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Mechanism of Functional Inactivation of a Li-Fraumeni Syndrome p53 That Has a Mutation Outside of the DNA-binding Domain[1]

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ABSTRACT

The majority of p53 mutations are located in the DNA-binding domain of the protein. However, recently a family suffering from Li-Fraumeni syndrome (LFS) has been discovered, some of whom harbor a *p53* mutation in exon 4, outside of the core domain. How this mutation affects p53 function and subsequently leads to malignant transformation is not yet clear. Interestingly, the p53 mutation found in this LFS family is localized to the p53 region that we have recently identified as necessary for Mdm2-mediated p53 degradation. We therefore endeavored to study further the LFS-associated p53 mutation at the molecular level by creating an equivalent lesion in a p53 expression construct and functionally characterizing it. Here we demonstrate that a mutation in this region is associated not only with resistance of the mutant p53 to Mdm2-mediated degradation, but also with an impaired response of mutant protein to DNA damage. In addition, the p53(LFS) mutant was found to be defective in its transactivation function, which correlated with its inability to suppress cell growth and to induce apoptosis. The molecular basis for p53(LFS) functional impairment appears to be its predominantly cytoplasmic localization caused by faulty nuclear import mechanism, which, at least in part, resulted from the mutant's decreased affinity to importin.

INTRODUCTION

LFS[3] is a rare autosomal-dominant disorder that features the onset of tumors under the age of forty-five, with a wide variety of tumor types represented. Usually, several family members are affected (1). The underlying genetic lesion in most LFS families is a mutation in the tumor suppressor gene *p53*, which is consistent with the finding that >50% of human cancers are associated with mutation in *p53* (2). The majority of *p53* mutations have been found in the DNA-binding domain of the protein, which results in a loss of p53 transcriptional function. Birch *et al.* (3), however, reported a LFS family with a *p53* mutation in exon 4, outside of the DNA-binding domain. This mutation involved deletion of 11 bp and insertion of 5 bp that corresponds to a change in codons 108–111 from Gly-Phe-Arg-Leu to Ile-Gln, but which did not lead to an alteration of the reading frame. The same mutation was detected in the proband and his affected mother, indicating that this mutation indeed accounts for the high incidence of cancer in the family. How a mutation outside of the DNA-binding domain can affect p53 function and cause malignant transformation remains unclear.

In response to genotoxic stress, the tumor suppressor p53 is induced, and initiates a series of cellular responses to prevent lesions caused by the stress of persisting in the genome. Inactivation of p53 results in a failure of cells to respond properly to genotoxic stress, which in turn leads to replication or segregation of damaged DNA and subsequent genomic instability (4). p53 induction by genotoxic stress is attributable, in large part, to an increased half-life of the p53 protein (5). An important regulator of p53 protein stability is Mdm2, which has been reported to function as an E3 ubiquitin ligase, thereby promoting ubiquitination and subsequent degradation of p53 (6). We recently identified a unique sequence within p53 that is required for Mdm2-mediated degradation (7). Interestingly, the LFS-associated exon 4 *p53* mutation is located in the same region of the p53 molecule. We therefore set out to characterize the p53(LFS) mutant and to investigate how this mutation leads to the inactivation of p53 function.

MATERIALS AND METHODS

Cell Culture, Transfection, and Luciferase Assay. H1299 cells, SAOS-2 cells (American Type Culture Collection), NIH 3T3 (J. B. Little, Harvard School of Public Health, Boston, MA), $p53^{-/-}$ MEFs (P. Leder, Harvard Medical School, Boston, MA), and Rat/Myc embryonic fibroblasts (S. Kharbanda, Dana-Farber Cancer Institute, Boston, MA) were maintained in MEM supplemented with 10% fetal bovine serum. Cells were transfected by a calcium-phosphate method as described (7). For luciferase assay, cells were seeded in 60-mm plates and transfected with one of the expression vectors (200 ng of each) and a luciferase reporter construct containing a p53-responsive element. Luciferase activity was measured 24 h posttransfection as described previously (7). The p53(LFS) mutant and point mutants were generated with the method described previously (7).

Preparation of Whole Cell Extracts and Immunoprecipitation Analysis. Cells were transfected in 60-mm plates with 8 μg of DNA and harvested at 24 h posttransfection. Cells were lysed in 100 μl of lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors] by incubating on ice for 30 min, and the extracts were centrifuged at 13,000 rpm for 15 min to remove cell debris. Protein concentrations were determined by the Bio-Rad protein assay (Hercules, California). GST-binding assays were performed by incubation of the GST-fusion protein with cell lysate at 4°C for 4 h. Then the beads were washed four times with 0.8 ml of lysis buffer, and after removing the excess liquid, 30 μl of 2× loading buffer were added. Then the samples were incubated at 95°C for 5 min and resolved on a SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) and probed with the indicated antibody. Proteins were visualized with an enhanced chemiluminescence detection system (NEN).

Colony Formation Assay. $p53^{-/-}$ MEFs, NIH3T3, or H1299 cells were transfected with hygromycin-containing vector expressing wild type or p53(LFS) (10 μg/100-mm plate). Empty vector was included as a control. The cells were split (1:3) into selection media 48 h posttransfection. The plates were fixed and stained after 14 days. Colonies containing >50 cells were scored.

Apoptosis Assay. Rat/Myc cells were transfected in 60-mm plates with 5 μg of expression vector containing either GFP-tagged wild-type or mutant p53. At 48 h posttransfection, cells were observed under fluorescence (Nikon Diaphot 300), and images were photographed with an Pptronics 3CCD camera linked to a PC. For each condition, three plates were used, and 500 GFP-positive cells were counted in randomly selected fields from each plate. Among the GFP-positive populations, apoptotic cells were identified by the presence of apoptotic bodies and membrane blabbing.

Subcellular Distribution Assay. Cells were grown on Chamber Slides (Nunc, Naperville, IL) and transfected with GFP-p53(WT) or GFP-p53(LFS).

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[3] The abbreviations used are: LFS, Li-Fraumeni syndrome; LMB, leptomycin B; aa, amino acid; NLS, nuclear localization sequence; NES, nuclear export sequence; MEF, mouse embryonic fibroblasts; GST, glutathione *S*-transferase; GFP, green fluorescent protein; WT, wild type.

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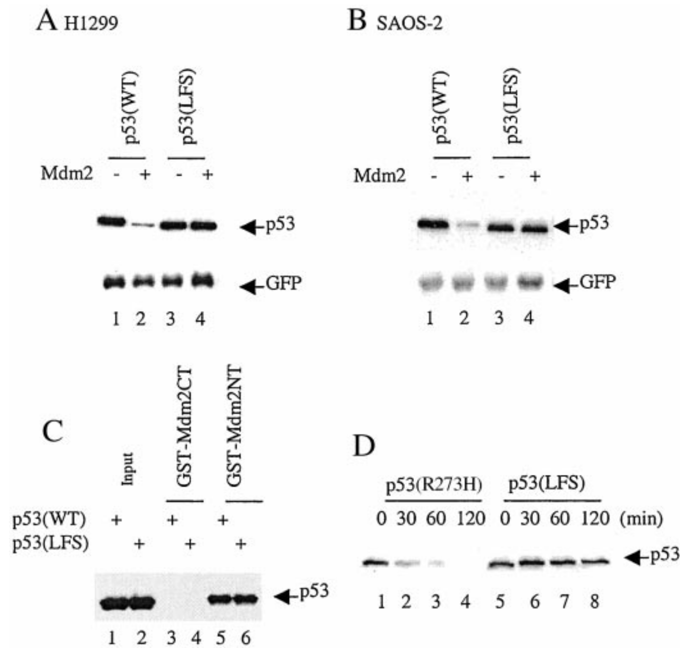

Fig. 1. Resistance of the p53(LFS) mutant to Mdm2-targeted degradation. *A*, Flag-tagged p53(WT) (*Lanes 1* and *2*) or p53(LFS) mutant (*Lanes 3* and *4*) (1 μg) expression vector was cotransfected with pCMV-empty (*Lanes 1* and *3*) or pCMV-Mdm2 vector (*Lanes 2* and *4*) (3 μg) into H1299 cells. pGFP-C1 (Clontech; 0.5 μg) was included as a transfection efficiency control. The transfectants were harvested 24 h posttransfection, and protein levels were analyzed by Western blot with anti-Flag (M5, Sigma) or anti-GFP (Clontech). *B*, similar experiment to *A* was performed in SAOS-2 cells. *C*, lysates prepared from cells expressing Flag-tagged p53(WT) (*Lanes 1*, *3*, and *5*) or p53(LFS) mutant (*Lanes 2*, *4*, and *6*) were incubated with GST-Mdm2 c-terminus (GST-Mdm2CT, *Lanes 3* and *4*) or GST-Mdm2 n-terminus (GST-Mdm2NT, *Lanes 5* and *6*). The adsorbates were analyzed by Western blot with an anti-Flag antibody. One-tenth of the whole cell lysates was loaded as a blotting control (*Lanes 1* and *2*). *D*, Flag-tagged vector expressing p53(R273H) (*Lanes 1–4*) or the LFS p53 mutant (*Lanes 5–8*) (5 μg) were transfected into H1299 cells. Cyclohexamide (20 μg/ml) was added into the media at 24 h posttransfection. The cells were harvested at 0, 30, 60, and 120 min after addition of the drug and then subjected to Western analysis as described in "Materials and Methods."

Twenty-four h after transfection, cells were washed with cold PBS and fixed with 4% paraformaldehyde for 30 min at 4°C. After the PBS wash and quenching with 50 mM NH_4Cl for 5 min, coverslips were washed again with PBS and mounted with antifade solution. Specimens were examined under a fluorescent microscope. Where indicated, cells were treated with 100 nM LMB for 4 h at 24 h posttransfection.

RESULTS

The p53(LFS) Mutant Is Resistant to Mdm2-mediated Degradation. To characterize the p53(LFS) mutant at the molecular level, we generated a p53 mutant equivalent to that found in the LFS patient that had a change in aa residues 108–111 from Gly-Phe-Arg-Leu to Ile-Gln and subcloned it into pCDNA3-Flag expression vector. Restriction enzyme digestion and DNA sequence were used to confirm its identity (not shown). We showed previously that substitution or deletion of the aa 92–112 of p53 resulted in a protein that is resistant to Mdm2-mediated degradation (7). Therefore, it was of interest to determine whether the LFS-associated mutation had any effect on p53 susceptibility to degradation by Mdm2. Vectors expressing p53(LFS) or wild-type p53 were cotransfected with pCMV-Mdm2 or pCMV-empty vector into H1299 cells, which are null for *p53*. Cell lysates were prepared 24 h posttransfection, and the p53 protein levels were determined by Western blot analysis. As expected, wild-type p53 was efficiently degraded by Mdm2 (Fig. 1*A*, *Lanes 1* and *2*). In contrast, no apparent decrease of the p53(LFS) level was detected in the Mdm2-expressing H1299 cells (*Lanes 3* and *4*). The resistance of the p53(LFS) to Mdm2-mediated degradation was also observed when SAOS-2 cells were used (Fig. 1*B*), indicating that this resistance to degradation by Mdm2 was not cell-type specific. Because binding of p53 to Mdm2 is required for Mdm2-mediated degradation, the resistance to Mdm2 associated with this p53 mutation could be attributable to a defect in the binding of the p53 mutant to Mdm2. To test this possibility, a GST pull-down assay was performed to assess the binding of the p53(LFS) mutant to Mdm2. A GST fusion protein of Mdm2 NH_2 terminus was incubated with cell lysates of H1299 cells expressing the p53(LFS) mutant or wild-type p53. A GST fusion protein of the Mdm2 COOH-terminus was included as a control. Consistent with the location of the p53-binding motif at the Mdm2 NH_2 terminus, analysis of the adsorbates revealed strong binding of p53 to the GST-Mdm2 NH_2 terminus, but not the COOH terminus. The p53(LFS) mutant displayed comparable binding affinity to Mdm2 (Fig. 1*C*), indicating that resistance of the p53(LFS) mutant to the Mdm2-mediated degradation was not a result of defective Mdm2 binding.

To test whether the resistance to Mdm2-mediated degradation would correlate with altered protein stability, we measured the half-life of the p53(LFS) mutant. Vectors expressing p53(R273H) and p53(LFS) were transfected into H1299 cells. Cycloheximide, which inhibits *de novo* protein synthesis, was added to the medium at 24 h posttransfection, and the cells were harvested at 0, 30, 60, and 120 min after the addition of the cycloheximide for Western blot analysis. As shown in Fig. 1*D*, the half-life of the p53(LFS) mutant was significantly prolonged when compared with that of p53(R273H), indicating that resistance of the p53(LFS) to Mdm2-mediated degradation corresponded to its increased stability.

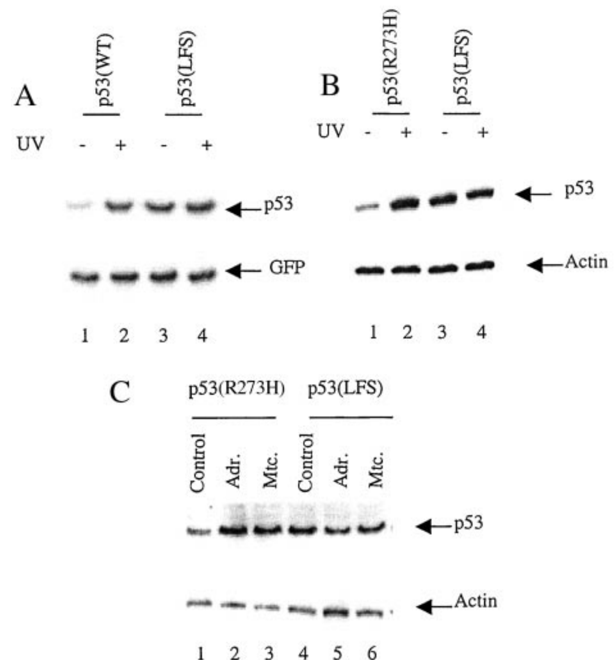

Fig. 2. Failure of the p53(LFS) mutant to be induced by DNA damage. *A*, Flag-p53(WT) (*Lanes 1* and *2*) or Flag-p53(LFS) (*Lanes 3* and *4*) (0.5 μg/60-mm plate) were transfected into $p53^{-/-}$ MEFs. 0.5 μg of pGFP vector was included as a transfection control. The cells were irradiated with UV light (15 J/cm^2). After incubation for an additional 8 h at 37°C, the cells were harvested and analyzed by Western blot with anti-Flag or anti-GFP. *B*, Flag-p53(R273H) (*Lanes 1* and *2*) or p53(LFS) (*Lanes 3* and *4*) vector containing hygromycin-resistant marker was transfected into $p53^{-/-}$ MEFs. The cells were selected with hygromycin-containing media (400 μg/ml) 48-h posttransfection. Two weeks later, the positive cells were pooled and expanded for UV-induced p53 induction as described in *A*. Immunoblotting with an anti-actin antibody was included as a loading control. *C*, the stable transfectants were treated with Adriamycin (2 μg/ml) or mitomycin C (10 μg/ml) for 6 h. The cells then were analyzed by Western blot.

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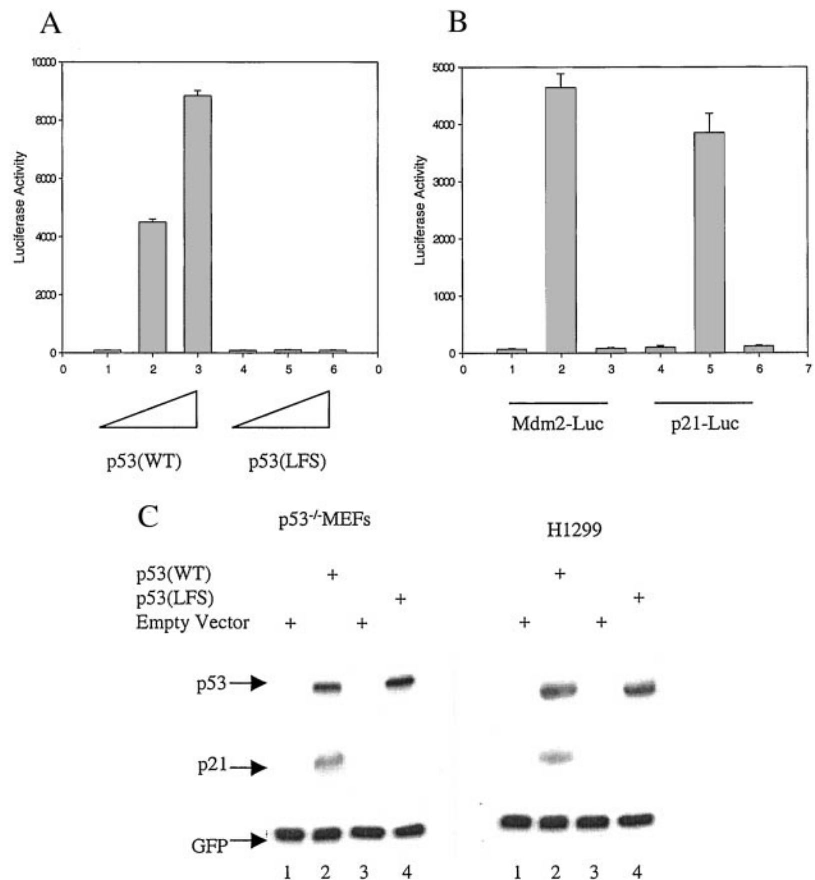

Fig. 3. An impaired transcription function of the p53(LFS) mutant. *A*, Flag-p53(WT) (0, 0.5, or 1 μg) or Flag-p53(LFS) (0, 0.5, or 1 μg) were cotransfected into $p53^{-/-}$ MEFs with pGL13-Luc plasmid-containing *luciferase* gene and p53-responsive elements. *Luciferase* activity was assayed 24 h later using Promega Rapid Detection System. Experiments were repeated twice with all samples assayed in duplicates. *B*, similar luciferase assays were performed with reporter construct with Mdm2 or p21-binding site [*Lanes 1* and *4* are empty vector, *Lanes 2* and *5* are p53(WT), and *Lanes 3* and *6* are p53(LFS)]. *C*, Flag-p53(WT) (*Lane 2*) or Flag-p53(LFS) (*Lane 4*) (1 μg) were transfected into $p53^{-/-}$ MEFs (*left panel*) or H1299 cells (*right panel*). The cells were analyzed for p53 and p21 expression 24-h posttransfection.

The p53(LFS) Mutant Is Not Induced in Response to DNA Damage. Because Mdm2-mediated degradation has been suggested to be a principle mechanism in DNA damage-induced p53 accumulation, we asked whether the resistance to Mdm2-mediated degradation would compromise the p53 induction by DNA damage. Flag-tagged vectors expressing wild-type p53 or p53(LFS) were transiently transfected into $p53^{-/-}$ MEFs and then UV-irradiated 24 h posttransfection. After incubation for an additional 8 h, the cellular p53 levels were determined by Western blot analysis. In contrast to the wild-type p53, which was markedly induced by the UV treatment, the p53(LFS) mutant failed to display any appreciable induction in response to UV irradiation (Fig. 2*A*). To confirm this finding, stable MEF cell lines expressing p53(R273H) (the ability of p53 to suppress cell growth impedes a stable expression of wild type p53) or p53(LFS) mutant were generated to assess the UV-induced p53 accumulation. As shown in Fig. 2*B*, UV treatment was associated with a significant induction of the p53(R273H) but not the p53(LFS) mutant. To examine if this defect is damage-type specific, additional DNA damage agents, Adriamycin and mitomycin C, were included. Again, unlike the p53(R273H)-expressing clone, exposure of the p53(LFS)-expressing cells to either Adriamycin or mitomycin C did not result in any noticeable induction of the p53 mutant protein (Fig. 2*C*, *Lanes 4–6*). In addition, the p53(LFS)-expressing cells were found to have elevated basal levels of p53. These results demonstrate that the mutation found in p53(LFS) leads to increased p53 protein levels, which are not induced further in response to DNA damage.

The p53(LFS) Mutant Exhibits an Impaired Transactivation Function and Inability to Suppress Cell Growth and Induce Apoptosis. The transactivation activity of p53 is crucial to its tumor-suppressor function. Although the mutation found in the p53(LFS) mutant is outside of the DNA-binding domain, it was of interest to examine whether this mutation had any effect on its transcriptional activity. To accomplish this, the p53(LFS) expression vector was cotransfected with a reporter construct containing the *luciferase* gene driven by a p53 enhancer from the PG13 element into *p53*-deficient MEFs. As expected, expression of wild-type p53 activated the luciferase activity in a dose-dependent fashion. The p53(LFS) mutant, however, lacked the ability to stimulate luciferase expression (Fig. 3*A*). When assayed with a luciferase reporter containing the p21 or Mdm2 promoter, similar results were obtained (Fig. 3*B*), indicating a generally compromised transactivation function associated with this mutation. Western blot analysis of p21 induction confirmed the results obtained from the luciferase assays. As shown in Fig. 3*C*, expression of p53(LFS) mutant in $p53^{-/-}$ MEFs did not result in any detectable induction of p21, whereas wild-type p53-expressing cells exhibited an increase in p21 expression (Fig. 3*C*, *left*). An almost identical result was seen in H1299 cells (Fig. 3*C*, *right*).

Because generally there is a strong correlation between transactivation activity of p53 and the suppression of cell proliferation, we next performed a colony formation assay to assess the efficiency of growth suppression by the p53(LFS) mutant. Parallel transfection of $p53^{-/-}$ MEFs with the *hygromycin resistance* gene-containing vector expressing wild-type p53 or p53(LFS) mutant were performed. Examination of the plates 2 weeks after selection with hygromycin demonstrated that wild-type p53 efficiently suppressed cell growth as expected, whereas the p53(LFS) mutant-expressing cells failed to display growth inhibition (Fig. 4*A*). Similar results were obtained when H1299 and NIH 3T3 cells were tested (Fig. 4*A*). Quantitative analysis of the colony numbers revealed a moderate outgrowth in the p53(LFS)-expressing cells (Fig. 4*B*). Hence, the inability of the p53(LFS) mutant to suppress growth is probably attributable to its impaired transactivation function.

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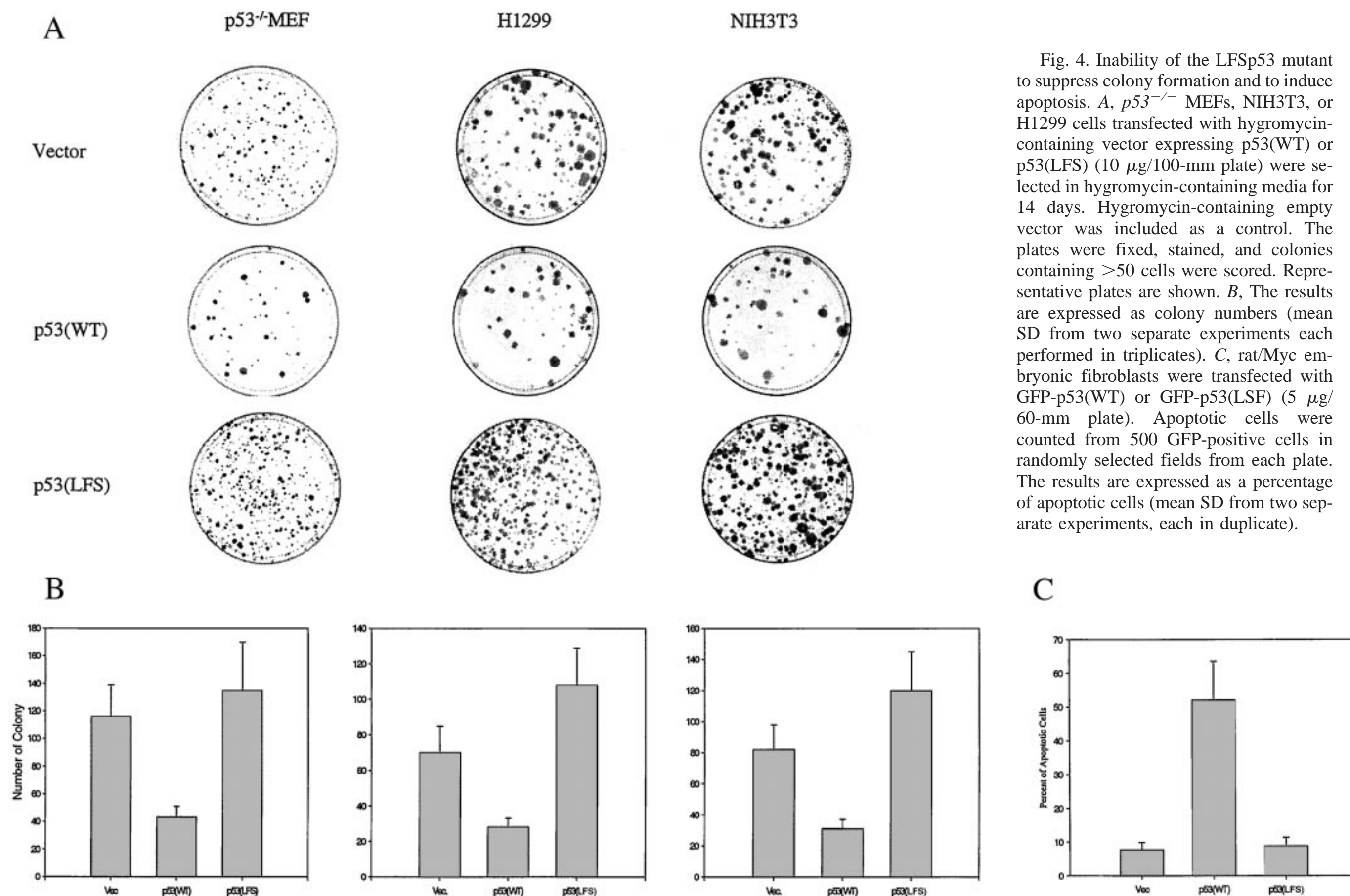

Fig. 4. Inability of the LFSp53 mutant to suppress colony formation and to induce apoptosis. *A*, *p53*$^{-/-}$ MEFs, NIH3T3, or H1299 cells transfected with hygromycin-containing vector expressing p53(WT) or p53(LFS) (10 μg/100-mm plate) were selected in hygromycin-containing media for 14 days. Hygromycin-containing empty vector was included as a control. The plates were fixed, stained, and colonies containing >50 cells were scored. Representative plates are shown. *B*, The results are expressed as colony numbers (mean SD from two separate experiments each performed in triplicates). *C*, rat/Myc embryonic fibroblasts were transfected with GFP-p53(WT) or GFP-p53(LSF) (5 μg/60-mm plate). Apoptotic cells were counted from 500 GFP-positive cells in randomly selected fields from each plate. The results are expressed as a percentage of apoptotic cells (mean SD from two separate experiments, each in duplicate).

Another important cellular response mediated by p53 is apoptosis. Although impaired in its transactivation function, the p53(LFS) mutant may still retain apoptotic activity, because p53-mediated apoptosis can be transcriptionally dependent or independent (4). To test this, a GFP-tagged vector expressing wild-type p53 or the p53(LFS) was transfected into Myc-expressing rat embryonic fibroblasts to assess its apoptotic potential. Among the GFP-positive populations, apoptotic cells were identified under a fluorescent microscope by the presence of apoptotic bodies and membrane blabbing. In sharp contrast with the expression of wild-type p53, which induced extensive apoptotic response in positively transfected Rat/Myc fibroblasts, no significant apoptosis was associated with the expression of the p53(LFS) mutant (Fig. 4*C*), indicating its failure to induce programmed cell death.

The p53(LFS) Mutant Is Predominantly Cytoplasmically Localized. Appropriate subcellular localization of p53 has been demonstrated to be essential for its stability as well as its function. The p53 mutation found in the LFS family is located outside of the DNA-binding domain, which could not explain its impaired function. In addition, despite binding to Mdm2, the p53(LFS) was not degraded by it. We therefore asked whether the mutation caused an abnormal subcellular distribution of the p53(LFS) protein. When transfected into *p53*$^{-/-}$ MEFs, the GFP-tagged wild-type p53, as expected, was mainly localized in the nucleus, with a small population in the cytoplasm (Fig. 5*A*, *top*). Surprisingly, however, the p53(LFS) mutant was found predominantly in the cytoplasm. A similar observation was recorded when H1299 cells were used (Fig. 5*A*, *middle*), which proved this phenomenon to be independent of cell type. Cytoplasmic localization of p53 could be either the result of an impaired nuclear import or faster nuclear export. LMB, a nuclear-export inhibitor, was used to differentiate these two possibilities. If the cytoplasmic localization were attributable to an impaired nuclear import, the p53(LFS) mutant should remain in the cytoplasm after the LMB treatment. Otherwise, a nuclear retention of the mutant would be expected from the action of LMB if a hyperactive nuclear export were responsible. The *p53*$^{-/-}$ MEFs transfected with a GFP-tagged vector expressing wild type or the p53(LFS) mutant were incubated with 5 ng/ml LMB for 6 h, and the subcellular distribution of p53 was examined. In agreement with previous reports (8), LMB treatment was associated with a nuclear retention of wild-type p53. The p53(LFS) mutant, however, remained cytoplasmically localized after incubation with LMB (Fig. 5*A*, *bottom*), excluding the possibility of hyperactive nuclear export. Because mutation in this p53(LFS) involves a change of only four aa residues, codons 108–111, we sought to examine further which of the four residues is responsible for the altered cellular localization. To do this, site-directed mutagenesis was used to substitute each of the four aa residues individually, and the p53 mutant was again subcloned into the GFP-tagged expression vector for assessing its subcellular distribution. As shown in Fig. 5*B*, substitution of Phe or Arg, but not Gly (not shown) or Leu, resulted in a protein predominantly cytoplasmically localized that is similar to that of p53(LFS), indicating that Phe and Arg are the two aa residues critical to regulate the subcellular distribution of p53. Nuclear import of p53 has been shown to be mediated by a family of transporter proteins named importins. Because the binding of p53 to importin α is crucial for its nuclear import (9), we asked whether compromised nuclear import of the p53(LFS) mutant was attributable to a defect in importin-binding. To test this, a GFP-tagged vector expressing importin α was generated for co-transfection with Flag-p53(WT) or p53(LFS) into H1299 cells. Anti-Flag immunoprecipitation was performed with cell lysates

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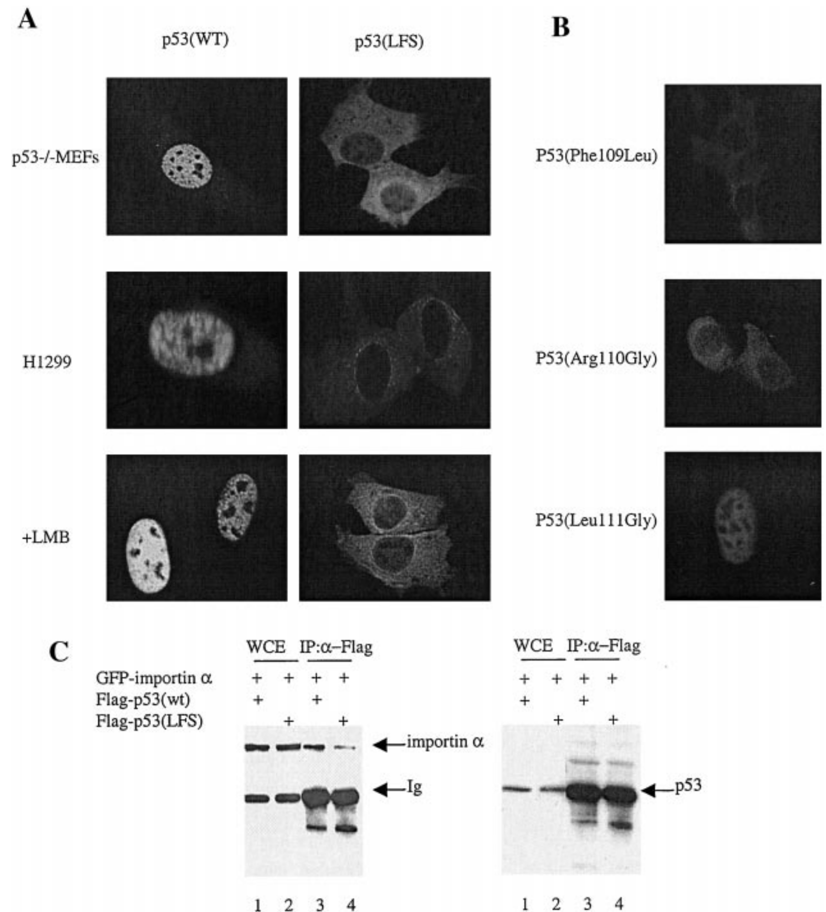

Fig. 5. Predominantly cytoplasmic localization of the p53(LFS). *A*, $p53^{-/-}$ MEFs *(top)* or H1299 cells (*middle*) grown in Chamber Slide were transfected with GFP-p53(WT) (*left*) or GFP-p53(LFS) (*right*). The cells were fixed, mounted with antifade solution 24 h posttransfection, and examined under fluorescence microscopy. The cells were treated with 100 nM LMB for 4 h at 24 h posttransfection (*bottom*) before mixing. *B*, the indicated p53 point mutant in GFP-tagged expression vector was transfected into $p53^{-/-}$ MEFs, and the cells were analyzed 24 h later as described in *A*. *C*, a GFP-tagged importin α construct was cotransfected with the vector expressing Flag-p53(WT) or p53(LFS) into H1299 cells. Anti-Flag immunoprecipitations (*IP*) were performed with cell lysates prepared from the transfectants 24 h after transfection. The whole cell extracts (*Lanes 1* and *2*) and the immunocomplexes (*Lanes 3* and *4*) were analyzed by Western blot with an anti-GFP antibody (*left*) or a polyclonal anti-p53 antibody (*right*; (C-FL; Santa Cruz).

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prepared from the transfectants after 24 h. Western blot analysis with an anti-GFP antibody (Fig. 5*C*, *left*) revealed that the amount of importin α detected in the p53(LFS) immunocomplex was less than half of that in the p53(WT) complex (*Lanes 3 versus 4*), whereas an anti-p53 immunoblot demonstrated that comparable levels of p53(WT) and p53(LFS) were brought down by the anti-Flag immunoprecipitations (Fig. 5*C*, *right*). Taken together, these data demonstrate that the abnormal cytoplasmic distribution of the p53(LFS) mutant was due to a defect in nuclear import, which, at least in part, resulted from the decreased affinity of the mutant p53 to importin α.

DISCUSSION

The p53 sequence element aa 92–112 has been previously shown by us to be essential for the Mdm2-mediated degradation of the p53 protein. Under normal conditions, Mdm2 is responsible for keeping p53 at low levels in the cell (6). Upon the onset of genotoxic stress, Mdm2-mediated p53 degradation is inhibited and levels of p53 rise, triggering a variety of downstream pathways. In a subset of LFS patients, a p53 mutation is found in exon 4, within aa 92–112, and not in the DNA-binding domain. In this study, we demonstrate that LFS-associated mutation causes p53 to become resistant to the Mdm2-mediated degradation, even under normal conditions. The LFS mutant p53 acquires increased protein stability, abnormally accumulates in the cell, and fails to accumulate further in response to DNA damage.

Proper nuclear import/export has been shown to be essential for the Mdm2-mediated degradation as well as for p53 induction by DNA damage (8). Functioning as a transcription factor, p53 has to be translocated into the nucleus to induce target-gene expression. It has been shown that nuclear import/export of p53 protein is controlled by a fast, energy-dependent pathway (10). Similar to other nuclear proteins, p53 contains both a NLS and a NES. The NLSs and NES are recognized by special transporter proteins, karyopherin α (importin α) or exportin 1 (CRM 1), respectively (11). Binding of the corresponding transporter to the NLSs or NES is essential to protein shuttling between the cytoplasm and the nucleus. We provide evidence for the impaired nuclear import of the LFS p53 mutant because of its decreased binding to importin α. Our findings, therefore, establish a molecular basis for the resistance of mutant p53 to Mdm2, its impaired response to DNA damage, and its inability to suppress cell growth.

Three putative NLSs have been identified in the COOH-terminus of p53 and proven to be necessary and sufficient for p53 nuclear import (12). However, a recent study reported an additional basic domain, Lys^{305}–Arg^{306}, which is also required for the p53 nuclear import (9). This basic domain and other NLSs form a bipartite NLS that enhances the binding of importin α to p53 and thereby mediates nuclear import of p53. We suggest that yet another binding site for importin α might be located in the region of aa 92–112 of p53. It remains to be tested whether impaired importin-binding of the LFS mutant is attributable to a general alteration in the p53 protein conformation or because the LFS-associated mutation modifies the importin-binding site in that region. In either case, our study reveals that aa 92–112 of p53, specifically aa 109 and aa 110, can contribute to the regulation of the subcellular distribution of p53. Mutation in this region results in impaired nuclear import and, as a consequence, in the inactivation of p53 function.

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