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Biochemical and Biophysical Research Communications 350 (2006) 114–119
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Expression of mouse Fbxw7 isoforms is regulated in a cell cycle- or p53-dependent manner

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Received 22 August 2006
Available online 12 September 2006

Abstract

Fbxw7 is the F-box protein component of an SCF-type ubiquitin ligase that contributes to the ubiquitin-dependent degradation of cell cycle activators and oncoproteins. Three isoforms (α, β, and γ) of Fbxw7 are produced from mRNAs with distinct 5′ exons. We have now investigated regulation of *Fbxw7* expression in mouse tissues. Fbxw7α mRNA was present in all tissues examined, whereas Fbxw7β mRNA was detected only in brain and testis, and Fbxw7γ mRNA in heart and skeletal muscle. The amount of Fbxw7α mRNA was high during quiescence (G_0 phase) in mouse embryonic fibroblasts (MEFs) and T cells, but it decreased markedly as these cells entered the cell cycle. The abundance of Fbxw7α mRNA was unaffected by cell irradiation or p53 status. In contrast, X-irradiation increased the amount of Fbxw7β mRNA in wild-type MEFs but not in those from p53-deficient mice, suggesting that radiation-induced up-regulation of p53 leads to production of Fbxw7β mRNA. Our results thus indicate that expression of Fbxw7 isoforms is differentially regulated in a cell cycle- or p53-dependent manner.

Keywords: Ubiquitin; SCF complex; F-box protein; Isoform, p53; Cancer; Cell cycle

The abundance of cyclins, cyclin-dependent kinase inhibitors, and many other regulators of the cell cycle is controlled by the ubiquitin–proteasome system. Various alterations in the ubiquitylation of cell cycle regulators are implicated in the etiology of many human malignancies [1]. Down-regulation of protein abundance by the ubiquitin–proteasome system occurs in two distinct steps [2]: the covalent attachment of multiple ubiquitin molecules to the protein substrate, and degradation of the polyubiquitylated protein by the 26S proteasome complex. The first of these steps is mediated by at least three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). Two major classes of E3s, the Skp1–Cul1–F-box protein (SCF) complex and the anaphase-promoting complex or cyclosome (APC/C), play a central role in cell cycle regulation [1]. The SCF complex consists of common subunits (Skp1, Cul1, Rbx1) and a variable substrate-recognition subunit (F-box protein). Three F-box protein components of the SCF complex—S phase kinase-associated protein 2 (Skp2), F-box and WD-40 domain protein 7 (Fbxw7), and β-transducin repeat-containing protein (β-TRCP)—have been thought to contribute primarily to cell cycle regulation.

Fbxw7 is the F-box protein of an SCF complex that targets several oncoproteins, including cyclin E, c-Myc, Notch, and c-Jun, for degradation [1]. Fbxw7 was first discovered by genetic screening as a negative regulator of the LIN-12 (Notch) signaling pathway in *Caenorhabditis elegans* [3]. We and others have generated mice that are deficient in Fbxw7 and found that the homozygous mutant embryos die in utero at embryonic day 10.5 manifesting marked abnormalities in vascular development [4,5]. Notch4 accumulates in $Fbxw7^{-/-}$ embryos, resulting in

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.09.003

increased expression of Hey1, a transcriptional repressor that acts downstream of Notch and is implicated in vascular development. These observations suggest that Fbxw7 plays an essential role in mammalian vascular development by regulating Notch stability during embryogenesis.

Given that Fbxw7 is responsible for degradation of the above-mentioned oncoproteins, it is thought to function as a tumor suppressor. Indeed, mutations in *FBXW7* have been identified in human ovarian [6], breast [7,8], endometrial [9,10], and colorectal [11] cancers. The loss of Fbxw7 in cultured cells also results in genetic instability [11]. In addition, *Fbxw7*$^{+/-}$ mice exhibit an increased susceptibility to radiation-induced tumorigenesis even though most tumors retain and express the wild-type allele, indicating that *Fbxw7* is a haploinsufficient tumor suppressor gene [12].

The *FBXW7* locus maps to human chromosomal region 4q32, which is frequently deleted in a wide range of human tumor types [13]. This locus encodes three protein isoforms (Fbxw7α, Fbxw7β, Fbxw7γ) [7,9,14,15], each of which is translated from an mRNA with a unique 5′ exon and 10 shared exons. Each isoform of Fbxw7 exhibits a distinct subcellular distribution: Fbxw7α is localized in the nucleus, Fbxw7β shows a cytoplasmic distribution suggestive of localization to the endoplasmic reticulum (ER) and Golgi apparatus, and Fbxw7γ is predominantly nucleolar [16,17]. Northern blot analysis of human tissues revealed that Fbxw7α mRNA is widely distributed, whereas Fbxw7β/γ mRNA (the probe did not distinguish between the two transcripts) is restricted to brain, heart, and skeletal muscle [9]. Expression of Fbxw7β was shown to be up-regulated by p53, and a potential p53 binding site is present in exon 1b of human *FBXW7* [18]. However, the regulation of Fbxw7α/γ expression has remained uncharacterized. Furthermore, most studies of Fbxw7β expression have been performed with cultured human cell lines, and the results therefore require verification in other species with primary cultured cells.

With the use of tissues and primary cultured cells, including embryonic fibroblasts (MEFs) and freshly isolated T cells, from wild-type or *p53*$^{-/-}$ mice, we have now shown that Fbxw7β and Fbxw7γ mRNAs exhibit different tissue and cell distributions. Furthermore, we found that Fbxw7α mRNA is abundant in quiescent NIH 3T3 cells and T cells but is down-regulated on entry of these cells into the cell cycle. Whereas the level of Fbxw7α mRNA in MEFs does not appear to be affected by p53 status, that of Fbxw7β mRNA is regulated in a p53-dependent manner. These observations suggest that the spatiotemporal control of *Fbxw7* expression in mice is mediated in an isoform-specific manner.

Materials and methods

Cells. Wild-type or *p53*$^{-/-}$ MEFs as well as freshly isolated lymphocytes were prepared as described previously [19,20]. For radiation treatment, cells were exposed to 0, 2, 4, or 8 Gy of ionizing radiation and then incubated at 37 °C for 4 h (MEFs) or 2 h (thymocytes). Splenic T cells were isolated to a purity of ~90% with the use of a T Cell Enrich column (R&D Systems); they were stimulated for the indicated times with plate-bound antibodies to (anti-) CD3ε (coated at 5 μg/ml; 145-2C11, BD Bioscience Pharmingen) in 96-well plates.

Quantitation of mRNA by RT-PCR. Total RNA was extracted from cells by the guanidinium thiocyanate–phenol-chloroform method, purified, and subjected (1 μg) to reverse transcription (RT) with random hexanucleotide primers (ReverTra Ace α, Toyobo). The resulting cDNA was then subjected to quantitative polymerase chain reaction (PCR) analysis with 1× SYBR Green PCR master mix (Applied Biosystems) and 200 nM gene-specific primers. Assays were performed in triplicate with an ABI Prism 7700 sequence detector (Applied Biosystems). The amplification protocol comprised initial incubation at 60 °C for 31 s and 95 °C for 5 s followed by 40 cycles. The sequences of the various primers (sense and antisense, respectively) were 5′-GCCTAAGATGAGCGCAAGTTG-3′ and 5′-TACTAGGCAGATGGCCACAGG-3′ for hypoxanthine phosphoribosyltransferase (HPRT), 5′-CTCACCAGCTCTCCTCTCCATT-3′ and 5′-GCTGAACATGGTACAAGGCCA-3′ for Fbxw7α, 5′-TTGTCAGAGACTGCCAAGCAG-3′ and 5′-GACTTTGCATGGTTTCTTTCCC-3′ for Fbxw7β, 5′-AACCATGGCTTGGTTCCTGTTG-3′ and 5′-CAGAACCATGGTCCAACTTTC-3′ for Fbxw7γ, and 5′-TGTCTGAGCGGCCTGAAGATTC-3′ and 5′-GCAGAAGACCAATCTGCGCTTG-3′ for p21. Each reaction was performed concurrently on the same plate with an HPRT control, and the results were normalized relative to HPRT mRNA abundance.

Flow cytometry. Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU, which was from BD Bioscience Pharmingen, was used for flow cytometry. All analyses were performed with FACSCalibur instrument (Becton–Dickinson). For cell cycle analysis, purified splenic T cells or NIH 3T3 cells were exposed to 10 μM bromodeoxyuridine (BrdU) during the last 1 h of incubation, washed with phosphate-buffered saline, fixed in 70% ethanol at 4 °C, and incubated for 30 min at room temperature with 2 M HCl containing 0.5% Triton X-100. After neutralization with 0.1 M sodium tetraborate (pH 8.5), the cells were washed with phosphate-buffered saline containing 1% bovine serum albumin and 0.5% Tween 20, stained with FITC-conjugated anti-BrdU, washed again, stained with propidium iodide (PI), and then analyzed by flow cytometry.

Statistical analysis. Quantitative data are expressed as means ± SD and were analyzed by Student's *t* test. A P value of <0.05 was considered statistically significant.

Results

Tissue distribution of Fbxw7 isoform mRNAs in mice

We investigated the distribution of Fbxw7 isoform mRNAs in mouse tissues by semiquantitative RT-PCR analysis with sets of specific primers corresponding to the distinct 5′ exon of each mRNA. Fbxw7α mRNA was detected in all tissues examined (Fig. 1). In contrast, Fbxw7β and Fbxw7γ mRNAs were found to be restricted to brain and testis, and to heart and skeletal muscle, respectively. Fbxw7α and Fbxw7β mRNAs, but not Fbxw7γ mRNA, were also detected in MEFs. In the following experiments, we attempted to characterize the regulation of Fbxw7α and Fbxw7β mRNA abundance in cultured mouse cells.

Fbxw7α mRNA is enriched in quiescent cells

Given that Fbxw7 is thought to contribute to the ubiquitin-dependent degradation of cell cycle activators such as

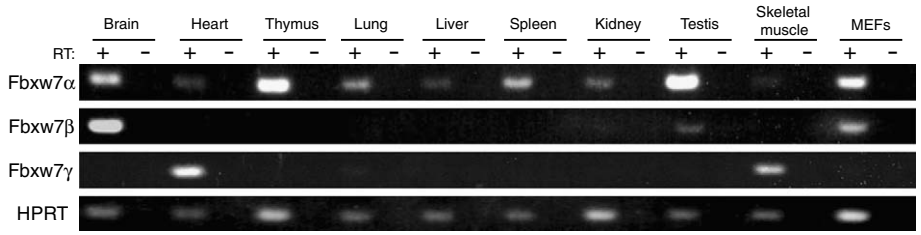
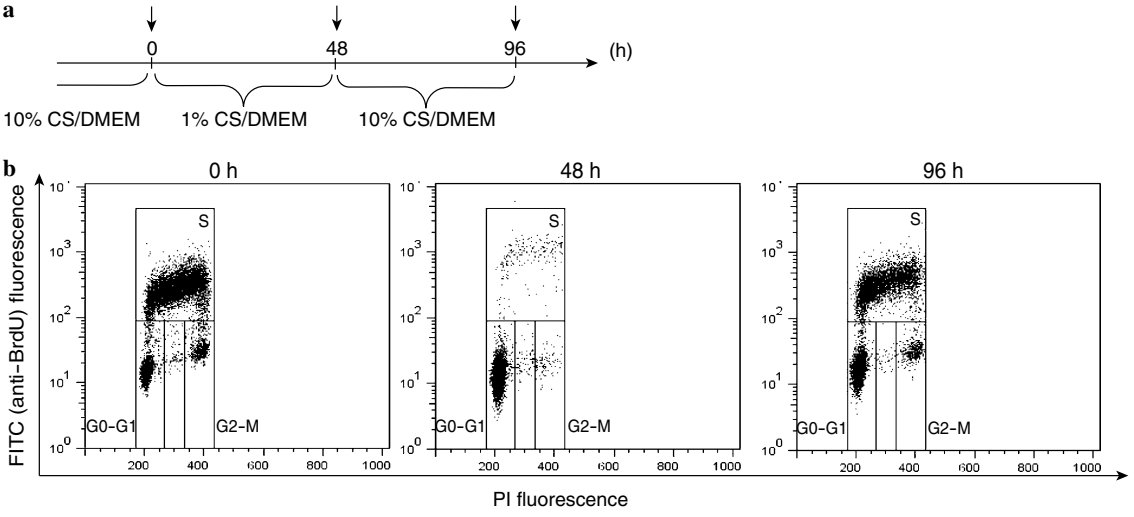


Fig. 1. Distribution of Fbxw7 isoform mRNAs in mouse tissues. The amounts of Fbxw7α, Fbxw7β, and Fbxw7γ mRNAs in the indicated tissues and cells were determined by semiquantitative RT-PCR analysis. RT (+) and RT (−) indicate reactions performed with or without reverse transcriptase, respectively.



Time (h)	G0–G1 (%)	S (%)	G2–M (%)
0	24.1	68.4	6.6
48	95.1	2.7	1.2
96	49.6	45.3	4.5

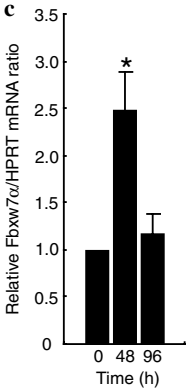


Fig. 2. Cell cycle-dependent regulation of Fbxw7α mRNA in NIH 3T3 cells. (a) Protocol for induction of arrest and subsequent reactivation of the cell cycle. Cells cultured in Dulbeccos's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS) were incubated for 48 h in DMEM containing 1% CS and then for 48 h in DMEM containing 10% CS. They were harvested at time 0, 48, and 96 h as indicated. (b) Flow cytometric analysis of cell cycle status. The percentages of cells in G_0 or G_1, in S, and in G_2 or M phases of the cell cycle at each time point are indicated. (c) RT and real-time PCR analysis of Fbxw7α mRNA at the indicated time points. Data are means ± SD of triplicates from a representative experiment. $^*P < 0.01$ versus value for time 0.

cyclin E, c-Myc, and c-Jun, we hypothesized that Fbxw7 might be expressed specifically in quiescent cells. To test this hypothesis, we induced quiescence in NIH 3T3 cells by depriving them of serum for 48 h and then examined the level of Fbxw7α mRNA by RT and real-time PCR analysis (Fig. 2a). Cell cycle status was monitored by flow cytometric analysis (Fig. 2b). The amount of Fbxw7α mRNA was increased in response to serum deprivation, and it subsequently returned to its original level on stimulation of the cells to re-enter the cell cycle by replenishment of serum (Fig. 2c).

We also measured the level of Fbxw7α mRNA in freshly isolated splenic T cells. Flow cytometric analysis revealed that only a small proportion (0.8%) of these cells was in

S phase, indicative of cell cycle arrest (Fig. 3a). Mitogenic stimulation with anti-CD3ε increased the proportion of cells in S phase in a time-dependent manner. The amount of Fbxw7α mRNA was significantly reduced 24 h after the initiation of stimulation with anti-CD3ε (Fig. 3b), when 5.2% of the cells had entered S phase (Fig. 3a). The decrease in the level of Fbxw7α mRNA was more pronounced at 48 h, when 45.4% of cells were in S phase. Together, these results showed that Fbxw7α mRNA is most abundant in quiescent cells and undergoes down-regulation as cells enter the cell cycle. The abundance of Fbxw7β and Fbxw7γ mRNAs is too low to be detected in NIH 3T3 cells and splenocytes by RT-PCR.

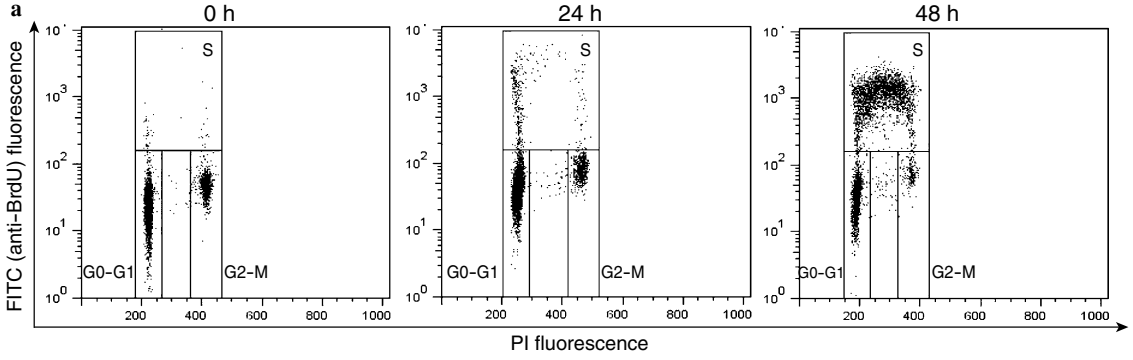
Genotoxic stress up-regulates Fbxw7β mRNA in a p53-dependent manner

We exposed MEFs or mouse thymocytes to 0, 2, 4, or 8 Gy of X-radiation and then examined the abundance of Fbxw7α and Fbxw7β mRNAs by quantitative RT-PCR analysis at 4 h (MEFs) or 2 h (thymocytes) thereafter. Fbxw7α mRNA was detected in both MEFs and thymocytes, but its abundance in these cells was not affected by X-radiation (Fig. 4a). In contrast, Fbxw7β mRNA was detected only in MEFs, and its abundance in these cells was increased by X-radiation in a dose-dependent manner (Fig. 4b). As a positive control, p21 mRNA was shown to be up-regulated by X-radiation in both cell types, although its abundance was markedly greater in MEFs than in thymocytes (Fig. 4c).

To examine whether the up-regulation of Fbxw7β mRNA by X-radiation in MEFs is mediated by p53, we compared the effects of irradiation between MEFs derived from wild-type or $p53^{-/-}$ mice. The amount of Fbxw7α mRNA in $p53^{-/-}$ MEFs was similar to that in the wild-type cells and was not affected by X-radiation (Fig. 4a). In contrast, the basal level of Fbxw7β mRNA in $p53^{-/-}$ MEFs was greatly reduced compared with that in wild-type MEFs, and it was not increased in response to X-irradiation (Fig. 4b). Similar results were obtained with p21 mRNA (Fig. 4c), the gene for which is p53 inducible. Fbxw7γ mRNA was not detected in MEFs by RT-PCR. Our data suggest that the production of Fbxw7β mRNA, but not that of Fbxw7α mRNA, is activated by p53.

Discussion

Fbxw7 has been implicated as a key regulator of the cell cycle and an oncosuppressor protein, given that most proteins targeted by Fbxw7 for degradation are cell cycle



Time (h)	G0–G1 (%)	S (%)	G2–M (%)
0	86.0	0.8	12.9
24	85.6	5.2	8.5
48	49.6	45.4	3.9

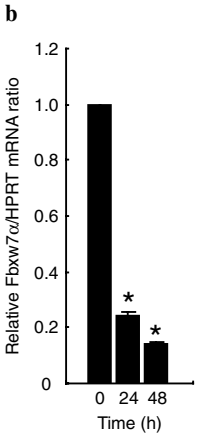


Fig. 3. Down-regulation of Fbxw7α mRNA on entry of splenic T cells into the cell cycle. (a) Splenic T cells were stimulated with anti-CD3ε in RPMI-1640 supplemented with 10% fetal calf serum for 0, 24, or 48 h, at which times cell cycle status was determined by flow cytometry. The percentages of cells in G_0–G_1, S, and G_2–M phases of the cell cycle at each time point are shown. (b) RT and real-time PCR analysis of Fbxw7α mRNA at the indicated time points. $^*P < 0.01$ versus value for time 0.

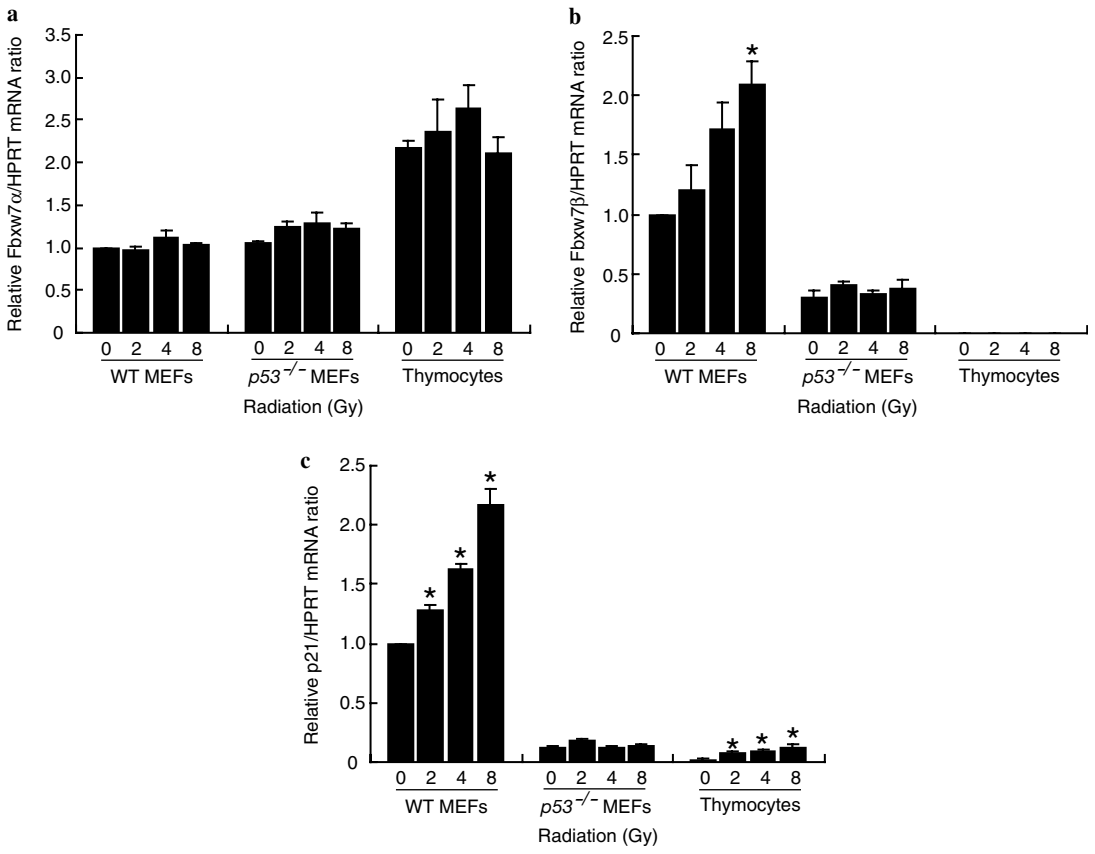


Fig. 4. Up-regulation of Fbxw7β mRNA by X-radiation in a p53-dependent manner. Wild-type (WT) or $p53^{-/-}$ MEFs as well as wild-type thymocytes were exposed to 0, 2, 4, or 8 Gy of ionizing radiation and then incubated for 4 h (MEFs) or 2 h (thymocytes) at 37 °C in DMEM (MEFs) or RPMI-1640 (thymocytes) supplemented with 10% fetal calf serum. The abundance of Fbxw7α (a), Fbxw7β (b), and p21 (c) mRNAs was determined by RT and real-time PCR analysis. Data for each mRNA are expressed relative to the corresponding value for nonirradiated wild-type MEFs. $^{*}P < 0.01$ versus value for corresponding nonirradiated cells.

activators, such as cyclin E, c-Myc, and c-Jun [1]. Consistent with this notion, mutations in *FBXW7* have been detected in certain human malignancies [6–11,15]. Like cyclin E, c-Myc, and c-Jun, Fbxw7α is localized to the nucleoplasm. Our results now indicate that the abundance of Fbxw7α mRNA is highest in quiescent cells and decreases as cells enter the cell cycle. This expression pattern is consistent with an antiproliferative action of Fbxw7α. The mechanism underlying this cell cycle-dependent regulation of Fbxw7α expression remains to be determined.

Fbxw7β is localized to the ER membrane in the cytoplasm [16,17], a localization likely mediated by a putative transmembrane domain that is present near the NH_2-terminus and encoded by the β isoform-specific 5′ exon [14]. Although cytoplasmic cyclin E has been suggested as a target of Fbxw7β [17], the bona fide targets and functions of Fbxw7β remain unknown. In contrast, Fbxw7γ, which is localized to the nucleolus, was shown to contribute to control both of the amount of c-Myc in the nucleolus and of cell size [16]. The tissue distribution patterns of Fbxw7β and Fbxw7γ may provide insight into their functions. Fbxw7β mRNA is largely restricted to the brain, suggesting that the targets of Fbxw7β might be ER-associated proteins in neurons. Parkin, mutations in the gene for which are responsible for an autosomal recessive, early onset form of Parkinson's disease, has been shown to interact with Fbxw7 [21]. Among potential targets of parkin, the Pael receptor is an ER-resident protein and is ubiquitylated by the ER-associated protein degradation pathway [22,23]. These observations thus implicate Fbxw7β in degradation of the Pael receptor. Fbxw7γ mRNA is largely restricted to muscle tissue, including the heart and skeletal muscle. Given the role of Fbxw7γ in regulation both of the nucleolar level of c-Myc and of cell size and that muscle fibers are large compared with other cell types, Fbxw7γ might contribute to muscle differentiation through regulation of c-Myc-dependent cell growth.

We previously identified mouse *Fbxw7* as a p53-dependent tumor suppressor gene with the use of a mammalian genetic screen for p53-dependent genes involved in tumorigenesis [12]. $Fbxw7^{+/-}$ mice manifest an increased susceptibility to radiation-induced tumorigenesis, although most of the induced tumors retain and express the wild-type allele. Loss of Fbxw7 also alters the spectrum of tumors that develop in p53-deficient mice to include those of epithelial tissues in the lung, liver, and ovary. Radiation-

induced lymphomas in $p53^{+/-}$ mice, but not those in $p53^{-/-}$ mice, also show frequent deletion or mutation of *Fbxw7*. We have now shown that Fbxw7α mRNA is present in T cells, but that its abundance in these cells appears to be independent of p53. In contrast, the amount of Fbxw7β mRNA in MEFs was shown to be controlled by p53, but this transcript was not detected in thymocytes. These results suggest that the total amount of Fbxw7 isoforms is unlikely to be reduced more in $Fbxw7^{+/-}$;$p53^{+/-}$ T cells than in $Fbxw7^{+/-}$ T cells. It thus remains to be determined why a decrease in *Fbxw7* expression results in tumorigenesis in a p53-dependent manner.

Acknowledgments

We thank Masaaki Nishiyama for p21 primers for quantitative RT-PCR; Koji Oyamada and Naoko Nishimura for technical assistance; Ryosuke Tsunematsu, Takamichi Sato, Etsuo Susaki, Toru Saiga, Tadashi Nakagawa, and other members of our laboratory for comments on the manuscript; and Akane Ohta and Mihoko Kimura for help in preparation of the manuscript.

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