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Serine 312 phosphorylation is dispensable for wild-type p53 functions *in vivo*

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Cellular stimulation results in phosphorylation of the tumor suppressor p53 on multiple residues, though the functional relevance is not always clear. It is noteworthy that the serine (S) 315 residue is unique, as it has been suggested to be phosphorylated not only by genotoxic signals, but also during cell-cycle progression and by endoplasmic-reticulum stress. However, *in vitro* data have been conflicting as phosphorylation at this site was shown to both positively and negatively regulate p53 functions. We have thus generated knock-in mice expressing an unphosphorylable S312 (equivalent to human S315), by substitution with an alanine (A) residue, to clarify the conflicting observations and to evaluate its functional relevance *in vivo.* Born at Mendelian ratios, the $p53^{S312A/S312A}$ mice show no anomalies during development and adulthood. p53 activation, stability, localization and ability to induce apoptosis, cell-cycle arrest and prevent centrosome amplification are not compromised in $p53^{S312A/S312A}$ cells. $p53^{S312A/S312A}$ mice are unable to rescue $mdm2^{-/-}$ lethality, and tumorigenesis – both spontaneous and irradiation/oncogene-induced – is not accentuated. Taken together, the results show that the S312 phosphorylation site is not in itself necessary for efficient p53 function, and advocates the possibility that it is neither relevant in the mouse context nor important for p53 functions *in vivo*.

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Regulation of p53 function is extremely crucial for the proper functioning of a cell, and thus the organism, as p53 activation restricts growth, whereas its functional loss leads to tumorigenesis.[1,2] Expectedly, p53 is found inactivated in most tumors,[3] and activation of p53 is only noted in extreme conditions when cells are subjected to stress, as physiologically, cells cannot tolerate activated p53.[4,5] Deregulated p53 activation leads to embryonic death, as shown in the case of the Mdm2 and Mdm4 deficient mice,[1] and also results in a dwarfed phenotype observed in mice expressing a mutant hyperactive p53.[1] Collectively, all these findings highlight the need for precise regulation of p53 function for physiological processes to ensue.

Not surprisingly, p53 is a labile protein whose levels are kept minimal primarily by the ubiquitin ligase Mdm2.[6] Several post-translational modifications (PTMs) including acetylation and phosphorylation have also been suggested to regulate p53's turnover.[7] Acetylation at the carboxyl-terminal lysines have been shown to be important for Mdm2-dependent degradation.[8] Furthermore, phosphorylation at the amino-terminal sites have been shown to be crucial for disrupting Mdm2 binding, and hence, critical for p53 stabilization.[9]

Most of the phosphorylation sites on p53 are clustered in its amino- and carboxyl-terminals.[7] Critical amino-terminal sites suggested to regulate p53 function include the serine (S) residues 15, 20 and 46.[10] Among the carboxyl-terminal sites, S392 had been shown to be specifically regulated by ultra violet (UV) irradiation (but not by IR) – highlighting signal specificity – and seems to have a part in regulating optimal p53 functions.[11] Other carboxyl-terminal sites, such as S315, S373 and S376 have also been implicated to varying extents.[10,12] Many of the phosphorylation sites are often modified in response to stress signals,[7] consistent with the notion that phosphorylation regulates p53 functions during the SOS response. However, the S315 site has been suggested to be unique, being regulated during cell-cycle progression and also upon endoplasmic-reticulum (ER) stress, an event in which p53 is not upregulated, besides genotoxic stress.[10,12–16] Two seemingly opposite sets of results have emerged from the analysis of the S315 site in mouse and human cell lines – one which highlights the importance of this phosphorylation site for proper p53 functioning, and the other which highlights its role in leading to p53 degradation.[12,13] One possible explanation for these conflicting findings is that these observations have been

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Abbreviations: A, alanine; BrdU, bromodeoxyuridine; ER, endoplasmic-reticulum stress; ES, embryonic stem; IR, ionizing radiation; MEF, mouse embryonic fibroblasts; PTM, post-translational modification; S, serine; WT, wild-type; UV, ultra-violet

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made using various phospho-mutant cDNAs, and therefore, the results may not absolutely reflect the physiological significance of the phosphorylation events because of the unusually high p53 levels in the overexpression systems. Thus, the solution to faithfully recapitulate physiological conditions rely on the recent advances in knock-in mouse models whereby the phosphorylable serine residues have been substituted to alainine residues – to mimic an unphosphorylated state – in the germ-line of mice.[1] We have therefore generated a knock-in S312A mutant mouse strain, in which the serine residue has been substituted with an unphosporylable alanine (A) residue, to evaluate the physiological importance of the S315 phosphorylation site. The detailed characterization of the $p53^{S312A/S312A}$ mice is presented.

Results

Generation of $p53^{S312A/S312A}$ knock-in mice. We first analyzed the protein sequence of p53 of various species by sequence alignment, which revealed that the S315 site and its neighboring sequences in human p53 are highly conversed among different species (Supplementary Figure 1A), with the S312 residue in mouse p53 being the corresponding site (Figure 1a).[17] S312 has also been shown to be phosphorylated on transformation in murine cell-based studies.[18,19] To confirm this, we transfected $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) with wild-type (WT) or S312A mouse *p53* cDNAs and treated them with the ER-stress inducer thapsigargin (TG)[12] and analyzed the phosphorylation status. We observed increased phosphsorylation of WT p53 over time, whilst this was not the case in the S312A-transfected cells (Supplementary Figure 1B), indicating that mouse S312 can indeed be phosphorylated *in vivo*.

We therefore constructed the gene-targeting vector harboring the S312A mutation to generate 'knock-in' mice to investigate the effects of the lack of this phosphorylation site *in vivo* (Figure 1b). Homologous recombinants in embryonic stem (ES) cells were identified by both PCR and Southern blot hybridization (data not shown), and the neomycin cassette was excised to obtain $p53^{+/S312A}$ ES cells that were blastocyst injected to generate the knock-in mice. Expression of the knock-in allele was confirmed by sequencing (Figure 1c) and by RT-PCR, wherein only the mutant *p53* transcripts were detected in homozygous $p53^{S312A/S312A}$ knock-in mice after *BstUI* digestion – which is specific to the S312A substitution – of the RT-PCR product (Figure 1d). We have also sequenced the entire p53 transcript and found no additional mutations (data not shown).

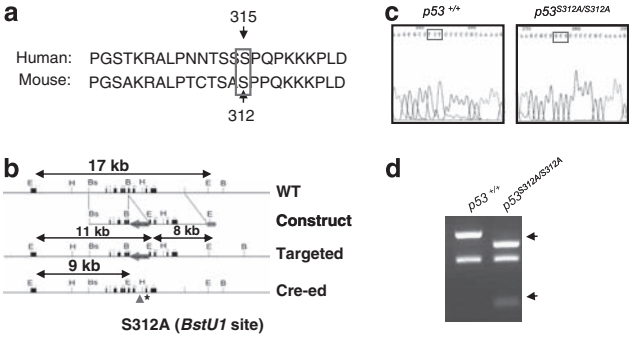

Figure 1 Generation of $p53^{S312A/S312A}$ knock-in mice. (**a**) Amino acid sequence alignment of the C-terminus of human and mouse p53 protein. Conserved serine (S) residues (315 and 312 in human and mouse, respectively) are indicated. (**b**) Schematic of the endogenous wild-type (WT) *p53* allele, the targeting construct, the targeted and final *S312A* knock-in allele after Cre-mediated neomycin cassette excision are shown. (**c**) Sequencing results of p53 transcripts from $p53^{+/+}$ and $p53^{S312A/S312A}$ cells. (**d**) *BstUI* digestion of RT-PCR product of mouse p53 transcript from $p53^{+/+}$ and $p53^{S312A/S312A}$ cells. The arrows indicate the fragments resulting from *BstUI* digestion of the S312A mutant transcript

$p53^{S312A/S312A}$ mice are viable and mice of all genotypes were born at normal Mendelian and gender ratios (Table 1 and data not shown). Of the 394 pups born, 104 were $p53^{S312A/S312A}$, in line with the expected number of 98.5 ($P = 0.2018$; Table 1). Moreover, $p53^{S312A/S312A}$ mice are fertile and give birth with normal litter size (data not shown). Macroscopic analysis of homozygous mutant mice revealed no significant alterations, both during embryogenesis and in the adult stage up to 2 years of age (data not shown).

The S312A p53 is functional in both cultured cells and tissues. We first investigated if the $p53^{S312A/S312A}$ mice could rescue the p53-dependent lethality due to Mdm2-deficiency. Offspring analysis from the $p53^{S312A/S312A}mdm2^{+/-}$ intercross revealed that the $p53^{S312A/S312A}$ mutants could not rescue the Mdm2-deficiency-dependent lethality, as there were no $p53^{S312A/S312A}mdm2^{-/-}$ offsprings born, unlike the presence of the $p53^{-/-}mdm2^{-/-}$ animals from control crosses (Table 2), suggesting that the S312A mutant p53 was not functionally compromised. We therefore investigated the biochemical properties of the S312A p53 protein using primary cells and tissues. The S312A p53 protein was phosphorylated at both S15 and S20 residues as efficiently as the WT protein, and showed a concomitant increase in the steady-state levels over time on γ-irradiation, both in primary MEFs and thymocytes (Figure 2a and Supplementary Figure 2A). Similar results were also obtained after UV irradiation of MEFs (Figure 2b). Furthermore, whole body γ-irradiation led to increased S312A p53 levels to similar extends as WT p53 in thymus and liver tissues (Figure 2c), confirming that the induction of S312A protein is not compromised both *in vitro* and *in vivo*. It is noteworthy that p53 can be induced in livers of young mice[20] (Supplementary Figure 2B), unlike in the older mice which are found to be radioresistant.[21]

To evaluate whether S312A p53 is truly a functional protein, we investigated the expression of some classical p53-target genes in various organs (thymus, spleen and liver) and MEFs, which showed p53-dependent transactivation to similar extends and with similar kinetics in both WT and $p53^{S312A/S312A}$ tissues and cells on γ-irradiation (Figure 2d). Moreover, cell-death rates were similar between $p53^{S312A/S312A}$ and $p53^{+/+}$ thymocytes on γ-irradiation over time and over various doses (Figure 2e and data not shown), and were unlike $p53^{-/-}$ cells, indicating that the S312A protein is indeed functional.

Analysis of cell-cycle progression also revealed that the growth rates were comparable between the $p53^{S312A/S312A}$ and the $p53^{+/+}$ MEFs, unlike the fast growing $p53^{-/-}$ MEFs (Figure 2f). Bromodeoxyuridine (BrdU) pulse-chase experiments confirmed these data ($p53^{S312A/S312A}$ *versus* $p53^{+/+}$ *versus* $p53^{-/-}$: 20.76% *versus* 22.57% *versus* 40.69%; Supplementary

Figure 3A). Furthermore, cycling kinetics of the BrdU$^+$ cells was found to be similar between *p53*$^{S312A/S312A}$ and *p53*$^{+/+}$ MEFs (Supplementary Figure 3B). Analysis of total p53 levels, its subcellular localization and the expression of p53 target-genes and cyclins in synchronous cultures of *p53*$^{S312A/S312A}$ and *p53*$^{+/+}$ MEFs also revealed no differences (Supplementary Figure 3C and data not shown), suggesting that phosphorylation at S312 is not essential for cell-cycle progression under normal culture conditions. We therefore extended our investigations to evaluate whether stress-induced cell-cycle arrest was affected. Doxorubicin treatment or γ-irradiation was found to effectively reduce the number of BrdU$^+$ cells in both *p53*$^{S312A/S312A}$ and *p53*$^{+/+}$ populations (*p53*$^{S312A/S312A}$ *versus p53*$^{+/+}$ *versus p53*$^{-/-}$ cells $\rightarrow$ doxorubicin: 0.34 ± 0.04 *versus* 0.48 ± 0.16 *versus* $16.94 \pm 0.44\%$; γ-irradiation: $1.43 \pm 0.45\%$ *versus* $2.00 \pm 0.86\%$ *versus* $25.24 \pm 2.30\%$; *p53*$^{S312A/S312A}$ *versus* *p53*$^{+/+}$: $P = 0.2381$ for doxorubicin and $P = 0.3871$ for γ-irradiation; Figure 2g), suggesting that S312 phosphorylation is also dispensable for stress-induced cell-cycle arrest. All these results together indicate that S312 phosphorylation is not required for proper p53 functioning under normal and stress conditions.

p53 stability and ubiquitination is not affected in *p53*$^{S312A/S312A}$ cells. As S315 phosphorylation was previously shown to reduce p53 stability,[12] we investigated the stability of S312A mutant p53. The turnover rate of both WT and S312A p53 were found to be similar in cycloheximide pulse-chase experiments in unstressed MEFs ($T_{\frac{1}{2}}$ (mins) – S312A *versus* WT: 15.79 *versus* 16.51, $P = 0.4168$; Figure 3a). After γ-irradiation, the half-life of p53 protein was significantly increased between 3 and 6 h in both cases (Figure 3b), together suggesting that degradation of p53 may not be affected by the status of the S312 residue. As p53 is degraded in an ubiquitin/Mdm2-dependent manner,[22–24] we assayed for ubiquitination and found both WT and S312A p53 to be ubiquitinated to the same extend in unstressed cells (Figure 3c). The ubiquitination was also reduced to similar extends on doxorubicin treatment. We further tested the physiological effects of inhibiting the association between p53 and Mdm2, using nutlin which disrupts this association, which revealed that the numbers of BrdU$^+$ cells were reduced to similar extends in both *p53*$^{S312A/S312A}$ and *p53*$^{+/+}$ cultures, unlike *p53*$^{-/-}$ MEFs (*p53*$^{S312A/S312A}$ *versus p53*$^{+/+}$ *versus p53*$^{-/-}$: $0.48 \pm 0.31\%$ *versus* $0.725 \pm 0.08\%$ *versus* $23.57 \pm 3.78\%$; *p53*$^{S312A/S312A}$ *versus p53*$^{+/+}$: $P = 0.3703$; Figure 3d). These data together show that the S312 residue is not required for regulation of p53 ubiquitination, stability and function, including that which is mediated by Mdm2.

Table 1 S312A mice are born at Mendelian ratio

	p53$^{+/+}$	*p53*$^{S312A/+}$	*p53*$^{S312A/S312A}$
Observed number of mice	80	210	104
Expected number of mice (ratio of 1:2:1)	98.5	197	98.5

The number of mice of various genotypes obtained by the *p53*$^{+/S312A}$ intercross is shown. The expected number of mice was calculated according to the total number of mice born and based on the expected Mendelian 1:2:1 ratio.

S312 phosphorylation is dispensable for ER stress-induced cytoplasmic localization. Phosphorylation of S376 and S315 of human p53 has been shown to be necessary for the cytoplasmic localization on ER stress.[12] However, we have recently reported that S373 phosphorylation on mouse p53 (the equivalent site of S376 of human p53) is dispensable for this process.[25] We thus tested whether the presence of an unphosphorylable S312A p53 would affect its cytoplasmic localization on ER stress. Nuclear p53 levels were reduced on treatment with the ER stress-inducer TG in *p53*$^{+/+}$ MEFs (Figure 4a, left). A similar reduction was also observed in *p53*$^{S312A/S312A}$ MEFs (Figure 4a, left), though p53 levels did not clearly and distinctly increase in the cytosolic fractions in both WT and *p53*$^{S312A/S312A}$ cells (Figure 4a, right). Therefore, to ascertain that the ER stress-induced nuclear p53 reduction is indeed due to increased nuclear export and not due to reduced translation, we used leptomycin B to block nuclear export of p53. Leptomycin B treatment completely abrogated the reduction of p53 protein by ER stress in both *p53*$^{+/+}$ and *p53*$^{S312A/S312A}$ MEFs (Figure 4b), suggesting that both WT and S312A p53 proteins can be exported to cytosol effectively on ER stress, and perhaps not detected in the cytoplasmic fractions due to accelerated degradation.

Consistently, TG treatment reduced nuclear p53 accumulation that occurred on UV-irradiation in both the WT and *p53*$^{S312A/S312A}$ MEFs (Figure 4c). Immunoblot analysis revealed that both WT and S312A p53 protein induction was compromised to similar extends in cells treated with TG before UV (Figure 4d) or γ-irradiation (data not shown), as previously published.[12] Furthermore, analysis of p53 levels after TG

Table 2 S312A mutant does not rescue Mdm2-dependent lethality

	Offsprings of *p53*$^{S312A/S312A}$*mdm2*$^{+/-}$ × *p53*$^{S312A/S312A}$*mdm2*$^{+/-}$			
Genotype	*p53*$^{S312A/S312A}$*mdm2*$^{+/+}$	*p53*$^{S312A/S312A}$*mdm2*$^{+/-}$	*p53*$^{S312A/S312A}$*mdm2*$^{-/-}$	Total
Observed number of mice	19	42	0	61
	***Offsprings of p53*$^{-/-}$*mdm2*$^{+/-}$ × *p53*$^{-/-}$*mdm2*$^{+/-}$**			
Genotype	*p53*$^{-/-}$*mdm2*$^{+/+}$	*p53*$^{-/-}$*mdm2*$^{+/-}$	*p53*$^{-/-}$*mdm2*$^{-/-}$	Total
Observed number of mice	25	58	24	107

The number of offsprings obtained for the various *Mdm2* genotypes from the *p53*$^{S312A/S312A}$*; mdm2*$^{+/-}$ and *p53*$^{-/-}$*; mdm2*$^{+/-}$ intercrosses are shown. A total of 61 mice born from *p53*$^{S312A/S312A}$*; mdm2*$^{+/-}$ intercross and 107 mice born from *p53*$^{-/-}$*; mdm2*$^{+/-}$ intercross.

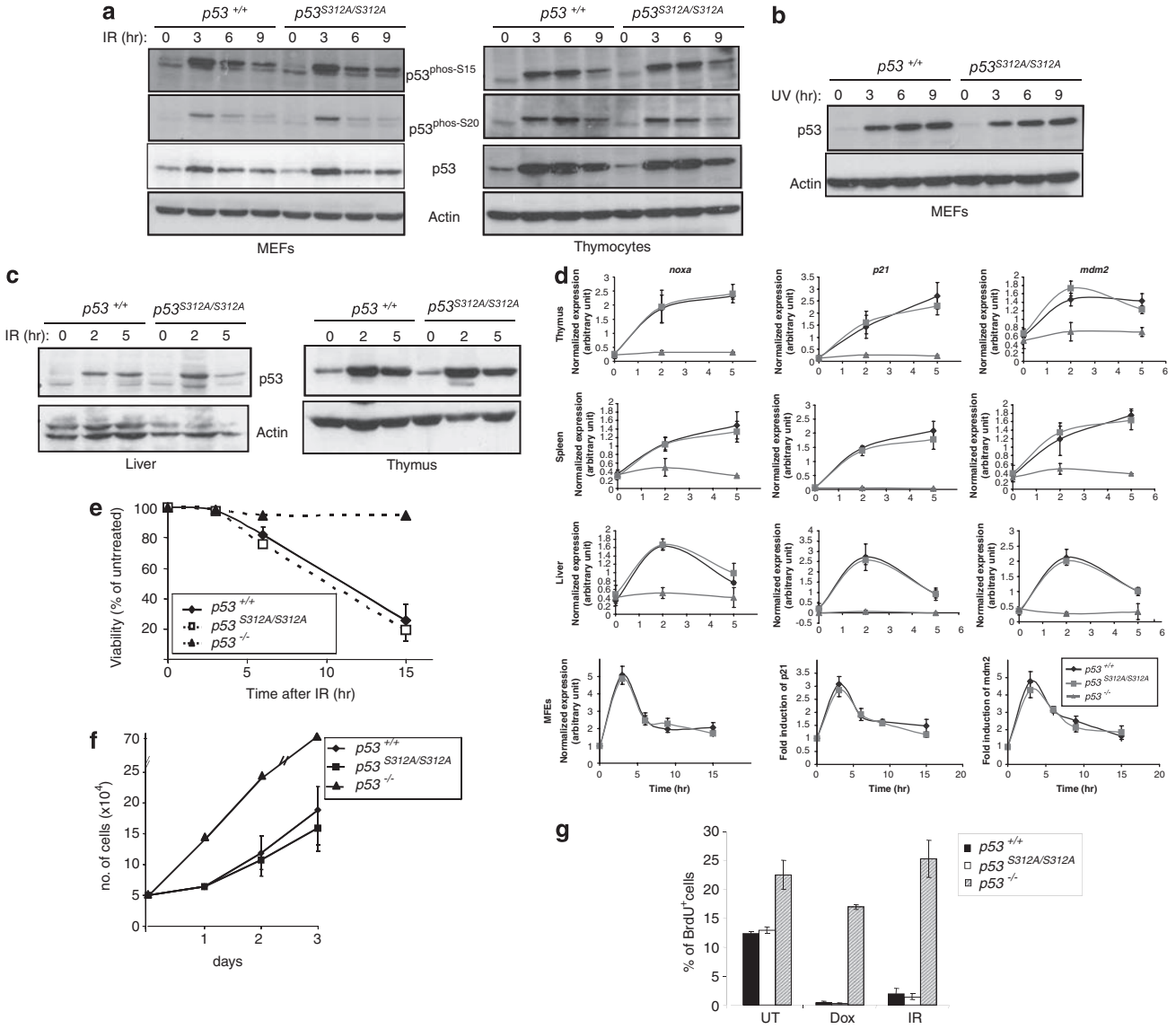

Figure 2 Activation and functional analysis of S312A p53 upon genotoxic stresses. (**a**, **b**) MEFs and thymocytes were γ-irradiated (IR) at 10 and 5 Gy, respectively, and the phospho-p53 and total p53 levels were analyzed by western blotting at various time points (**a**). MEFs were UV irradiated (60 J/m^2) and analyzed in (**b**). Thymocytes from three age-matched (5-week-old) mice and three independent MEF clones were analyzed and representative results were shown. (**c**) Organs from 4-week-old $p53^{+/+}$ and $p53^{S312A/S312A}$ mice (3–4 mice/genotype/time point) were whole body γ-irradiated (10 Gy) and harvested at indicated time-points and analyzed for p53 expression. Representative blots are shown. (**d**) mRNA from organs of whole-body γ-irradiated mice described in (**c**) and MEFs (4–5 independent clones/genotype) were used for analysis of p53 target genes (*noxa*, *p21* and *mdm2*) by qRT-PCR and normalized against *gapdh* expression. Data represent mean ± S.D. (**e**) Viability of thymocytes were determined by annexin-V/PI staining after 5 Gy irradiation. Data represent mean ± S.D. from one of the three independent experiments using thymoctyes isolated from three mice/genotype. (**f**–**g**) Primary MEFs were plated at 5x10^4 cells/well in 6-well plates and were counted for 3 consecutive days to monitor growth rate (**f**). These cells were exposed to doxorubicin (0.5 μg/ml) or IR (10 Gy) for 24 h and were subsequently treated with 10 μM BrdU for 1 h before harvesting and analysis of BrdU$^+$ population by flow cytometry (**g**). Data represent mean ± S.D. from three independent clones/genotype

treatment followed by γ-irradiation confirmed that this phenomenon also occurs in thymocytes (Figure 4e). Taken together, it is evident that the S312A p53 can be exported out of the nucleus by ER stress regardless of stimuli and cell-type, suggesting that this phosphorylation site is not crucial in regulating the nuclear-cytoplasmic shutling of p53.

Centrosome duplication and carcinogenesis are not accentuated by the presence of S312A p53. Human p53 has been shown to regulate centrosome duplication, partially through the phosphorylation of the S315 residue.[26] Hence, we tested whether centrosome duplication was affected in $p53^{S312A/S312A}$ MEFs. Most of the untreated WT or $p53^{S312A/S312A}$ MEFs have one or two centrosome(s) per cell, whereas the $p53^{-/-}$ cells showed a slight increase in the number of cells having three or more centrosomes (percentage of cells with three or more centrosomes in $p53^{S312A/S312A}$ *versus* $p53^{+/+}$ *versus* $p53^{-/-}$ MEFs: 4.326 ± 0.71 *versus* 4.043 ± 0.88 *versus* 11.77 ± 1.46; $p53^{S312A/S312A}$ *versus* $p53^{+/+}$: $P = 0.918$, and $p53^{+/+}$ *versus* $p53^{-/-}$:

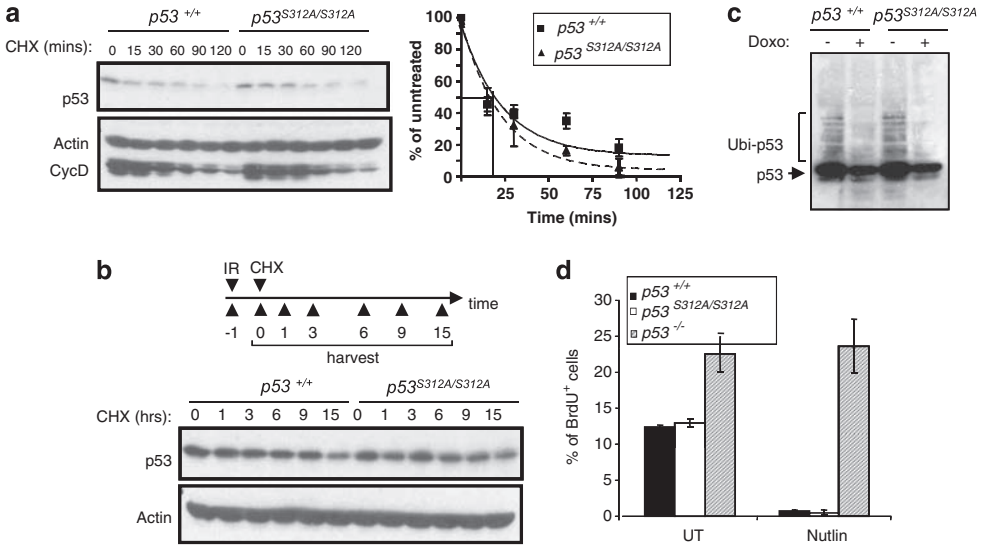

Figure 3 Stability and degradation of S312A p53. (**a**, **b**) Unstressed (**a**) or γ-irradiated (10 Gy, 1 h) (**b**) $p53^{+/+}$ and $p53^{S312A/S312A}$ MEFs were treated with 50 μg/ml cycloheximide and harvested for p53 analysis. Cyclin D and actin were used as controls (positive and loading, respectively). The experiment was repeated with a total of three clones of MEFs/genotype and the normalized p53 level was plotted as mean ± S.E.M. The exponential decay curve was determined by nonlinear regression and used to calculate the half-life of the p53 protein (**a**, right panel). Schematic of order of treatment for irradiated samples is shown in top panel (**b**). (**c**) $p53^{+/+}$ and $p53^{S312A/S312A}$ MEFs were treated without or with 0.5 μg/ml doxorubicin for 1 h and the ubiquitinated p53 levels were determined by immunoblotting. (**d**) Primary MEFs were treated with 10 μM nutlin for 24 h followed by 10 μM BrdU incubation for 1hr before harvesting and analysis by flow cytometry. Data represent mean ± S.D. from three independent clones/genotype

$P<0.0001$; Figure 5a). Treatment with hydroxyurea leads to inhibition of DNA synthesis and arrest in S-phase, although centrosome duplication continues, and p53 has been shown to suppress the re-duplication of duplicated centrosomes.[26] Hydroxyurea treatment resulted in majority of the $p53^{S312A/S312A}$ and $p53^{+/+}$ MEFs having two centrosomes per cell, whereas most $p53^{-/-}$ MEFs had three or more centrosomes per cell (percentage of $p53^{S312A/S312A}$ *versus* $p53^{+/+}$ *versus* $p53^{-/-}$ MEFs with one centrosome per cell: 12.25 ± 1.71 *versus* 16.5 ± 1.18 *versus* 4.22 ± 0.98; with two centrosomes per cell: 54.47 ± 4.19 *versus* 54.95 ± 2.22 *versus* 13.11 ± 5.9%; and with three or more centrosomes per cell: 27.71 ± 3.39 *versus* 22.33 ± 1.46 *versus* 82.67 ± 5.87; $p53^{S312A/S312A}$ *versus* $p53^{+/+}$: $P=0.0632$ and $p53^{+/+}$ *versus* $p53^{-/-}$: $P<0.0001$ for three or more centrosomes per cell; Figure 5a). These results indicated that functional p53 is indeed important for regulating centrosome duplication, and the presence of the S312A p53 was sufficient for this process.

We next evaluated whether the $p53^{S312A/S312A}$ mice were susceptible to cancer formation, in three different contexts: spontaneous tumor formation, after DNA-damage by γ-irradiation and in the presence of the *Eμ-myc* oncogene. Unlike $p53^{-/-}$ mice that succumb to spontaneous tumors between 3–6 months of age, $p53^{S312A/S312A}$ homozygous mice did not develop any spontaneous tumors up to 2 years (data not shown). Therefore, the effect of γ-irradiation (4 Gy) was investigated by irradiating 5-week-old mice and monitoring over time. Though there was a decrease in the survival of irradiated mice, there was no statistically significant difference between the median survival of the $p53^{S312A/S312A}$ and $p53^{+/+}$ mice (median survival (weeks) of $p53^{S312A/S312A}$ *versus* $p53^{+/+}$ mice after IR: 44.9 *versus* 45.7, $P=0.8822$; Figure 5b), suggesting that S312 phosphorylation is not important for the tumor suppressive activity of p53 on genotoxic insult.

Finally, the effects of the S312A protein were analyzed in oncogene-induced carcinogenesis, using the *Eμ-myc* transgenic mice. Whereas the survival of the $E\mu\text{-}myc^{Tg}$; $p53^{+/-}$ mice was dramatically reduced compared with that of $E\mu\text{-}myc^{Tg}$; $p53^{+/+}$ mice (median survival (weeks) of $E\mu\text{-}myc^{Tg}$; $p53^{+/-}$ *versus* $E\mu\text{-}myc^{Tg}$; $p53^{+/+}$ mice: 6.36 *versus* 19.0, $P<0.0001$), there was no significant difference in the survival between $E\mu\text{-}myc^{Tg}$; $p53^{S312A/S312A}$ and $E\mu\text{-}myc^{Tg}$; $p53^{+/+}$ mice (median survival (weeks) of $E\mu\text{-}myc^{Tg}$; $p53^{S312A/S312A}$ *versus* $E\mu\text{-}myc^{Tg}$; $p53^{+/+}$ mice: 19.00 *versus* 19.57, $P=0.7903$; Figure 5c), indicating that the S312A p53 is indeed functionally active as WT p53. Nevertheless, we performed microarray analysis to detect any subtle differences using B-lymphoma cells, and found the gene expression profiles from the $E\mu\text{-}myc^{Tg}$; $p53^{S312A/S312A}$ and $E\mu\text{-}myc^{Tg}$; $p53^{+/+}$ mice to be almost identical (Supplementary Figure 4). These data lead us to conclude that the S312 phosphorylation of mouse p53 is dispensable for its tumor-suppressor functions.

Discussion

The data presented in this study, show that altering the S312 residue of mouse p53 does not affect any of the known functions of p53 both *in vitro* and *in vivo*, indicating that this phosphorylation site may not be relevant in the regulation of p53 function.

S312 phosphorylation was noted more than two decades ago in mouse NIH3T3 cells, specifically on SV40-transformation.[18,19]

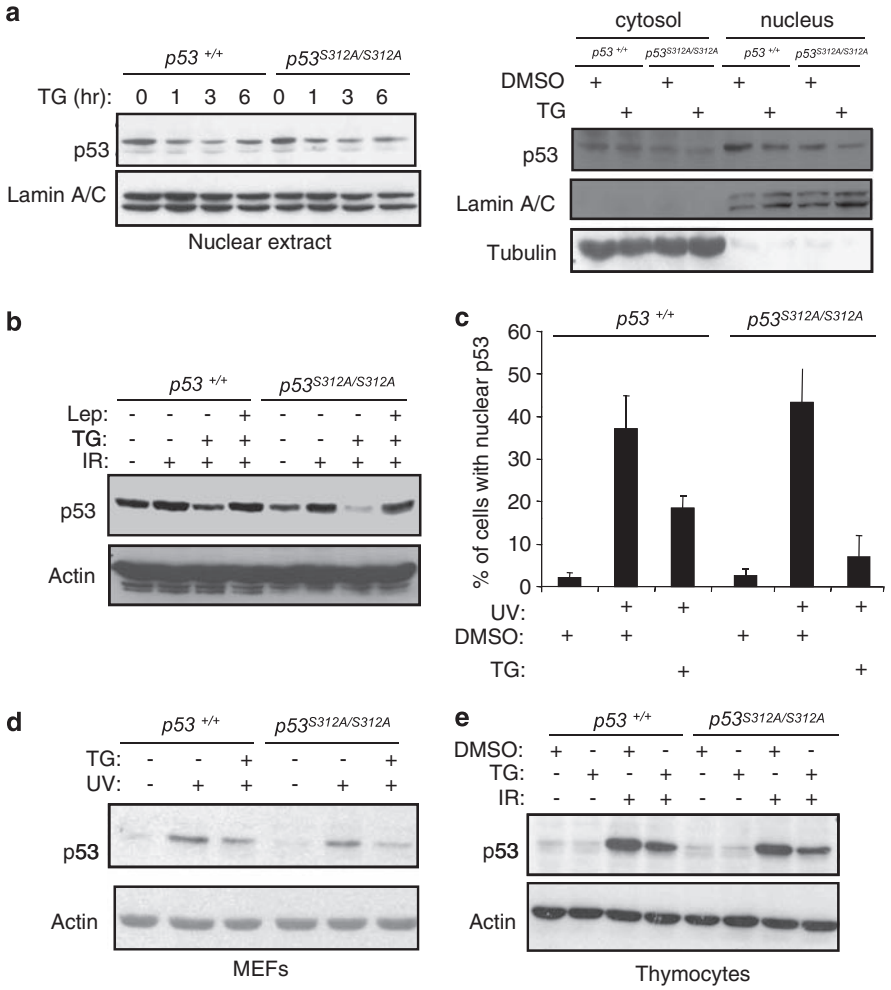

Figure 4 S312A mutant p53 is not defective in nuclear export on ER stress. (**a**) Unstressed $p53^{+/+}$ and $p53^{S312A/S312A}$ MEFs (three clones of MEFs/genotype) were treated with 1 μM thapsigargin (TG) and 100 μg of nuclear extract was used for p53 analysis (left). Both nuclear and cytosolic proteins were fractionated from MEFs treated with 1 μM TG for 3 h, and analyzed by immunoblotting (right). (**b**) MEFs (three clones of MEFs/genotype) were pretreated with 1 μM TG and 30 ng/ml leptomycin B for 1 h, before γ-irradiation (5 Gy, 3 h) and immunoblot analysis. (**c**) MEFs (as above) were pretreated with 1 μM TG or DMSO (control) before 20 J/m^2 UV treatment and collected 15 h later for analysis of subcellular localization of endogenous p53 protein by fluorescence immunocytochemistry. Approximately 100 cells were counted per genotype. (**d**, **e**) MEFs (**d**) and thymocytes (**e**) were UV (20 J/m^2, 15 h) or γ (5 Gy, 3 h) irradiated and whole cell lysates were used for p53 analysis. Experiment was repeated with three clones of MEFs/genotype or three mice/genotype for thymi and representative blots are shown in all cases

Subsequent studies have identified several kinases that phosphorylate this site and documented the effects of this phosphorylation event, with results being not congruent at all times. For example, initial studies indicated that S315 was phosphorylated by the cell-cycle regulated p34^{cdc2} kinase,[16] as well as on UV irradiation.[13] Nevertheless, both the S312A and the S315A mutants were found to be defective neither in their transactivation ability,[10,27] nor in their stability and cytoplasmic-nuclear translocation,[28–30] leading to the conclusion that phosphorylation at S312/S315 may regulate other functions of p53. However, subsequent studies suggested that phosphorylation on this site led to decreased tetramer stability,[31] and that the S315A mutant had reduced transactivation potential.[13] Recent studies have suggested a critical role for this site in regulating centrosome duplication,[26] and in the nuclear retention and binding affinity of p53 to target genes.[14] Conversely, ER stress was reported to activate GSK3β, which was shown to phosphorylate S315, leading to its cytoplasmic export.[12] Consistently, Aurora A was shown to phosphorylate p53 at this site leading to ubiquitination by Mdm2,[32] and consequently, S315A was shown to be retained in the nucleus and less ubiquitinated. Collectively, these *in vitro*-derived data indicated that the S312/S315 phosphorylation may be important in the regulation of p53 function, though to varying degrees and probably in a context-dependent manner.

We therefore embarked on evaluating the effect of this phosphorylation site *in vivo* by generating a knock-in mutant mouse strain. To our surprise, we did not observe any defects in transactivation, cell-cycle arrest and apoptosis, inhibition of centrosome amplification and tumor formation under various conditions. Moreover, the S312A mutant p53 was also unable to rescue embryonic lethality due to Mdm2 deficiency, altogether indicating that the S312A mutant behaves

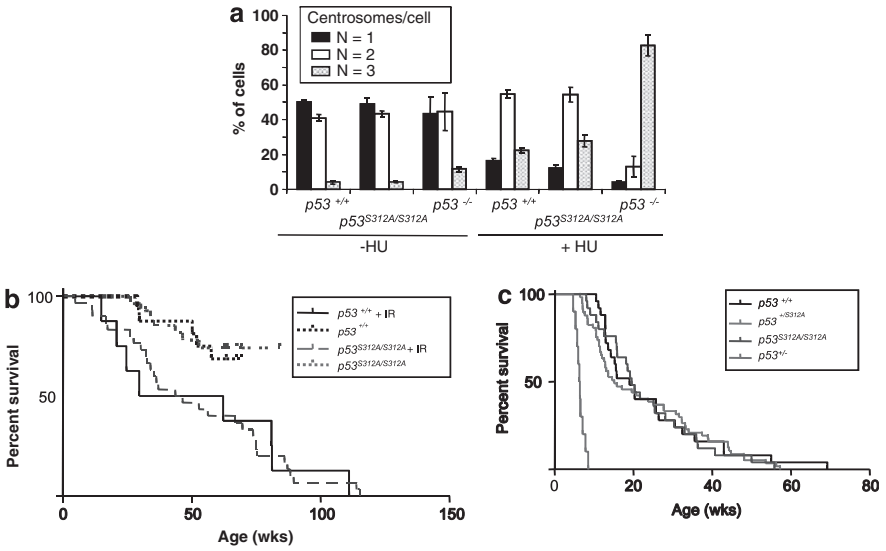

Figure 5 S312A mutant p53 does not accentuate centrosome duplication and tumor susceptibility. (**a**) MEFs of various *p53* genotypes were incubated without or with 2 mM hydroxyurea for 48 h and centrosomes were stained by anti-γ-tubulin antibody and visualized by fluorescence microscopy. Data represent mean ± S.D. from six independent clones per genotype. (**b**) Kaplan–Meier survival curves of unirradiated and irradiated $p53^{+/+}$ (16 unirradiated and 8 irradiated) and $p53^{S312A/S312A}$ (28 unirradiated and 30 irradiated) mice are shown. (**c**) Kaplan–Meier survival curves of Eμ-myc transgenic mice with various *p53* genotypes (25 $p53^{+/+}$ mice, 57 $p53^{+/S312A}$ mice, 25 $p53^{S312A/S312A}$ mice and 10 $p53^{+/-}$ mice) are shown

absolutely as a WT p53 protein in all aspects we have tested. These findings have several implications. First, this phosphorylation site may not be important in the physiological context on its own, as has been suggested by some of the *in vitro* studies. This conclusion has several parallels. Abrogation of S23 (human S20 equivalent) and S389 (human S389 equivalent) phosphorylation sites did not significantly alter the general response to irradiation,[1,33–36] except for UV irradiation in the later context, suggesting that most of the major functions of p53 can be carried out regardless of the phosphorylation status at these residues. Thus, the general effectiveness of the phospho-mutant proteins suggests that these phosphorylation events may not be essential in regulating acute p53 functions by themselves.

Although single phospho-mutant p53 knock-in mice behaved very similar to the WT mice, the S18/23A mutant, in which both S18 and S23 residues are unphosphorylatable, is almost completely defective in apoptosis and developed an array of spontaneous tumors,[1,33–36] showing that mutating multiple phosphorylation sites have synergistic effects on p53 functions. Therefore, it is possible that mice in which multiple phosphorylation sites such as S18/23/312A are altered may reveal the functional significance of S312, and requires further investigation.

Second, as the S315 phosphorylation site is one that is not regulated by DNA damage only,[12,14] it is not surprising that lack of this phosphorylation site does not alter any DNA damage-dependent functions of the knock-in mice, even after 2 years of age. This is unlike the S23A and S389A mice which develop tumors much later in life, probably on accumulation of genetic anomalies over time.[1,33] Nevertheless, no defects on ER stress were also observed in these mutant cells, raising the possibility that this site is indeed dispensable for all tested p53 functions. However, we cannot exclude the possibility that a phenotype may be apparent in sensitized backgrounds.

A salient point that requires consideration is whether the phosphorylation sites of mouse p53 are indeed functionally equivalent to their human counterparts. As noted earlier, the S15, S20 and S392 sites are thought to be critical residues in regulating p53 function, but knock-in mice do not show major defects.[34–36] Thus, it is not inconceivable that the phosphorylation sites may have evolved to be functionally more relevant in humans and are largely dispensable in mice or other lower organisms. In support of this idea, mouse ES cells expressing a human-mouse hybrid (hupki) S315A mutant p53 protein (S315 residue is located in the region of human p53) expressed from the endogenous locus seems to have some defects in p53-mediated gene regulation,[37] suggesting that the observed defects may be due to a combinatorial effect of the presence of human p53 sequence and unphosphorylable S315 residue. Moreover, Drosophila p53 is phosphorylated on stress but the protein levels remain unchanged,[38] contrary to the dogma that phosphorylation is generally critical for regulating p53 stability in mouse and human cells.[9,17] Furthermore, acetylation of p53 was shown to be critical in human cells, but ablating all seven acetylatable lysine residues did not have any major effect on p53 functions in mice.[1] In addition, the codon 72 polymorphism that seems to differentially affect the apoptotic activity of human p53 is absent in mouse p53.[39] Altogether, subtle modifications on p53 by PTMs or otherwise seem to be more relevant in the human cellular context rather than the mouse environment – probably because of the proposed weaker activity of human p53 compared with mouse p53[39] – and hence, elaborated to regulate human p53 activity exquisitely.

In conclusion, the findings presented in this study show that altering the S312 to an unphosphorylable alanine residue does not affect any of the known major functions of p53 in mice. This suggests that either single residue substitutions may not be sufficient to discern a phenotype, or that the S312

phosphorylation is neither relevant in the mouse context nor important for p53 functions *in vivo*.

Materials and Methods

Generation and breeding of $p53^{S312A/S312A}$ knock-in mice. The targeting construct and screening strategy were described previously,[40] with the exception that the construct here carries a S312A (TCT to GCG) mutation and E14.1 feeder-dependent ES cells were used. The neomycin selection cassette was removed by transcient Cre expression in ES cells before microinjection. As the S312A mutation generates a novel *BstUI* restriction site, the presence and the expression of S312A allele were confirmed by *BstUI* digestion of genomic PCR and RT-PCR product, respectively. All mice were then maintained on a hybrid background of C57BL/6 and Sv129Ola. $p53^{-/-}$, $E\mu$-*myc* and $mdm2^{-/-}$ mice were obtained from the Jackson Laboratory Jackson Laboratory, Bar Harbour, ME, USA and the MMHCC Repository (NCI-Frederick, Frederick, MD, USA).

Five-week-old mice were $\gamma-$irradiated with a single dose of 4 Gy and followed up till $\sim$100 weeks. For the B-cell lymphoma study, $p53^{+/-}$ or $p53^{S312A/S312A}$ mice were bred with $E\mu$-*myc* transgenic mice. All animal experiments were approved by and performed in accordance with the guidelines of the Singhealth's Animal Care and Use Committee.

Cell culture and biochemical analysis. Details of cell culture, treatment, immunoblotting, RNA analysis, fluorescence immunohistochemistry for centrosome counting are described in detail in the Supplementary Information.

Contributions. MKL designed the experiments, performed the research work, analyzed the data and helped with writing of the paper. WMT and ZQW helped with ES cell targeting and generation of the chimaeric mice. KS designed the experiments, analyzed the data and wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

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