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Author(s)	Yamane, Takuya; Murao, Sato; Kato-Ose, Izumi; Kashima, Lisa; Yuguchi, Motoki; Kozuka, Miyuki; Takeuchi, Keisuke; Ogita, Hisakazu; Ohkubo, Iwao; Ariga, Hiroyoshi
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Transcriptional regulation of the legumain gene by p53 in HCT116 cells

Takuya Yamane^{a,d,*}, Sato Murao^a, Izumi Kato-Ose^a, Lisa Kashima^{a,b}, Motoki Yuguchi^c,
Miyuki Kozuka^a, Keisuke Takeuchi^d, Hisakazu Ogita^d, Iwao Ohkubo^e, and Hiroyoshi
Ariga^a

*^aGraduate School of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo
060-0812, Japan*

*^bCardiovascular Research Institute, University of California, San Francisco, San
Francisco, CA. 94143-3118, USA*

*^cDivision of Bioscience, Hokkaido System Science Co. Ltd., Kita-ku, Sapporo 001-0932,
Japan*

*^dDivision of Molecular Medical Biochemistry, Department of Biochemistry and
Molecular Biology, Shiga University of Medical Science, Seta, Otsu 520-2192, Japan*

*^eDepartment of Nutrition, School of Nursing and Nutrition, Tenshi College, Higashi-ku,
Sapporo 065-0013, Japan*

* Corresponding author:

Takuya Yamane

Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812,
Japan

Tel: +81-11-706-3711; Fax: +81-11-706-4988; E-mail: t-yamane@pharm.hokudai.ac.jp

Abstract

Legumain (EC 3.4.22.34) is an asparaginyl endopeptidase. Strong legumain activity was observed in the mouse kidney, and legumain was found to be highly expressed in tumors. We previously reported that bovine kidney annexin A2 was co-purified with legumain and that legumain cleaved the N-terminal region of annexin A2 at an Asn residue *in vitro* and *in vivo*. In this study, we found a p53-binding site in intron 1 of the human *legumain* gene using computational analysis. To determine whether transcription of the *legumain* gene is regulated by p53, HCT116 cells were transfected with p53 siRNA and the effect of knockdown of p53 expression on legumain expression was examined. The results showed that expression levels of both legumain mRNA and protein were decreased in the siRNA-treated cells. Furthermore, enzyme activity of legumain was also increased by doxorubicin and its activity was reduced by knockdown of p53 in HCT116 cells. These results suggest that legumain expression and its enzyme activity are regulated by p53.

Key words: Legumain, p53, doxorubicin

1. Introduction

Legumain (EC 3.4.22.34) is an asparaginyl endopeptidase belonging to the cysteine peptidase C13 family [1]. Legumain activity has been detected in a number of mammalian tissues, including the kidney, placenta, spleen, liver and testis, and the highest level of activity was detected in the kidney [2]. We previously reported that legumain is mainly expressed in proximal tubules of the rat kidney [3]. We further reported that legumain might have an important role in remodeling of the extracellular matrix through degradation of fibronectin in renal proximal tubular cells [4]. It has been suggested that legumain plays an important role in tumor growth/metastasis, carotid artery-atherosclerosis development [5-12], hemophagocytic syndrome [13] and formation of human unstable carotid plaque [14]. Recently, we reported that degradation of annexin A2 was reduced by knockdown of legumain in the mouse kidney [15].

p53 tumor suppressor is a well-known transcription factor that modulates the expression of numerous genes involved in various cellular functions, including anti-angiogenesis, cell cycle arrest and induction of apoptosis [16,17].

In this study, we found a p53-binding site in intron 1 of the human *legumain* gene using computational analysis. The results showed that both legumain mRNA and protein expression levels and legumain activity were decreased by p53 knockdown and that legumain activity was increased by doxorubicin, an inducer of p53 expression. These results suggest that legumain expression and its enzyme activity are regulated by p53.

2. Materials and Methods

2.1. Materials

Sequences of siRNA targeting human p53 were designed by Hokkaido System Science (Sapporo, Japan) using the B-Algo™ algorithm. Nucleotide sequences of the siRNA used were as follows: 5'-AGACUGACCCUUUUUGGACTT-3' (sense) and 5'-GUCCAAAAGGGUCAGUCUTT-3' (antisense). The sense and antisense strands of siRNAs were also chemically synthesized by Hokkaido System Science. HCT116 cells were purchased from American Tissue Culture Collection (Manassas, VA, USA). *RNAlater* and *RNeasy Mini* were purchased from Qiagen (Venlo, Netherlands). Lipofectamine 2000, PrimeScript RT Master Mix and Z-Ala-Ala-Asn-MCA were obtained from Invitrogen (St. Louis, MO, USA), Takara (Seta, Shiga, Japan) and Peptide Institute (Osaka, Japan), respectively. Anti-legumain and p53 antibodies were purchased from Santa Cruz (CA, USA). All other chemicals were of analytical grade and purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Cell culture and knockdown of p53 expression

HCT116 cells were cultured in Macoy's 5A medium supplemented with 10% calf serum at 37°C in a humidified atmosphere containing 5% CO₂. The cells were transfected with p53 siRNA and negative control siRNA (Qiagen). Twenty-four hours after transfection, cells were treated with 0.5 µg/ml of doxorubicin for 24 hours. Total RNAs and proteins were then extracted from the cells.

2.3. Overexpression of p53 in H1299 cells

H1299 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10%

calf serum. The cells were transfected with pcDNA3-p53-FLAG, and proteins were extracted from the cells at 48 hours after transfection.

2.4. Quantification of mRNA level by reverse transcription polymerase chain reaction

Total RNAs were prepared from HCT116 cells using an RNeasy mini kit. Reverse transcription was carried out in a mixture containing 500 ng of total RNAs and specific primers under the conditions of 95°C for 30 sec, 39 cycles of 95°C for 10 sec, and 60°C for 30 sec by using an SYBR *Premix Ex Taq* II (Takara) and a real-time PCR system (MiniOpticon, Bio-Rad, Hercules, CA, USA). β -actin (ACTB) mRNA was also amplified as an internal control. Nucleotide sequences of oligonucleotides used for real-time PCR primers were as follows: hLGMN-F: 5'-GTGAGAAGTCTCCGCTGCTC-3', hLGMN-R: 5'-AAAAGACTGGGGAAGCAGGT-3', TP53-F: 5'-CCACTGGATGGAGAATATTTACCCTTCAG-3', TP53-R: 5'-TGGCTCCTTCCCAGCCTGGGCATC-3', CDKN1A-F: 5'-CTGGAGACTCTCAGGGTCGAA-3', and CDKN1A-R: 5'-GATGTAGAGCGGGCCTTTGA-3'.

2.5. Western blotting and antibodies

To examine the maturation and expression levels of legumain in HCT116 cells, proteins were extracted from cultured cells in a solution containing 50 mM Tris-HCl (pH 7.4) and 0.5% NP-40. Proteins were then separated on a 12.5% polyacrylamide gel and subjected to Western blotting with respective antibodies. Proteins on the membrane were reacted with an IRDye 800- (Rockland, Philadelphia, PA, USA) or Alexa Fluor

680-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) and visualized by using an infrared imaging system (Odyssey, LI-COR, Lincoln, NE, USA). Antibodies used were anti-legumain (1:1000, R&D Systems, Minneapolis, MN, USA) and anti-p53 (1:1000, Santa Cruz, CA, USA) antibodies.

2.6. Identification of a potential p53-binding site in the legumain gene

A potential p53 binding site was identified using the p53scan algorithm [18].

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assays using cultured HCT116 cells were performed according to the protocol of the ChIP Assay Kit (Millipore, Billerica, MA, USA). Briefly, after proteins had been cross-linked with DNA, cell pellets were resuspended in an SDS-lysis buffer and sonicated on ice using a sonicator (UR-20P, TOMY, Tokyo, Japan) 3 times for 20 seconds each time. Genomic DNA was sheared to 300 to 1200 base pairs of length. Chromatin solution from 1×10^6 cells/dish was preincubated with salmon sperm DNA and Protein A-agarose and incubated with species-matched IgG or with specific antibodies overnight at 4°C. DNA fragments immunoprecipitated were then used as templates for PCR with HS taq (Hokkaido System Science) and reacted for 15 min at 96°C, 32 cycles of 0.5 min at 96°C, 0.5 min at 60°C, 0.5 min at 72°C and 2 min at 72°C. Nucleotide sequences of oligonucleotides used for real-time PCR primers were as follows: ChLGMN1-F: 5'-AGTACTCTGGGAGGCCGAAG-3', ChLGMN1-R: 5'-CATTGAGCGACATTTTTGGA-3', ChLGMN2-F: 5'-ACGCGTGAGAAACGAGAAAC-3', and ChLGMN2-R: 5'-ATTTTTCCCGTCCCTTTCTG-3'.

2.8. Avidin-biotin complex DNA assay

Avidin-biotin complex DNA assays were carried out as described previously [19, 20]. Briefly, a reaction mixture containing 50 µg of HCT116 cell nuclear extract, 20 mM Hepes (pH 7.9), 20 mM KCl, 2 mM MgCl₂, 0.2 mM EDTA, 20% glycerol and a biotin-conjugated probe was incubated. DNA-protein complexes formed in the mixture were precipitated using Dynabeads M-280 Streptavidin (Invitrogen) according to the manufacturer's instructions. Eluted proteins were then loaded onto a 15% polyacrylamide gel containing SDS and subjected to Western blotting using an anti-p53 antibody. Nucleotide sequences of oligonucleotides used for probes were as follows: LGMN-s: 5'-biotinyl-TATCTTGCCCAGGCCAGTCTC-3', LGMN-as: 5'-GAGACTGGCCTGGGCAAGATA-3', LGMNm-s: 5'-biotinyl-TATATTACCCAGGCCAGTCTC-3', and LGMNm-as: 5'-GAGACTGGCCTGGGTAATATA-3'.

2.9. Proteolytic activity of legumain

Enzyme activity of legumain was examined by measuring the fluorometrical number (excitation, 380 nm; emission, 440 nm) of liberation of 7-amino-4-methylcoumarin (AMC) in a mixture containing 10 µl of 10 mM Z-Ala-Ala-Asn-MCA, 100 µl of 0.5 M sodium citrate buffer (pH 5.0), 5 µl of 1 M 2-mercaptoethanol, 20 µl of enzyme solution and water (18 mΩ) in a total volume of 1 ml. After incubation of the mixture at 37°C for 30 min, 2 ml of 0.2 M acetic acid was added to the mixture to stop the reaction. One unit of activity was defined as the amount of enzyme that hydrolyzed 1 µmol of the substrate per min.

2.10. Statistical analyses

Data are expressed as means \pm S.E. Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by unpaired Student's *t*-test.

3. Results

3.1. Effect of doxorubicin and p53 siRNA on legumain expression in HCT116 cells

To confirm regulation of legumain gene expression by p53, HCT116 cells were treated for 24 hours with doxorubicin, an inducer of p53, and expression levels of legumain, p53, p21 and actin mRNA were examined by quantitative real-time PCR. Actin mRNA was used as a loading control. As shown in Figs. 1A-1C, the expression levels of legumain, p53 and p21 mRNA in doxorubicin-treated cells were increased to about 50%, 40% and 120%, respectively, of the levels in untreated cells. When doxorubicin-treated cells were transfected with p53 siRNA, however, expression levels of legumain, p53 and p21 mRNA were reduced to about 20%, 90% and 80%, respectively, of those in untransfected HCT116 cells (Figs.1D-1F).

3.2. Regulation of legumain activity by p53

To examine the effect of doxorubicin on legumain activity, HCT116 cells were treated with 0.5 μ g/ml of doxorubicin for 24 hours and cell extracts were prepared by using 50 mM sodium citrate buffer (pH 5.0). Legumain activity was then measured against Z-Ala-Ala-Asn-MCA and subjected to Western blotting. As shown in Fig.2A, legumain activity in doxorubicin-treated cells was increased to 2.7-fold compared to that in untreated cells. The results by Western blotting showed that the expression level of the

mature form of legumain as well as that of p53 were also increased in doxorubicin-treated HCT116 cells (Fig. 2B). To examine whether doxorubicin-induced legumain activity is affected by p53 expression, HCT116 cells were first transfected with siRNA targeting p53 and negative control siRNA and then treated with doxorubicin. As shown in Fig. 2C, legumain activity in p53 siRNA-transfected cells was reduced to 0.4-fold of that in control siRNA-transfected cells. The results by Western blotting showed that the expression level of the mature form of legumain as well as that of p53 were also decreased in p53 siRNA-transfected cells (Fig. 2D). To further examine the effect of p53 on legumain expression, p53-null H1299 cells were transfected with a pcDNA3-p53-FLAG expression vector. As shown Figs. 2E and 2F, both the precursor and mature forms of legumain levels were increased by p53. These results indicate that p53 positively regulates legumain expression and its enzymatic activity.

3.3. Association of p53 with intron 1 of legumain

A computational analysis of the p53-binding site in the *legumain* gene revealed that the site was in intron 1 of the *legumain* gene. To examine the association of p53 with intron 1, chromatin immunoprecipitation assays were carried out. Chromatin extracted from HCT116 cells was reacted with an anti-p53 antibody or non-specific IgG, and two regions spanning -15,600 to -15,459 and spanning -12,390 to -12,261 were amplified by PCR with specific primers and precipitated DNA as a template. As shown in Fig. 3A, the anti-p53 antibody, but not IgG, specifically precipitated the region spanning -12,390 to -12,261, and no amplified band in the region spanning -15,600 to -15,459 was observed, indicating that p53 bound to the region spanning -12,390 to -12,261. To further confirm that p53 binds to the putative p53-binding site present in intron 1 of the

legumain gene, avidin-biotin complex DNA assays were carried out using nuclear extracts from HCT116 cells and biotin-labeled wild-type p53-recognition sequence that is present in intron 1 of the *legumain* gene as a probe. A mutated p53-recognition sequence was also used. After reaction of labeled wild-type and mutant p53- recognition sequences with nuclear extracts, bound proteins were eluted and analyzed by Western blotting with the anti-p53 antibody. As shown in Fig. 3B, p53 bound more strongly to the wild-type p53-recognition sequence than to the mutant p53-recognition sequence, indicating that p53 specifically binds to intron 1 of the *legumain* gene.

4. Discussion

In this study, we first found that p53 positively regulates *legumain* gene expression at the transcriptional level (Fig. 1). Increased expression of the *legumain* gene was observed in HCT116 cells that had been treated with doxorubicin, an inducer of p53, and reduced expression of the *legumain* gene was observed in p53-knocked-down HCT116 cells (Fig. 1). We then found that p53 binds to the putative p53-binding site present in intron 1 of the *legumain* gene using CHIP and avidin-biotin complex DNA assays (Fig. 3). This is the first finding of participation of p53 in transcriptional regulation of the *legumain* gene. Legumain is expressed as an inactive proenzyme of 56 kDa, which is then self-cleaved to 47-kDa inactive and 46-kDa active forms under the condition of acidic pH. Protein is further processed to a mature active form of 36 kDa by other proteases [21]. Western blotting analysis showed that the level of the mature form of legumain with 36-kDa protein was increased by doxorubicin-induced p53. The mature form of legumain with 36 kDa was, on the other hand, decreased by knockdown of p53. Overexpression of p53 increased legumain expression (Fig. 2). Furthermore,

legumain activity against Z-Ala-Ala-Asn-MCA was increased by doxorubicin and decreased by knockdown of p53 (Fig. 2). These results indicate that transcription of legumain and its activity were positively regulated by p53. Legumain plays an important role in tumor growth/metastasis [5-11], and cystatin E/M suppresses legumain activity and its processing [22, 23]. The amount of legumain expression regulated by p53 in tumor cells may regulate legumain maturation and activation. It would therefore be interesting to further analyze the effects of various p53 mutations found in tumor cells on the expression level and maturation of legumain and the effect of cystatin E/M on p53-regulated legumain functions.

Abbreviations: Z, benzyloxycarbonyl; MCA, methylcumarinamide; DXR, doxorubicin.

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Figure legends

Fig. 1. Effect of p53 siRNA on legumain expression and activity.

A-C. HCT116 cells were treated with doxorubicin for 24 hours and expression levels of legumain, p53 and p21 mRNAs were examined by quantitative RT-PCR (real-time PCR). p21 and actin mRNAs were used as positive and loading controls, respectively. Relative expression level of each mRNA toward that of actin mRNA is shown. Number of experiments (n) was 4.

D-F. HCT116 cells were transfected with p53 siRNA or negative control siRNA and then treated with doxorubicin for 24 hours. At 48 hours after transfection, expression levels of legumain, p53 and p21 mRNAs were examined by quantitative RT-PCR. Relative expression level of each mRNA toward that of actin mRNA is shown. Number of experiments (n) was 4.

From A to F, statistical analyses were carried out using Student's *t*-test. Significance: * $p < 0.05$, ** $p < 0.01$

Fig. 2 Effect of doxorubicin and p53 siRNA on legumain expression and activity.

A. HCT116 cells were treated with doxorubicin for 24 hours. Proteins were extracted from the cells and legumain activity against Z-Ala-Ala-Asn-MCA was measured as described in Materials and methods. Statistical analyses were carried out using Student's *t*-test. Significance: *** $p < 0.001$. Number of experiments (n) was 4.

B. Proteins were extracted from doxorubicin-treated HCT116 cells as described in the legend for Fig. 2A and analyzed by Western blotting with anti-legumain, anti-p53 and β -actin antibodies. β -actin was used as a loading control.

C. HCT116 cells were transfected with p53 siRNA or negative control siRNA and then

treated with doxorubicin for 24 hours. At 48 hours after transfection, legumain activity was measured as described in the legend for Fig. 2A. Statistical analyses were carried out using Student's *t*-test. Significance: ** $p < 0.01$. Number of experiments (n) was 4.

D. Proteins were extracted from doxorubicin-treated HCT116 cells as described in the legend for Fig. 2C and analyzed by Western blotting with anti-legumain, anti-p53 and β -actin antibodies.

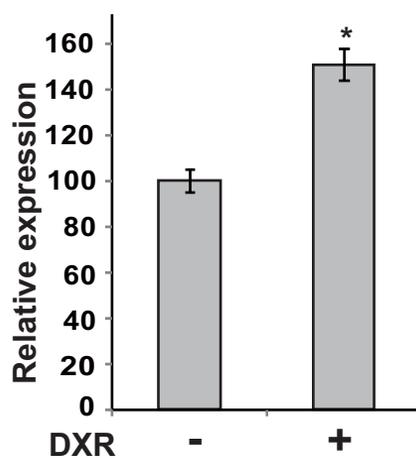
E and F. Proteins were extracted from p53-transfected H1299 and analyzed by Western blotting with anti-legumain, anti-p53 and β -actin antibodies (E). Quantification of the intensity of bands is shown Fig. 2F. Statistical analyses were carried out using Student's *t*-test. Significance: * $p < 0.05$. Number of experiments (n) was 4.

Fig. 3. Association of p53 with intron 1 of the *legumain* gene.

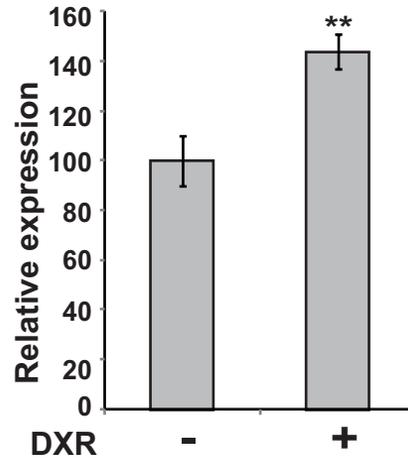
A. Chromatin immunoprecipitation assays were carried out using chromatin prepared from HCT116 cells. Chromatin was immunoprecipitated with an anti-p53 antibody or non-specific IgG. After extraction of DNA from precipitated chromatin, two regions spanning -12,390 to -12,261 and spanning -15,600 to -15,459 were amplified by real-time PCR with specific primers and with amplified DNA as described in Materials and methods. Statistical analyses were carried out using Student's *t*-test. Number of experiments (n) was 3.

B. HCT116 nuclear extracts were mixed with biotin-labeled wild-type and mutated oligonucleotides containing the p53-recognition sequence present in intron 1 of the *legumain* gene. After reaction, proteins were eluted and subjected to Western blotting analysis with an anti-p53 antibody.

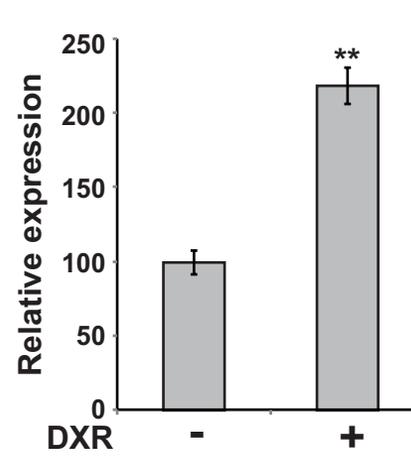
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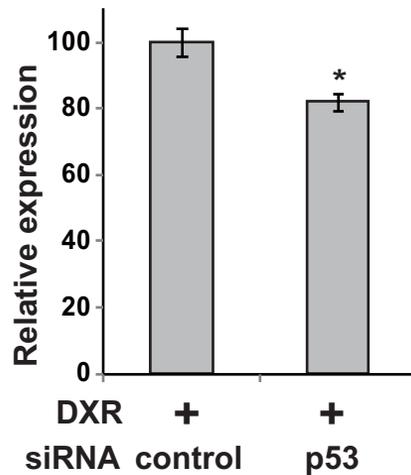
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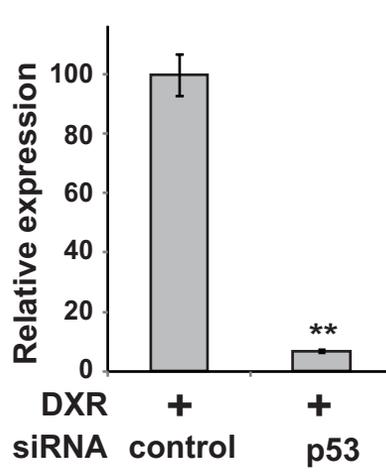
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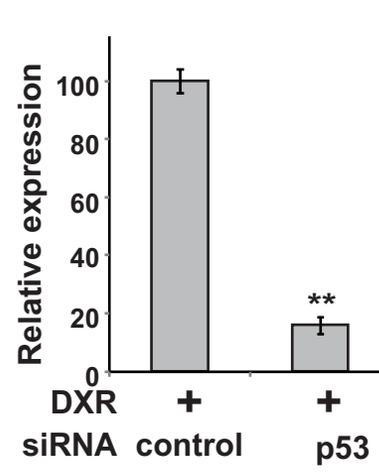
D. Legumain

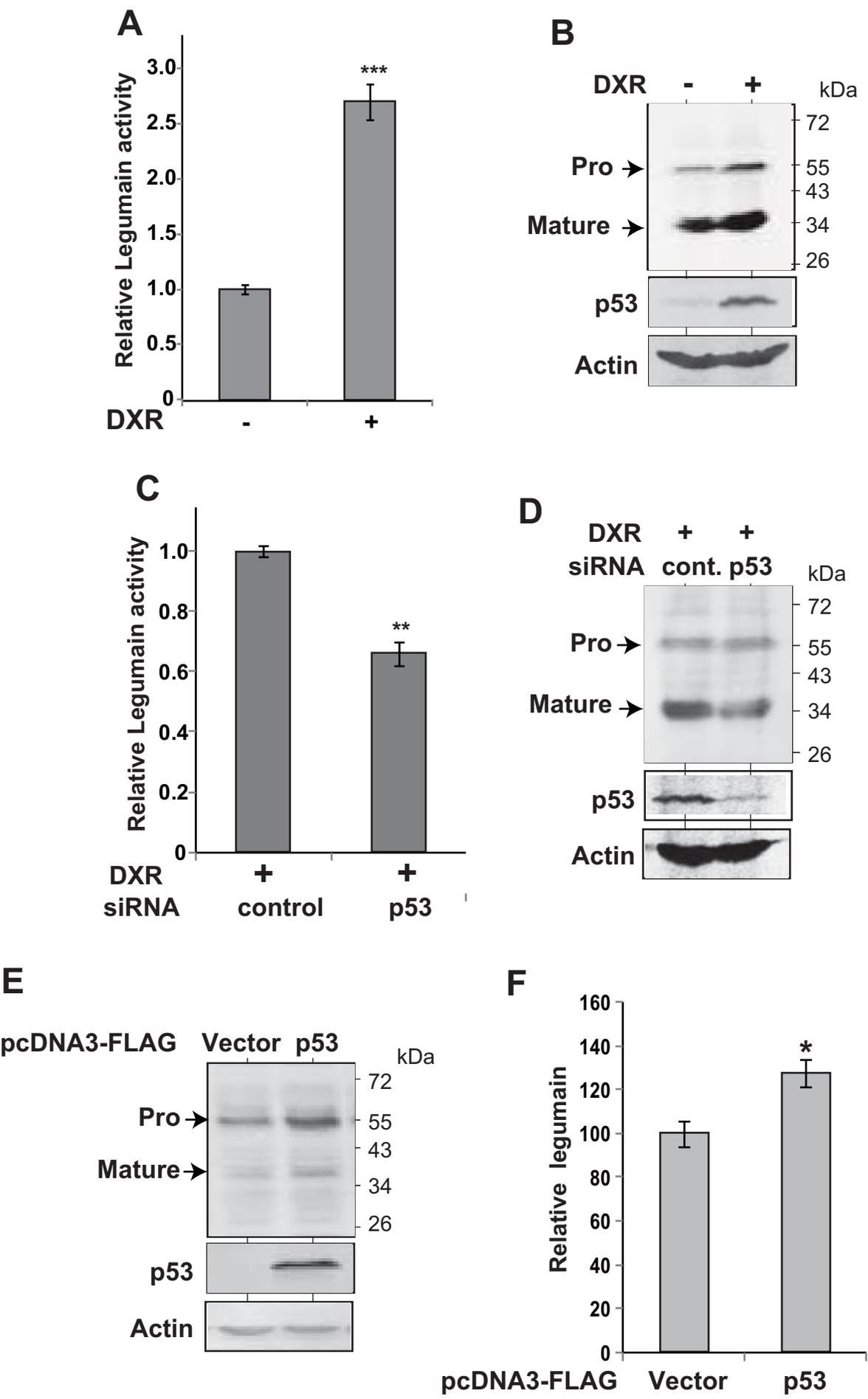


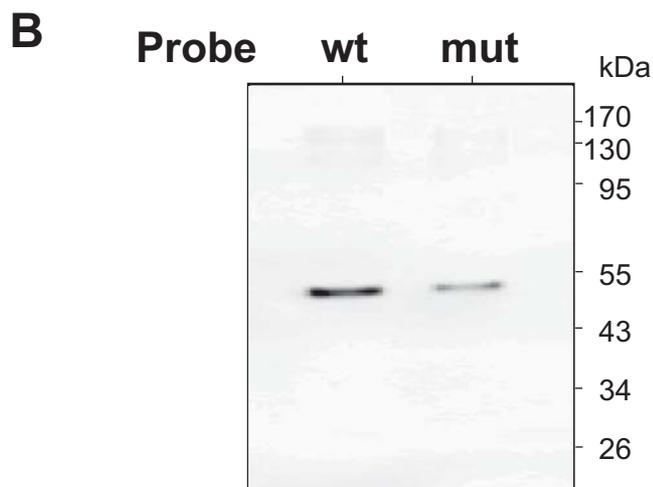
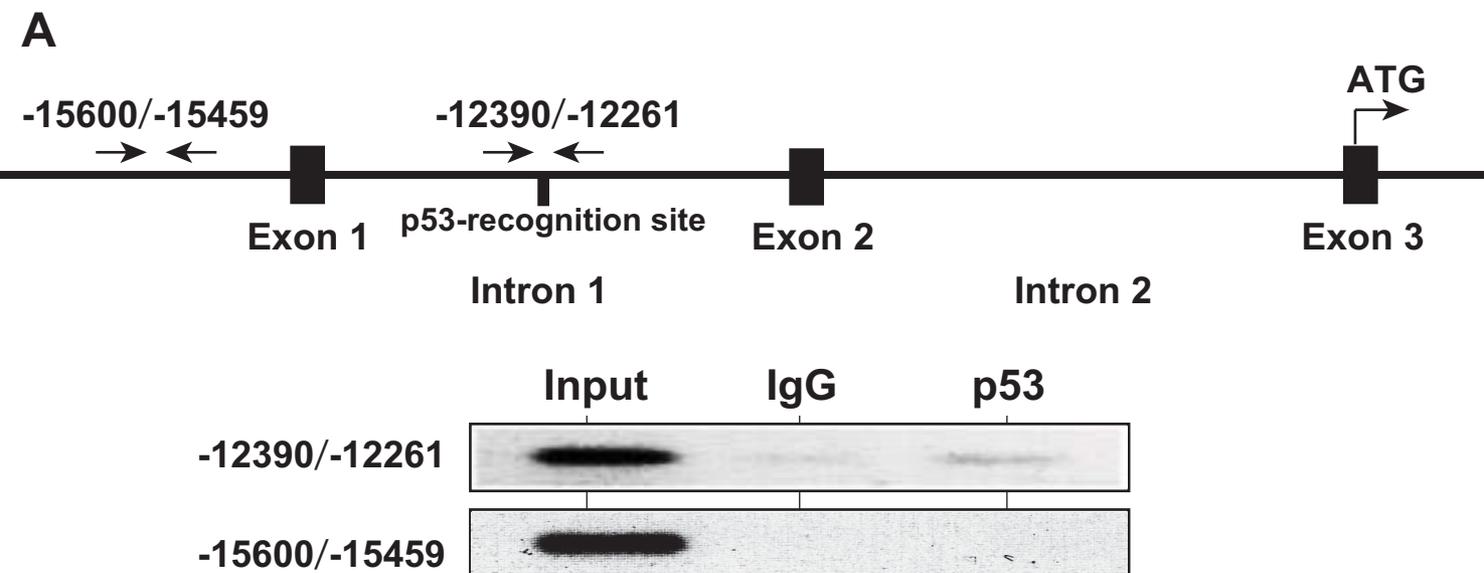
E. p53



F. p21







p53 wt: TATCTTGCCAGGCCAGTCTC

p53 mt: TATATTACCCAGGCCAGTCTC