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# Identification of two serine residues important for p53 DNA binding and protein stability

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**Abstract The p53 core DNA binding domain has been implied in Mdm2-mediated protein degradation. Here we show that the substitution of the serine residues 116 and 127 with alanine residues (S116/127A) has no effect on p53 DNA binding and protein stability. However, the substitution of the serine residues with the aspartic acid (S116/127D) abolished p53 DNA binding and led to protein stabilization. Importantly, we have shown that S116/127D exhibits a structural mutant conformation that results in a loss of p53-dependent transcription and Mdm2-mediated protein degradation.**



*Key words:* p53; DNA binding; Stability; Ser-116; Ser-127

## 1. Introduction

Wild-type p53 exerts its tumor suppression activity by binding to specific DNA sequences and functioning as a transcription factor [1]. The wild-type p53 protein is maintained at low levels in most cells because of the short p53 protein half-life [2]. In contrast, the p53 protein accumulates dramatically in response to various cellular stresses that include DNA damage [3]. The cellular protein Mdm2 functions as a major negative regulator of p53 by acting as a ubiquitin E3 ligase, promoting p53 ubiquitination and subsequent degradation [4]. Conversely, Mdm2 is also a transcriptional target of p53 and the expression of Mdm2 is activated by p53 at the transcription level [5,6]. This allows the formation of a unique autoregulatory feedback loop in restraining the p53 protein levels and activity. The physiological relationship between p53 and Mdm2 is further supported by Mdm2 gene knockout studies in mice. Disruption of the murine *Mdm2* locus results in early embryonic death, which can be rescued by the further inactivation of *p53* [7,8]. Mutation of p53 therefore results in the loss of DNA binding required for Mdm2 activation and subsequent protein stabilization.

The p53 protein can be divided functionally and structurally into five regions: the acidic N-terminal transactivation domain, the proline-rich stretch, the hydrophobic core DNA binding domain which bears 95% of 10 000 somatic tumorigenic TP53 mutations [9], the oligomerization domain, and the C-terminal domain [10]. In addition to having a role in DNA binding, the N-terminal region of the core domain has been implied in Mdm2-mediated protein degradation. Mutation of the COP9 signalosome (CSN) phosphorylation site (Thr155) to valine (T155V) results in p53 stabilization [11]. The amino acid residues 92–112 of p53 have been suggested to inhibit p53 by acting as a signal for Mdm2-mediated protein degradation [12]. Results from our laboratory have also revealed a potential inhibitory domain between amino acids 92 and 109 (Abela and Liu, unpublished results). Of note, the c-jun protein also contains an inhibitory domain capable of silencing its activation function. Furthermore, the c-jun inhibitory domain has been shown to interact with the c-jun N-terminal kinase (JNK). JNK is a member of the mitogenic activated protein kinase (MAPK) family, which can regulate c-jun protein levels through phosphorylation of serine residues 63 and 73. A comparison of the regulatory region of c-jun has revealed its homology with a p53 inhibitory domain, particularly with two serine residues at positions 116 and 127 (Fig. 1B), which prompted us to examine the function of the two serines in p53 DNA binding and protein degradation.

Here, we show that the substitution of the serine residues with the alanine (S116/127A) has no effect on p53 DNA binding and protein stability. However, the substitution of the serine with the aspartic acid residues (S116/127D) abolished p53 DNA binding and led to p53 stabilization. Importantly, we have shown that the S116/127D mutant exhibits a structural mutant conformation that can be recognized by anti-mutant p53 but not anti-wild-type p53 antibody. Furthermore, the S116/127D mutant can be degraded by overexpression of Mdm2. These results suggest that the serine residues 116 and 127 are important for p53 DNA binding, and the substitution of the serine residues with aspartic acid leads to a loss of p53-dependent transcription and Mdm2-mediated protein degradation.

## 2. Materials and methods

### 2.1. Plasmid

p53 S116/127A and S116/127D were generated by polymerase chain reaction (PCR) using pcDNA-p53 as a template and confirmed by sequencing analysis. The luciferase reporter plasmid pRGCE4luc was as described [13].

### 2.2. Transfection, luciferase and Western blot analysis

Various combinations of plasmids were transfected into Saos2 cells by use of the calcium phosphate method. The amounts of plasmids used for transfection of 60 mm plates were as follow: 0.5 μg of pRGCE4Luc, 0.2 μg of wild-type p53 or mutants (S116/127A, S116/127D, R175H and R248W). Luciferase assays were normalized for β-galactosidase activity from a cotransfected control expression vector

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as described [14]. We used Lipofectamine (Invitrogen) for transfection in p53/Mdm2 double knockout mouse embryonic fibroblast cells (*p53*−/−; *Mdm2*−/−MEF). A total of 0.2 μg of wild-type p53 or various mutants in the presence or absence of 1.2 μg pCMVMdm2 was used for transfection on 60 mm plates. 28–32 h posttransfection, the cells were lysed in 'L' lysis buffer (50 mM Tris–HCl pH 8.0, 120 mM NaCl, 0.5% NP-40). The Mdm2 and p53 proteins were detected by use of monoclonal antibodies SMP14 (anti-Mdm2) and DO1 (anti-p53, Santa Cruz).

### 2.3. Electrophoretic mobility shift assay (EMSA)

pcDNA-p53 and various mutant plasmids (0.5 μg each) were transfected into Saos2 cells on 60 mm plates with use of Lipofectamine 2000 reagent (Invitrogen). 30 h posttransfection, nuclear proteins were isolated as described [15]. In brief, cells were allowed to swell on ice in a hypotonic buffer, the nuclei were then collected, and nuclear proteins were extracted in a high-salt buffer. To minimize proteolysis, all buffers contained freshly added 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml antipain. The nuclear extracts were snap-frozen in liquid $N_2$ and stored at −80°C. The amounts of the protein used in each EMSA were normalized to wild-type p53. EMSA was performed as described [16]. The ribosomal gene cluster (RGC) p53 binding site probe (5′-AGCTTGCCTCGAGCTTGCCTGGACTTGCCTGGTCGACGC-3′) was $^{32}$P-labeled with the Klenow polymerase. Binding reactions contained 60 mM KCl, 12% glycerol, 5 mM $MgCl_2$, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.2 μg bovine serum albumin (BSA), 1 μg poly(dG-dC) and 100 cpm $^{32}$P-labeled probe. Reactions were incubated at 30°C for 40 min and analyzed on a 5% polyacrylamide gel electrophoresis (PAGE) gel containing 0.5×triborate EDTA (TBE). DNA–protein complexes were visualized with a phosphorimager with use of the Adobe Photoshop software.

### 2.4. Immunoprecipitation

Wild-type p53 and various p53 mutants were transcribed and translated in vitro using [$^{35}$S]methionine and the coupled in vitro TNT system from Promega. The translated proteins were then pre-cleared with protein G Sepharose and immunoprecipitated using 1 μg p53 antibodies (Ab-5 or Ab-3, Oncogene). The $^{35}$S-labeled immunoprecipitates were subjected to 10% sodium dodecyl sulfate (SDS)–PAGE, dried and exposed to X-ray film. Wild-type p53 and various p53 mutants were also expressed in p53/Mdm2 double knockout MEF cells, immunoprecipitated and assayed on Western blotting with the rabbit polyclonal anti-p53 antibody, FL-393 (Santa Cruz).

## 3. Results and discussion

### 3.1. Role of Ser116 and 127 in p53-dependent transcription

In order to study whether the serine residues 116 and 127 may have any effect on the transcriptional function of p53, we substituted the serine residues with either alanine (S116/127A) or aspartic acid (S116/127D). Transient transfection assay was performed in p53 null Saos2 cells, in which wild-type and mutant p53 were tested for their ability to stimulate the expression of the luciferase gene under the control of a promoter containing one p53 binding site (RGC) and the E4 TATA box (pRGCE4Luc). The S116/127A mutation retained its ability to activate transcription (Fig. 1D). However, the serine-to-aspartic acid mutant, S116/127D, revealed a loss of transcription activity compared with the wild-type p53. The p53 R175H and R248W mutants were included as controls (Fig. 1D). We performed Western blot analysis to ensure that the mutant proteins were expressed at comparable levels to the wild-type p53 (Fig. 1E). The S116/127D mutant, like the R175H and R248W mutants, was expressed at higher levels. This is consistent with the finding that without p53-dependent transcription of Mdm2, mutant p53 accumulates in high levels. These results suggest that the serine residues 116 and 127 are important for p53 transcription activity.

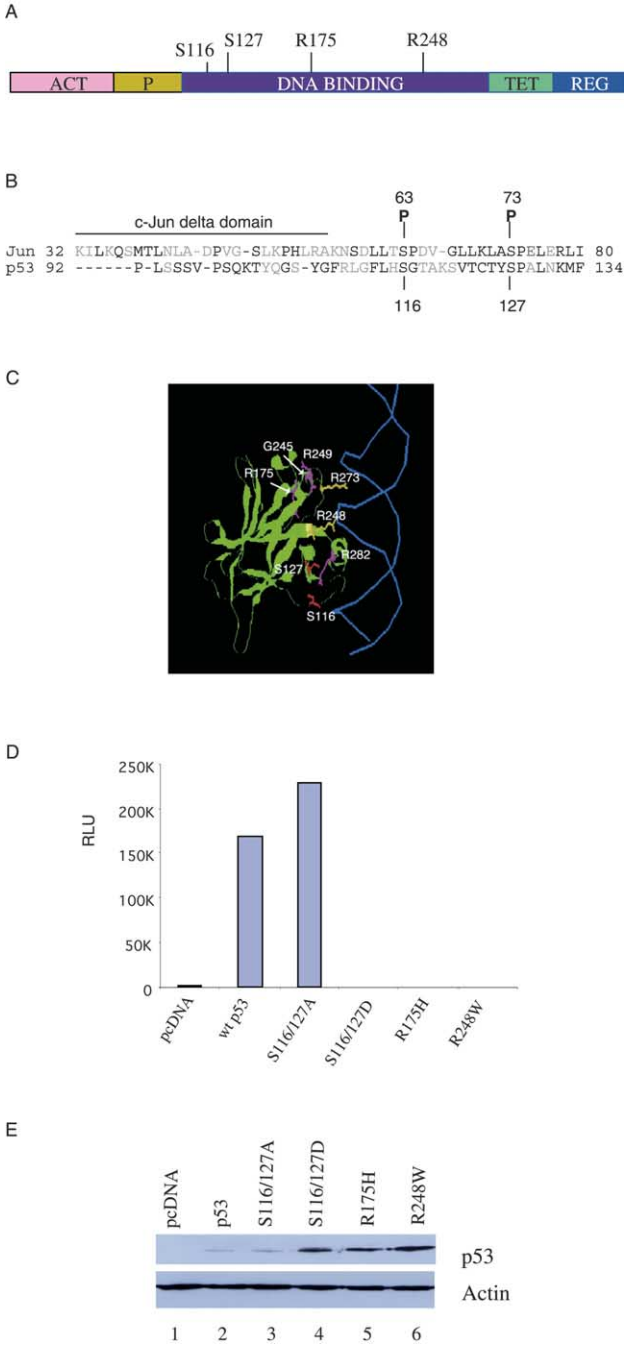


Fig. 1. A: Functional domains of p53 and two serine residues, Ser116 and 127, identified by sequence comparison with the c-jun delta domain (B). C: Crystal structure of the p53 core domain–DNA complex. The side chains of the six most frequently mutated residues of p53 are purple or gold and Ser116 or 127 are red. D: Saos2 cells were transiently transfected with the plasmid indicated below. 28–32 h after transfection, the luciferase activity was assayed after normalization with β-galactosidase. E: Transiently transfected Saos2 cells were lysed with 'L' buffer and analyzed by Western blotting using the anti-p53 antibody, DO1.

### 3.2. Ser116 and 127 are important in p53 DNA binding

The fact that S116/127D is defect in p53 transcription prompted us to examine the abilities of the mutants to bind DNA. We performed a gel shift assay using a probe containing the p53 binding site identified in the RGC as described [13]. The p53 mutants and the wild-type protein were ex-

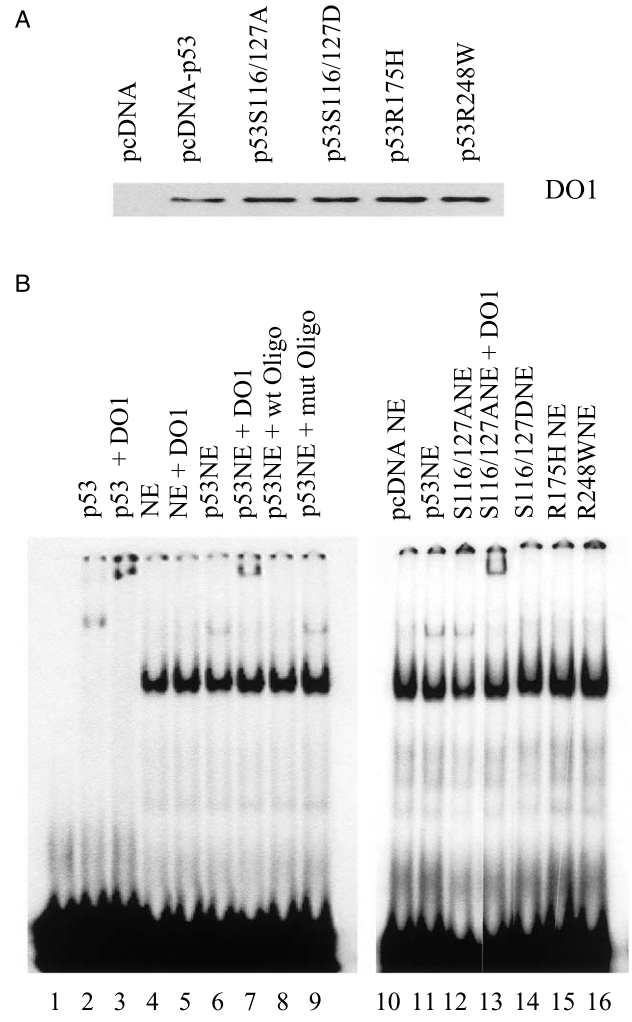


Fig. 2. p53 S116/127A but not S116/127D binds to DNA. A: Saos2 cells were transiently transfected with various plasmids listed above. The nuclear extracts were isolated and normalized to ensure equal amounts of p53 protein in the gel shift assay. B: S116/127A but not S116/127D binds to DNA: lanes 2 and 3 show DNA binding of purified baculovirus-expressed p53. Lanes 4 and 5 show DNA binding of nuclear extracts from Saos2 cells transfected with an empty vector. Lanes 6–16 show DNA binding of nuclear extracts from wild-type p53 and Saos2 cells transfected with various mutant p53.

pressed in Saos2 cells, from which nuclear lysates were prepared. As expected, 50 ng of purified baculovirus-expressed p53 bound to this probe and produced a retarded p53–DNA complex (Fig. 2B, lane 2) which can be supershifted by the addition of an anti-p53 antibody (DO1, lane 3). A slowly migrated p53–DNA complex was also observed when wild-type p53-transfected nuclear extracts were incubated with the RGC probe (lane 6) and this complex was supershifted by the addition of an anti-p53 antibody (DO1, lane 7). Results of two control experiments demonstrated the specificity of p53 DNA binding. Nuclear extracts obtained from empty vector-transfected Saos2 cells did not show a band at the p53–DNA complex position (lanes 4 and 5). Also, the p53 DNA binding observed in lane 6 could be competed by the addition of the excess unlabeled wild-type but not mutant probes (lanes 8 and 9). Next, we tested the ability of the p53 mutants to bind DNA. Similar to results for the wild-type p53, the addition of S116/127A-transfected nuclear extracts resulted in a slowly migrated band (Fig. 2B, lane 12) and a supershifted band when the anti-p53 antibody DO1 was added (lane 13). In contrast, S116/127D was significantly impaired in its ability to bind DNA (lane 14). In these experiments, R175H and R248W were used as negative controls, as they carry mutations in the DNA binding region (lanes 15 and 16). The amounts of protein used in each reaction are shown in Fig. 2A.

### 3.3. S116/127D exhibits a structural mutant (Pab240+) conformation

Two classes of p53 mutations, 'DNA contact' and 'structural', have been described previously [9]. The contact mutants preferentially bind to the Pab1620 antibody (raised against native p53), as they have a native conformation, whereas the structural mutants specifically bind to the Pab240 antibody (raised against denatured p53), which recognizes an epitope inaccessible in the native conformation. Because the serine residues 116 and 127 positioned close to R282 (Fig. 1C), we postulated that the addition of positive charges to these residues might contribute to the disruption of the surrounding structure of R282 and abolished p53 DNA binding. We then examined the mutant type of S116/127D using the Pab1620 and Pab240 antibodies. In these experiments, the S116/127A and S116/127D mutants were either translated in vitro or transiently expressed in vivo and immunoprecipitated with the Pab1620 and Pab240 antibodies. As shown in Fig. 3, S116/127A was only recognized by Pab1620 antibody, indicating it was in its native conformation state. In contrast, S116/127D was recognized only by the Pab240 antibody, which suggests that this mutant exhibits the structural mutant (Pab240+) conformation. As controls, wild-type p53 and the reported 'DNA contact' mutant R248W were recognized by Pab1620 but not Pab240 antibodies, whereas the 'structural' mutant R175H was recognized by Pab240, but not Pab1620 antibodies. These results show that the S116/127D mutant exhibits the structural mutant conformation and therefore is unable to bind DNA.

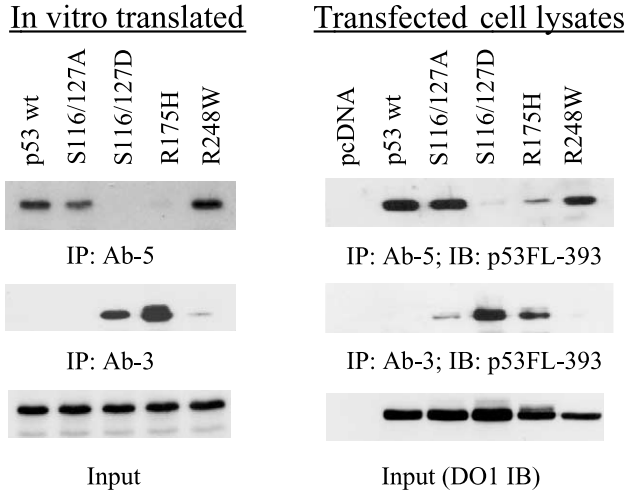


Fig. 3. p53 S116/127D exhibits a structural mutant conformation. Left panel: In vitro transcription and translated lysates were immunoprecipitated with Ab-5 (Pab1620) or Ab-3 (Pab240). Right panel: Lysates from cells transfected with wild-type or mutant p53 were immunoprecipitated with either Ab-5 (Pab1620) or Ab-3 (Pab240), and p53 was detected with an anti-p53 polyclonal antibody following immunoprecipitation.

### 3.4. S116/127D is not resistant to Mdm2-mediated protein degradation

The N-terminus of the p53 core DNA binding domain has been implied in Mdm2-mediated protein degradation. Because the S116/127D mutant exhibits the structural mutant conformation, we examined whether this conformation would result in a loss of p53 sensitivity to Mdm2-mediated protein degradation. To test this, we first transfected the p53 S116/127D mutant into p53 and Mdm2 double knockout MEF cells (*p53*−/−; *Mdm2*−/−) and performed Western blotting. As shown in Fig. 4A, the protein levels of S116/127D were comparable to that of the wild-type p53, which suggests that a lack of Mdm2-mediated protein degradation plays a critical role in S116/127D stabilization. Mdm2 has been shown to interact with the N-terminal domain of p53, and this interaction is not greatly affected by point mutations in the p53 DNA binding domain [17]. To determine whether Mdm2 can affect the protein stability of the inactive S116/127D mutant, we cotransfected Mdm2 with wild-type p53 in the *p53*−/−; *Mdm2*−/− cells. Similar to results for the wild-type p53, cotransfection of Mdm2 indeed leads to S116/127D, R175H and R248W protein degradation (Fig. 4B). Similar results have been observed in Saos2 cells (Fig. 4C). These results suggest that the S116/127D mutant, although exhibiting the structural mutant conformation, is not resistant to Mdm2-mediated protein degradation.

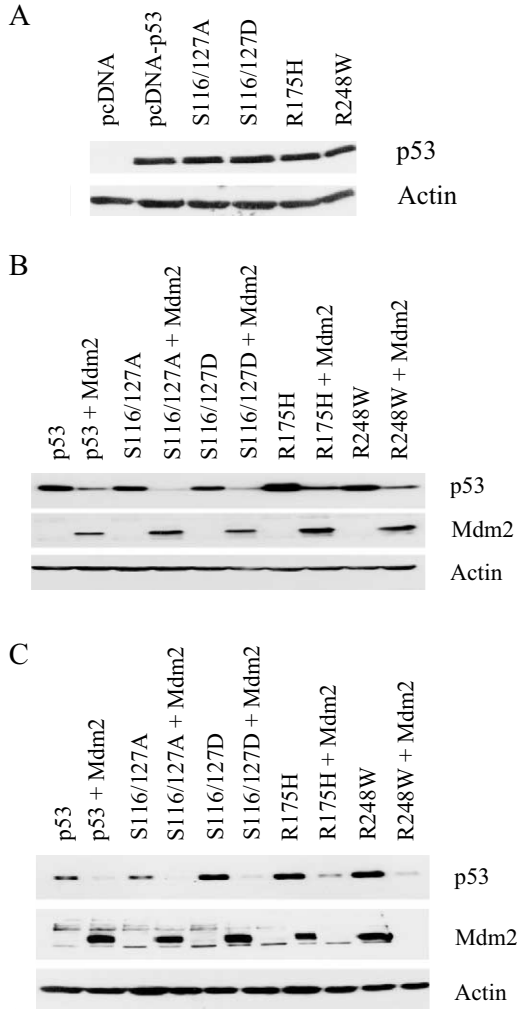


Fig. 4. The S116/127D mutant is subjected to Mdm2-mediated protein degradation. A: Double knockout cells were transfected with various plasmids as listed. Wild-type p53 and various p53 mutants were cotransfected with Mdm2 into either double knockout MEF cells (B) or Saos2 cells (C). The p53 protein expression was detected with the DO1 antibody.

Together, our results suggest that the serine residues 116 and 127 are important for p53 DNA binding and that the substitution of the serine residues with aspartic acid leads to a possible loss of p53-dependent transcription of Mdm2, which results in a loss of protein degradation. Importantly, we have shown that S116/127D exhibits a structural mutant conformation. We consider the possibility that the simultaneous introduction of two large charged aspartate residues into the compact core domain of p53 would result in its unfolding. However, two lines of evidence suggest that this is unlikely. First, the crystal structure of p53 reveals that Ser116 and 127 residues are either completely or partially on the surface of the core domain. Second, similar to other p53 mutants with the structural mutant conformation, the S116/127D mutant can be degraded by the overexpression of Mdm2. Notably, a recent study has suggested that COP9 signalosome (CSN) phosphorylates p53 within the core domain of the protein (Thr155) and targets it to ubiquitin-mediated protein degradation [11]. Mutation of Thr155 with valine (T155V) is sufficient to stabilize p53. Interestingly, the stabilized T155V mutant also exhibits the structural mutant conformation (Pab240+). Therefore, the alteration of charges in the core domain, potentially through phosphorylation/dephosphorylation, could trigger a conformational change in the p53 molecule and affect its DNA binding and stability. Although we have not yet obtained direct evidence of a phosphorylation at Ser116 and 127 in vivo, our mutagenesis studies seemed to support this notion.

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