM2, Pirh2, COP1, and ARF-BP1 each possess an intrinsic E3 ligase activity that leads to lower p53 activity, and the genes that encode for MDM2, Pirh2, and COP1 are all transcriptionally activated by p53. Our previous report indicated that MDM2, Pirh2, COP1, and ARF-BP1 are not yet the whole pack of ubiquitin ligase complexes that control the cellular abundance of p53. We showed that JFK also interacts with p53 and promotes p53 turnover in a ubiquitin- and proteasome-dependent fashion (11). However, unlike MDM2, Pirh2, COP1, and ARF-BP1, which all possess an intrinsic E3 ligase activity, JFK, as a member of the F-box family, targets p53 via assembly an
SCF complex. This means that cells employ all kinds of E3 ligase arsenals, including the single subunit RING finger type (MDM2, Pirh2, and COP1), the multisubunit RING finger type (JFK), and the HECT domain type (ARF-BP1), in guarding p53. In addition, JFK is also transcriptionally regulated by p53. If our interpretation is correct, this means that there are at least five ubiquitin ligase complexes and four autoregulatory negative feedback loops involved in p53 regulation in cells.

JFK is the only Kelch domain-containing F-box protein encoded by the human genome. The Kelch motif was initially discovered as a 6-fold tandem element in the sequence of the Drosophila Kelch ORF1 protein and predicts a conserved tertiary structure, a β-propeller, that contains multiple potential protein-protein interaction sites (36). Indeed, we showed that this motif is required for the interaction of JFK with p53 (11). In their interactions with p53, MDM2 binds to the 52 N-terminal amino acid residues and Pirh2 binds to the central core domain (residues 82–292) of p53 (8). Although the exact binding sites for COP1 and ARF-BP1 remain to be determined, they do not appear to interact with the N-terminal domain of p53 (9, 10, 37). Our data indicate that JFK also binds to the central core region (residues 113–236) (11). Whether or not the binding of these proteins with p53 involves cooperative or competitive mechanisms is currently unknown. At least in vitro, JFK, ARF-BP1, COP1, Pirh2, and MDM2 are each capable of promoting ubiquitination of p53.

The similarity in functional relationships between these regulators and p53 is striking, and this kind of functional redundancy is intriguing. Several possibilities may explain the existence of the functional redundancy among these ubiquitin ligases, as reviewed in the literature (1, 5, 38). But the message is clear: the guardian of the genome must be carefully guarded with all available forces in the genome. In fact, in addition to MDM2, Pirh2, COP1, ARF-BP1, and JFK, other protein factors have been reported to negatively regulate p53 through ubiquitin-dependent or -independent mechanisms. For example, JNK targets p53 for ubiquitin-mediated degradation, although the MDM4 (39, 40), hSIRT1 (33), and the HDAC1 complex (41) exert negative control over p53 at the functional level. Moreover, ubiquitin-dependent destruction of p53 is often explored by viral pathogens. For instance, the human papillomavirus E6 protein targets p53 for ubiquitin-mediated degradation via the cellular E6-AP ubiquitin ligase, and the adenovirus E1B55K and E4orf6 proteins function together to promote p53 degradation through a Cul5-containing E3 ubiquitin ligase complex.

One possible rationale for this level of redundancy is that these gene products are expressed or act optimally in different cell or tissue types or even at different stages of development. The studies on MDM2, Pirh2, COP1, and ARF-BP1 and our data for JFK indicate that the gene expression of these regulators exhibits a nonoverlapping tissue distribution pattern. Specifically, JFK is highly expressed in heart and skeletal muscle, although the highest level of Pirh2 mRNA was detected in liver and heart (8); the highest expression of COP1 was found in testes (42); and the highest expression of ARF-BP1 was seen in skeletal muscle (10). Therefore, tissue-specific action of these ubiquitin ligases on p53 is a distinct possibility. Alternatively, different E3 ligases may be activated at different stages of development. For example, knock-out of MDM2 in mice is lethal at about 6 days after fertilization, at the time of implantation of the blastocyst (43, 44), leading to a suggestion that the MDM2 protein acts without any backup ubiquitin ligase activity in the blastocyst stage, but the other proteins might exert more normal function at later stages of development (1, 5, 37, 38). Moreover, it is proposed that the predominant role of MDM2 was not to mediate p53 degradation in unstressed cells but instead to control p53 level and activity during the stress response (1). This hypothesis might also explain the fact that MDM2 null embryonic cells would proliferate without any obvious growth arrest for up to 6 days and then die because p53 is activated by an undefined stage-specific developmental stress, and its proapoptotic functions remain incessant in the absence of MDM2 feedback inhibition.

There is also the possibility that one or more of the ubiquitin ligases are responsible for the maintenance of p53 levels in the nonstressed or basal state, although others target only stress-induced p53. In this regard, it is worth noting that activated p53 and stress-induced p53 proteins have very different protein modifications. Therefore, each of the ubiquitin ligases may be associated with distinct proteins in a cell, connecting them to different regulatory circuits and thus influencing different cellular functions of p53. Furthermore, the fact that p53 functions as a tetramer, that it has several variants or isoforms, that it can be differentially modified post-translationally in response to various stress signals, and that it associates with various cellular and viral proteins, all imply that different forms of p53 species co-exist within a cell. It is likely that these different forms of p53 are targeted by different ligases in a particular cellular micro-milieu.

Most substrates require phosphorylation to interact with the F-box protein in an SCF complex. Within the context of a complex cellular environment, phosphorylation endows substrate discrimination by the SCF complex due to its ability to specifically recognize phosphorylated motifs or phosphodegrons, thereby linking the substrate to the conjugation machinery. Additionally, phosphorylation offers temporal regulation of substrate degradation, even if SCF complexes are constitutively active throughout the cell cycle. As stated above, our previous data indicate that the p53 central core region is responsible for its binding to JFK (11). This region contains three phosphorylation sites, Ser-149, Thr-150, and Thr-155, all of which are potential targets for CSN-associated kinase (23, 30). Indeed, we showed that CSN-associated kinase is required for the association of p53 with JFK and for JFK-promoted p53 degradation. In support of these observations, we demonstrated that JFK was unable to destabilize p53 S149A/T150V/T155V in p53+/−/MDM2+/− MEFs. In the TP53 web site, it is indicated that mutation(s) at Ser-149, Thr-150, or Thr-155 do occur in human cancers, such as head and neck, lung, and pancreatic cancers. Our experiments (Fig. 3A) indicated that the transactivation activity of p53 S149A/T150V/T155V, p53 S149D/T150E/T155E, and wild-type p53 is about the same. However, the stability of these mutants increases, which could lead to a gain-of-function effect.
CSN is an eight-subunit complex and is highly homologous to the lid of the 26 S proteasome regulatory particle (20, 22). In addition to its associated kinase activity, CSN directly interacts with and is required for the proper function of SCF ubiquitin ligases by virtue of its isopeptidase activity residing in CSN subunit 5 (CSN5 or Jab1) that deconjugates the essential ubiquitin-like Nedd8 modification from Cul1 (20, 22). As such, CSN has been intimately linked to proteasome activity. Interestingly, genetic knock-out of Csn2 (45), Csn3 (46), or Csn5 (47, 48) in mice all resulted in an early embryonic lethality, massive cell apoptosis/death, and p53 accumulation, and several studies have indeed linked the CSN-associated kinase or CSN complex to p53 destabilization. Specifically, CSN5 has been reported to induce the translocation of p53 from the nucleus to the cytoplasm (32, 49) and has been observed to be able to inhibit MDM2 self-ubiquitination, which in turn promotes p53 ubiquitination (23, 24, 32, 45, 50). However, intriguingly, it was previously reported that p53 S149A/T150V/mutates p53 ubiquitination (23, 24, 32, 45, 50). However, in this study, it is likely that future work will extend the functional connection/coordination between CSN and the SCF ubiquitin ligase but also provides a molecular basis for the functional link between CSN and p53.

Further studies will be needed to investigate the cellular microenvironment and to determine the molecular mechanisms governing the functional regulation and substrate specification of MDM2, Pirh2, COP1, ARF-BP1, and JFK. It is also worthy of noting that p53 is not the only substrate for these ubiquitin ligases. Therefore, it is likely that future work will identify additional targets for JFK. Perhaps more relevant to our current study, it is critical to explore the scope of the CSN-associated kinase and the variety of other kinases in specifying substrates for JFK. Such efforts will be essential to better understanding of the biological function of JFK.

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