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Mice defective in p53 nuclear localization signal 1 exhibit exencephaly

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Abstract

p53 is a major suppressor of human malignancy. The protein levels and activity are tightly regulated in cells. Early experiments identified nuclear localization signal 1 (NLS1) as a regulator of p53 localization. We have generated mice bearing a mutation in *p53^{NLS1}*, designated *p53^{NLS1}*. Our experiments confirm a role for NLS1 in regulating p53 function. Murine embryonic fibroblasts generated from homozygous *p53^{NLS1}* animals are partially defective in cell cycle arrest and do not respond to inhibitory signals from oncogenic *Ras*. In addition, p53-dependent apoptosis is abrogated in thymocytes. Contrary to predicted results, fibroblasts from homozygous *p53^{NLS}* animals have a greater rate of proliferation than *p53-null* cells. In addition, *p53^{NLS}* cells are more resistant to UV-induced death. Surprisingly, the homozygous *p53^{NLS1}* animals exhibit embryonic and peri-natal lethality, with a significant portion of the animals developing exencephaly. Thus,

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p53^{NLS1/NLS1} embryos exhibit a reduced viability relative to *p53-null* mice. These studies indicate that the NLS1 is a major regulator of p53 activity in vivo.

Keywords

p53; Nuclear localization signal 1; Exencephaly; Apoptosis; Embryonic lethality

Introduction

p53 is a major suppressor of human malignancy (Hainaut and Hollstein 2000). p53 is activated in cells in response to various stimuli, including DNA damage, oncogenic transformation and environmental stress. The protein levels and activity are under tight regulation in the cells (Liang and Clarke 2001). In unstressed cells, p53 protein half-life is short and the protein is targeted for degradation in the cytoplasm. This process is regulated by the E3 ligase Mdm2, as well as additional newly identified E3 ligases (Brooks and Gu 2006). Mdm2 binds and ubiquitinates p53 whereby p53 is exported to the cytoplasm and degraded by the proteasome (O'Brate and Giannakakou 2003). In response to DNA damage, inhibition of the p53-Mdm2 complex formation and p53 degradation results in elevated p53 protein levels. p53 is imported to or retained in the nucleus to undertake one of its primary roles as a transcriptional activator. Thus, in response to DNA damage, there is both inhibition of p53 degradation and increased nuclear accumulation of p53.

Early experiments demarcated the sequences involved in nuclear import and nuclear export. Three nuclear import sequences (NLS1, NLS2, and NLS3) were identified in the carboxyl terminus based on mutagenesis studies (Dang and Lee 1989; Shaulsky et al. 1990). NLS1 forms a basic core region similar to SV40 Large T-Antigen NLS and is conserved across species (Addison et al. 1990; Dang and Lee 1989; Shaulsky et al. 1990). In addition, a basic and spacer region has been shown to cooperate in regulating p53 cytoplasmic location, demonstrating NLS1 functions as a bipartite signal (Liang and Clarke 1999a, b; Liang et al. 1998). p53 nuclear import is mediated by binding of the NLS to the importin- α complex (Liang and Clarke 1999a). Proteins have been identified that act to tether p53 in the cytoplasm (Gannon and Lane 1991; O'Brate and Giannakakou 2003; Wadhwa et al. 1998). Nuclear localization is important for p53 activity. In an E1A-Ras transformation assay, the suppressive properties of wildtype p53 were dependent on an intact NLS1 (Shaulsky et al. 1991). In addition, nuclear retention has been shown to be critical for p53-dependent apoptosis (Ryan et al. 1994) and growth inhibition (Knippschild et al. 1996; Moll et al. 1996). However, p53 NLS1 has been shown to be a weak monopartite nuclear localization signal (Liang and Clarke 1999a, b).

Although critical in developing a model of p53 regulation, these in vitro expression studies have the disadvantage of analyzing p53 function in culture cells. In order to examine the role of p53 cellular localization in vivo, we have generated a knock-in mouse expressing a p53 protein with a mutated NLS1, designated *p53^{NLS1}*. Several p53 functions were defective in cells derived from homozygous *p53^{NLS1}* animals. However, additional characteristics were identified. For instance, *p53^{NLS1/NLS1}* cells grow more rapidly than *p53-null* cells.

Interestingly, the homozygous *p53^{NLS1}* mice exhibit embryonic and peri-natal lethality with a high degree of penetrance. Therefore, *p53^{NLS1}* animals are not simply recapitulating *p53-null* mice phenotype. These studies demonstrate a definitive role of the NLS1 in regulating p53 function and an additional role of the p53 NLS1 in the regulation of embryonic development.

Results

Generation of p53 NLS1 mutant animals

The p53 gene consists of 11 exons and spans about 14 kb. The sequences of murine p53 nuclear localization 1 (NLS1) include amino acids 313–322 in exon 9 (Fig. S1A). We disrupted the core domain by substituting Alanines for Lysines 316, 317 and 318 (Fig. 1a) and introduced a *NotI* restriction site (Fig. S1B). This substitution has previously been made in human p53 cDNA and, when overexpressed in MCF-7 cells, lead to a cytoplasmic localization and diffuse nuclear staining (Liang and Clarke 1999a). This mutant was shown to bind weakly to importin- α . NLS1 resides outside of the tetramerization domain, and does not affect the tetramerization of the protein (Liang and Clarke 1999a). To introduce an Alanine substitution at Lys316–318, we built a targeting vector that contained LoxP sites flanking a neomycin selection marker, and introduced the mutation by site-directed mutagenesis (Fig. 1b). The targeting vector was sequenced to confirm that no other mutations were present in the p53 sequence. We electroporated PC3 ES cells (O’Gorman et al. 1997). We identified 10 positive clones for recombination by southern analysis. The targeted allele is depicted in Fig. 1c.

One positive ES cell line was injected into blastocysts and chimeras were established. High degree chimeras were generated and mated with C57/B6 to produce progeny. The PC3 cells contain the Cre recombinase under the protamine reporter, which excises the neo in the male germline (O’Gorman et al. 1997). Therefore, after Cre excision, the resulting allele contained a loxP site and the exon containing the mutation of p53 (Fig. 1d). We identified animals that were heterozygous for the mutation. We confirmed excision of the floxed allele by southern analysis (Fig. 1e). The presence of the Alanine mutation in the mice tail DNA was confirmed by PCR and subsequent digestion of the PCR fragments with *NotI* (Fig. S1C). The mutation was also confirmed by sequencing the PCR product.

Growth characteristics of p53 NLS1- deficient embryonic fibroblasts

To explore the role of Lys316–318 (NLS1) in p53-mediated control of cell growth, we generated murine embryonic fibroblasts (MEFs) from *p53^{+/+}*, *p53* heterozygous (*p53^{+/-}*), *p53-null* (*p53^{-/-}*), *p53^{NLS1/+}* and *p53^{NLS1/NLS1}* embryos. Previous studies showed that *p53^{-/-}* MEFs are distinguishable from wildtype cells in their growth rate and plating efficiency (Harvey et al. 1993). Therefore, the growth rates of *p53^{NLS1/+}* and *p53^{NLS1/NLS1}* fibroblasts were compared with wildtype and *p53^{-/-}* cells (Fig. 2a). The *p53^{NLS1/+}* and *p53^{NLS1/NLS1}* MEFs had a faster proliferation rate than wildtype MEFs. There was no significant difference in the proliferation rates or saturation densities of *p53^{+/+}* and *p53^{NLS1/+}* MEFs. *p53^{-/-}* MEFs grew very rapidly and reached saturation on day 5. However, *p53^{NLS1/NLS1}* MEFs grew faster at low densities than *p53^{-/-}* cells and achieved

confluent densities at day 4. The increased growth rate was significant 2 and 3 days after plating. The cells were analyzed for bromodeoxyuridine (BrdU) uptake. $p53^{NLS1/+}$ and $p53^{NLS1/NLS1}$ cells demonstrated an increased S-phase population compared to wildtype (Fig. 2b). In addition, $p53^{-/-}$ cells exhibited an increase in S-phase compared to wildtype cells, however, it was not significantly greater than $p53^{NLS1/NLS1}$ cells. These data suggest that growth is abnormal in $p53^{NLS1/NLS1}$ MEFs and that the mutation in both alleles gives MEFs a growth advantage.

To determine the role for Lys316–318 (NLS1) in p53-mediated cell cycling, we analyzed the DNA-damage induced growth arrest in response to gamma irradiation (IR). Wildtype MEFs displayed a 70% reduction in S-phase population of cells following IR treatment (Fig. 2b). The $p53^{NLS1/+}$ cells arrested in response to IR, with a 60% reduction in S-phase. $p53^{NLS1/NLS1}$ cells exhibited a 14% reduction in S-phase. In contrast, $p53^{-/-}$ cells exhibit no reduction in S-phase (Fig. 2b). Therefore, $p53^{NLS1/NLS1}$ cells exhibited a partial response to DNA damage.

Western analysis was performed to examine the levels of p53 and p21, a downstream target of p53, in response to DNA damaging agents (Fig. 2c). In wildtype cells treated with IR, ultra-violet light (UV), and doxorubicin (dox), p53 protein levels were induced (Fig. 2c, lanes 2–4). Surprisingly, in untreated $p53^{NLS1/NLS1}$ cells, p53NLS1 protein levels were elevated (Fig. 2c, lane 5). There was no further induction of p53 in $p53^{NLS1/NLS1}$ cells in response to UV, IR, or doxorubicin treatment (Fig. 2c, lanes 6–8)).

Role of p53 NLS1 in Ras induced senescence

To further examine the effects of Lys316–318 (NLS1) in p53 regulated cell growth, we performed a senescence assay with oncogenic Ras, which causes cells to undergo p53-dependent senescence (Serrano et al. 1997). The effect of expressing oncogenic Ras was examined in wildtype, $p53^{-/-}$ and $p53^{NLS1/NLS1}$ MEFs (Fig. 3). Wildtype cells infected with a pBabe control vector doubled in population over 6 days and expression of oncogenic Ras inhibited this growth (Fig. 3a). In addition, the number of cells positive for senescence marker was increased with oncogenic Ras expression in wildtype cells (Fig. 3b, c). Wildtype cells infected with Ras demonstrated the flat cells phenotype characteristic of cells that have exited the cell cycle (Fig. 3b). $p53^{-/-}$ cells did not have any growth change in the presence of Ras (Fig. 3a) and very few senescent cells were detected (Fig. 3b, c). The $p53^{NLS1/NLS1}$ MEFs also did not respond to the growth-suppressive properties of oncogenic Ras (Fig. 3a), and very few senescent cells were present (Fig. 3b, c). Thus, $p53^{NLS1/NLS1}$ cells were able to escape Ras induced senescence.

$p53^{NLS1}$ homozygous cells have defective expression of p53

The increased levels of p53 under non-stressed conditions (Fig. 2c) suggested that basal p53NLS1 protein levels would be increased. Therefore, we examined the cellular location of the p53NLS1 protein. We performed immunofluorescence for the p53 protein on wildtype ($p53^{+/+}$) and $p53^{NLS1}$ homozygous ($p53^{NLS1/NLS1}$) cells (Fig. 4). The p53 protein in wildtype cells is barely discernable under basal conditions (data not shown). In contrast, p53NLS1 protein was readily detected in non-stressed $p53^{NLS1/NLS1}$ cells (Fig. 4, top panel).

The detection of the protein at basal levels supports the observation of increased levels of p53 in the Western analysis (Fig. 2a, c). The staining pattern of p53 in the non-stressed *p53^{NLS1/NLS1}* cells was not uniform. The cells either had a predominantly cytoplasmic staining, with very little nuclear staining, or the cells had staining with distribution of p53 in the nucleus and the cytoplasm. Examples of both cells are labeled in Fig. 4. We examined p53 cellular distribution in 30 *p53^{NLS1/NLS1}* cells. We determined that 53% of the cells had cytoplasmic p53 staining and 46% had distribution of p53 in the cytoplasm and nucleus. Thus, under basal conditions, the protein can be detected in the cytoplasm and/or nucleus. This observation supports previous studies that additional sequences (such as NLS2, NLS3 and the basic region, Fig. S1A) are also contributing to nuclear localization of the p53^{NLS1} protein (Liang and Clarke 1999a, b).

In order to examine p53 localization in response to DNA damage, we examined p53 location after UV treatment in wildtype and *p53^{NLS1}* cells. There was a dramatic increase in p53 in wildtype cells treated with UV with greatly induced nuclear staining (Fig. 4, middle panels). The distribution of p53 in UV-treated *p53^{NLS1/NLS1}* cells varied from cell to cell (Fig. 4, bottom panels). We detected the same expression pattern that had either a diffuse staining (cytoplasmic and nuclear), or a predominantly cytoplasmic staining such as observed in basal conditions (Fig. 4, top panels). In addition, very few *p53^{NLS1/NLS1}* cells exhibited nuclear stabilization of p53. In response to UV, 16% of the cells showed the predominantly cytoplasmic staining, whereas 75% had a diffuse staining, and another 16% had nuclear stabilization of p53.

***p53^{NLS1}* homozygous thymocytes are resistant to p53-mediated death**

In order to explore the effect of loss of p53 NLS1 on p53-dependent apoptosis in vivo, we examined thymocyte apoptosis in response to DNA-damage. CD4⁺/CD8⁺ thymocytes undergo p53-dependent apoptosis following IR (Clarke et al. 1993; Lowe et al. 1993b). We compared the relative population of CD4⁺/CD8⁺ cells in thymocytes from wildtype (*p53^{+/+}*), *p53^{-/-}*, *p53^{NLS1/NLS1}* and *p53^{NLS1/+}* mice, treated or untreated with IR (8 Gy). CD4⁺/CD8⁺ staining was performed 24 h post-IR. A significant depletion of CD4⁺/CD8⁺ thymocytes was observed in wildtype mice (Fig. 5a). *p53^{NLS1/+}* cells showed a decrease in depletion of CD4⁺/CD8⁺ population in response to IR compared to wildtype thymocytes. The partial depletion of CD4⁺/CD8⁺ in *p53^{NLS1/+}* cells was similar to that previously observed in *p53^{-/-}* animals (Sluss et al. 2004). This result suggests the presence of the *p53^{NLS1}* allele in the heterozygous animal was not exerting a dominant negative effect on wildtype p53. *p53^{NLS1/NLS1}* CD4⁺/CD8⁺ thymocytes were resistant to death, similar to that observed in *p53^{-/-}* cells (Fig. 5a).

The fraction of non-apoptotic thymocytes was also measured at multiple time points post-irradiation by staining cells for annexin-V and 7AAD (Fig. 5b). annexin-V negative and 7AAD negative populations were determined, and compared to untreated thymocytes. Following DNA damage, a significant decrease in non-apoptotic cells and concomitant increase in Annexin V positive cells was observed in wild-type thymocytes (Fig. 5b). *p53^{NLS1/NLS1}* thymocytes displayed almost 100% survival following DNA damage,

indicating no apoptosis was occurring (Fig. 5b). These results demonstrated that loss of p53 Lys316–318 abrogated p53-dependent death in thymocytes.

p53NLS1 exerts a gain of function phenotype

To further examine the effects of p53NLS1 on cell survival, we examined the survival of E1A-transformed MEFs in response to UV. MEFs are normally resistant to death by radiation and chemotherapeutic agents (Lowe et al. 1993a). However, MEFs co-expressing the adenovirus E1A protein undergo apoptosis when treated with DNA-damaging agents or chemotherapeutic agents (Attardi et al. 2004; Lowe et al. 1993a). E1A—transformed cells exhibit p53-dependent and independent death (a portion of E1A-*p53*^{-/-} MEFs die). We examined viability of cells at 0, 18, and 24 h after treatment with 5 J/m² of UV (Fig. 5c). Whereas wildtype cells were lost viability with treatment, *p53*^{NLS1/NLS1} cells were resistant to UV-induced death. In addition, we observed a significant increase in survival of *p53*^{NLS1/NLS1} cells compared to *p53*^{-/-} cells. Thus, there was an increase in the portion of cells resistant to death at low doses of UV, suggesting a gain-of-function for the p53^{NLS1} allele.

In order to examine a possible mechanism of this defect, we found that p53NLS1 cells exhibited very little induction of NOXA, compared to wildtype cells. There was a 27-fold reduction in the amount of UV-induced NOXA in the mutant cells compared to wildtype (Fig. 5d). Therefore, although nuclear p53NLS1 was able to induce p21, it was severely defective in inducing NOXA.

p53^{NLS1} animals exhibit embryonic lethality and develop exencephaly

In addition to studies in cells, we examined the effects of p53 NLS1 on whole animals. Mice heterozygous for the p53^{NLS1} allele (*p53*^{NLS1/+}) are viable (Table 1). These mice were intercrossed and we noted that mice homozygous for the mutant allele were recovered at low frequency (Table 1). There was a 90% reduction in the expected number of *p53*^{NLS1/NLS1} offspring at birth. This surprising result suggested the homozygous p53^{NLS1} allele could be causing embryonic lethality. We performed an analysis on timed embryos to determine how early we could detect homozygous *p53*^{NLS1} embryos. We detected almost the expected ratio of *p53*^{NLS1/NLS1} embryos at embryonic day 14.5 and 12.5 (83 and 78%, respectively). We detected *p53*^{NLS1/NLS1} embryos as young as embryonic day 9.5, although there was no reduction in expected ratio of *p53*^{NLS1/NLS1} embryos. This suggested *p53*^{NLS1/NLS1} embryos were dying mid to late gestation. During the examination of embryos day 9.6–14.5, we detected embryos that exhibited hindbrain exencephaly (Fig. 6a, b). Examination of embryonic day 14.5 embryos revealed that 40% *p53*^{NLS1/NLS1} embryos exhibited hindbrain exencephaly (Table 1). This percentage was similar in other embryos. Most of the females exhibited exencephaly (75%), whereas one out of six males exhibited exencephaly. Exencephaly is observed in a low frequency of *p53*^{-/-} animals (Armstrong et al. 1995; Sah et al. 1995). Unlike *p53*^{NLS1/NLS1} mice, *p53*^{-/-} mice are mostly viable. Thus, the reduced viability of *p53*^{NLS1/NLS1} mice relative to *p53*^{-/-} mice could be attributed to an increase in the rate of exencephaly. Exencephalic *p53*^{-/-} embryos show defects in apoptosis (Sah et al. 1995). Studies are underway to examine apoptosis in the *p53*^{NLS1/NLS1} exencephalic embryos.

Discussion

Early experiments showed the p53 protein translocates to the nucleus via a NLS1 (Dang and Lee 1989; Shaulsky et al. 1990), a secondary NLS2 and NLS3 (Shaulsky et al. 1990), and a basic and spacer sequences adjacent to NLS1 that are critical for nuclear import (Liang and Clarke 1999a, b; Liang et al. 1998) (s). The importance of nuclear import of p53 is underscored by the observation that p53 function can be inactivated in tumors by cytoplasmic sequestration (Bosari et al. 1995; Moll et al. 1996; Ryan et al. 1994). Although many studies have demonstrated nuclear localization is central to p53 function, recent studies have implicated a role for cytoplasmic p53 in p53 function (Green and Kroemer 2009). Therefore, to address the contribution of NLS1 to p53 function, we generated a mouse knock-in mutation in the p53 locus that abolished the primary Lysines in the NLS1 (Lys316–318). We have utilized gene targeting in mouse ES cells to generate mice bearing a NLS1 defective p53 allele, designated p53^{NLS1}. Our results confirm a critical role for p53 NLS1 in p53 function.

Interestingly, the mutant protein was stabilized in basal conditions, and could be detected by Western and immunofluorescence, whereas wildtype protein can not be detected. We detected either a diffuse staining pattern or a predominantly cytoplasmic staining pattern in unstressed p53^{NLS1/NLS1} cells (Fig. 4). This observation has been shown in in vitro overexpression studies. Cells with expressing a p53cDNA with the three Lysines in the NLS1 mutated to Alanine exhibited a cytoplasmic and partial nuclear p53 staining (Liang and Clarke 1999a).

The elevated p53NLS1 protein in basal conditions was unexpected as wildtype p53 is under tight control in the non-stressed cells and maintained in low levels. It is interesting that the p53NLS1 protein is not degraded in the cytoplasm, as that is the major site of p53 degradation (Liang and Clarke 2001). Thus, p53NLS1 is likely not being targeted for degradation, despite its cytoplasmic localization (Yu et al. 2000). The site in the NLS1 is also a site for ubiquitination, therefore, it is possible the protein is not receiving the proper signal for degradation, such as mono-ubiquitination by Mdm2 (Li et al. 2003). Studies are underway to look at the ubiquitination status of the p53NLS1 protein.

In addition, we observed very little nuclear accumulation/stabilization of p53 in p53^{NLS1/NLS1} cells in response to UV (Fig. 4, bottom panels). Whereas almost 100% of wild type cells had strong induction of p53 in the nucleus, 16% of p53^{NLS1/NLS1} cells were observed to have nuclear induction. These observation suggests that the NLS1 is a predominant site for control of subcellular localization for p53 upon UV treatment.

Supporting a role for NLS1 in the regulation of p53 activity, cells derived from p53^{NLS1/NLS1} mice exhibit loss of p53 function. MEFs from p53^{NLS1/NLS1} animals had a faster proliferation rate than wildtype MEFs (Fig. 2a). p53^{NLS1/NLS1} cells grew faster than p53^{-/-} cells, although they reached similar saturation densities. It is not clear why this occurred, however, basal p53 levels are greater in p53^{NLS1/NLS1} cells. This would argue that increased p53 should inhibit cell growth. It is possible the mutation in p53NLS1 is providing a gain-of-function, in either the cytoplasm or nucleus (where it can be detected in non-stressed

cells). There is data that the p53NLS1 protein can have some normal function. For instance, *p53^{NLS1}* cells exhibited a partial defect in cell cycle arrest in response to gamma radiation and induced p21 (Fig. 2b, c). However, several p53 functions were disrupted. *p53^{NLS1/NLS1}* cells were able to escape *Ras*-induced senescence (Fig. 3). Thymocytes from *p53^{NLS1/NLS1}* animals exhibited complete survival in response to gamma radiation (Fig. 5a, b). In addition, *p53^{NLS1/NLS1}* E1A-MEFs were more resistant to death than *p53^{-/-}* E1A-MEFs (Fig. 5c). The loss of apoptosis correlated with defective induction of *NOXA* (Fig. 5d). This observation, along with the cell growth advantage, suggests that the p53 NLS1 mutation imparted a gain of function phenotype to the cells.

p53 can mediate a cytoplasmic death program [reviewed in (Fuster et al. 2007)]. In one model, p53 exerts a BH3-like role. Studies have suggested this is transcription-independent or dependent on priming by induction of PUMA (Chipuk et al. 2005). Another model of cytoplasmic p53-dependent death involves translocation of p53 to the mitochondria. Recent work suggests that monoubiquitination of p53 allows for mitochondrial targeting (Marchenko et al. 2007). Studies have demonstrated Mdm2 can ubiquitinate proteins in the cytoplasm (Yu et al. 2000). Therefore, it is interesting the *p53^{NLS1/NLS1}* cells do not die even with increased levels of p53 protein in the cytoplasm. The mutation lies within ubiquitination sites that are important to mitochondrial targeting as well as nuclear targeting.

We observed a dramatic loss of induction of *NOXA* in response to UV (Fig. 5d). Therefore, this mutation may be contributing to the loss of function of the residual p53^{NLS1} protein in the nucleus. In support of this possibility, transgenic mice have been generated wherein the PCAF1/NLS1 site in p53 was mutated, and Lys317 was replaced with an Arg residue (Chao et al. 2006). Increased apoptosis and *NOXA* and PUMA expression was observed in these *p53^{Lys317Arg}* cells, suggesting that acetylation negatively regulates p53-dependent transcription of *NOXA*. Thus, Alanine, which can mimic neutralization imparted by acetylation (Ren and Gorovsky 2001), would be predicted to have less expression *NOXA*. It is interesting to speculate that the protein may also exert some functions as a mimic of charge patch neutralization. Irrespective of the model, the p53^{NLS1} protein is not competent of undergoing p53-dependent cytoplasmic/mitochondrial death. Furthermore, it appears that p53NLS1 mutation interferes with induction of p53-mediated apoptotic genes.

Surprisingly, we found that the loss of the NLS1 lead to embryonic and peri-natal lethality. *p53^{NLS1/NLS1}* animals develop exencephaly as early as embryonic day 9.5. A subset of *p53^{-/-}* mice exhibit exencephaly (Armstrong et al. 1995; Sah et al. 1995). It is possible loss of p53-mediated gene expression during embryonic development induces exencephaly, which would suggest the nuclear p53NLS1 protein is mimicking a *p53-null* allele (where there is no nuclear p53 expression). Mice deficient in the p53 transactivation domain (*p53^{QS}* animals) also exhibit embryonic lethality (Johnson et al. 2005). However, these mice die earlier than *p53^{NLS1/NLS1}* mice, and mostly reabsorptions are detected. Thus, it is unlikely that the exencephaly observed in *p53^{NLS1/NLS1}* embryos was due solely to the loss of p53-transcriptional activity. The *p53^{NLS1/NLS1}* mice will be important for examining the layers of complex regulation that govern p53 functions during development and growth suppression.

Methods

Generation of targeting vector

The targeting vector was generated using a portion of the p53 gene (129SVBrd-strain) spanning an *Xho*I to *Hind*III fragment 5496 base pair from exon 2 to intron 11 of the p53 gene. Lysines 316–318 in exon 9 were mutated using oligonucleotide site directed mutagenesis (QuickChange Kit, Stratagene) with a sub-fragment of the targeting vector. The subfragment was sequenced to confirm the mutation and subcloned back into the targeting vector. Additional sequencing of the targeting vector revealed no mutations (data not shown). A Neo^R cassette flanked by LoxP sites was introduced in an *Avr*2 restriction endonuclease site. An MC1-TK gene was added to the 3' end of the targeting vector.

Generation of targeted ES cells and p53^{NLS1} mice

PC3 ES cells (O'Gorman et al. 1997) were electro-porated with the targeting vector and drug selected with G418 and FIAU. ES cells were screened and correctly targeted clones were identified by Southern blot and PCR analysis of *Eco*R1-digested genomic DNA using 5' and 3' probes external to the targeting vector. We identified 10 positive ES clones for recombination. The presence of the Alanine mutation in the ES clones was confirmed by PCR as described below. Chimeric mice were created by microinjection of the ES cells into E3.5 blastocysts (C57BL/6 strain) and the embryos were surgically implanted into pseudopregnant foster mice using standard procedures. The male chimeric mice were bred with female C57BL/6J mice (The Jackson Laboratories). Excision of the Neo^R cassette in the F1 generation was confirmed by digestion with *Eco*RI and *Not*I and Southern analysis using a p53 intron 1 probe (data not shown). All mice were on a mixed 129/Sv × C57BL/6 background. All research with mice complies with federal and institutional policies, as well as guidelines established by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School.

PCR genotyping and sex determination

Lysine 316, 317 and 318 were mutated to an Alanine. A *Not*I restriction site was introduced by mutagenesis. The presence of the Alanine mutation in the mice tail DNA was confirmed by PCR using oligonucleotide primers to exon 8 forward (5'-GAGAGACCGCCGTACAGAAG -3') and intron 9 reverse (5'-CCATCCCCAGTGGAGGAGCACCTGTTTAT G-3') and subsequent digestion of the PCR fragments with *Not*I. The wild-type PCR product was digested to yield one fragment, 477 bp. The mutated PCR product yielded 279 bp and 198 bp DNA fragments following *Not*I digestion (Fig. S2B).

The PCR to determine embryo sex was done with Forward primer: CCGCTGCCAAATTCTTTGG and Reverse primer: 5'-TGAAGCTTTTGGCTTTGAG-3'. PCR of a male will give 2 bands and female one band at 300 bp (Mroz et al. 1999).

Cell growth studies and immunofluorescence

Murine embryonic fibroblasts (MEFs) were generated and maintained as described (Sluss et al. 2004). Growth curves were performed with triplicate plating of wildtype, p53^{NLS1/+},

p53^{NLS1/NLS1}, *p53^{-/+}*, and *p53-null* early passage MEFs (E13.5). Cells were plated (5×10^5 cells per 10 cm dish) in triplicate and cell number was determined each day 7 days post-plating using a hemocytometer. Proliferation assays were done with two independent lines of each genotype, and one representative experiment is presented.

Immunofluorescence was performed on low passage primary MEFs. Cells were either untreated or treated (60 J/m^2 UV) and fixed 18 h post treatment. Cells were stained with sheep anti-p53 (Ab-7) and biotin anti-sheep (Calbiochem), and then p53 was visualized with Streptavidin-Alexa 488. DNA was stained with DAPI. Cells were analyzed on a Leica Confocal Microscope.

Ras transformation assay

Retroviral transduction studies were performed with recombinant viruses generated using Bosc cells and the vectors pBabe-Puro and pBabe-*HaRas61L*-Puro as described (Armata et al. 2007). Transduced MEFs were plated at 25,000 cells/well (12-well multi-dish). Relative viability was determined at days 2, 4, and 6 post-plating by trypan blue staining. The number of senescent cells was determined by staining for the senescence marker acidic β -galactosidase (Dimri et al. 1995).

Protein analysis and westerns

For western analysis, MEFs were treated with DNA damaging agents [UVC light (50 J/m^2), gamma irradiation (IR 20 Gy) and doxorubicin (Dox $50 \mu\text{g/ml}$)] and harvested 8 h post treatment. Following treatment, cells were lysed in NP-40 lysis buffer and western performed for p53 as described (Armata et al. 2007). p53 antibody (Ab-7) was obtained from Calbiochem, anti- α -Tubulin from Sigma and anti-p21 from Santa Cruz. Bound Antibody was visualized by enhanced chemiluminescence (ECL, Amersham).

DNA damage response

For growth arrest, cells were treated with 8 Gy, labeled with bromodeoxyuridine (BrdU) for 3 h and harvested 18 h post-treatment. Replicative DNA synthesis was quantified using bivariate flow cytometry from propidium iodide-complexes as described (Sluss et al. 2004) on a FACStar flow cytometer. Cell cycle distribution was determined using FLOW Jo software. Similar numbers of cells were analyzed for each sample.

Thymocyte CD4/CD8 and viability analysis

For CD4/CD8 staining, animals were irradiated (8 Gy) with a Gammacell 40 irradiator and thymocytes harvested (24 h post-treatment). Thymocytes were stained with anti-CD4-PE and anti-CD8-FITC (Oncogene Research Products) as described (Sluss et al. 2004) and analyzed by FACS. After staining, the fraction of CD4/CD8-double positive thymocytes were quantified using FLOW Jo software. For viability analysis, thymocytes were removed from untreated and treated (8 Gy) animals 16 h post-treatment. Thymocytes were stained with fluorescein isothiocyanate-labeled annexin V antibody (Pharmingen) and PI (Sigma) as described (Sluss et al. 2004). Relative amounts of apoptotic cells were determined by binding of annexin V and subsequent FACS analysis. The fraction of non-apoptotic

thymocytes post irradiation was measured by calculating annexin-V- and 7AAD- negative populations and compared to untreated thymocytes.

Generation and analysis of E1A-MEFs

Retroviral transduction studies were performed as described (Armata et al. 2007) with recombinant viruses generated from the vectors pBabe-*Puro* and pBabe-*E1A*. Cells were selected with puromycin. For death assays, E1A-expressing cells were plated in triplicate 3–4 days prior to treatment, at 1.25×10^5 cells/6-well. Cells were treated with 5 J/m² of UV light. Relative cell number was obtained by the crystal violet staining method.

RNA preparation and analysis

RNA was prepared from tissues collected in RNA-later (Ambion) and snap frozen in liquid nitrogen. Total RNA was prepared with RNeasy kits (Qiagen) following manufacturer's instructions. The purified RNA was subjected to an additional DNase treatment (Ambion) to ensure removal of contaminating genomic DNA prior to final column purification. The relative expression of mRNA was examined by quantitative PCR analysis. cDNA was prepared using Superscript III (Invitrogen) with random hexamers and 0.5–1 µg of RNA per tissue. Quantitative realtime PCR was performed on a Biorad iCycler using SyBr Green master mix (Biorad). The primer sequences for the murine genes were: *Gapdh* (5'-CT TCACCACCATGGAGAAGGC-3'; 5'-GGCATGGA CTGTGGTCAT-3'); *p53* (5'-TGAAACGCCGACC TATCCTTA-3'; 5'-GGCACAAACACGAACCTCA AA-3'); and *NOXA* (5'-AGGAAGGAAGTTCCGCC G-3', 5'-AGCGTTTCTCTCATCACATCACA-3'). All samples were examined in triplicate and values were normalized for baseline expression and for expression of *Gapdh*. Calculations of values were made using the DDCT method. Statistical significance was calculated using CT values.

Data analysis

To calculate statistical changes, statistically significant differences ($P < 0.05$) between groups were examined using the two-tailed Student's *t*-test. Microsoft Excel was used for statistical calculations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Mutation of Lysines 316–318 (NLS1) in the *p53* locus. **a** Organization of the *p53* locus. Filled boxes represent exons. Restriction enzymes sites are indicated. **b** The targeting vector encodes a triple Lysine to Alanine missense mutation in exon 9. A floxed *Neo^R* gene was introduced in intron 9 to permit positive selection of ES cells. **c** Homologous recombination of the targeting vector in ES cells is illustrated. Probes used for Southern blot analysis are shown (hatched boxes). **d** The *Neo^R* gene is excised in the chimera due to expression of a protamine-Cre transgene. **e** Southern blot analysis of targeted ES cell DNA using a 5' external probe. *EcoR1* digest of ES cell DNA indicates the presence of the mutant allele (unexcised *Neo^R* gene, 11 kb) and the wildtype allele (14 kb)

Fig. 2.

Growth characteristics of *p53^{NLS1}* cells. **a** Effects of p53 NLS1 mutation on cell growth. Cells were plated in triplicate and cell number was determined at the indicated times. **b** Loss of p53 NLS1 leads to defects in cell cycle arrest. Cell cycle arrest of heterozygous and homozygous *p53^{NLS1}* MEFs in response to gamma radiation, compared to wild type and *p53^{-/-}* MEFs. Cells were harvested 18 h post-treatment. The decrease in S-phase in *p53^{NLS1/NLS1}* MEFs is significant (*, $P < 0.05$). **c** Expression of p53NLS1 protein. Western analysis of wildtype (*left panel*) and *p53^{NLS1/NLS1}* cells (*right panel*) in response to various genotoxic stress [UV (50 J/m²), gamma irradiation (IR 20 Gy) and doxorubicin (Dox 50 µg/ml)] and harvested at 8 h post-treatment. α-tubulin is the loading control

Fig. 3. *p53^{NLS1/NLS1}* cells escape *Ras* induced cellular senescence. **a** *Ras* infection does not reduce viability of *p53^{NLS1/NLS1}* cells. Cells were infected with pBabe (vector) or pBabe-*HaRas* were plated and growth recorded at the indicated time points. **b** *p53^{NLS1/NLS1}* cells do not senesce in response to *Ras*. Phase-contrast micrograph of cells infected with oncogenic *HaRas* stained with senescence marker β -galactosidase. **c** Number of cells staining positive for β -galactosidase in cells in response to oncogenic *Ras*

Fig. 4.

Analysis of $p53^{NLS1}$ localization. Representative staining in cells with or without UV treatment. Staining of untreated $p53^{NLS/NLS1}$ cells (*top panels*), wildtype cells treated with UV (*middle panels*), and UV-treated $p53^{NLS1/NLS1}$ cells (*bottom panels*). Cells were stained 18 h post-treatment with the anti-p53 antibody Ab-7 and DAPI. Nuclear accumulation in response to UV is defective in $p53^{NLS1}$ cells

Fig. 5.

Loss of p53 Lys316–318 (NLS1) abrogates p53-dependent death. **a** Protection of CD4⁺/CD8⁺ thymocytes in *p53^{NLS1/NLS1}* animals. Mice were treated in vivo with IR (8 Gy) or untreated and the thymocytes harvested 24 h post treatment. Cells were stained for CD4 and CD8. **b** Loss of p53 NLS1 leads to increased survival in thymocytes. Thymocytes were removed from untreated and treated (8 Gy) animals 16 h post-treatment. The percent of viable cells is presented (cells negative for annexin-V and 7AAD). **c** Loss of p53 NLS1 leads to increased survival in E1A-MEFs. MEFs were exposed to UV radiation (5 J/m²) and survival was measured at 18 and 24 h post-treatment. All numbers are normalized to the number of cells in untreated samples. Increased survival in *p53^{NLS1/NLS1}* MEFs compared to *p53^{-/-}* MEFs is significant (*, $P < 0.05$). **d** Defective NOXA induction in response to UV in *p53^{NLS1}* cells. The expression of NOXA mRNA was measured by quantitative real time PCR analysis of UV-treated MEFs (6 h post-treatment). The amount of *Gapdh* mRNA in each sample was used to calculate relative mRNA expression (mean $\pm$ S.E.M.; $n = 3$). The induction of NOXA was presented as the ratio of induced in wildtype/induced in *p53^{NLS1}* MEFs

Fig. 6. *p53^{NLS1/NLS1}* animals develop exencephaly. **a** Embryonic Day 15.5 embryos from a heterozygous *p53^{NLS1}* cross. Wildtype animal (on left), with complete closure of hindbrain. *p53^{NLS1/NLS1}* animals in the middle (female, NLS1-1) and right (male, NLS1-2), showing hindbrain exencephaly. **b** *p53^{NLS1/NLS1}* exencephalic embryo showing the eversion and exposure of neural tissue (arrows) pictured in frontal section (*left panel*) and back sagittal section (*right panel*)

Table 1Genotype of embryos from $p53^{NLS1}$ heterozygous intercross

Genotype	$p53^{+/+}$	$p53^{NLS1/+}$	$p53^{NLS1/NLS1}$	
Birth ($n = 131$)				
Obtained	61	67	3	
Expected	32.75	65.5	32.75	
Genotype	$p53^{+/+}$	$p53^{NLS1/+}$	$p53^{NLS1/NLS1}$	Reabsorptions
Embryo Day 14.5 ($n = 48$)				
Obtained	12	26	10	3
Expected	12	24	12	
Genotype	$p53^{+/+}$	$p53^{NLS1/+}$	$p53^{NLS1/NLS1}$	Reabsorptions
Embryo day 12.5 ($n = 46$)				
Obtained	12	25	9	6
Expected	11.5	23	11.5	
Genotype	$p53^{+/+}$	$p53^{NLS1/+}$	$p53^{NLS1/NLS1}$	Reabsorptions
Embryo day 9.5 ($n = 36$)				
Obtained	6	18	10	0
Expected	9	18	9	
Ratio of $p53^{NLS1/NLS1}$ embryos exhibiting exencephaly				
Embryo day 14.5				
Males/exencephaly			6/1	
Females/exencephaly			4/3	