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Reconstitution of the mitochondrial Hsp70 (mortalin)-p53 interaction using purified proteins – Identification of additional interacting regions

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ABSTRACT

Previous studies have shown that the mammalian mitochondrial 70 kDa heat-shock protein (mortalin) can also be detected in the cytosol. Cytosolic mortalin binds p53 and by doing so, prevents translocation of the tumor suppressor into the nucleus. In this study, we developed a novel binding assay, using purified proteins, for tracking the interaction between p53 and mortalin. Our results reveal that: (i) P53 binds to the peptide-binding site of mortalin which enhances the ability of the former to bind DNA. (ii) An additional previously unknown binding site for mortalin exists within the C-terminal domain of p53.

Structured summary:
MINT-7557591: *p53* (uniprotkb:P04637) *binds* (MI:0407) to *DnaK* (uniprotkb:P0A6Y8) by *affinity chromatography technology* (MI:0004)
MINT-7557644: *mortalin* (uniprotkb:P38646) *binds* (MI:0407) to *p53* (uniprotkb:P04637) by *pull down* (MI:0096)
MINT-7557580, MINT-7557611: *p53* (uniprotkb:P04637) *binds* (MI:0407) to *mortalin* (uniprotkb:P38646) by *affinity chromatography* technology (MI:0004)

1. Introduction

The mitochondrial 70 kDa heat-shock protein (Sscl in yeast and mortalin in mammals) plays a key role in the biogenesis of mitochondrial proteins [1–3]. Several lines of experimental evidence showed that mortalin performs additional, diverse extra-mitochondrial functions [4]. The most apparent evidence for this is the fact that mortalin has been found to associate with a large number of unrelated cytosolic proteins. The list includes Mps1 kinase, fibroblast growth factor-1 (FGF-1), cytosolic J proteins, p53 and others (for a review see [5]).

Several observations suggested that mortalin is involved in the transformation of normal cells to cancer-cells, as well as in the resistance of cancer-cells to chemotherapy:

(1) Mortalin was found to be overexpressed in tumor cells of various origins [6].
(2) Mortalin was found to change its subcellular location from mitochondria, in normal cells, to the cytosol, in cancerous cells [7].
(3) Mortalin was found to interact with p53 and this interaction was suggested to promote sequestration of p53 in the cytoplasm, thereby inhibiting its nuclear activity [5,8,9] and inducing the resistance of some tumors to radiotherapy and chemotherapy.

The p53-mortalin interaction has been the subject of several studies, which mapped the binding sites between mortalin and p53. For example, one site on p53 that binds mortalin was suggested to be localized to the tetramerization domain, namely amino acids 323–337 [10]. Conversely, the binding site for p53 on mortalin was mapped to amino acids 253–282, in the ATPase domain [11]. However, these studies were performed using a combination of purified proteins and cell lysates. In this study, we reconstituted the interaction between p53 and mortalin using purified proteins and mapped the domains of interaction between them.

2. Methods

2.1. Cloning and purification of proteins

DnaK and mortalin were cloned in bacterial expression vectors and engineered to carry an octa-histidine tag, which is removable upon treatment with TEV protease. Proteins were initially purified

Abbreviations: mtHsp70, 70-kDa mitochondrial heat-shock protein; PAGE, polyacrylamide gel electrophoresis; ssDNA, salmon sperm DNA; FL-p53, full-length p53; NLS, nuclear localization signal

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on Ni-agarose and subsequently separated using a HiLoad 16/60 Superdex 200 gel filtration column.

The p53 used in this study is a super-stable quadruple mutant form of the protein (M133L/V203A/N239Y/N268D) which is stabilized by the mutations, as described in [12–14]. Full-length super-stable p53 (residues 1–393) in a modified pET24a vector, served as a template for PCR-cloning of the various p53 constructs. P53 constructs were purified using a method previously described [15].

2.2. Pull down of the p53-mortalin complex using heparin-sepharose beads

Pull-down assays were performed by incubating full-length p53 (FL-p53) or p53 mutants, with the various mortalin constructs. The proteins were incubated in 200 μl of binding buffer (50 mM Tris–HCl, pH 7.4, 45 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 5 mM DTT, 1 mg/ml BSA) for 30 min at room temperature. Then, 25 μl of heparin-sepharose beads (Amersham Bioscience) was added to the reaction and the mixture was incubated at 4 °C for 1 h on a rotating plate. The beads were washed three times with 1 ml of binding buffer and further incubated at 4 °C in elution buffer (binding buffer containing 500 mM KCl) for 30 min. Proteins eluted from the beads were analyzed using 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

2.3. Pull down of the p53-mortalin complex using Nickel-agarose beads

Proteins were incubated in 200 μl of binding buffer (50 mM Tris–HCl, pH 7.4, 100 mM KCl, 100 mM NaCl, 5 mM $MgCl_2$, 5% glycerol, 40 mM imidazole, 10 mM β-mercaptoethanol) for 30 min at room temperature. Then, 15 μl of nickel-agarose beads (GE Healthcare) was added to the reaction and the mixture was incubated at 4 °C for 1 h on rotating plate. The beads were washed three times with 1 ml of binding buffer and further incubated at 4 °C in elution buffer (binding buffer containing 500 mM imidazole) for 30 min. Proteins eluted from the beads were analyzed on 10% SDS–PAGE.

2.4. Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was carried out according to a previously published protocol with slight modifications [16] (see the legend of Fig. S1 for a detailed description).

3. Results

3.1. The effect of nucleotides on complex formation between p53 and mortalin

We developed a new method to track formation of the p53-mortalin complex and monitor the effect of various co-factors on the interaction. First, purified mortalin and p53 are incubated together to allow for complex formation. It is known that p53 can bind to heparin-sepharose [17]. Thus, the second step involves incubating the complex with heparin-sepharose beads, and then washing the beads three times to remove non-specifically bound proteins. P53 and any bound mortalin are eluted from the heparin beads using 0.5 M KCl and analyzed by SDS–PAGE. The interaction between mortalin and p53 that we detected using the heparin pull-down assay is specific since: (i) BSA, which is included in all binding buffers, did not bind significantly either to p53 that was bound to heparin beads or to heparin beads alone. (ii) Mortalin did not show significant binding to heparin beads, in the absence of p53 (Fig. 1A, lane 2). (iii) A specific interaction between p53 and the bacterial homologue of 70-kDa mitochondrial heat-shock protein, DnaK, was also previously reported [18,19]. Consistent with this, a complex between DnaK and p53 was detected using the heparin pull-down assay (Fig. 1B).

The interaction between Hsp70 chaperones and their client proteins is modulated by nucleotides. Thus, we examined the effect of nucleotides on the p53-mortalin interaction. Without added nucleotides, a very strong association was observed between mortalin and p53. Strong complex formation was also observed in the presence of ADP. In contrast, in the presence of ATP, the p53-mortalin complex did not form at all. Dissociation of the p53-mortalin complex was also observed in the presence of AMP-PNP, a non-hydrolyzable analogue of ATP, albeit less than in the presence of ATP (Fig. 1A). Notably, a similar effect of various nucleotides was also observed when we examined the DnaK-p53 interaction (Fig. 1B). In summary, we were able to observe an interaction between purified p53 and mortalin using the method of pull-down with heparin-sepharose beads. The interaction is sensitive to the presence of nucleotides. As such, the p53-mortalin complex was found to partially dissociate in the presence of AMP-PNP and completely in the presence of ATP.

3.2. P53 binds directly to the peptide-binding site of mortalin

The aforementioned method enabled us to examine directly which domain of mortalin – the ATP-binding or peptide-binding domain – associates with p53. For this purpose, we expressed the ATP-binding and the peptide-binding domains of mortalin individually in bacteria and purified them. Next, we carried out the heparin pull-down assay, as previously described. Interestingly, we found that the peptide-binding domain (Fig. 2B, lanes 3–8), but not the ATP-binding domain (Fig. 2B, lane 11), was able to associate with p53, in a concentration dependent manner. These results are in contrast to results obtained using cell lysates, which suggested that the association of p53 occurs via the ATP-binding domain of mortalin [11]. We next examined whether the association of mortalin with p53 is affected by mutating the peptide-binding region of mortalin. To this end, we created a mutant of mortalin, V482F, which is expected to exhibit a defective interaction with substrate proteins, based on a similar DnaK mutant [20]. Indeed, the V482F mutant of mortalin did not bind p53 at all, when examined using the heparin-binding assay (Fig. 2C). Finally, when mortalin was preincubated with p5, a peptide that was shown to bind strongly to Hsp70 chaperones [21], binding of p53 was significantly reduced (Fig. S2). We conclude that purified p53 binds purified mortalin via its peptide-binding site, through an interaction that is sensitive to the presence of nucleotides.

3.3. Mortalin binding affects the ability of p53 to bind DNA

Chaperone proteins were shown to affect the ability of p53 to bind DNA [18,22,23]. Such an effect of mortalin on p53 was never demonstrated. Consequently, the next step was to examine the ability of p53 to bind radio-labeled DNA using an Electrophoretic Mobility Shift Assay (EMSA). As shown in Fig. 3, p53 alone binds DNA. In agreement with previous reports, which demonstrated that the C-terminal region of p53 plays an important role as a regulator of specific DNA binding by the protein, association was dramatically increased upon deletion of the C-terminal domain of the protein (p53ΔCTD) (Fig. 4A) [18]. Addition of excess, non-specific, unlabeled DNA as binding competition decreased the binding of double-stranded radio-labeled DNA by p53. Interestingly, a profound increase in the ability of p53 to bind DNA was observed when mortalin was included in the reaction mixture (Fig. 3, lane 11). When binding was examined in the presence of the V482F mutant of mortalin, the specific DNA binding by p53 was ~63% less than binding in the presence of wild type mortalin (Fig. S1). The

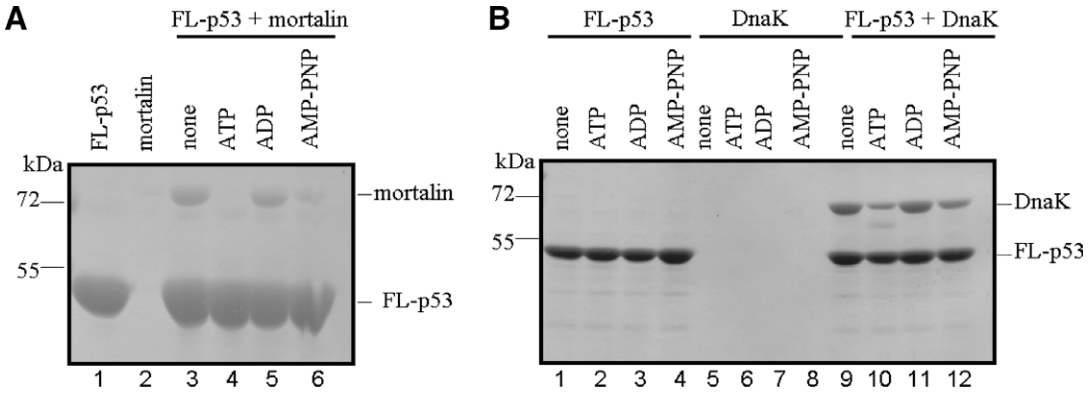

Fig. 1. Effect of nucleotides on the p53-mortalin interaction. FL-p53 was subjected to the heparin-bead pull-down assay (see Section 2) in the presence of (A) Mortalin or (B) DnaK, together with various nucleotides (1 mM) as indicated. The concentration of all proteins in all the reactions was 10 μM.

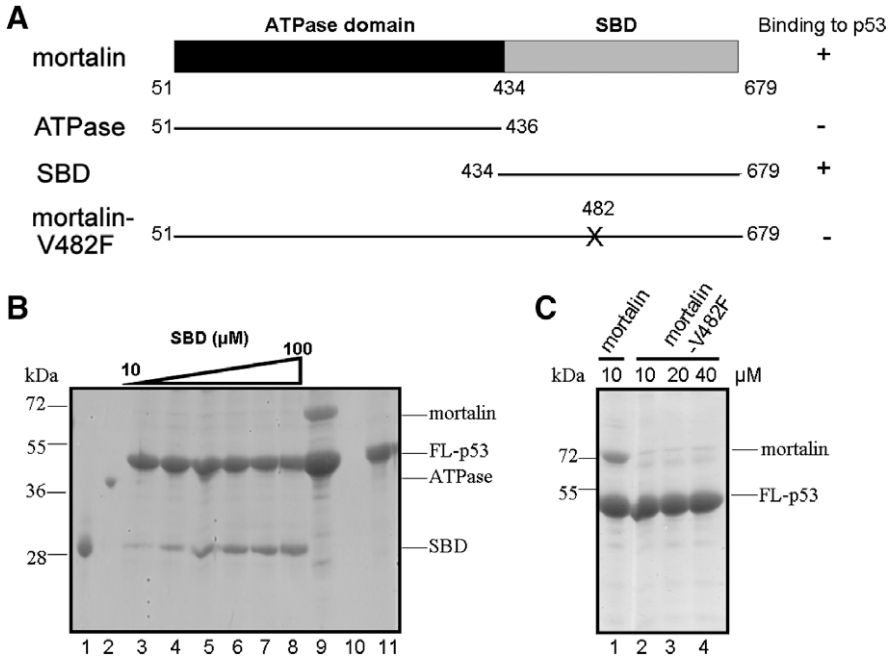

Fig. 2. P53 binds to the substrate-binding domain of mortalin. (A) Schematic representation of mortalin constructs used in this study. Constructs that bind to p53 are designated with a "+" to the right of the panel. In B and C, the heparin-bead pull-down assay was carried out as described under Section 2. (B) 5 μg of SBD (lane 1) and 2.5 μg of the ATPase domain (lane 2). Lanes 3–8 correspond to 10, 20, 40, 60, 80, and 100 μM SBD, that was incubated with FL-p53 (10 μM). In lane 9, FL-p53 was incubated with 10 μM of mortalin. Lane 10: 10 μM of SBD were incubated with heparin beads alone. Lane 11: FL-p53 was incubated with 40 μM of the ATPase domain. (C) FL-p53 (10 μM) was incubated with the indicated concentrations of mortalin or mortalin-V482F.

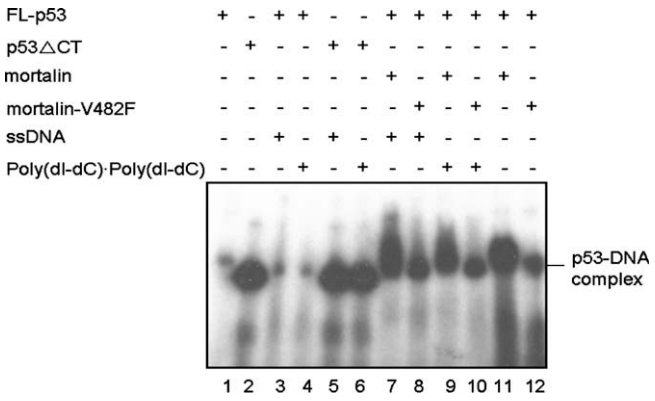

Fig. 3. Mortalin affects the ability of p53 to bind DNA. EMSA was carried out as described in the legend to Fig. S1. Protein concentrations were 0.1 mg/ml for the indicated p53 constructs and 2 mg/ml for mortalin constructs.

fact that the V482F mutant still had some effect on DNA binding by p53 (Fig. S1) suggests that the mutation did not completely abolish an interaction between the two. This interaction was not observed in the heparin-binding experiments due to the extensive washes used in our binding assay (Fig. 2C). In summary, we have demonstrated for the first time that mortalin regulates the ability of p53 to bind DNA.

3.4. P53 harbors two distinct binding sites for mortalin

Our successful reconstitution of the interaction between p53 and mortalin from purified components enabled us to examine the binding site for mortalin on p53. To this end, we investigated the ability of several modified p53 constructs to bind purified mortalin (Fig. 4A). The first was a deletion of the C-terminal domain (CTD) which was previously shown to bind DnaK and other Hsp70 family members [18,24]. Notably, deletion of this region did not abolish the ability of p53 to bind mortalin (Fig. 4B). Another

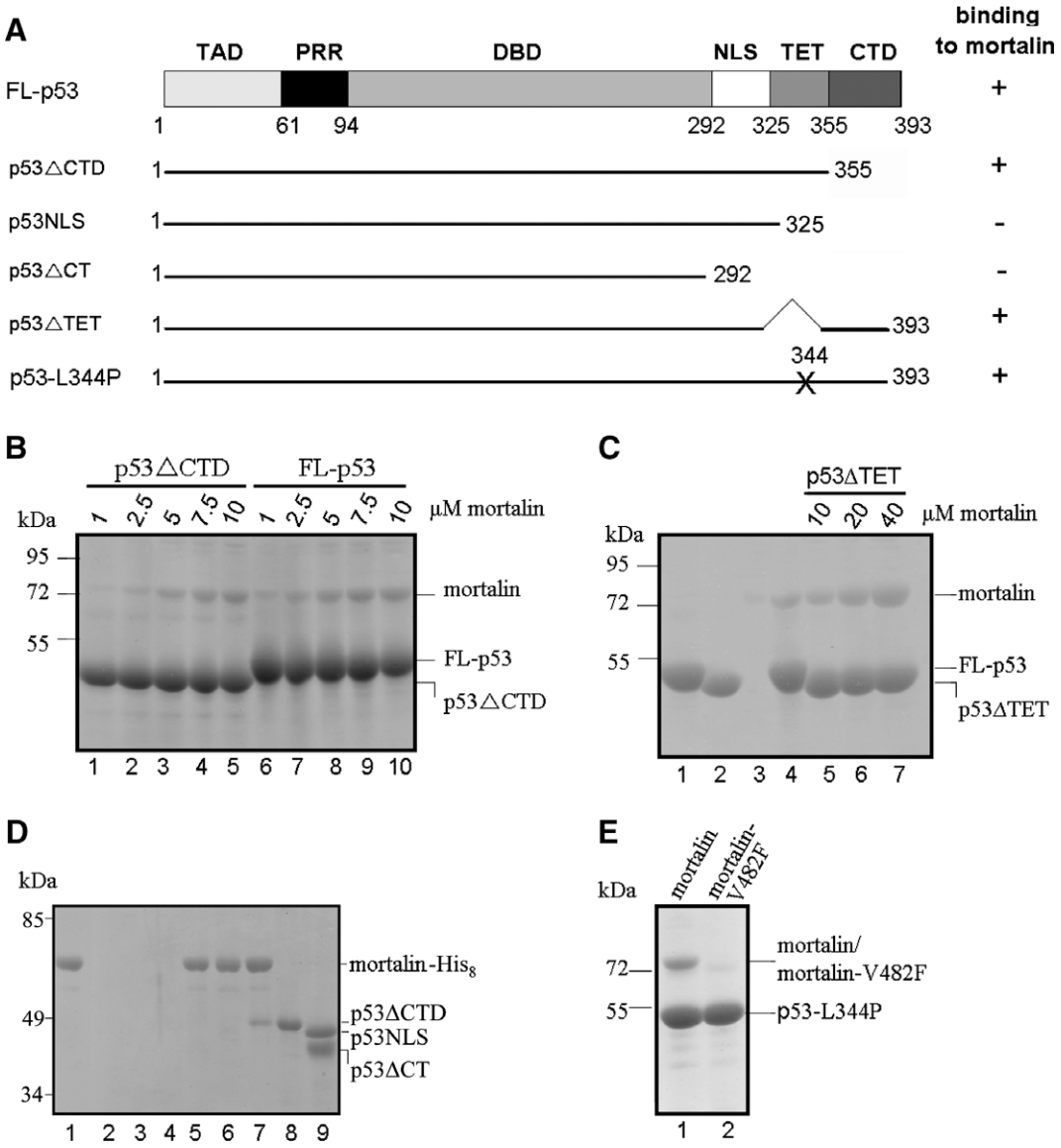

Fig. 4. Two sites for mortalin binding on p53. (A) Schematic representation of the various p53 constructs used in this study. Constructs that bind to mortalin are designated with a "+" to the right of the panel. In B, C and E, the heparin-beads pull-down assay was carried out as described under Section 2. When included, the p53 concentration was 10 μM. (B) The indicated mortalin concentrations were incubated with either p53ΔCTD (lanes 1–5) or with FL-p53 (lanes 6–10). (C) Lanes 1–3: FL-p53, p53ΔTET and 20 μM mortalin were incubated alone with heparin beads, respectively. Lane 4: incubation of FL-p53 with 10 μM of mortalin. Lanes 5–7: the indicated mortalin concentrations were incubated with p53ΔTET. (D) A Nickel-bead pull-down assay was carried out as described under Section 2. Lane 1: 10 μM of mortalin-his_8 were incubated alone with nickel beads. Lanes 2–4: 10 μM of either p53ΔCT, p53NLS or p53 ΔCTD were incubated alone with nickel beads, respectively. Lanes 5–7 correspond to 10 μM of mortalin-his_8 that were incubated with 10 μM of either p53ΔCT, p53NLS or p53ΔCTD, respectively. Lane 8: 5 μg of p53ΔCTD. Lane 9: 5 μg of p53ΔCT and p53NLS. (E) p53-L344P was incubated with either 10 μM of mortalin (lane 1) or mortalin-V482F (lane 2) and protein complexes were subjected to the heparin pull-down assay.

region of p53, the tetramerization domain (TET), was suggested to be the region mediating interaction with mortalin [10]. Interestingly, a purified construct of p53 that harbors a deletion of the TET domain was also able to bind mortalin (Fig. 4C). These results suggest that p53 harbors two binding sites for mortalin, one in the TET domain and the second in the CTD. To examine this possibility, we designed and purified two additional constructs, both of which lack the TET domain and the CTD. The first is p53NLS, which contains the nuclear localization signal (NLS) area (a.a. 292–325) and the second is p53ΔCT which additionally lacks the NLS domain [25]. In contrast to full-length p53, purified p53NLS and p53ΔCT did not bind to heparin beads and did not bind non-specifically to Ni-agarose beads (not shown). Thus, we used octa-histidine tagged mortalin to examine its binding to purified p53NLS and p53ΔCT by pull-down experiments with Ni-agarose. As shown in Fig. 4D, neither purified p53NLS nor p53ΔCT bound to mortalin in the Ni-agarose pull-down assay. As a positive control, we used p53ΔCTD, which bound mortalin in the heparin-sepharose assay (Fig. 4B), and did not bind to Ni-agarose in a non-specific manner (Fig. 4D, lane 4). As expected, from the heparin pull-down experiments, p53ΔCTD was detected bound to mortalin using the Ni-agarose pull-down assay (Fig. 4D, lane 7). All together, these results suggest that p53 harbors two binding sites for mortalin, one in the C-terminal domain and the other in the TET domain. Each one of these domains is sufficient to allow binding of mortalin to p53. In a final experiment, we examined whether the tetrameric structure of p53 is necessary for binding mortalin. To examine this possibility, we created a mutant of p53 (L344P) that was previously shown to be impaired in its ability to form tetramers [26]. Purified p53 (L344P) was able to bind mortalin similarly to the wild type

protein (Fig. 4E). We conclude that the tetrameric structure of p53 is not required for its association with mortalin.

4. Discussion

P53 is a tumor suppressor that is essential for the prevention of cancer development [27]. This function of p53 requires that it be translocated to the nucleus. Factors preventing the movement of p53 into the nucleus should therefore prevent its anti-tumor function and thereby enhance cancer development [28–31]. Mitochondrial localization and interaction of p53 have been also reported [32]. Mortalin was shown to bind p53 in the cytosol and was suggested to prevent its translocation into the nucleus [7]. In this study, we developed a pull-down assay using heparin beads to map domains that mediate the p53-mortalin interaction. We showed that (i) p53 binds to the peptide-binding domain of mortalin and not to the ATP-binding site as previously reported [11]. Our results are in accord with previous results showing that Hsp70 chaperones bind p53 as a substrate protein [23]. (ii) P53 harbors two binding sites for mortalin. The first one was previously determined to be in the tetramerization domain [10], and the second is found in the C-terminal domain. Interestingly, the binding site for DnaK was also shown to be in the CTD [19]. The differences between the results of this *in vitro* study and the results of previous studies may be explained by possible modification or additional interacting proteins which may exist in cell lysates and may affect the character of the interaction between p53 and mortalin. Further study will be required to determine which of these possibilities is the case.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.02.019.

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