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# A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking

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**Appropriate subcellular localization is crucial for regulating p53 function. We show that p53 export is mediated by a highly conserved leucine-rich nuclear export signal (NES) located in its tetramerization domain. Mutation of NES residues prevented p53 export and hampered tetramer formation. Although the p53-binding protein MDM2 has an NES and has been proposed to mediate p53 export, we show that the intrinsic p53 NES is both necessary and sufficient for export. This report also demonstrates that the cytoplasmic localization of p53 in neuroblastoma cells is due to its hyperactive nuclear export: p53 in these cells can be trapped in the nucleus by the export-inhibiting drug leptomycin B or by binding a p53-tetramerization domain peptide that masks the NES. We propose a model in which regulated p53 tetramerization occludes its NES, thereby ensuring nuclear retention of the DNA-binding form. We suggest that attenuation of p53 function involves the conversion of tetramers into monomers or dimers, in which the NES is exposed to the proteins which mediate their export to the cytoplasm.**

*Keywords*: MDM2/neuroblastoma/nuclear export/p53/tetramerization

## Introduction

The p53-mediated stress response is among the most frequent targets of inactivation in human cancer. This signal-transduction cascade produces cell cycle arrest or apoptosis in response to a variety of agents or conditions that damage DNA, affect chromosome replication and segregation, or generate inappropriate proliferative signals (for reviews see Ko and Prives, 1996; Levine, 1997; Sherr, 1998). In contrast, cells lacking this pathway are more resistant to chemotherapeutic agents and exhibit increased genomic instability, allowing them to gain a selective advantage during tumor progression (Wahl *et al*., 1997; Chernova *et al*., 1998; Paulson *et al*., 1998).

The identification of inactivating lesions in the p53 pathway has helped to uncover the mechanisms by which p53 contributes to tumor suppression. These lesions fall into three classes. The first and most frequent class consists of altered residues within the *p53* gene that perturb the structure of its DNA-binding domain (Pfeifer and Holmquist, 1997). These alterations incapacitate it as a tumor suppressor, since the ability of p53 to transcriptionally regulate target genes involved in growth arrest and apoptosis is dependent on its ability to bind DNA (Crook *et al*., 1994; Pietenpol *et al*., 1994). A second class comprises extragenic lesions affecting proteins that interact with or modify p53. For example, the MDM2 oncoprotein binds to p53 and abrogates the p53-mediated stress response by inhibiting its transcriptional activity and facilitating its degradation (Oliner *et al*., 1993; Haupt *et al*., 1997; Kubbutat *et al*., 1997). The *MDM2* oncogene is amplified in multiple tumor types, resulting in the constitutive inhibition of wild-type p53 (Momand *et al*., 1998).

The third and least understood class of inactivating lesions results in nuclear exclusion of wild-type p53 and has been observed in diverse neoplasms (Moll *et al*., 1992, 1995; Sun *et al*., 1992; Bosari *et al*., 1995; Ueda *et al*., 1995; Lou *et al*., 1997; Schlamp *et al*., 1997) and embryonic stem (ES) cells (Aladjem *et al*., 1998). Cells with cytoplasmically sequestered wild-type p53 are less responsive to signals that would normally induce its nuclear retention. For example, neuroblastoma and ES cells have cytoplasmic, wild-type p53 and an impaired $G_1$ arrest in response to genotoxic stresses (Moll *et al*., 1996; Aladjem *et al*., 1998). Cytoplasmic sequestration of p53 in tumor cells is associated with poor long-term patient survival and may be an important mechanism of tumorigenesis in the substantial fraction of tumors that do not have mutated p53 (Sun *et al*., 1992; Stenmark-Askmalm *et al*., 1994). In contrast, p53 in normal, unstressed cells is predominantly nuclear in $G_1$ and is largely cytoplasmic during S and $G_2$ (Shaulsky *et al*., 1990a; David-Pfeuty *et al*., 1996), consistent with its role as a mediator of $G_1$ checkpoints. In response to stress, however, p53 is stabilized and retained in the nucleus where it induces the expression of genes involved in cell cycle arrest or apoptosis. These observations indicate that the ability to retain p53 in the nucleus is critical for a cell to mount a p53-mediated stress response. Conversely, an effective means of downregulating p53 activity is through its spatial separation from its downstream effector genes. The mechanisms for regulating p53 subcellular localization in normal cycling cells and in neoplastic growth are currently unknown, but may involve either tethering to cytoplasmic or nuclear structures or regulated nuclear import and export (Middeler *et al*., 1997; Klotzsche *et al*., 1998).

This report demonstrates that an intrinsic nuclear export signal (NES) mediates the subcellular localization and

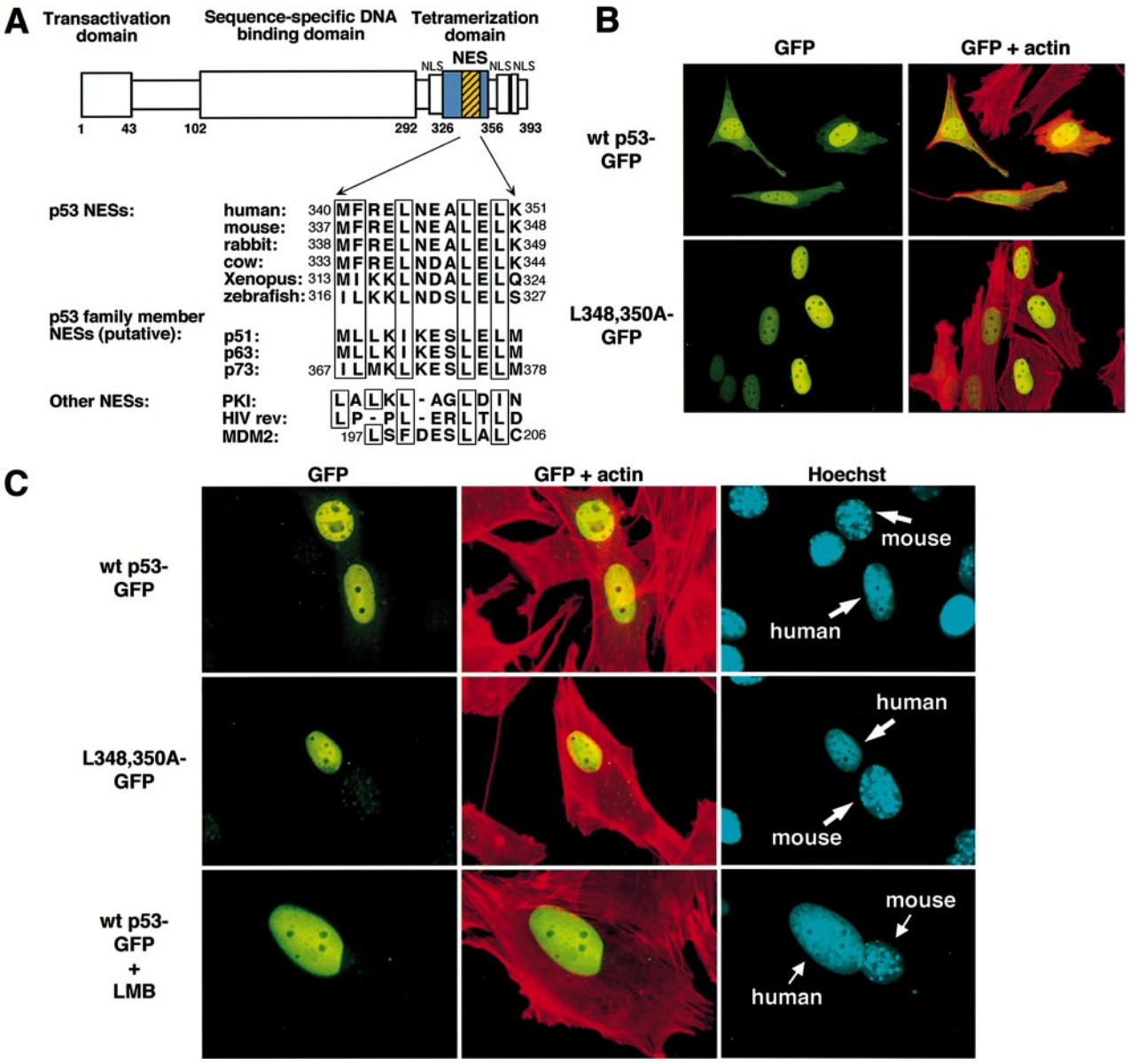


**Fig. 1.** A conserved Rev-like NES in the C-terminal tetramerization domain mediates p53 nuclear export. (**A**) At residues 340–351, the p53 NES lies within the tetramerization domain and between two nuclear localization signals (NLSs) in the C-terminus. The NES is depicted in gold stripes and the tetramerization domain is in blue. The p53 NES is aligned with homologous sequences of p53 family members p51, p63 and p73, and the NESs of PKI, HIV REV and MDM2 (Fischer *et al*., 1995; Wen *et al*., 1995; Kaghad *et al*., 1997; Osada *et al*., 1998; Roth *et al*., 1998; Yang *et al*., 1998). (**B**) Subcellular localization of wild-type and NES-mutant p53. Wild-type p53–GFP or L348,350A–GFP constructs were electroporated into exponentially growing Saos-2 cells. The fixed cells were stained with Texas Red–phalloidin to visualize cytoplasmic actin, and images of representative cell fields were captured on an epifluorescence microscope. More than 1000 cells were analyzed for each construct. (**C**) Mutations in the NES prevent nuclear–cytoplasmic shuttling. Wild-type p53–GFP or L348,350A NES mutant p53–GFP constructs were electroporated into human Saos-2 cells, pre-treated with cycloheximide alone or LMB and cycloheximide, and fused to non-expressing mouse Balb/c 3T3 cells. LMB-treated fusions were then incubated in the presence of leptomycin B (LMB) and cycloheximide for an additional 3 h. GFP fluorescence in the mouse nucleus is indicative of export of p53–GFP from the human nucleus. Mouse nuclei exhibit a mottled appearance when stained with Hoechst, while the human nuclei stain evenly, enabling the two to be distinguished easily. Cells were fixed 3 h after fusion, and stained with Texas Red–phalloidin to reveal cytoplasmic actin filaments, facilitating detection of heterokaryons with two or more nuclei. Each image is representative of >50 heterokaryons analyzed on each coverslip.

nuclear-cytoplasmic shuttling of p53 through an association with an export receptor, probably CRM1. Although the p53-binding protein MDM2 has its own NES and has been proposed to mediate p53 nuclear export (Roth *et al*., 1998), we show that MDM2 is not required for p53 to exit the nucleus under the experimental conditions employed. The p53 NES lies within a highly conserved region in the C-terminal tetramerization domain, and mutation of residues involved in export concurrently decreases the efficiency of tetramer formation. We further show that endogenous wild-type p53 that is cytoplasmic in neuroblastoma cells is translocated and retained in the nucleus when bound to a peptide consisting of the p53 tetramerization domain. These observations lead us to propose a model in which p53 subcellular localization is established through tetramerization-regulated exposure of

the NES to the export machinery, providing a previously unrecognized juncture at which p53 activity can be regulated. This would allow for simultaneous nuclear retention and tetramerization of p53, both of which must occur for it to elicit stress responses by gene transactivation. The general applicability of a model linking functional control of diverse proteins through structure-regulated access to their nuclear export and import signals will be discussed.

## Results

### *A highly conserved NES lies within the C-terminal tetramerization domain of p53*

Proteins >40 kDa must use a nuclear export receptor complex to pass through the nuclear pore (Gerace, 1995; Görlich and Mattaj, 1996). Because p53 is known to shuttle between the nucleus and cytoplasm (Middeler *et al*., 1997), we examined the p53 primary amino acid sequence to determine whether it contains a leucine-rich sequence of conserved spacing and hydrophobicity which fits the criteria established for an NES (Bogerd *et al*., 1996; Kim *et al*., 1996). We observed that the C-terminal residues between amino acids 340 and 351 conform to this motif, as indicated by their similarity to other known NESs such as HIV REV (Figure 1A; Fischer *et al*., 1995). The putative NES lies within the tetramerization domain, which is between the first and second of three nuclear localization signals spanning amino acids 316–325, 369–375 and 379–384 (Shaulsky *et al*., 1990b). This sequence is highly conserved in widely divergent species, and in the recently discovered p53 homologues p51, p63 and p73 (Figure 1A; Soussi and May, 1996; Cheng *et al*., 1997; Kaghad *et al*., 1997; Osada *et al*., 1998; Yang *et al*., 1998).

We generated constructs that link the green fluorescent protein (GFP) to the C-terminus of p53 to test whether the putative NES contributes to p53 subcellular localization. Normal p53 functions, including transactivation of a reporter construct, inhibition of colony formation and recognition by various conformation-specific p53 antibodies, are unaffected by the C-terminal attachment of GFP (Norris and Haas, 1997). We constructed a mutant NES p53–GFP fusion consisting of leucine to alanine conversions at residues 348 and 350 in the putative p53 NES, since analogous mutations in other NES-containing proteins have been reported to prevent nuclear export (Bogerd *et al*., 1996; Kim *et al*., 1996). Wild-type or mutant NES–GFP constructs were cloned in retroviral vectors and plasmids, and used in infection, transfection and microinjection assays. We used these three approaches to ensure that the method of gene transfer did not affect p53 localization due to, for example, the activation of a stress response pathway or to excessive transgene expression. The patterns of expression were equivalent with each method used (data not shown).

Wild-type p53–GFP (wt p53–GFP) exhibited a significantly different subcellular distribution than the putative NES mutant (Figure 1B). Approximately 77% of log-phase Saos-2 cells expressing wt p53–GFP showed fluorescence in both the nucleus and cytoplasm, while fluorescence in the remaining 23% was exclusively nuclear. This variability in subcellular distribution of wt p53–GFP may be related to normal cell cycle regulation and is consistent with other reports (Shaulsky *et al*., 1990a; David-Pfeuty *et al*., 1996; Liang *et al*., 1998). In contrast, 98% of the Saos-2 cells expressing the p53 NES mutant L348,350A–GFP had exclusively nuclear p53. These expression patterns were similar in primary and immortal cells (human WS1 or murine $p53^{-/-}$ fibroblasts and Saos-2, HS683, U251, MCF-7 or MDAH041), in cells whose endogenous p53 was wild-type, mutant or absent, and in cells with cytoplasmically sequestered p53 (MCF-7 or SK-N-SH). Thus, the NES consensus sequence of p53 is an important determinant of p53 subcellular localization, independent of species, cell type, p53 status or extrinsic genomic mutations that confine the endogenous p53 to the cytoplasm.

The above data suggest that the p53 NES mediates its nuclear-cytoplasmic shuttling. This was tested using a heterokaryon assay in which human Saos-2 cells transiently transfected with wild-type or mutant p53–GFP were fused to mouse Balb/c 3T3 cells. If nuclear export occurs, p53–GFP should exit the human nucleus, traverse the cytoplasm and enter the mouse nucleus. Conversely, there should be no fusion protein in the mouse nucleus if the L348,350A mutation prevents export from the human nucleus or if its export is otherwise blocked. Mouse nuclei were readily distinguished from human nuclei by the punctate fluorescence they exhibited when stained with Hoechst 33342 (Figure 1C) (Moser *et al*., 1975). The fused cells were treated with cycloheximide to prevent *de novo* synthesis of p53–GFP. By 3 h post-fusion, wt p53–GFP was seen in both the mouse and human nuclei, as expected of an export-competent molecule (Figure 1C). In contrast, L348,350A–GFP was detected in only the human nuclei indicating that this construct was not exported.

In addition, we determined whether the export of wt p53 is dependent on its association with the export receptor CRM1 by treating the heterokaryons expressing wt p53–GFP with LMB. This drug inhibits the formation of complexes consisting of CRM1, RanGTP and NES-containing proteins, thus non-specifically blocking nuclear export (Fornerod *et al*., 1997; Ossareh-Nazari *et al*., 1997; Ullman *et al*., 1997; Wolff *et al*., 1997). In heterokaryons treated with LMB, wt p53–GFP was seen only in the human nuclei, indicating that its export requires an association with the export receptor CRM1 (Figure 1C). Together, these data show that p53 contains a CRM1-dependent NES which mediates its nuclear–cytoplasmic shuttling.

### *The intrinsic p53 NES is sufficient to mediate nuclear export*

The data above do not address the possibility that an NES-containing p53-binding partner may participate in the nuclear export of p53. One potential shuttling partner is the p53 negative regulator, MDM2, which binds to p53 and facilitates its degradation by cytoplasmic proteasomes (Haupt *et al*., 1997; Kubbutat *et al*., 1997; Freedman and Levine, 1998). MDM2 contains a leucine-rich NES, the mutation of which compromises its ability to mediate p53 degradation (Roth *et al*., 1998). Therefore, it is possible that formation of an MDM2–p53 complex is required for both p53 nuclear export and degradation. This possibility was tested using p53–GFP fusion constructs containing

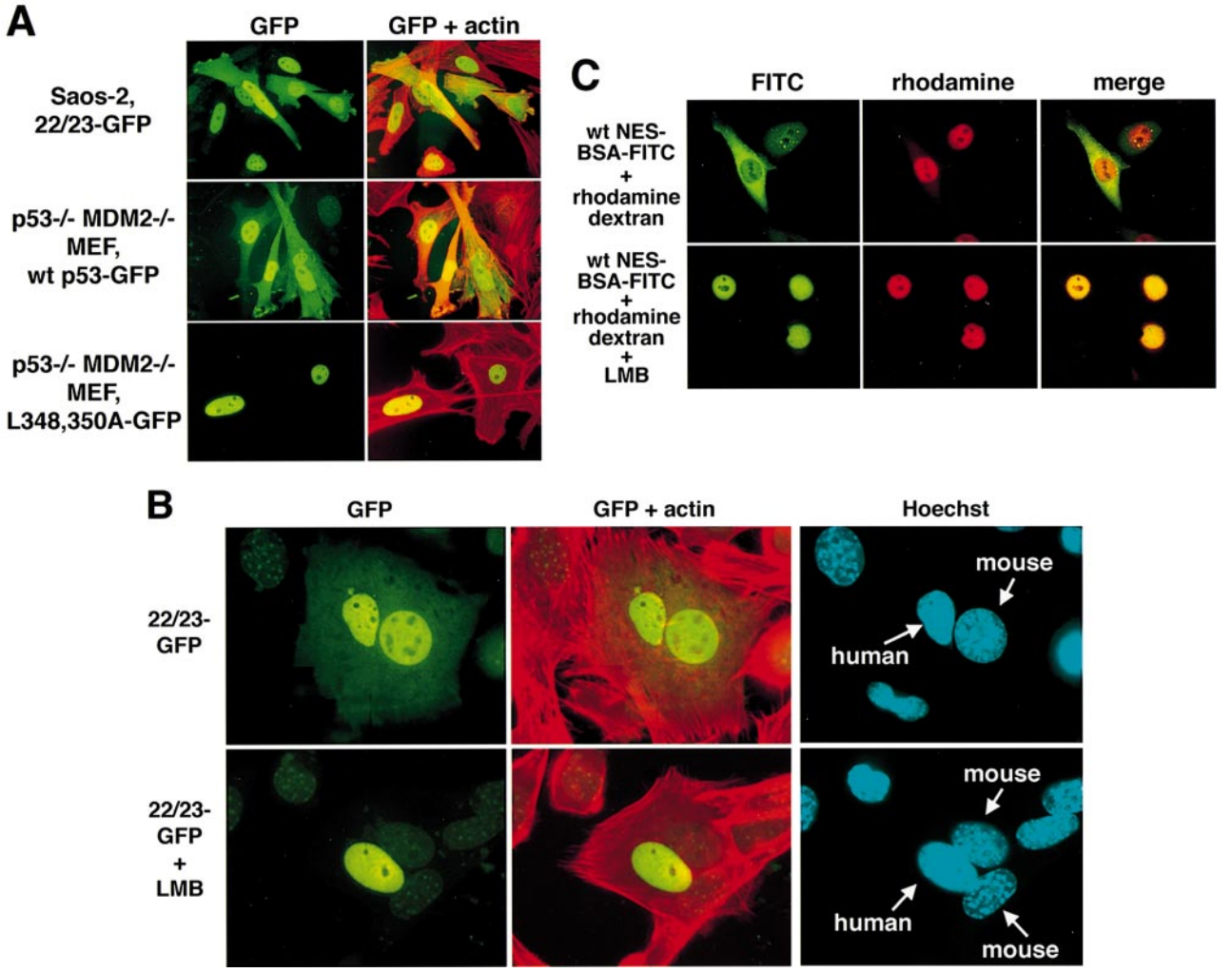


**Fig. 2.** The intrinsic p53 NES is sufficient to mediate nuclear export. (**A**) Subcellular localization of p53 without MDM2. Plasmid expression constructs encoding 22/23–GFP (MDM2-binding deficient), wild-type p53–GFP or L348,350A–GFP were electroporated into Saos-2 *or* $p53^{-/-}$/$mdm2^{-/-}$ mouse embryonic fibroblasts (MEF) and fixed and stained with Texas Red–phalloidin to visualize cytoplasmic actin. Images of representative cell fields were captured on an epifluorescence microscope. More than 1000 cells were analyzed for each construct. (**B**) Shuttling of a p53 mutant which does not bind MDM2. Saos-2 cells (human) were electroporated with 22/23–GFP, pre-treated with cycloheximide alone or with LMB and cycloheximide, and then fused to non-expressing Balb/c 3T3 cells (mouse) in the presence of cycloheximide. Fused cells were incubated and fixed as described in Figure 1C. Each image is representative of >50 fusions on each coverslip. (**C**) Shuttling of a heterologous protein mediated by the p53 NES. A wild-type peptide consisting of amino acids 339–352 was covalently conjugated to FITC-labeled BSA. wt NES–BSA–FITC was then microinjected along with 70 kDa rhodamine–dextran (an inert marker which enables localization of the injection site) into the nuclei of untreated or LMB pre-treated HeLa cells. The injected cells were incubated for 4 h with or without the drug at 37°C to allow sufficient time for export. Images were captured on a confocal microscope.

mutations at amino acids 22 and 23 (22/23–GFP), which prevent binding of MDM2 to p53 (Lin *et al*., 1994). The subcellular distribution of this mutant was similar to that of wild-type: 75% of the Saos-2 cells had varying degrees of both nuclear and cytoplasmic 22/23–GFP and 25% had exclusively nuclear fluorescence (Figure 2A). In addition, wt p53–GFP showed the same balance of nuclear and cytoplasmic localization in $p53^{-/-}/mdm2^{-/-}$ mouse embryonic fibroblasts. These results indicate that the absence of MDM2 binding does not alter the subcellular localization of p53 and provide further evidence for the sufficiency of the p53 NES in its own export.

We tested whether the p53 mutant that cannot bind MDM2 can still undergo nuclear–cytoplasmic shuttling in heterokaryons consisting of 22/23–GFP-expressing Saos-2 cells fused to non-expressing Balb/c 3T3 cells. Figure 2B shows that 22/23–GFP localizes to both the human and mouse nuclei, indicating that it was fully capable of being exported from the human nucleus. In addition, the export-inhibiting drug LMB blocked the export of this protein as demonstrated by the absence of fluorescence in the murine nucleus. These results reveal a mechanism of p53 nuclear export that is independent of MDM2 binding, but requires CRM1.

If the p53 NES is sufficient to mediate p53 nuclear export without the assistance of an NES-containing binding partner, then a peptide consisting of the minimal p53 NES should confer the ability to shuttle upon a heterologous, non-shuttling protein. To test this prediction, we covalently cross-linked peptides consisting of the wild-type NES (residues 339–352) to FITC-labeled bovine serum albumin (BSA) (wt NES–BSA–FITC). At 68 kDa, BSA is too large to passively diffuse through the nuclear pore; therefore, its nuclear export must be mediated by an exogenous NES.

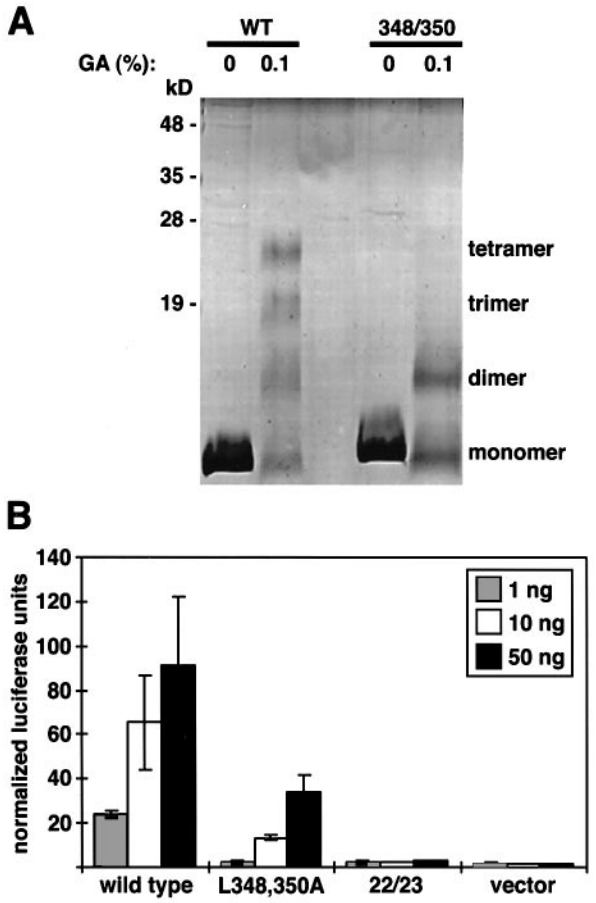


**Fig. 3.** Mutation of NES residues that impair nuclear export also reduces tetramerization efficiency. (**A**) Tetramerization of an NES mutant. Wild-type or L348,350A mutant proteins consisting of the tetramerization domain (amino acids 326–357) were expressed in *E.coli* as GST fusions. Twenty micrograms of GST-cleaved protein was incubated at 37°C with or without 0.1% glutaraldehyde for 30 min, then fractionated on a 20% SDS–PAGE gel to distinguish monomers from oligomers. (**B**) Transactivation by an NES mutant. Saos-2 cells were transiently transfected with 1, 10 or 50 ng of each p53–GFP construct and 750 ng of p21 promoter-driven luciferase reporter, and normalized luciferase units were quantified relative to β-galactosidase activity. Data shown are representative of four experiments.

The purified conjugate was microinjected into the nuclei of HeLa cells along with a high molecular weight rhodamine–dextran (70 kDa), which was used to assess the site of injection and the post-injection integrity of the nucleus. As expected, unconjugated BSA–FITC remained exclusively nuclear throughout the course of the experiment (data not shown). However, the wt NES–BSA–FITC conjugates moved from the nucleus to the cytoplasm, indicating that the attached p53 NES peptides were sufficient to mediate the nuclear export of the non-shuttling protein (Figure 2C). LMB blocked the export of these NES–BSA conjugates, further demonstrating that the p53 NES itself is the target of the export receptor, CRM1, rather than an NES-containing p53 binding partner.

### *The p53 NES residues mediate both nuclear export and tetramerization*

The p53 NES consists of residues 340–351, which lie within the tetramerization domain. This domain consists of a β-strand at residues 326–333 which aligns with the β-strand of another p53 monomer to form a dimer, and an α-helix at residues 335–356, which mediates the interaction of two p53 dimers to form a tetramer (see Figure 5A; Lee *et al*., 1994; Clore *et al*., 1995; Jeffrey *et al*., 1995). Interestingly, three of the key hydrophobic residues of the putative NES, L344, L348 and L350, localize to the dimer interface and mediate the interaction of the p53 dimers to form a tetramer (Lee *et al*., 1994; Clore *et al*., 1995; Jeffrey *et al*., 1995; Waterman *et al*., 1995; Mateu and Fersht, 1998), suggesting that the NES mutations may also be affecting tetramerization.

We tested the effects of NES mutations on p53 oligomerization using an *in vitro* cross-linking assay. *Escherichia coli* expression constructs were generated in which glutathione *S*-transferase (GST) was fused to either wild-type or L348,350A p53 peptides in the context of the entire p53 tetramerization domain (amino acids 326–357). It has been shown previously that peptides consisting of the wild-type p53 tetramerization domain can form tetramers *in vitro* (Wang *et al*., 1994). We separated the GST from the p53 and incubated the p53 peptides in glutaraldehyde to covalently cross-link any dimers and tetramers that formed. Figure 3A shows that the wild-type peptide formed tetramers. In contrast, the double point-mutant peptide effectively formed dimers, but formed tetramers much less efficiently than wt p53. These data are consistent with structural studies of this region (Waterman *et al*., 1995; Mateu and Fersht, 1998). This indicates that the crucial residues for NES function and tetramer formation are shared and that the regulation of nuclear export of p53 may be linked to the association or disassociation of p53 monomers.

Because p53 binds its DNA response elements most efficiently as a tetramer (Friedman *et al*., 1993; Halazonetis and Kandil, 1993; Hainaut *et al*., 1994; Hupp and Lane, 1994; McLure and Lee, 1998), we assayed the ability of the p53 NES mutants to transactivate p53-responsive genes. We transiently co-transfected Saos-2 or MDAH041 cells with the p53–GFP constructs and a luciferase reporter and assayed for luciferase activity. The construct encoding the L348,350A mutations was consistently less active than wild-type *p53* (Figure 3B; data not shown), exhibiting ~10% of wild-type activity at the lowest doses and 40–50% of wild-type at the highest doses. Consistent with earlier studies, p53–GFP with the 22/23 mutation was almost inactive (1–8% of wild type) due to its reduced ability to bind the basal transcription machinery and served as a negative control (Lin *et al*., 1994). We also used a colony formation assay to test the efficiency of growth suppression by the p53 NES mutant. The results of this assay were consistent with those of the transient transfection studies described above: at low virus titers (m.o.i. = 2) the NES mutant was about 10–16% as effective as wild type at suppressing colony formation, and at high virus titers (m.o.i. = 10) the effectiveness of growth suppression increased to 25–50% (data not shown). The observation that the NES mutant has a decreased capacity for transactivation and growth suppression relative to wild type is consistent with the requirement for tetrameric p53 to suppress growth and to bind and activate p53 response elements (Friedman *et al*., 1993; Halazonetis and Kandil, 1993; Hainaut *et al*., 1994; Hupp

and Lane, 1994; Pietenpol *et al*., 1994; Tarunina *et al*., 1996; McLure and Lee, 1998).

### *p53 in neuroblastoma cells is hyperactively exported but can be retained in the nucleus when bound to a tetramerization domain peptide*

The neuroblastoma cell lines SK-N-SH, CHP134, IMR32 and LAN5 have cytoplasmically sequestered wt p53 and an impaired $G_1$ arrest in response to DNA-damaging agents (Moll *et al*., 1996). The mechanisms conferring constitutive cytoplasmic localization of p53 in these and other tumor lines are currently unknown. p53 in these cells may be tethered to a cytoplasmic structure, which prevents its release and subsequent movement into the nucleus. Alternatively, cytoplasmic localization of p53 may reflect the balance of a dynamic nuclear import/export process, such that p53 is mobile and can enter the nucleus but is rapidly exported to the cytoplasm. To distinguish between these hypotheses, we treated SK-N-SH (Figure 4A) and CHP134 cells (data not shown) with the export-inhibiting drug, LMB, and examined p53 subcellular localization via immunofluorescence staining with the p53 antibody, pAb 1801. If p53 in these cells is tethered in the cytoplasm, it should remain there upon treatment with LMB. However, if p53 is rapidly shuttling between the nucleus and cytoplasm, LMB should trap it in the nucleus. In untreated neuroblastoma cells, p53 is predominantly cytoplasmic, but in the LMB-treated cells, p53 is retained in the nucleus (Figure 4A). The shuttling molecule IκBα also contains an LMB-responsive NES (Bachelerie *et al*., 1997; Ossareh-Nazari *et al*., 1997; C.Johnson and T.Hope, in preparation) and served as a positive control for LMB treatment. It has been reported previously that LMB does not induce a p53 stress response (Freedman and Levine, 1998). We verified this by comparing the localization of p53 and IκBα in cells treated with the DNA-damaging agent, actinomycin D (ActD). p53 entered the nuclei of cells treated with this drug, but IκBα did not (Figure 4B). Because IκBα is unlikely to be induced by stresses that activate p53, the nuclear localization of p53 observed in the LMB-treated cells is not likely to reflect a stress response, as indicated by the differential subcellular localization of IκBα and p53 after ActD treatment. Taken together, these results demonstrate that in these neuroblastoma cells, p53 shuttles between the nucleus and cytoplasm but nuclear export predominates, resulting in the appearance of cytoplasmic sequestration.

Our data show that the p53 NES lies within the tetramerization domain; therefore, p53 nuclear export and retention may depend on its oligomeric state. Interestingly,

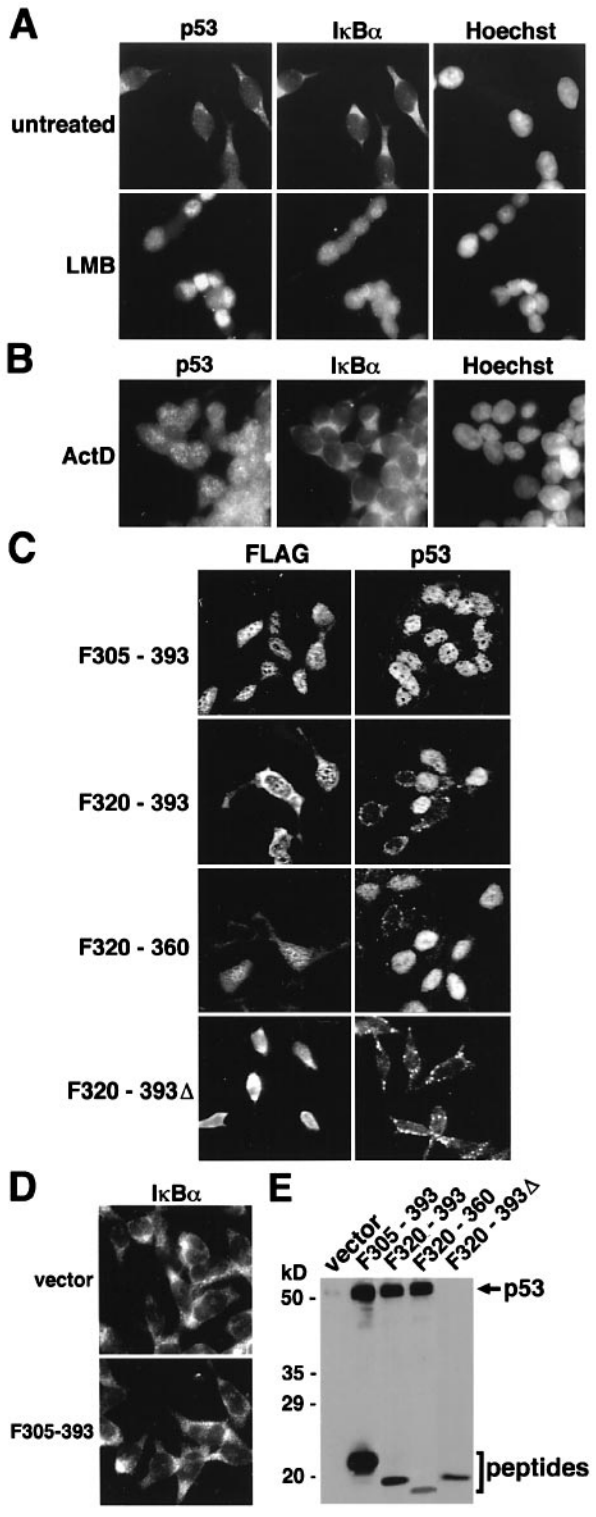


**Fig. 4.** Cytoplasmically localized p53 in neuroblastoma cells is subject to NES-mediated nuclear-cytoplasmic shuttling. (**A**) LMB traps p53 in the nuclei of neuroblastoma cells. Exponentially growing untreated SK-N-SH cells or cells treated with 20 nM LMB for 8 h, were fixed and stained with p53 or IκBα antibodies to ascertain subcellular localization. Cells were stained with Hoechst to reveal nuclei. (**B**) As in (A), but cells were treated with 7.2 nM ActD for 24 h before fixation. (**C**) The smallest peptide able to retain endogenous p53 in the nucleus consists of the tetramerization domain, residues 320–360. C-terminal Flag-tagged p53 construct F305–393 was stably transfected into SK-N-SH cells, and constructs F320–393, F320–360 and F320–393Δ were transiently transfected into LAN-5 cells. Transfected cells were fixed and stained with antibodies to the Flag-tagged peptide (FLAG) or to endogenous p53 (pAb 1801). Cells were visualized using a confocal microscope. (**D**) C-terminal peptide mediated nuclear retention is specific for p53. Endogenous IκBα localization was examined by immunofluorescence staining with an IκBα antibody in SK-N-SH cells stably transfected with the F305–393 construct or empty vector. (**E**) Heterocomplex formation between C-terminal peptides and p53 correlates with nuclear accumulation. LAN-5 cells transiently transfected with vector alone or the indicated C-terminal peptide constructs (except lane 1, in which SK-N-SH cells were stably transfected with the F305–393 construct) were immunoprecipitated using a Flag antibody. Immunoblotting was performed using a polyclonal p53-specific antiserum.

endogenous cytoplasmic wt p53 in neuroblastoma cells can be relocated to the nucleus by a p53 peptide consisting of the entire C-terminus, including both the tetramerization domain and the NES (Ostermeyer *et al*., 1996). We determined the minimal portion of the C-terminus capable of mediating p53 nuclear retention by constructing a series of plasmids that encode Flag-tagged human p53 C-termini of various lengths. SK-N-SH and LAN-5 cells were transfected with these constructs, then stained with an anti-Flag antibody to visualize the C-terminal peptide or with pAb 1801 to visualize endogenous p53. In cells transfected with empty vector, endogenous p53 localizes to discrete cytoplasmic structures readily visible by confocal microcopy (data not shown). However, in the cells expressing human F305–393, which consists of the entire p53 C-terminus, the export of endogenous cytoplasmic p53 was inhibited (Figure 4C). A similar result was observed in cells expressing peptides encompassing the beginning of the tetramerization domain to the end of the C-terminus (F320–393), the beginning of the C-terminus to the end of the tetramerization domain (F305–360), or solely the tetramerization domain (F320–360; Figure 4C; data not shown). It is possible that the cytoplasmic retention of p53 in these cells results from the titration of export factors by the NES in the over-expressed C-terminal peptides. If that were the case, the export of all shuttling proteins would be blocked. However, we observed that the localization of IκBα was unaffected by the expression of the F305–393 peptide (Figure 4D). Therefore, the inhibition of export mediated by these peptides is specific for p53. Taken together, these data show that the minimal domain necessary for disrupting nuclear export of p53 in these cells is the tetramerization domain, which includes the p53 NES.

The C-terminal mutant construct F320–393Δ, consisting of a point mutation (R335P) and an internal deletion of the tetramerization domain (Δ336–345) failed to inhibit the export of endogenous p53 (Figure 4C). As this mutant should be unable to bind to endogenous p53, this result, along with the data described above, is consistent with the hypothesis that the formation of hetero-oligomers between wt p53 and the C-terminal fragments results in nuclear retention. We examined this possibility using a co-immune precipitation analysis to detect hetero-oligomers. Endogenous p53 was precipitated by an anti-Flag antibody from lysates of SK-N-SH cells transfected with F305–393, F320–393, F305–360 or F320–360, which retain p53 in the nucleus (Figure 4E; data not shown). In contrast, wt p53 was not precipitated from extracts prepared from cells expressing F320–393Δ, which does not retain p53 in the nucleus (Figure 4E). This was also observed in a reciprocal co-immunoprecipitation using pAb 1801 (data not shown). These results demonstrate that forced oligomerization with an intact tetramerization domain peptide is sufficient for the disruption of p53 export in these cells, thus implicating a role for tetramerization in p53 nuclear retention.

## Discussion

In an unstressed cell, the p53 tumor suppressor is maintained at low levels in an inactive state. When the cell is challenged by stress, however, p53 is activated through post-translational mechanisms that increase its stability, convert the 'latent' form into a DNA-binding 'active' form, and retain it in the nucleus. The failure to retain p53 in the nucleus can lead to tumorigenesis, as p53 is incapable of accessing and transactivating the genes required for inducing $G_1$ arrest or apoptosis. This report demonstrates that an intrinsic leucine-rich NES mediates the subcellular localization of p53. Nuclear export of p53 is likely to be achieved through its direct interaction with the export receptor CRM1 rather than through an NES-containing binding partner since the export of a heterologous, non-shuttling protein is mediated by the p53 NES in an LMB-sensitive manner. This report also demonstrates that the cytoplasmic localization of p53 in neuroblastoma cells is due to a defect in p53 nuclear retention resulting from its hyperactive export. p53 in these cells can be trapped in the nucleus by the export-inhibiting drug LMB or by binding a p53 tetramerization domain peptide that includes the NES. The p53 NES is embedded within the tetramerization domain and modification of its primary amino acid sequence affects both nuclear export and tetramerization. As the p53 NES alone mediates the export of a heterologous protein, we infer that the positioning within this domain is not required for export. Rather, these data lead us to propose that the highly conserved positioning of the NES may have evolved to provide a mechanism for linking nuclear accumulation to p53 activation through formation of tetramers which mask the NES and prevent access to the nuclear export machinery.

### *The residues that comprise the NES are necessary for tetramerization*

p53 binds its DNA response elements most efficiently as a tetramer, and tetrameric p53 is most effective for transactivation (Friedman *et al*., 1993; Halazonetis and Kandil, 1993; Hainaut *et al*., 1994; Hupp and Lane, 1994; Pietenpol *et al*., 1994; Tarunina *et al*., 1996; McLure and Lee, 1998). Thus, amino acids that participate in tetramerization should be crucial for p53 to function as a transcriptional regulator. Mutagenesis studies have revealed that L344, L348 and to a lesser extent L350 are crucial for tetramer formation (Waterman *et al*., 1995; Mateu and Fersht, 1998). We found that mutations in L348 and L350 reduced tetramerization and consequently reduced transactivation and growth suppression. Although we did not examine L344, a Li-Fraumeni cancer predisposition family exists in which this leucine is converted to proline (Varley *et al*., 1996). This mutation prevents the formation of p53 tetramers (Ishioka *et al*., 1997), which presumably contributes to the cancer predisposition through p53 inactivation. We found that the L348 and L350 mutations not only prevent tetramer formation, but they also cause constitutive nuclear localization of p53, which is also observed in cells from the Li-Fraumeni patients with the L344P mutation (Varley *et al*., 1996). As the leucines occupying these positions are necessary for the interaction with a CRM1–RanGTP complex in other proteins with structurally related NESs (Fornerod *et al*., 1997; Ossareh-Nazari *et al*., 1997; Ullman *et al*., 1997; Askjaer *et al*., 1998), we infer that these mutations also prevent binding of the export receptor complex. Taken together, these data reveal a biologically

important link between p53 tetramerization, function and nuclear localization.

Because the NES mutations affect both tetramerization and export, an alternative hypothesis is that rather than abrogating the ability of the export receptor to recognize a consensus binding sequence, these mutations create an altered quaternary structure which is incapable of being bound by the export receptor. We disfavor this model, since fusion of an NES peptide lacking the β-strand portion of the tetramerization domain, which is required for p53 dimer formation (Figure 5A), enabled BSA to be exported (Figure 2C). Because the p53 tetramer forms from the interaction of two pre-existing p53 dimers, the NES peptides fused to BSA should be incapable of forming higher order structures. These data show that this consensus NES sequence still functions in nuclear export when separated from the tetramerization domain and placed in a context in which oligomers should not form.

### *A model for nuclear retention via NES-masking within the tetramer*

Three-dimensional structural analysis reveals that when p53 is monomeric or dimeric, the residues comprising the NES are exposed and should be a suitable substrate for an export receptor (Figure 5A and B; Lee *et al*., 1994; Clore *et al*., 1995; Jeffrey *et al*., 1995). Conversely, these residues are buried in the tetramer and it is difficult to see how the export machinery could gain access to them in this conformation. We propose that this placement of the NES enables the coordinated regulation of p53 tetramerization with nuclear retention, both of which are necessary for activation of a p53 stress response. The details of how this may be achieved are considered below.

Stresses, such as ionizing radiation, have long been known to stabilize p53 and lead to its nuclear accumulation (Kastan *et al*., 1991; Fritsche *et al*., 1993; Linke *et al*., 1996; Martinez *et al*., 1997). Stress-induced nuclear accumulation is probably due to an inhibition of nuclear export, as retained p53 is incapable of shuttling between nuclei in irradiated heterokaryons (Komarova *et al*., 1997). A mechanism by which p53 can be retained in the nucleus in response to stress is depicted in Figure 5C. DNA damage induces N-terminal modifications that inhibit MDM2 binding, thereby enhancing p53 transactivation and preventing its degradation (Shieh *et al*., 1997). Stress-induced activating modifications may also alter p53 structure to favor tetramerization. For example, UV radiation leads to phosphorylation of S392, resulting in increased DNA binding and tetramer formation *in vitro* (Hupp *et al*., 1992; Sakaguchi *et al*., 1997; Lu *et al*., 1998). Treatment of cells with PKC inhibitors, which prevents phosphorylation of the C-terminus, leads to DNA binding and nuclear accumulation (Chernov *et al*., 1998), both of which correlate with tetramer formation. Once p53 is tetrameric, the masking of its NES should lead to nuclear retention. This provides an efficient mechanism for ensuring that stress and cell cycle-induced modifications that create the DNA-binding form of p53 concurrently sequester p53 in

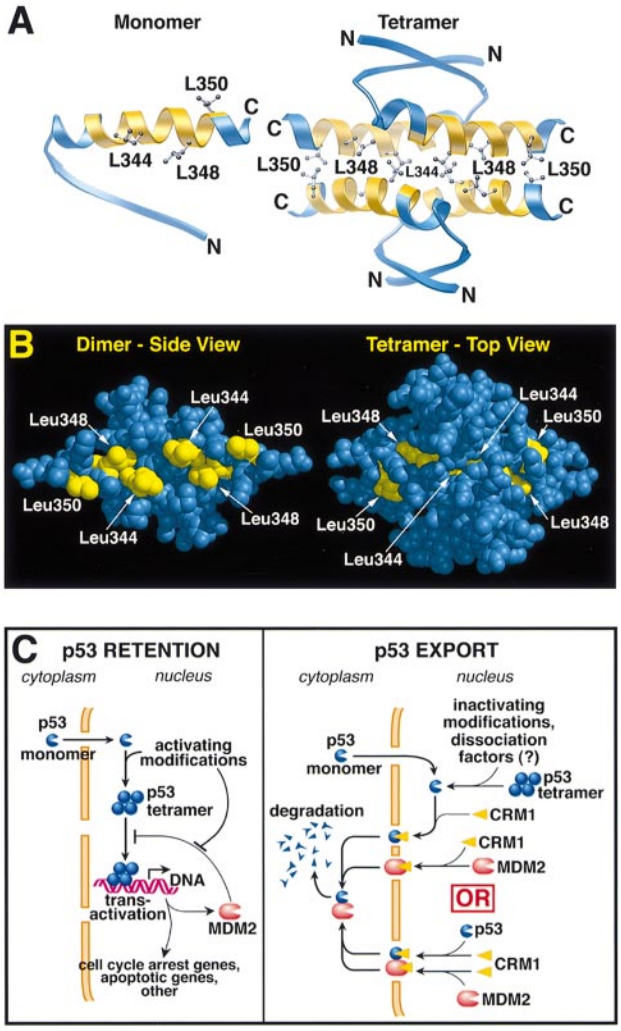


**Fig. 5.** A model for p53 subcellular localization based on oligomerization and NES masking. (**A**) Ribbon diagrams of the p53 tetramerization domain (residues 326–353) depicting both the monomer and tetramer. The β-strand enables the formation of dimers and the α-helix potentiates tetramer formation from a pair of dimers. The backbone NES sequence within the α-helix is colored gold, with the leucine side chains critical for export depicted as ball-and-stick figures. Produced with MOLSCRIPT (Kraulis, 1991), Raster 3D (Bacon and Anderson, 1988) and POVRAY (unpublished). Structural data were obtained from Lee *et al*. (1994). (**B**) Space-filling model depicting the p53 tetramerization domain as a dimer and a tetramer. The leucines crucial for nuclear export are highlighted in gold. These leucines are solvent-accessible in both the monomeric and dimeric forms and should be available for interaction with an export receptor. However, in the tetrameric form, the same leucine side chains are buried in the dimer–dimer interface, and should be unable to bind an export receptor. Produced with the conic option (Huang *et al*., 1991) of MIDAS (Ferrin *et al*., 1988). Structural data obtained from Lee *et al*. (1994). (**C**) Model for p53 nuclear export and accumulation based on NES accessibility. p53 retention: stress-induced activating modifications facilitate tetramer formation, resulting in the occlusion of the NES and the consequent nuclear retention of p53. Once in the nucleus and tetrameric, appropriately modified p53 binds DNA and transactivates downstream effectors of the p53 stress response. Some activating modifications are predicted to prevent MDM2-mediated inhibition of p53 transactivation (Shieh *et al*., 1997), thereby interrupting the MDM2-dependent autoregulatory feedback loop (Wu *et al*., 1993). p53 export: either in an unstressed cell, or in a cell recovering from a reversible p53-mediated stress response, the p53 monomer (or perhaps the dimer) is the proposed optimal substrate for nuclear export. Inactivating modifications or dissociation factors may facilitate tetramer dissociation, resulting in monomeric p53 with an exposed NES which can bind CRM1 and be exported to the cytoplasm where MDM2 mediates its proteasomal degradation. p53 may be exported independently of MDM2 or within an MDM2–p53 complex with the NES in each protein bound by its own CRM1.

the nucleus through the masking of the NES within the tetramer.

### *p53 export may occur only when the NES is unmasked*

p53 must also have a mechanism of regulated nuclear export, both for its cell cycle-dependent subcellular localization, and for its removal from the nucleus during the alleviation of a stress response. In both cases, the p53 monomer or dimer should be the preferred substrate for export, as these are the forms with an unmasked NES (Figure 5). Re-entry into the cell cycle subsequent to a $G_1$ arrest requires that p53 be inactivated as a transcription factor, which could result from modifications that reduce its capacity for DNA binding and mediate the dissolution of the p53 tetramer. Inactivating modifications or dissociation factors may separate the subunits of the p53 tetramer, exposing their NESs for binding by the export receptor, CRM1, which then mediates their removal from the nucleus.

It has been proposed that p53 nuclear export may occur through an association with its NES-containing negative regulator, MDM2 (Roth *et al*., 1998). MDM2 binds to p53 and facilitates its degradation by cytoplasmic proteasomes (Haupt *et al*., 1997; Kubbutat *et al*., 1997; Freedman and Levine, 1998); therefore, it is reasonable to hypothesize that an essential step in the regulation of p53 stability is its removal from the nucleus by MDM2. However, our data show that MDM2 is not necessary for p53 export (Figure 2A and B). Indeed, the p53 NES alone appeared to be sufficient for nuclear export (Figure 2C). It is possible that MDM2 and p53 exit the nucleus separately by their own NESs and meet in the cytoplasm where p53 is degraded through an association with MDM2. This model is compatible with the available data. However, an additional possibility is that MDM2 binding may enhance p53 export, such that the binding of MDM2 to p53 might create an additive export effect deriving from the NES in each protein (Figure 5C). This is an attractive model, as it would provide a mechanism for achieving a rapid reversal of stress responses when MDM2 levels are high. Experiments to distinguish between these possibilities are currently underway.

p53 in unstressed cells is present at low levels and is primarily cytoplasmic in S and $G_2$ phases (Shaulsky *et al*., 1990a; David-Pfeuty *et al*., 1996). Our data suggest that regulated nuclear export mediates the cell cycle-dependent localization of p53, since NES mutants are exclusively nuclear in unstressed cycling cells. It is possible that cdk2 and cdc2 are involved in the cell cycle-dependent localization of p53, since they phosphorylate p53 after the $G_1$ restriction point when p53 returns to the cytoplasm (Bischoff *et al*., 1990; Price *et al*., 1995). Consistent with this idea, phosphorylation at S315 results in reduced tetramer stability *in vitro* (Sakaguchi *et al*., 1997). cdk2- and cdc2-mediated tetramer destabilization in S and $G_2$ is consistent with our proposal that cytoplasmic localization of p53 results from the export of NES-accessible monomers or dimers.

### *p53 in neuroblastoma cells is hyperactively exported but retained in the nucleus by the masking of its NES*

Several tumor types have wt p53 which is inactive as a tumor suppressor due to its constitutive cytoplasmic localization (Moll *et al*., 1992, 1995; Sun *et al*., 1992; Stenmark-Askmalm *et al*., 1994; Bosari *et al*., 1995; Ueda *et al*., 1995; Lou *et al*., 1997; Schlamp *et al*., 1997). We determined that this defect in subcellular localization in neuroblastoma cells is not a result of a tether retaining p53 in the cytoplasm. Rather, p53 in these cells is subject to continuous nuclear–cytoplasmic shuttling but with predominant nuclear export, giving the appearance of static cytoplasmic 'sequestration'. The hyperactive export of p53 in neuroblastoma cells was blocked by the expression of p53 C-terminal peptides which contain the tetramerization domain, including the isolated tetramerization domain itself, and correlated with the ability of these peptides to oligomerize with endogenous p53 (Figure 4C and E). Interestingly, the tetramerization domain peptide F320–360 did not contain any of the three p53 nuclear localization signals, yet the heterocomplex formed between this construct and endogenous p53 was fully capable of entering the nucleus. This indicates that the p53 nuclear localization signals in these cells are active, and since our data show that LMB can trap endogenous p53 in the nucleus, demonstrates that the cytoplasmic localization of p53 in these cells is not due to a defect in nuclear import.

The inhibition of nuclear export of the p53 hetero-oligomers is consistent with our model that nuclear retention of p53 is due to the masking of the p53 NES. The nuclear heterocomplexes may have NESs that are buried within the interface of the peptides and the p53 tetramerization domains, and are therefore unable to bind an export receptor. An additional interpretation of these results is that the hetero-oligomers may have a quaternary structure that is not recognized by factors required for their export. For example, the exposure of the p53 NES to the export receptor may be due to a modification from a kinase which can only bind the p53 homotetramer or whose target is not present in the hetero-oligomer (see Figure 5C). The hetero-oligomer, therefore, would not be subject to these modifications and would not be exported. Alternatively, the export of p53 in these cells may require an export partner, such as MDM2. If the hetero-oligomers are incapable of binding this export partner, their export would be inhibited. However, because MDM2 is capable of low-efficiency binding to p53 mutants with altered quaternary structures (Lomax *et al*., 1998), and because the MDM2 binding domains in the p53 N-terminus should be freely accessible in these heterocomplexes (Kussie *et al*., 1996), it is more likely that p53 retention in these cells is due to NES occlusion resulting from the interaction of the α-helices in wt p53 and the C-terminal fragment tetramerization domains. Experiments to distinguish these possibilities are in progress.

### *Masking of nuclear export and import signals as a general mechanism of regulating cytoplasmic and nuclear localization*

The masking and unmasking of nuclear export and import signals within an oligomer may be a general mechanism for regulating subcellular localization. This could occur through either homo- or hetero-oligomerization. For example, a recent study identified a putative NES at the dimer interface of the MAP kinase ERK2 (Khokhlatchev *et al*., 1998). Phosphorylation of the ERK2 monomer induces homodimerization, and the resulting nuclear accu-

mulation may reflect the masking of an NES, as we observed in p53. The masking of nuclear import signals through oligomerization can lead to cytoplasmic retention, as observed in NF-AT4 (Zhu *et al*., 1998). While many of the examples reported thus far involve proteins that transduce growth or stress signals, we anticipate that the appropriate spatial and temporal regulation of a variety of cellular processes may involve the masking of nuclear export and import signals within protein–protein complexes. The activation of p53 by numerous stresses, combined with the high resolution structures available for the tetramerization domain, make it an attractive model for investigating the molecular chain reactions that alter subcellular distribution through changes in protein conformation.

## Materials and methods

### *Construction and mutagenesis of p53–GFP expression clones*

Mutations were introduced into wt p53 by two-step PCR mutagenesis with oligonucleotides containing base alterations to convert leucines 348 and 350 to alanines. For the double point mutant, the oligonucleotides 5′(1036)GAG GCC GCG GAA GCC AAG GAT GCC CAG GCT GGG(1069)3′ and 5′(1056)ATC CTT GGC TTC CGC GGC CTC ATT CAG CTC TCG(1024)3′ were used to create PCR products which were then co-amplified with p53 coding sequence 5′ and 3′ primers with *Sal*I and *Bam*HI restriction sites on the ends to create a full-length mutant construct without a stop codon. This PCR product was digested and ligated into pEGFP-N1 (Clontech) to create an in-frame C-terminal fusion with EGFP. The tetramerization domain portion of the p53 wild-type and mutant constructs were subsequently subcloned into pGEX-2T (Pharmacia) to create an in-frame N-terminal fusion with GST, the details of which can be provided upon request.

### *Cell culture and transfections*

Saos-2 (human primary osteogenic sarcoma, $p53^{-/-}$, ATCC HTB85), HeLa and Balb/c 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). $p53^{-/-}/mdm2^{-/-}$ mouse embryonic fibroblasts [kindly provided by Drs Allan Bradley and Stephen Jones (Jones *et al*., 1995)], were maintained in DMEM with 10% dialyzed FBS and non-essential amino acids. Each p53–GFP construct (4 μg) was electroporated into $4\times10^5$ exponentially growing Saos-2 or $p53^{-/-}/mdm2^{-/-}$ mouse embryonic fibroblasts at 0.25 kV and 960 mF using a Bio-Rad Gene Pulser, plated on collagen-coated 25 mm coverslips, and incubated at 37°C, 7% $CO_2$ for 36 h. Cells were fixed in 3.7% formaldehyde for 10 min at room temperature then stained with Texas Red–phalloidin (Molecular Probes), and the cover slips were mounted on microscope slides. GFP- and Texas Red-staining were visualized on a Zeiss Axioplan 2 epifluorescence microscope using a 63× objective and images were captured with a CCD camera using WinView/32 imaging software (Princeton Instruments) with 0.1–0.2 s exposure times for Texas Red and 1.0–4.0 s exposures for FITC. For luciferase assays, Saos-2 cells ($3\times10^5$ in 6-well TC plates) were transfected with 750 ng of WWP-luciferase (p21 promoter driving luciferase expression, kindly provided by Dr Bert Vogelstein), 500 ng of CMV110 (a β-galactosidase-expressing plasmid, used as an internal transfection control) and 1, 10 or 50 ng of each p53–GFP plasmid or pEGFP-N1 using SuperFect reagent (Qiagen). Cells were lysed and luciferase reporter and β-galactosidase transfection control assays were performed 48 h later as described previously (Heyman *et al*., 1992).

### *Generation and microinjection of BSA–peptide conjugates*

A peptide consisting of the wild-type p53 NES (C-339-EMFRELN-EALELKD-352) was conjugated to BSA (Boehringer Mannheim) using a modification of the protocol of Fischer *et al.* (1995). BSA (6 ml, 5 mg/ml) in phosphate-buffered saline (PBS) pH 7.4, was incubated with 50 mg of sulfosuccinmidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC, Pierce Chemical) for 1 h at room temperature. The BSA was separated from the unreacted sulfo-SMCC over a G-50 Sephadex (fine) column (Pharmacia). Approximately 5 mg of the purified sulfo-SMCC BSA (1 ml) was mixed with 1 ml of 40% DMSO in PBS pH 6.5, and incubated with 10 mg of peptide for 16 h at 4°C. The BSA–peptide conjugate was then separated from free peptide and DMSO over a G-50 Sephadex column and conjugation was assessed by SDS–PAGE. Altered mobility revealed that each BSA molecule contained ~15–20 peptides. The conjugate was labeled with 5-iodoacetamidofluorescein (5-IAF, Molecular Probes) in PBS pH 7.4 for 2 h at room temperature, then separated from unincorporated label by G-50 Sephadex chromatography followed by concentration in a Centricon C-50 unit (Amicon). At least 24 h before microinjection of the conjugate, HeLa cells were plated at ~50% confluency onto collagen-coated 25 mm glass coverslips. LMB (kindly provided by Barbara Wolff, Novartis) treatment involved growing cells in the presence of 5 nM LMB for 4 h before and during injection. Approximately 50 fl of a mixture of 5-IAF labeled p53 wt NES–BSA conjugate (~4 mg/ml) and 70 kDa rhodamine–dextran (1.5 mg/ml, Sigma) was injected into the nuclei of the HeLa cells using an Eppendorf microinjection system. Cells were incubated at 37°C, 7% $CO_2$ for 4 h, then fixed with 4% paraformaldehyde. Localization of the conjugate in the fixed cells was visualized by confocal microscopy.

### *Heterokaryon preparation and analysis*

Saos-2 cells ($5\times10^5$) were electroporated with 5 μg of each p53–GFP construct and immediately plated with Balb/c 3T3 cells ($5\times10^5$) onto collagen-coated 25 mm glass coverslips in 6-well TC plates and incubated at 37°C, 7% $CO_2$. The following day, cells were pre-treated for 30 min with 100 μg/ml cycloheximide, fused for 2 min with 50% PEG 3350/PBS pre-warmed to 37°C, washed five times with PBS, then incubated in DMEM, 10% FBS for 3 h. LMB-treated fusions were treated as above, but with the addition of a 3 h pre-treatment with 20 nM LMB before cycloheximide treatment, and the inclusion of LMB in the post-fusion incubation. Cells were fixed with room temperature 3.7% formaldehyde for 10 min then stained with Texas Red–phalloidin and Hoechst 33342 (50 μg/ml, Sigma). Cells were visualized on a Zeiss Axioplan 2 epifluorescence microscope as above, using 0.001–0.01 s exposure times for DAPI.

### *Protein production and cross-linking*

GST fusion constructs consisting of the tetramerization domain of p53 (amino acids 326–357), either wild-type or L348,350A NES mutant, were expressed in *E.coli* BL21 cells (Stratagene), and purified and cleaved with thrombin protease using Pharmacia's Bulk GST Purification Module. Twenty micrograms of the cleaved tetramerization domain proteins were then incubated in 200 mM $NaPO_4$ pH 7.5 with or without 0.1% glutaraldehyde (Sigma) at 37°C for 30 min, then resolved on a 20% SDS–PAGE gel and stained with Coomassie Brilliant Blue.

### *Analysis of drug-treated neuroblastoma cell lines*

Human neuroblastoma cell lines SK-N-SH and CHP134 were cultured in RPMI 1640, 10% heat inactivated fetal calf serum (FCS) at 37°C and 5% $CO_2$. Cells were cultured in collagen-coated 8-well chamber slides and incubated in the presence of 20 nM LMB for 8 h or 7.2 nM ActD for 24 h. Cells were fixed in room temperature acetone:methanol (1:1) for 3 min, air dried for 10 min, and stained with primary antibodies pAb 1801 (p53 mouse monoclonal, Santa Cruz Biotech) and C-21 (IκBα rabbit monoclonal, Santa Cruz Biotech) and secondary antibodies FITC-goat-anti-mouse and Texas Red-donkey-anti-rabbit and visualized on a Zeiss Axioplan 2 epifluorescence microscope as described above. Three independent experiments were performed for each condition.

### *Generation and analysis of neuroblastoma cells expressing p53 C-terminal peptides*

Human neuroblastoma cell lines SK-N-SH and LAN5 were cultured as above and transiently or stably transfected with expression plasmids encoding human p53 C-terminal peptides. The cDNAs encoding the N-terminally tagged p53 C-terminal peptides were generated by multiple-step PCR amplifications, the details of which can be provided upon request. pF320–393Δ was constructed by an *Eco*47III–*Stu*I 30 bp in-frame deletion of pF320–393, resulting in R335P mutation followed by a 10-amino-acid deletion. pCMV NeoBam3 was used as a vector control in all transfection experiments. For immunofluorescence staining, subconfluent cells were grown on P100 dishes overnight and re-fed 4 h prior to calcium phosphate-mediated transfection with 20 μg each of p53 polypeptide or vector-only DNA constructs. After overnight incubation in DNA mixtures, cells were dispensed onto polylysine-coated three-chamber culture slides (Becton-Dickinson) and grown for an additional 12–36 h. Cells were rinsed in PBS, fixed for 3 min in acetone:methanol (1:1) and air-dried for 10 min. After blocking in 10% normal goat serum for 20 min, parallel culture chambers were incubated in the appropriate

mouse primary antibodies overnight at 4°C. Staining was detected with biotinylated goat anti-mouse IgG (ZYMED) followed by streptavidin-FITC (Gibco-BRL). Cells were mounted with Antifade (Molecular Probes) and examined with a Nikon Confocal Scanning Laser microscope. The data shown are representative of three or four independent experiments performed for each construct. For co-immunoprecipitation, cell pellets were obtained by scraping cells from one 10 cm plate for each cell line transfected with p53 polypeptide or vector-only DNA constructs. The pellets were sonicated in 300 µl IP buffer (50 mM Tris pH 7.4, 2 mM EDTA, 2 mM EGTA, 0.3% NP-40, 0.2% Triton X-100, 150 mM NaCl, 25 mM NaF containing a protease inhibitor cocktail of 1 mg/ml aprotinin and 1 mM PMSF). After centrifugation at 12 000 *g* for 15 min, the supernatant was pre-cleared with 30 µl protein G–agarose beads (Gibco-BRL). Equal amounts of total protein (1.5 mg) were immunoprecipitated with 1.5 µg of pAb 1801 or Flag antibody overnight at 4°C. The beads were washed four times in SNNTE (50 mM Tris pH 7.4, 5 mM EDTA, 5% sucrose, 1% NP-40, 0.5 M NaCl) buffer. Samples were solubilized by boiling in SDS–PAGE running buffer before loading onto an 8% gel. The data are representative of five independent experiments performed for each construct.

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