

Stress-mediated nuclear stabilization of p53 is regulated by ubiquitination and importin- α 3 binding

ND Marchenko^{*,1}, W Hanel¹, D Li¹, K Becker¹, N Reich² and UM Moll^{1,3}

The activity of p53 as an inducible transcription factor depends on its rapid nuclear stabilization after stress. However, surprisingly, mechanism(s) that regulate nuclear p53 accumulation are not well understood. The current model of stress-induced nuclear accumulation holds that a decrease in p53 nuclear export leads to its nuclear stabilization. We show here that regulated nuclear import of p53 also has a critical function. p53 import is mediated by binding to the importin- α 3 adapter and is negatively regulated by ubiquitination. p53 harbors several nuclear localization signals (NLS), with the major NLS I located at amino-acids 305–322. We find that direct binding of p53 to importin- α 3 depends on the positive charge contributed by lysine residues 319–321 within NLS I. The same lysines are also targets of MDM2-mediated ubiquitination. p53 ubiquitination occurs primarily in unstressed cells, but decreases dramatically after stress. Importin- α 3 preferentially interacts with non-ubiquitinated p53. Thus, under normal growth conditions, ubiquitination of Lys 319–321 negatively regulates p53-importin- α 3 binding, thereby restraining p53 import. Conversely, stress-induced accumulation of non-ubiquitinated p53 in the cytoplasm promotes interaction with importin- α 3 and rapid import. In later phases of the stress response, blocked nuclear export also takes effect. We propose that p53 nuclear import defines an important novel level of regulation in the p53-mediated stress response.

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The p53 tumor suppressor serves as a pivotal surveillance factor guarding against genomic instability and transformation by inducing cell-cycle arrest, senescence and apoptosis. In unstressed cells, p53 levels are low because of its proteolytic turnover by the MDM2 E3 ligase. The activity of p53 as a rapidly inducible transcription factor in response to stress depends on its quick stabilization in the nucleus. However, the mechanism(s) that regulate stress-induced nuclear accumulation of p53 are not well understood. Nuclear import and export of large proteins is restricted, and transit through nuclear pore complexes is mediated by binding to soluble transport carriers.^{1,2} The transport carriers, referred to as importins and exportins, recognize amino-acid sequences that function as nuclear localization signals (NLS) or nuclear export signals (NES). Under normal growth conditions, p53 and MDM2 are subject to nuclear-cytoplasmic shuttling. In unstressed cells, p53 nuclear export has been shown to be an MDM2-dependent process. Monoubiquitination of the C-terminus of p53 enhances sumoylation in this region, promoting nuclear export.^{3–5} The Crm1 exportin is the primary transporter for p53, and the specific Crm1 inhibitor leptomycin B (LMB) blocks p53 nuclear export.^{6,7}

Explorations of p53 nuclear accumulation in response to stress have so far been limited to the function of nuclear export in this process. In response to stress, numerous post-translational modifications on p53 and MDM2 lead to disruption of the physical complex between p53 and MDM2,

as well as to downregulation of MDM2 mRNA and protein. The current model of stress-induced nuclear accumulation holds that these general events lead to a decrease in p53 nuclear export and thereby increased nuclear stabilization.⁸ The resulting rise in nuclear p53 levels stimulates p53 tetramerization, which masks the C-terminal NES that is strategically located within the tetramerization domain.⁵ A second NES located at the N-terminus of p53 within the MDM2-binding domain is inhibited by serine phosphorylations induced in response to DNA damage.⁹

In contrast to nuclear export, it is unknown whether p53 import is subject to regulation, and whether import has a function in the stress-induced p53 stabilization in the nucleus. Nevertheless, the importance of p53 nuclear import is highlighted by aberrant-cytoplasmic localization of wild-type p53 (wtp53) in some tumors, which leads to impaired function of p53 as a transcription factor (as well as impaired function as a mitochondrial permeabilization factor),¹⁰ thereby promoting tumorigenesis. For example, constitutive-cytoplasmic sequestration of p53 occurs in inflammatory breast cancer,^{11,12} neuroblastoma,¹³ retinoblastoma and some colon carcinomas¹⁴ and correlates with poor prognosis and resistance to therapy.

Analyses of p53 nuclear import have identified three NLS sequences in its C-terminus. The dominant NLS I at amino-acids 305–322 conforms to a bipartite basic sequence. NLS II and III at residues 366–372 and 376–381, respectively, are

¹Department of Pathology, Stony Brook University, Stony Brook, NY 11794, USA; ²Department of Molecular Genetics and Microbiology, Stony Brook University, Stony Brook, NY 11794, USA and ³Department of Molecular Oncology, University of Göttingen, Göttingen 37077, Germany

*Corresponding author: ND Marchenko, Department of Pathology, Stony Brook University, Stony Brook, NY 11794-8691, USA.

Tel: +631 444 3520; Fax: +631 444 3424; E-mail: nmarchenko@notes.cc.sunysb.edu

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Abbreviations: NLS, nuclear localization signal; NES, nuclear export signal; LMB, leptomycin B; siRNA, short-interfering RNA; UbAL, ubiquitin aldehyde; UBP, ubiquitin-specific processing protease

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weaker motifs with single stretches of basic amino acids.^{15,16} Basic NLS sequences are commonly recognized by an adapter family of importin- α proteins.¹⁷ Importin- β 1 mediates transit of importin- α with its cargo into the nucleus. The presence of canonical NLS signals in p53 strongly suggests that nuclear import of p53 occurs through the classic importin- α /importin- β 1 pathway. Here, we provide evidence that nuclear import of p53 is indeed mediated through this importin pathway, specifically using importin- α 3. p53 directly binds to importin- α 3 and this binding requires the basic amino acids contributed by lysine residues 319–321 within the major NLS I. Moreover, p53 import is negatively regulated by ubiquitination. This scenario simultaneously explains the predominant accumulation of ubiquitinated p53 in the cytoplasm and the low levels of p53 in the nucleus of unstressed cells. In contrast, stress-mediated modifications of MDM2 and p53 rapidly increase the non-ubiquitinated p53 pool, exposing NLS I for efficient importin- α 3 recognition and enhanced import. In summary, our data imply that ubiquitin-regulated nuclear import of p53 is a control switch and an important determinant for rapid nuclear p53 stabilization after stress.

Results

Under normal growth conditions p53 ubiquitination and degradation occur primarily in the cytoplasm. Ubiquitin-mediated degradation of p53 through MDM2 E3 ligase and 26S proteasomes has been firmly established. It is less clear, however, in which subcellular compartment at a given physiologic condition p53 ubiquitination and degradation take place.¹⁸ Earlier studies addressing this question relied on ectopic import/export mutants of p53 and MDM2 or on nuclear export block. They found that when forced together, both nuclear and cytoplasmic compartments are capable of p53 degradation.^{19,20} In agreement, we earlier showed that both nuclear and cytoplasmic proteasomes efficiently degrade p53 during the down-regulatory phase of the p53 DNA-damage response.²¹

These *ectopic* studies implied that the cytoplasm is the primary site of p53 ubiquitination and degradation in unstressed cells.^{6,18} To test whether this also holds for *endogenous* p53 in unmanipulated cells, we performed careful subcellular fractionations and examined the ubiquitination patterns of nuclear and cytoplasmic p53. To this end, we used a panel of unstressed human cancer cell lines that harbor wt p53 and analyzed them by immunoblots whose loadings were normalized for comparable amounts of non-ubiquitinated p53. As shown in Figure 1a, ubiquitination of endogenous p53 occurs predominantly in the cytoplasm of all cell lines, with little or no ubiquitinated p53 detectable in the nucleus. Thus, in agreement with earlier findings,⁶ this strongly suggests that endogenous p53 degradation occurs mainly in the cytoplasm of unstressed cells. In further support, we find differential stability of p53 in unstressed cells, with a much shorter half-life of cytoplasmic p53 (20 min) compared with a longer half-life of nuclear p53 (60 min) (Supplementary Figure 1A). Moreover, even after inhibition of nuclear export by LMB, p53 ubiquitination occurs preferentially in the cytoplasm (with only minimal increase of ubiquitinated p53

in the nucleus), further indicating that p53 degradation occurs outside the nucleus under normal growth conditions (Figure 1b). Similarly, after coexpression of p53 and MDM2 in p53-null cells (H1299), p53 ubiquitination occurs mainly in the cytoplasm, even after LMB treatment (Supplementary Figure 1B). The latter results exclude the possibility that ubiquitinated cytoplasmic p53 is primarily because of nuclear export, but instead is generated locally in the cytoplasm. In contrast, DNA damage eliminates ubiquitinated p53 species in the cytoplasm and induces nuclear accumulation of p53 (Figure 1b).

Together, these data indicate that ubiquitination and degradation of endogenous p53 occur mainly in the cytoplasm of unstressed cells. Similar to p53, MDM2 is located in both the nucleus and cytoplasm, and a high stoichiometric ratio of MDM2 to p53 is a key determinant of low p53 stability. To gain further insight into the underlying reason for preferential cytoplasmic ubiquitination of p53, nuclear and cytoplasmic fractions representing the same *number of cells* were blotted for MDM2 and p53. In agreement with an earlier report, endogenous MDM2 is preferentially located in the cytoplasm of unstressed cells,²² whereas p53 is either evenly distributed between nucleus and cytoplasm or more nuclear (Figure 1c). Moreover, MDM2 interacts with p53 predominantly in the cytoplasm rather than the nucleus of unstressed cells, as shown by reciprocal immunoprecipitations. In addition, consistent with increased interaction, ubiquitinated p53 is mainly detected in the cytoplasm (Figure 1d, left and right). Furthermore, Hausp (the major p53 deubiquitinase) and the amount of p53–Hausp complexes are equally distributed in nucleus and cytoplasm (Figure 1e). Moreover, deubiquitinating HAUSP activity is not higher in the nucleus (Supplementary Figure 1C), excluding the alternative possibility of preferential p53 deubiquitylation in the nucleus of unstressed cells. In summary, the relative abundance of MDM2 to p53 in the cytoplasm, as opposed to the nucleus, seems largely responsible for enhanced ubiquitination of p53 in the cytoplasm.

On the other hand, proteasome inhibition by ALLN stabilizes p53 mainly in the *nucleus* of RKO cancer cells, whereas cytoplasmic p53 accumulates to a far lower extent (Figure 1f, top), consistent with earlier reports.^{18,23} Surprisingly, however, even in the presence of ALLN, nuclear p53 is largely *non-ubiquitinated*, in sharp contrast to cytoplasmic p53, which stabilizes its ubiquitinated species (Figure 1f, bottom). Moreover, normal human fibroblasts (MRC5) produce the same result on ALLN inhibition (Figure 6g, top and bottom). Similar to ALLN, Nutlin, a specific inhibitor of the MDM2–p53 interaction that blocks p53 degradation,²⁴ induces strong p53 accumulation primarily in the *nucleus* (Figure 1f, top). Yet again, after Nutlin treatment, the nuclear p53 species is largely *non-ubiquitinated*, whereas ubiquitinated p53 is only detectable in the cytoplasm (Figure 1f, bottom). (Note, Nutlin only *partially* disrupts MDM2–p53 complexes and does not completely eliminate ubiquitinated p53 species, as earlier shown.^{25–27}) So, why – given that the post-stress nucleus is capable of degrading endogenous p53 – do we not see *ubiquitinated* p53 in the nucleus, not even under optimal conditions when p53 degradation is blocked by ALLN or Nutlin? We reasoned that a plausible explanation for

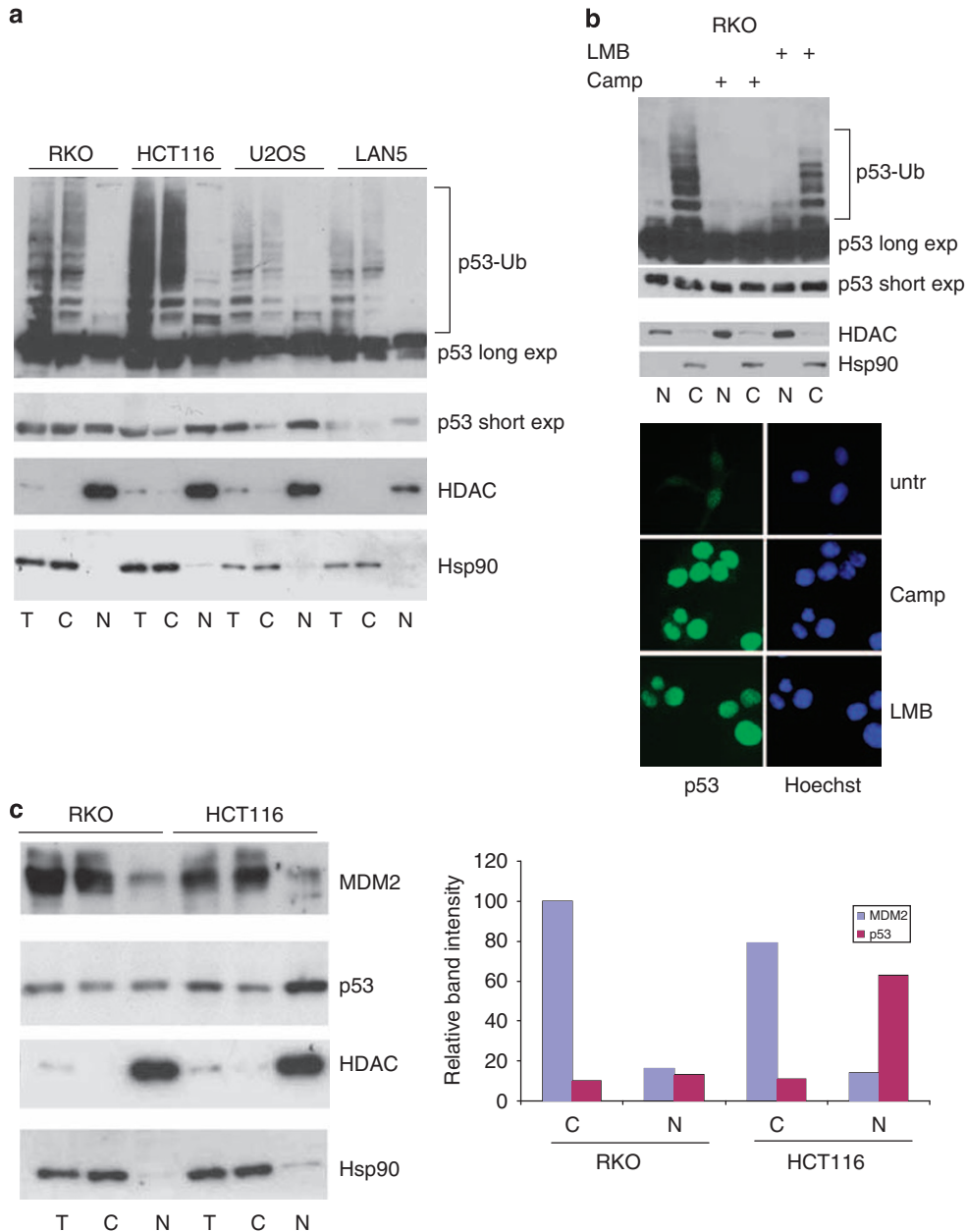


Figure 1 In unstressed cells, p53 ubiquitination and degradation occur primarily in the cytoplasm. **(a)** Ubiquitination of wtp53 occurs in the cytoplasm. RKO, HCT116, U2OS and LAN5 cells were carefully fractionated into cytoplasmic and nuclear compartments and immunoblotted for p53 with DO1 antibody. Short and long exposures are shown. Loadings were normalized for comparable amounts of non-ubiquitinated p53 between cytoplasm (C) and nucleus (N). T, total cell lysates. Hsp90 and HDAC are markers for the purity of cytoplasmic and nuclear fractions, respectively. **(b)** Even after inhibition of nuclear export by LMB, p53 ubiquitination preferentially occurs in the cytoplasm. *Top* – RKO cells were treated with camptothecin (Camp) or LMB, followed by fractionation and immunoblotting. Loadings were normalized for equal amounts of non-ubiquitinated p53 in cytoplasm and nucleus. *Bottom* – nuclear accumulation of p53 after camptothecin or LMB treatment. p53 immunofluorescence. Hoechst counterstain. Endogenous p53 in unstressed cells is barely detectable. **(c)** Endogenous MDM2 is mainly located in the cytoplasm of unstressed cells, whereas p53 is either evenly distributed between nucleus and cytoplasm or more nuclear, depending on the cell line. RKO and HCT116 cells were fractionated and aliquots corresponding to an equal cell number per fraction were immunoblotted as indicated. *Right* – densitometry of immunoblot shown on the left. Hsp90 and HDAC are purity controls of cytoplasmic and nuclear fractions, respectively. **(d)** Mdm2 interacts with p53 predominantly in the cytoplasm of unstressed cells. After stress, cytoplasmic p53–Mdm2 complexes rapidly decrease. RKO cells were left untreated or treated with camptothecin for 60 min and cells (1×10^7 each) subjected to fractionation, followed by immunoprecipitation of each fraction for MDM2 and subsequent immunoblotting for MDM2 and p53 (*left*). Reciprocal immunoprecipitation for p53 was carried out on proteasome inhibitor-treated RKO cells, using a cocktail of ALLN + MG132 (*right*). **(e)** Hausp, the major p53 deubiquitinase, is equally distributed in nucleus and cytoplasm ('input' panel) and the amount of p53–Hausp complexes are equal in nucleus and cytoplasm ('IP' panel), excluding the possibility of preferential p53 deubiquitylation in the nucleus of unstressed cells. RKO cells were fractionated and each fraction immunoprecipitated with an antibody to Hausp or irrelevant IgG, followed by immunoblotting for p53 and Hausp. **(f, g)** Despite p53 accumulation in the nucleus, proteasome inhibition (ALLN) and Nutlin treatment induces ubiquitinated p53 species only in the cytoplasm, whereas nuclear p53 is non-ubiquitinated. RKO cells (**f**) and normal human fibroblasts (MRC5, **g**) were treated with ALLN for 3 h or Nutlin for 6 h, followed by fractionation. Nuclear and cytoplasmic fractions normalized for equal total protein (*top*) or equal amounts of non-ubiquitinated p53 (*bottom*) were immunoblotted. Hsp90 and HDAC are as markers for cytoplasmic and nuclear fractions, respectively

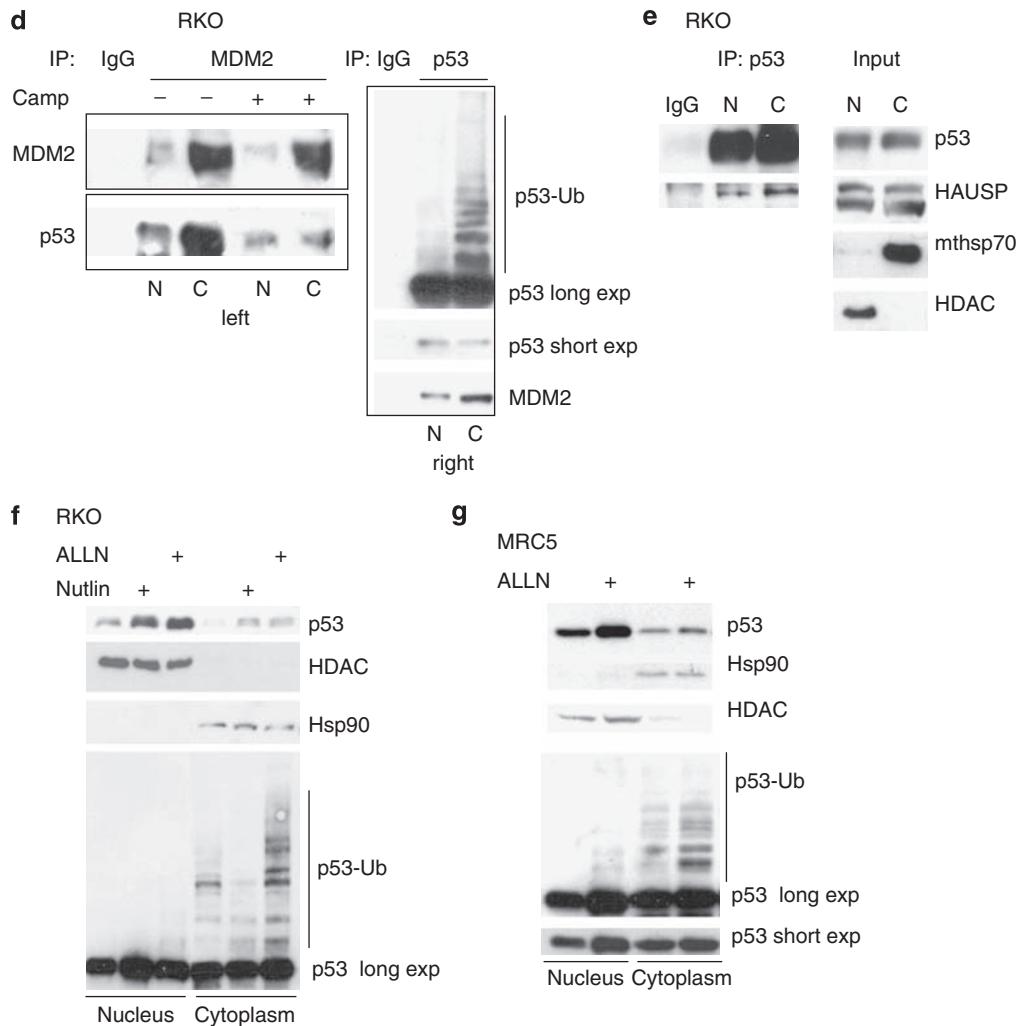


Figure 1 Continued

this phenotype is differential import: blocking p53–MDM2 complex formation or p53 degradation accumulates both ubiquitinated and non-ubiquitinated p53 in the cytoplasm. Although ubiquitinated p53 is blocked from import, non-ubiquitinated p53 is efficiently imported into the nucleus. This scenario could readily explain the strong accumulation of non-ubiquitinated, but lack of ubiquitinated p53 species in the nucleus after various types of degradation blockade. We, therefore, set out to test this hypothesis.

Nuclear import of p53 occurs through the canonical importin- α/β route. The presence of three canonical NLS in p53¹⁵ suggests that nuclear import occurs through the classic import route. Experimentally, this had not been tested in detail. To determine whether and under what conditions p53 nuclear import occurs through the classic importin- α/β pathway, we first used short-interfering RNA (siRNA) against importin- β , the generic component of the importin- α/β route. Indeed, downregulation of importin- β (Figure 2a) results in cytoplasmic retention with concomitant decrease in nuclear localization in 69% of H1299 cells expressing ectopic wtp53, compared with 5% of control cells

transfected with scrambled siRNA (Figure 2b; representative example of raw data shown in Figure 2c). Together, this indicates that import of endogenous p53 occurs through the canonical importin- α/β route and this route is essential for nuclear localization of p53.

p53 binds to importin- $\alpha 3$ and this interaction depends on the positive charge contributed by lysines 319–321 of NLS I of p53. In classic nuclear import, the interaction between importin- β and its cargo is mediated by an adaptor protein, importin- α , which determines substrate specificity and interacts with the NLS of the cargo. In mammalian cells, six importin- α proteins are known to bind to classic NLS motifs of cargos and facilitate their import.²⁸ To investigate whether any of these importin- α proteins specifically recognize p53, we performed *in vitro* binding assays using endogenous wtp53 derived from HCT116 cell lysates and a panel of recombinant importin- α proteins tagged with GST.

Importantly, in GST pull-down assays, very strong interaction of p53 was specifically detected with importin- $\alpha 3$. In contrast, p53 did not interact with empty GST or importin- $\alpha 1$ and $\alpha 7$, and only very weakly with importin- $\alpha 5$ and $\alpha 6$ (Figure 3a, left).

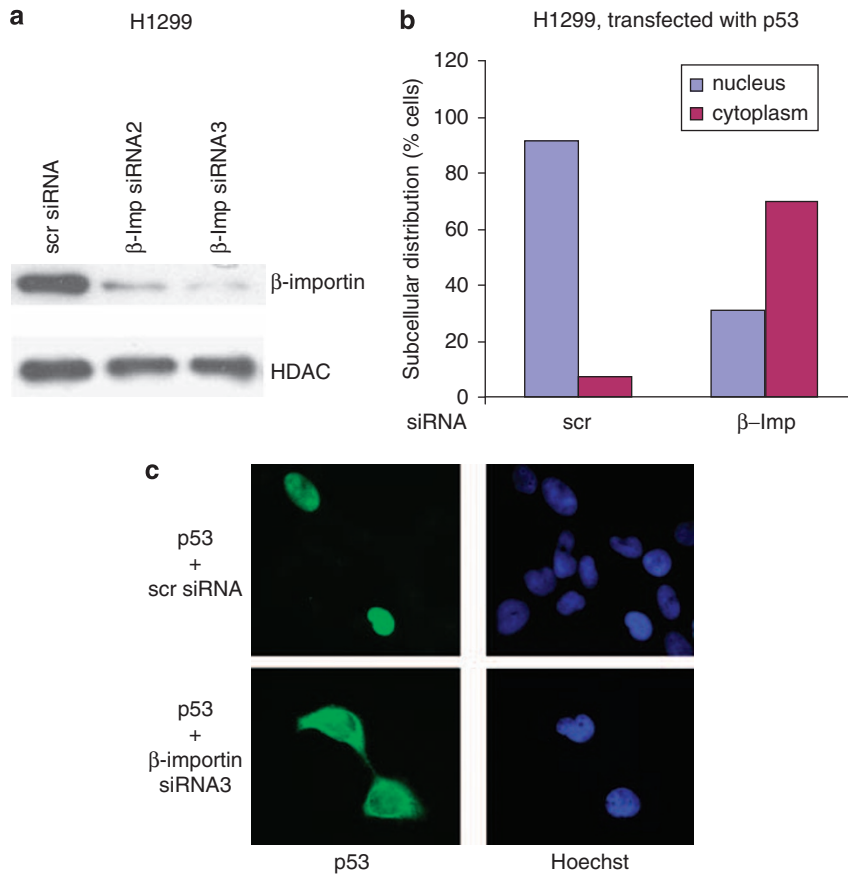


Figure 2 Nuclear import of p53 occurs through the canonical importin- α/β route. (a–c) Downregulation of importin- β by siRNA results in cytoplasmic retention of ectopic p53. H1299 cells were transfected with either scrambled or importin- β siRNA. (a) Total lysates were immunoblotted for β -importin. HDAC as loading control. After 24 h post-transfection with siRNA, cells were transfected with wtp53, followed by p53 immunofluorescence. (b) Four hundred random transfected cells were scored for p53 localization. The results are shown as percentage of nuclear *versus* cytoplasmic localization in scrambled and importin- β transfected cells. (c) Representative examples of transfected cells stained for p53 and viewed by epifluorescence (Zeiss Axiomat)

Moreover, GST-tagged importin- $\alpha 3$ shows strong interaction with purified bacterial p53, confirming that p53 directly binds to importin- $\alpha 3$ (Figure 3a, right). Of note, importin- $\alpha 3$ is ubiquitously expressed,²⁹ supporting the notion that it is the primary carrier for p53 nuclear import in most if not all cell types. Furthermore, specific downregulation of endogenous importin- $\alpha 3$ by siRNA results in cytoplasmic retention of endogenous p53 in unstressed and stressed HCT116 cells (Figure 6e).

To investigate which specific NLS of p53 is responsible for importin- $\alpha 3$ binding, we performed *in vitro* binding assays with NLS mutants (Figure 3b, top). The 3R mutant of NLS I (the major NLS in p53) carries arginines in exchange for lysines at residues 319–321, but preserves the basic charge, whereas the 3A mutant carries alanine exchanges that neutralize the basic charge of NLS I. The 5R mutant is an exchange of lysines to arginines in NLS II and III, but preserves NLS I. Lysates from H1299 cells (p53 null) transfected with these NLS mutants were incubated with GST-importin- $\alpha 3$ and immunoblotted for p53. In parallel, p53-transfected H1299 cells were analyzed by immunofluorescence. Mutating NLS I lysines to arginines (3R) partly reduces interaction of p53 with importin- $\alpha 3$ compared with wtp53 (Figure 3b, middle), although still supports nuclear accumulation, albeit not as

sharply as wtp53 (Figure 3b, bottom). In contrast, mutating NLS I lysines to alanines completely disrupts the interaction of p53 with importin- $\alpha 3$ and blocks nuclear import (Figure 3b). Conversely, mutating NLS II/III lysines to arginines only had a minor disruptive effect (Figure 3b). These data identify NLS I of p53 as the critical site of interaction with importin- $\alpha 3$ and crucial for its nuclear import. Moreover, this interaction depends on the positive charge contributed by lysines 319–321 of p53.

p53 Lysines 319–321 of NLS I, critical for its binding to importin- $\alpha 3$, are also targets of MDM2-mediated ubiquitination. Earlier work identified Lys 370, 372, 381, 382 and 386 within the extreme C-terminus of p53 as endogenous targets of MDM2-mediated ubiquitination.³⁰ Remarkably, these lysines are all localized within NLS II and NLS III of p53 (Figure 4a). So far we showed that ubiquitinated p53 predominantly resides in the cytoplasm of unstressed cells and that p53 nuclear import critically depends on the positive charge of NLS I (Figures 1–3). Together, this prompted us to test whether the lysine residues at major NLS I are also targets of ubiquitination by MDM2. To this end, we used the following p53 mutants: 3R, 5R, 6R (all lysines to arginines at NLS II and III) and 9R (all

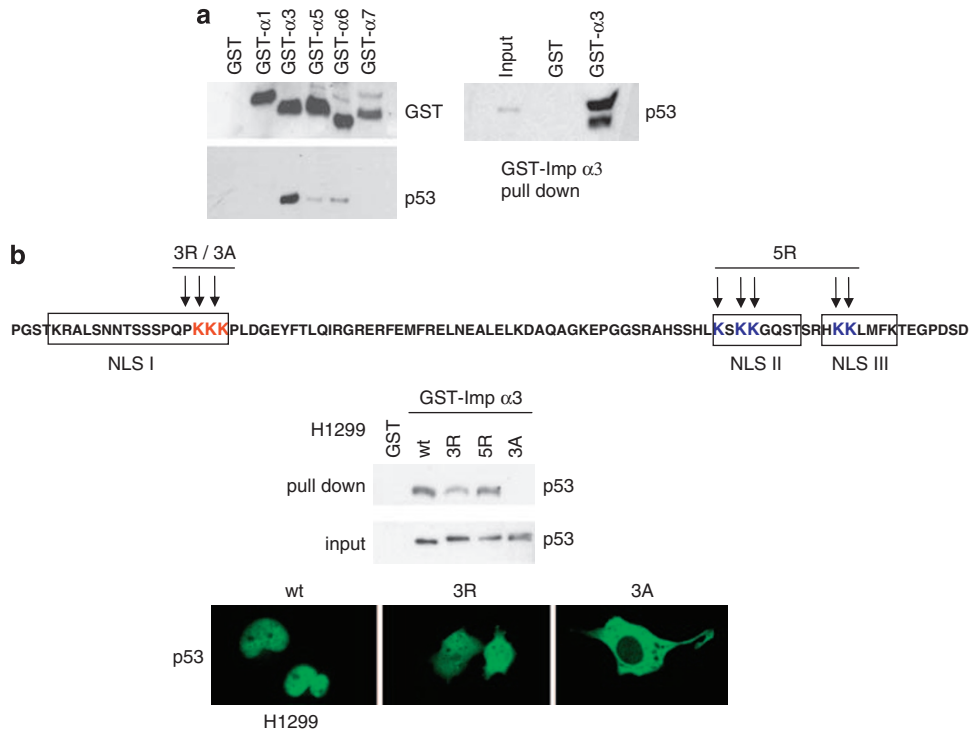


Figure 3 p53 binds to importin- α 3 and this interaction depends on the positive charge contributed by lysines 319–321 of p53. **(a)** Importin- α pull-down assays. *Left* – equal amounts of recombinant GST or GST-importin- α fusion proteins bound to glutathione agarose beads were incubated with equal amounts of HCT116 cell lysates. After washing, eluates were immunoblotted for GST and p53. *Right* – GST or GST-importin- α 3 bound to glutathione agarose beads were incubated with bacterially purified recombinant human p53. After washing, eluates were immunoblotted for p53. **(b)** Neutralizing the basic charge of NLS I results in nuclear exclusion of p53. *Top* – scheme of the p53 C-terminus with its three NLS motifs and the lysine point mutants of p53 that were generated in this study. *Middle* – importin- α pull-down assay as in **(a)**. GST or GST-importin- α 3 bound to glutathione agarose beads was incubated with lysates from H1299 cells that had been transfected either with wtp53 or the 3R, 3A or 5R mutants of p53. Eluates were immunoblotted for p53. *Bottom* – p53 immunofluorescence of the same H1299 cells, transfected as indicated. The 3A p53 mutant shows exclusive cytoplasmic p53 localization

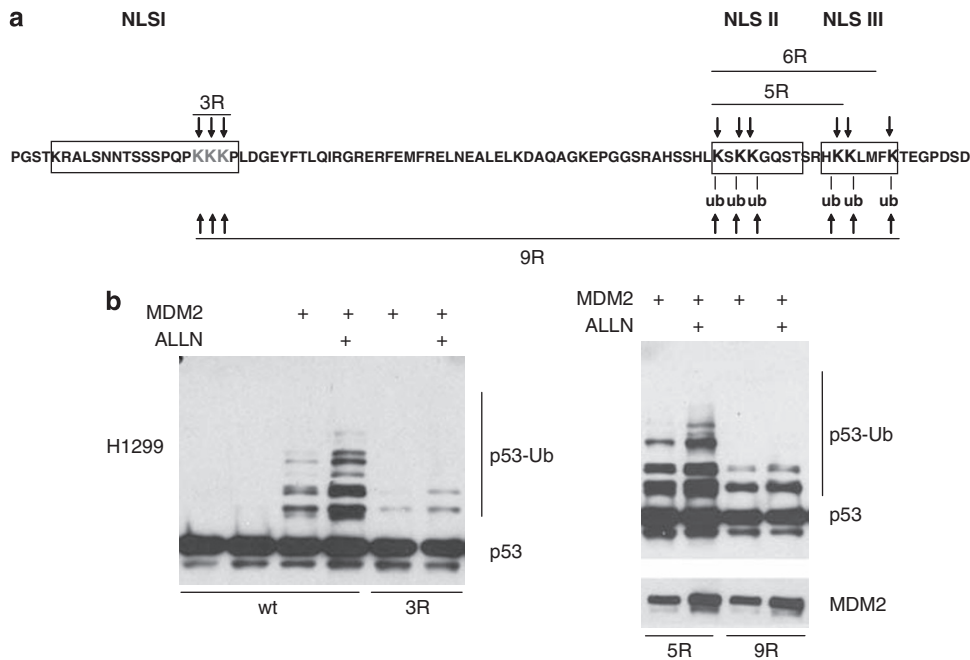


Figure 4 Lysines 319–321 of NLS I, critical for p53 binding to importin- α 3, are also targets of MDM2-mediated ubiquitination. **(a)** The p53 C-terminus depicting the p53 NLS mutants 3R, 5R, 6R and 9R used in this study. **(b)** Either wtp53 or the indicated NLS lysine mutants were transfected into H1299 cells along with MDM2. After 24 h, cells treated with ALLN for 3 h were indicated, followed by immunoblots. Loadings were normalized for equal amounts of non-ubiquitinated p53. See also Supplementary Figure 2

lysines to arginines in NLS I–III) (Figure 4a). These p53 mutants were coexpressed with MDM2 in H1299 cells side by side to compare their ability to undergo MDM2-mediated ubiquitination. Indeed, ubiquitination of 3R and 6R is greatly reduced compared with wtp53 (Figure 4b, left; Supplementary Figure 2). In addition, ubiquitination of 9R is significantly reduced compared with 5R (Figure 4b, right). The residual ubiquitination observed in the 9R mutant is due to ubiquitination of alternate lysine residues within the DNA-binding domain that were shown to be used as ‘escape’ ubiquitination targets when C-terminal lysines are mutated.³¹ Notably, neither ubiquitination of 3R nor of 9R is significantly increased after proteasome inhibition by ALLN, in contrast to wtp53 or the 5R mutant, which both stabilize on ALLN (Figure 4). This indicates that specifically the 3R and 9R mutants, which have in common the mutated Lys 319–321 residues, exhibit severe degradation defects. More importantly, these data confirm that Lys 319–321, which are critical for binding to importin- α 3, are also targets of MDM2-mediated ubiquitination.

Importin- α 3 preferentially interacts with non-ubiquitinated p53. The above data opens the possibility that nuclear import might be regulated by MDM2-mediated ubiquitination of p53 in the cytoplasm. It allows us to hypothesize that ubiquitination of Lys 319–321 may result in neutralization of the positive charge of NLS I with subsequent block of p53 nuclear import. To test this notion, we compared the binding ability of importin- α 3 for non-ubiquitinated or ubiquitinated p53 using pull-down assays. To this end, cytoplasmic fractions of H1299 cells transfected with p53 in the absence (containing only non-ubiquitinated p53) or presence (containing mixed species of p53) of MDM2 were used as input (Figure 5a, left) and incubated with GST-importin- α 3 beads. Indeed, importin- α 3 interacts with non-ubiquitinated, but not with ubiquitinated p53 (Figure 5a, right). To assess whether this is also the case for endogenous p53, the cytoplasmic fraction of unstressed HCT116 p53^{+/+} cells, containing a mixture of non-ubiquitinated and ubiquitinated endogenous p53, were split into two equal aliquots (Figure 5b). One half was incubated with GST-importin- α 3 beads for pull-down assays. The other half was immunoprecipitated for total p53, bringing down ubiquitinated and non-ubiquitinated forms. This excludes the possibility of a false positive result, that is the p53 might have become deubiquitinated under the conditions used. Consistent with ectopic p53 (Figure 5a), importin- α 3 selectively binds to endogenous *non*-ubiquitinated, but not ubiquitinated p53, although both were present in excess in the input material (Figure 5b). Moreover, ubiquitination of NLS I alone by MDM2 increases cytoplasmic retention of p53 *in vivo* by fivefold (Figure 5c; representative example shown on the right). Together, this supports our hypothesis that lack of ubiquitination of cytoplasmic p53 promotes nuclear import, whereas its presence blocks it.

Stress induces accumulation of non-ubiquitinated p53 in the cytoplasm, promoting interaction of p53 with importin- α 3 and nuclear import. The current model holds that a block of p53 export in the face of elevated nuclear p53

levels is the major determinant of nuclear p53 accumulation after stress. However, because our results clearly point to nuclear import as an important active component of stress-induced nuclear p53 stabilization, we next analyzed the functions of enhanced import *versus* blocked export. Thus, the relative kinetics of DNA damage induced nuclear p53 accumulation *versus* p53 export block turned out to be very informative. It suggests that nuclear accumulation occurs in two steps. Kinetic analysis clearly shows that camptothecin-induced nuclear p53 stabilization happens significantly faster (starts within 30 min) than LMB-induced nuclear p53 stabilization (detectable only at 4 h) (Figure 6a, compare left and right panels). Moreover, the rapid kinetics of camptothecin-induced nuclear p53 accumulation coincides with a rapid drop in cytoplasmic MDM2 levels and subsequent rapid drop in cytoplasmic p53 ubiquitination (Figure 6a, middle). Together, these data strongly favor the hypothesis that during the early stages of the p53 stress response, import of non-ubiquitinated p53 from the cytoplasm is an important factor that drives nuclear accumulation, contributing to its fast stabilization. This conclusion is further strengthened by the finding that stress-induced accumulation of p53 in the cytoplasm occurs with much delayed kinetics (2 h) compared with its nuclear accumulation (30 min) (Figure 6a, left), suggesting that the major pool of stabilized cytoplasmic p53 undergoes immediate import after the onset of stress, thereby maintaining low cytoplasmic levels for some time. In later phases of the stress response, blocked nuclear export takes effect and adds to nuclear accumulation of p53, as shown earlier.^{18,32} Next, we asked whether the stress-induced importin- α 3-mediated p53 import is an *active* process, or merely driven passively by increased cytoplasmic p53 concentration. To this end, we performed an independent side-by-side experiment as the most accurate way to compare the relative actions of camptothecin *versus* LMB, concomitantly analyzing cytoplasmic and nuclear fractions (Figure 6b). The relative band intensities of p53 induced by the two drugs in nucleus *versus* cytoplasm were quantified by densitometry. Importantly, we find again that camptothecin induces nuclear p53 accumulation rapidly within only 30 min and to a level not even reached after 4 h of nuclear export blockade by LMB. Notably, cytoplasmic p53 concentrations remain low and unchanged for 60 min. This data strongly supports the notion that nuclear import is an active process and crucial for the observed rapid nuclear p53 accumulation at the early stages of the stress response.

To further explore the function of import in p53's nuclear stabilization and activation after stress, we evaluated the interaction of endogenous p53 with importin- α 3 under physiologic conditions. As expected, DNA damage with camptothecin leads to elimination of ubiquitinated p53 in the cytoplasm (Figure 6c, left). Next, we incubated GST-importin- α 3 beads with equal amounts of *total* p53 from the cytoplasmic fractions of unstressed or stressed RKO cells. Of note, endogenous p53 from unstressed cells fails to show binding of ubiquitinated p53 species to importin- α 3 and also exhibits lower binding of non-ubiquitinated p53, compared with stressed cells (Figure 6c, right). This coincides with stress-induced disruption of MDM2-mediated p53 ubiquitination (Figure 1d). In further support, endogenous p53 in

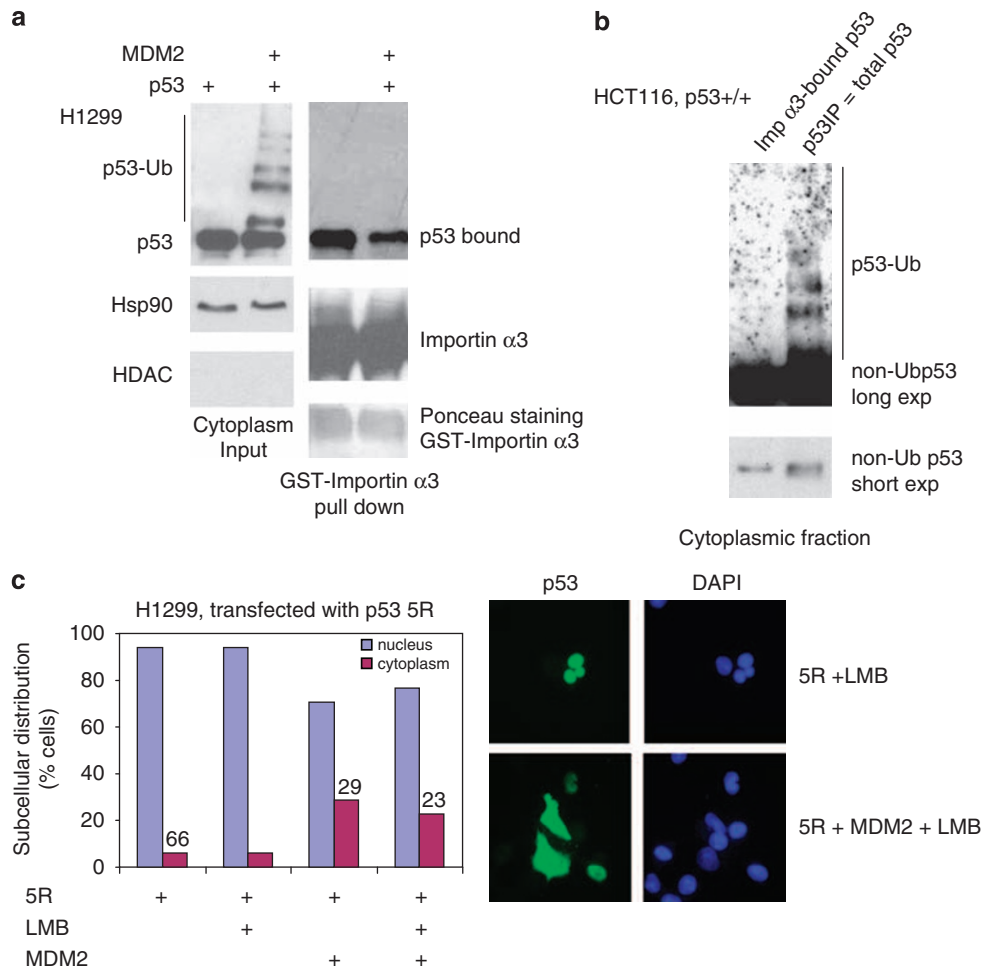


Figure 5 Importin- α 3 preferentially interacts with non-ubiquitinated p53. **(a)** Importin- α 3 interacts with non-ubiquitinated, but not with ubiquitinated p53. H1299 cells were transfected with p53 and MDM2, followed by fractionation. Cytoplasmic fractions from immunoblot (*left*, with Hsp90 as loading control and HDAC as purity control) (= input) were used for pull-down assays with GST-importin- α 3 bound to glutathione agarose beads (*right*). Eluates were immunoblotted for bound p53 and importin- α 3. Ponceau S staining of the membrane confirms equal loading of importin- α 3. **(b)** Importin- α 3 interacts with non-ubiquitinated, but not with ubiquitinated endogenous p53. Cytoplasmic fractions of unstressed HCT116 cells were split into two equal aliquots. One aliquot was incubated with GST-importin- α 3 (= bound p53) and the other aliquot was immunoprecipitated with p53 antibody CM1 (= control, total cytoplasmic p53). Both fractions were immunoblotted for p53 after normalizing loading for non-ubiquitinated p53. **(c)** Ubiquitination of NLS I of p53 promotes cytoplasmic retention *in vivo*. H1299 cells were transfected with the p53 5R mutant (lysines within NLS II and III are mutated to arginines). p53 5R retains wild-type NLS I and its requisite lysines as the only remaining ubiquitination sites. Cells were cotransfected with MDM2 where indicated. Twenty four hours post-transfection, cells were processed for p53 immunofluorescence and subcellular distribution of p53 was scored in 400 random transfected cells as in Figure 2b. *Right* – representative examples of transfected cells stained for p53 and viewed by epifluorescence

Camp-treated cells shows a time-dependent increase in its interaction with endogenous importin- α 3 (Figure 6d). These data are in agreement with results in Figure 5, and indicate that stress-induced deubiquitination promotes p53 import. However, it is possible that in addition to ubiquitination, p53 phosphorylation-mediated regulation also contributes to p53 nuclear import. Taken together, these data imply a higher rate of p53 import in stressed cells, contributing to its rapid nuclear accumulation on cell damage. Finally, to verify the functional relevance of importin- α 3 in the p53 stress response, we knocked down importin- α 3 by siRNA (Figure 6e, left). Knock-down of endogenous importin- α 3 indeed results in cytoplasmic retention of p53 in unstressed and even more so in stressed cells because of inhibition of nuclear import (Figure 6e, right), reflected by decreased transcriptional

activity, as determined by the prototypical p53 target gene p21 (Figure 6e, bottom).

In summary, our data support an expanded model whereby enhanced nuclear import of non-ubiquitinated p53 through importin- α 3 has an important function in rapid nuclear accumulation of transcriptionally active p53 during the early phase of the p53 stress response. Subsequently, blocked nuclear export then adds to nuclear accumulation of p53 during the later stage of the response.

Discussion

We show that ubiquitination and degradation of endogenous p53 occurs mainly in the cytoplasm of unstressed cells

(Figure 1). Although predicted by earlier studies using ectopic expression or nuclear export blockade, this had not been shown under physiologic conditions of undisturbed cells. More importantly, we find that p53 traffics to the nucleus through the canonical importin- α/β route (Figure 2). We identified importin- $\alpha 3$ from the multi-member family of importin- α adaptor proteins as the specific member facilitating p53 recognition by the import machinery (Figure 3). Interestingly, some human breast cancer cell lines with aberrant cytoplasmic localization of wtp53 were traced to truncation mutations in importin- α (the exact importin member was not determined), leading to severe defects of nuclear import of p53.³³ Selectivity for specific importin- α adaptors is mediated by specific NLS sequences in combination with structural features of the cargo.³⁴ Binding of an NLS sequence to importin- α requires close salt bridge interactions between the lysine ϵ -amino side chains within the NLS and pockets on the surface of the armadillo repeat sequences of importin- α .^{35,36}

Our data show that the positive charges contributed by the three basic lysine residues 319–321 within the major NLS of p53 are required for p53's direct physical interaction with importin- $\alpha 3$, presumably because they contribute to the close association with importin- $\alpha 3$.

We further show that lysines 319–321, critical for binding to importin- $\alpha 3$, are also the concomitant targets of negative regulation for p53 nuclear import. Regulation of these lysines occurs through MDM2-mediated ubiquitination (Figure 4). Thus, importin- $\alpha 3$ preferentially interacts with non-ubiquitinated p53 to mediate import (Figures 5 and 6). This explains the profound retention of ubiquitinated p53 in the cytoplasm of unstressed cells. Indeed, nuclear p53 is completely devoid of ubiquitinated p53 even after blocking degradation by ALLN or Nutlin (Figure 1f). Covalent addition of ubiquitin moieties, each with an isoelectric point of pH 6.79, to the three lysines in NLS I effectively neutralizes the positive charge of NLS I. In addition, linkage of ubiquitin chains to these lysines might covalently

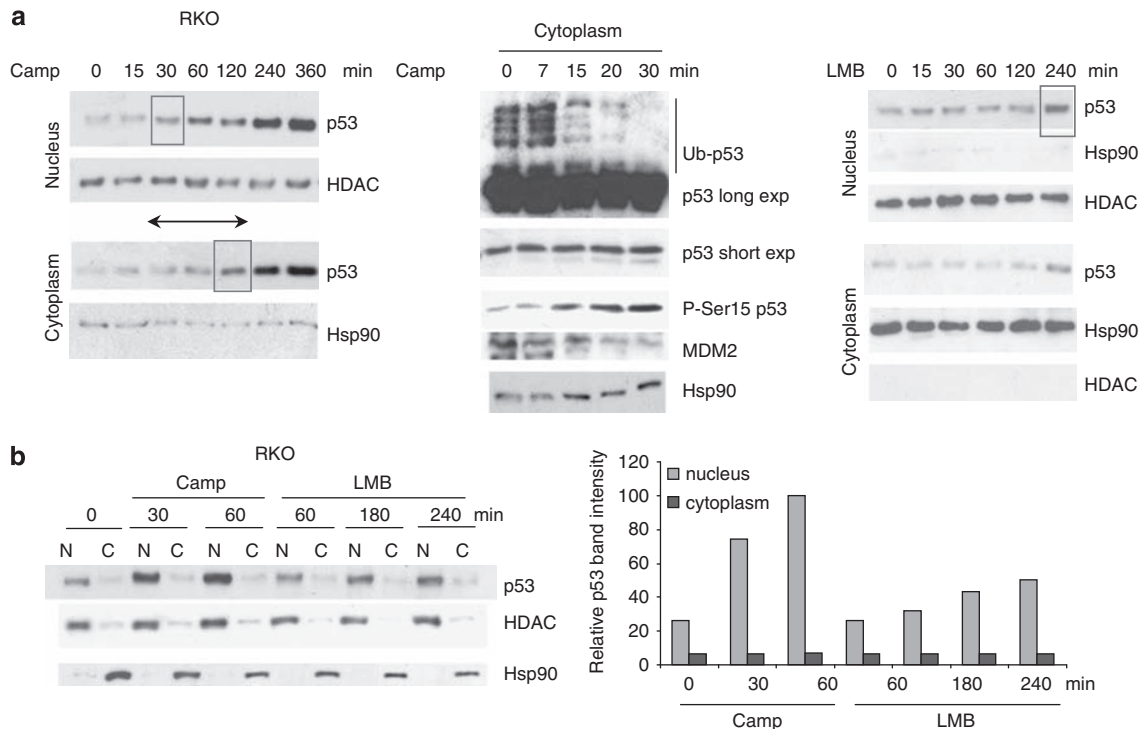


Figure 6 Stress induces accumulation of non-ubiquitinated p53 in the cytoplasm, which promotes its interaction with importin- $\alpha 3$ and p53 nuclear import. (a) Camptothecin-induced nuclear stabilization of p53 occurs significantly faster (within 30 min) than export block-induced nuclear stabilization (detectable at 4 h). Time course of endogenous p53 stabilization in nucleus and cytoplasm of RKO cells treated with 5 μ M Camptothecin (*left*) or 4 nM LMB (*right*). Immunoblot analyses. HDAC and Hsp90 as nuclear and cytoplasmic purity markers, respectively. *Middle* – time course of stress-induced loss of p53 ubiquitination in the cytoplasm. Cytoplasmic fractions (10 μ g protein) collected at the indicated times were immunoblotted for p53, S15-phosphorylated p53, MDM2 and Hsp90. (b) Stress-induced increase of p53-importin- $\alpha 3$ binding is an active process, and not a passive consequence of merely increased cytoplasmic p53 concentrations. Camptothecin induces significant nuclear p53 accumulation within 30 min to a level not reached even after 4 h of nuclear export blockade by 4 nM LMB. RKO cells were left untreated or treated as indicated, followed by fractionation. Equal aliquots of total protein were immunoblotted as indicated. *Right* – densitometry of nuclear and cytoplasmic p53 from immunoblot shown on the left. (c) RKO cells were treated with camptothecin or left untreated, followed by fractionation. *Left* – the cytoplasm of stressed cells is devoid of ubiquitinated p53. p53 immunoblots normalized for equal amounts of total protein. *Right* – these cytoplasmic fractions, normalized for equal amounts of total p53, were subjected to pull-down assays. Eluates from GST or GST-importin- $\alpha 3$ agarose beads were immunoblotted for p53 and importin- $\alpha 3$. (d) Time-dependent increase of p53-importin- $\alpha 3$ interaction in response to camptothecin-mediated stress. Crude lysates (equal total protein) of unstressed or 30–60 min camptothecin-stressed RKO cells were immunoprecipitated for importin- $\alpha 3$, followed by immunoblotting for p53 and importin- $\alpha 3$. IgG (1 μ g) as non-specific IP control. Immunoblot shows corresponding input. (e) Knockdown of importin- $\alpha 3$ results in cytoplasmic retention of p53 and decreased transcriptional activity. HCT116 cells were transfected with importin- $\alpha 3$ siRNA or a scrambled siRNA control. After 2 days, cells were either left untreated or treated for 4 h with 10 μ M camptothecin. *Left* – total cell lysates immunoblotted for importin- $\alpha 3$ and p53. PCNA as loading control. *Right* – purified cytoplasmic fractions of the same samples blotted for p53. Hsp90 as loading control. *Bottom* – corresponding p21 immunoblot on total cell lysates from HCT116 cells treated as indicated

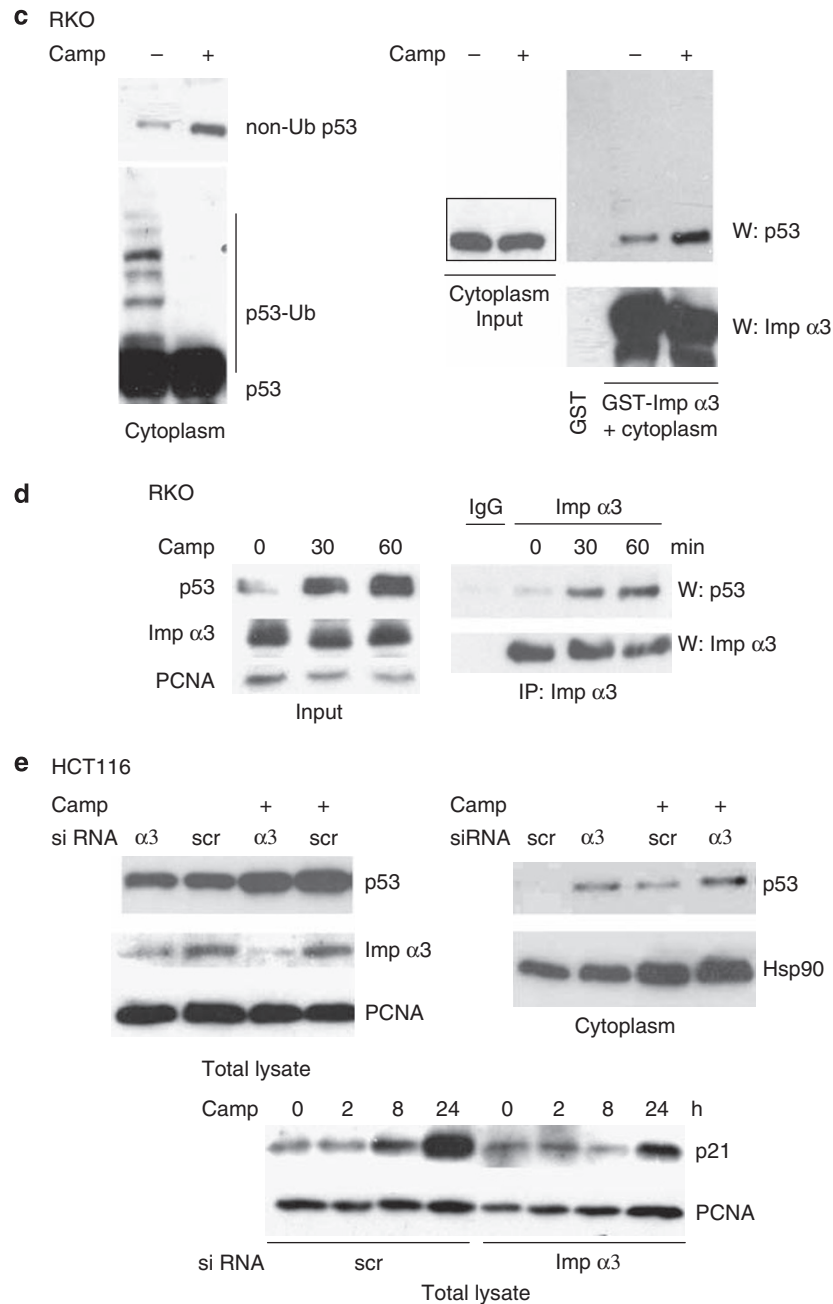


Figure 6 Continued

alter the structure of NLS I and impair binding to importin- α 3. In agreement, we showed earlier that aberrant hyper-ubiquitination of wtp53 contributes to its cytoplasmic sequestration in neuroblastoma, impairing p53 function.¹⁰

Thus, our findings add an important example to the growing significance of *non*-proteolytic ubiquitin modifications of p53. They occur at C-terminal lysines and are mediated by MDM2 and possibly other E3 ligases. One biological function lies in controlled subcellular trafficking and/or localization of p53. Interestingly, although p53 nuclear import is negatively regulated by ubiquitin modification, p53 nuclear export as well as translocation of cytoplasmic p53 to mitochondria are

positively regulated by ubiquitin.^{23,37,38} Interestingly, efficient delivery of cytoplasmic p53 to the importin- α/β complex before its nuclear import might rely on p53 transport by the microtubule cytoskeleton.^{39,40}

Until now, explorations on mechanisms of stress-induced nuclear p53 stabilization were limited to the function of nuclear export block. However, our analysis of the relative kinetics of enhanced nuclear import *versus* blocked export of p53 suggests that nuclear accumulation occurs in two steps (Figure 6a). Notably, blocking Crm1-mediated nuclear export of p53 by LMB is a slow event that takes hours before coming into effect and, therefore, cannot solely account for the rapid

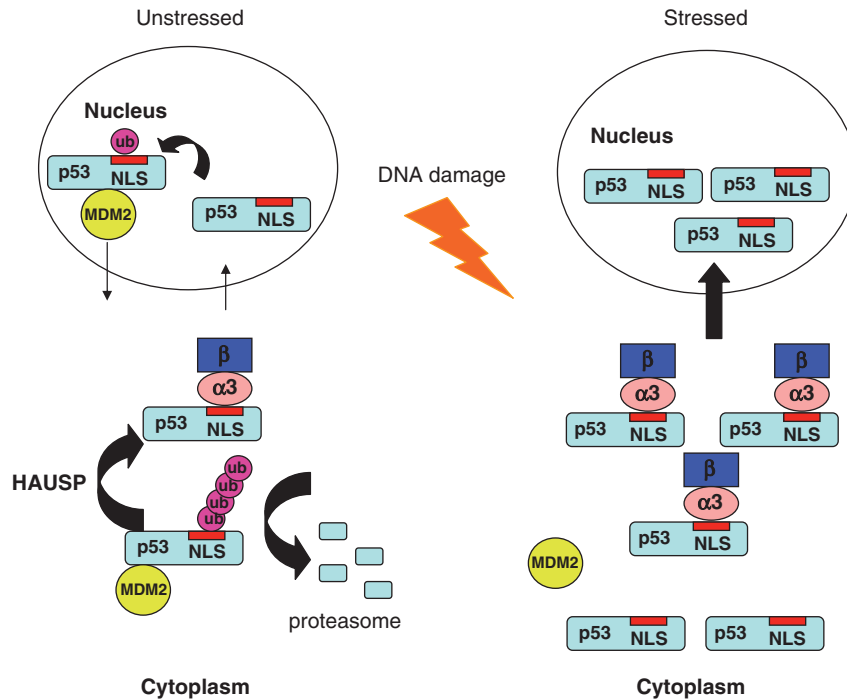


Figure 7 Model of regulating p53 nuclear import. In unstressed cells, the majority of p53 translated in the cytoplasm undergoes immediate ubiquitination and degradation by cytoplasmic proteasomes. Ubiquitination of NLS I of p53 prevents its recognition by importin- $\alpha 3$ and thus inhibits nuclear import, together explaining that ubiquitinated p53 is predominantly localized in the cytoplasm. The small pool of p53 that escapes NLS ubiquitination and/or is deubiquitinated by HAUSP interacts with the import machinery, supplying a constant, but low level of nuclear p53 in unstressed cells. After stress, MDM2 and p53 modifications quickly and dramatically reduce the level of ubiquitination and stabilize p53 in the cytoplasm. This exposes NLS I (and also NLS II and III) and enables importin- $\alpha 3$ recognition. This enhanced nuclear import contributes to rapid nuclear stabilization of p53 after stress, crucial for its transcription function. At a later stage of the stress response, induced tetramerization of p53 with subsequent masking of its NES and blocking of nuclear export further contributes to the stabilization of nuclear p53

nuclear p53 stabilization that starts within 30 min. The slow kinetics of nuclear export has been noted before.⁴¹ Our conclusion is supported by the fact that most NES motifs are weak and bind to the nuclear export receptor Crm1 with relatively low affinity. Interestingly, substitution of low affinity with high affinity NES impaired the efficient release of cargo proteins from the nuclear pore complex, providing an explanation why classical NES motifs have evolved to be weak.⁴² Furthermore, among many tested NES sequences from different nuclear proteins, the C-terminal (i.e. major) NES of p53 shows the weakest affinity to Crm1,⁴³ indicating that nuclear export of p53 is rather inefficient and alone cannot explain the rapid nuclear accumulation of p53 after stress.

On the basis of the aggregate data presented here, we propose an expanded *model* to explain the fast stabilization of p53 in the nucleus on DNA damage. It implies that enhanced nuclear import actively contributes to the early phase of nuclear p53 stabilization after stress (Figure 7). Conversely, in resting cells, the majority of p53 is translated in the cytoplasm and gets immediately ubiquitinated and degraded locally by cytoplasmic proteasomes. Ubiquitination of NLS I of p53 prevents its recognition by importin- $\alpha 3$ and inhibits nuclear import. The small pool of p53 that either escapes NLS I ubiquitination or is subsequently deubiquitinated by HAUSP interacts with the import machinery, supplying a constant, but low level of nuclear p53 in unstressed cells.

After stress, MDM2 and p53 modifications rapidly and dramatically reduce the level of ubiquitination, accumulating non-ubiquitinated p53 in the cytoplasm. This renders NLS I competent for efficient importin- $\alpha 3$ recognition. During later stages of the stress response, induced tetramerization with subsequent masking of NES and block of nuclear export further contributes to the stabilization of nuclear p53, crucial for its transcriptional function.⁵

In summary, our data imply that ubiquitin-regulated nuclear import of p53 is a control switch and an important determinant for both low nuclear p53 levels in the absence of stress and for rapid nuclear p53 stabilization in the presence of stress.

Materials and Methods

Cells. The colon carcinoma lines RKO and HCT 116 contain functional wtp53 while lung adenocarcinoma line H1299 is null for p53. All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Plasmids and recombinant protein purification. The 3R, 5R and 9R NLS mutants of human p53 were generated by using QuickChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA, USA) on a wtp53 pcDNA3 template. The CMVBamNeo-based human MDM2 plasmid was a gift of Dr. AJ Levine. For ubiquitination assays, p53-null H1299 cells were cotransfected with wtp53 or NLS mutant p53 and MDM2 in a plasmid ratio of 2:1. Under this condition, MDM2 preferentially induces monoubiquitination and stabilization, but not p53 degradation.³⁸ Cells were transfected with Lipofectamine (Invitrogen, Carlsbad, CA, USA). Recombinant importin- α proteins tagged with GST and deleted for the

importin- β -binding domain were constructed earlier and purified by standard binding to glutathione agarose beads (Sigma, St. Louis, MO, USA) as described. For importin- α protein-binding assays, purified recombinant GST-importin- α fusion proteins or GST protein were used at 25 μ g per reaction. Crude or cytoplasmic lysates (500 μ g) were incubated with importin- α proteins immobilized on Glutathione agarose beads, washed and eluted. Binding reactions with bacterially expressed p53 were performed similarly.

Treatments. Where indicated, cells were treated with 25 μ M ALLN (Calbiochem, Darmstadt, Germany) for 3 h before harvesting, 4 nM Leptomycin B (Biomol International, Plymouth Meeting, PA, USA) for 4 h, 10 μ M Camptothecin (Sigma) for 3 h. Cycloheximide (50 μ g/ml; Sigma) or 10 μ M Nutlin 3a (Calbiochem) were added to the medium for 6 h before harvesting where indicated. Ubiquitin aldehyde (UbAL) (BioMol), a specific inhibitor of the ubiquitin-specific processing protease (UBP) family of deubiquitinases, was routinely included in buffers for better visualization of ubiquitin conjugates.

Immunoblots and co-immunoprecipitation. For immunoblots, equal total protein of crude cell lysates (typically 2.5–5 μ g) were loaded. When loading was normalized for equal amounts of non-ubiquitinated p53, a first quantitation immunoblot was run before the second definitive immunoblot. Antibodies used were CM1 (Vector, Burlingame, CA, USA); DO1, FL393 (Santa Cruz, Santa Cruz, CA, USA) for p53; mthsp70, hsp90 and HDAC (Affinity Bioreagents, Golden, CO, USA); PCNA, rabbit IgG (Sigma); SMP14 for human Mdm2 (Santa Cruz); human HAUSP (Calbiochem); P-ATR and p53 Ser15-P (Cell Signalling, Boston, MA, USA); importin- β (Affinity Bioreagents) and importin- α (Imgenex, San Diego, CA, USA). For detecting endogenous complexes, crude lysates (500 μ g) were immunoprecipitated with 1 μ g antibody for 2 h at 4 °C and beads were washed three times with SNNT (50 mM Tris-HCl pH 7.4, 5 mM EDTA pH 7.4, 5% Sucrose, 1% NP-40, 0.5 M NaCl) plus two times by RIPA (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% NaDeoxycholate, pH 7.4) before immunoblotting.

Cell fractionation. Cells were harvested, rinsed with PBS and pelleted. Cells were resuspended in 5 vol of cold CARSB buffer (10 mM Tris pH 7.5, 1.5 mM CaCl₂, 10 mM NaCl, protease inhibitor cocktail (Sigma), 5 mM NEM, 5 nM ubiquitin aldehyde (to prevent deubiquitination of p53 during cell fractionations, the broad range deubiquitinase inhibitor ubiquitin aldehyde was included in all buffers), and allowed to swell on ice for 15 min, after which Triton X-100 was added to a final concentration of 0.3%. The homogenate was spun for 10 min at 1000 \times g. The supernatant, which comprises the cytoplasmic fraction was transferred into a fresh tube, and the salt concentration was adjusted to 200 mM with 5 M NaCl. The crude nuclear pellet was suspended in buffer C (10 mM Tris pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 400 mM NaCl, 0.5% Triton X-100, protease inhibitor cocktail, 5 nM ubiquitin aldehyde) and sonicated. The homogenate was centrifuged for 15 min at 16 000 \times g. This final supernatant comprises the nuclear fraction.

For Figure 1d right, to further control for potentially artifactual effects of different salt and detergent concentrations in nuclear and cytoplasmic fractions, final NaCl and Triton X-100 concentrations were made equal in both fractions NaCl (200 mM) and Triton (0.5%) and used for subsequent IPs.

RNA interference. Pools of siRNA duplexes specific for human importin- α 3, importin- β and scrambled siRNA (GeneSolution, Quiagen, Valencia, CA, USA) were transfected with Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were transfected with p53 and processed 14–16 h later. For statistical analyses in Figure 2, 200 random p53-expressing cells were counted.

Immunofluorescence. Cells were grown on eight-chamber polystyrene slides, fixed for 3 min in acetone-methanol and air-dried. After blocking in 10% normal goat serum for 1 h, cells were incubated in p53 antibody DO1 overnight at 4 °C. Staining was detected with FITC-anti-mouse secondary antibodies (Jackson, West Grove, PA, USA). Cells were mounted with Antifade (Molecular Probes, Carlsbad, CA, USA) and Immunomount (Thermo, Pittsburgh, PA, USA) and examined with an epifluorescent Zeiss Axioskop.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)