Requirement for phosphorylation of P53 at Ser312 in suppression of chemical carcinogenesis

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The p53 tumour suppressor is activated in response to a wide variety of genotoxic stresses, frequently via post-translational modification. Using a knock in mouse model with a Ser312 to Ala mutation, we show here that phosphorylation of p53 on Ser312 helps to prevent tumour induction by the alkylating agent MNU, which predominantly caused T cell lymphomas. This is consistent with our previous observation that p53312A/A mice are more susceptible to X-ray induced tumourigenesis. Phosphorylation on Ser312 aids p53's interaction with E2F1, and enhances p53-mediated apoptosis. Loss of E2F1 alone does not affect tumour susceptibility to MNU, but its absence partially rescues tumour formation in p53312A/A mice, thus reflecting the oncogenic properties of E2F1. Our data confirms the participation of Ser312 phosphorylation in tumour suppression by p53.

The p53 pathway is one of the main lines of defence in response to carcinogenic stress, and p53 inactivating mutations frequently arise in tumours. Damage to DNA from radiation or carcinogens causes the stabilisation of p53, which then prevents the transmission of this damage by inducing cell cycle arrest, apoptosis or senescence. p53 is mutated in around 50% of cancers, and the familial Li-Fraumeni syndrome, which results in an increased susceptibility to cancers, is caused by the inheritance of mutations in the TP53 gene. The importance of p53 in tumour suppression is also reflected in the susceptibility of p53 null mice to spontaneous tumour formation, and the decreased resistance of p53−/− mice to γ- radiation exposure1-3.

E2F1, like p53, is a transcription factor that impacts upon the cell cycle, senescence, apoptosis and tumour growth, and both are pivotal in two canonical tumour suppression pathways. E2F1 drives entry of the cell into S-phase, and is restrained by its interaction with the tumour-suppressive retinoblastoma protein (Rb). Phosphorylation of Rb by cyclin dependent kinases releases E2F1, allowing it to activate genes required for entry into S-phase. The Rb/E2F pathway is frequently disrupted in tumours, either through loss of Rb, or increased phosphorylation of Rb either by inactivation of the cyclin dependent kinase inhibitor p16INK4a or amplification of cyclin D14. Because of its role in driving entry into the cell cycle, E2F1 was originally thought to be oncogenic. However, mice lacking E2F1 unexpectedly showed aberrant cell proliferation and tumour development, indicating that E2F1 also has tumour suppressive roles5,6.

There is much evidence of cross talk between the p53 and Rb/E2F1 pathways7. Both, for example, are regulated by the CDKN2A locus, which encodes the proteins p16INK4a and p19ARF. Whereas p16INK4a inhibits E2F1 by preventing phosphorylation of Rb, p19ARF stabilises p53 by inhibiting Mdm2, an E3 ligase that causes proteasomal degradation of p53. In turn, E2F1 can activate the expression of p19ARF and cause p53 stabilisation8. Co-operation between p53 and E2F1 can also occur independently of p19ARF. The N-terminus of E2F1 can interact directly with a region towards the C-terminus of p53, resulting in increased nuclear retention of p53 and p53-mediated transcription and apoptosis. This is inhibited by competition between p53 and cyclin A at the binding site within E2F19,10. The interaction between p53 and E2F1 is enhanced by phosphorylation of p53 on Ser315, a residue within the E2F1 binding region that is phosphorylated by cyclin D1 kinases such as cdk1, cdk2, cdk9 and Aurora kinase A11-15.

Ser315 is one of approximately 20 serine/threonine phosphorylation sites that have been identified within p53 that constitute part of a complex regulatory network of post-translational modifications16. Many of these sites are phosphorylated by kinases activated in response to cellular stress, and are located at the N-terminus, the region of p53 that is involved in its stability and transcriptional regulation17. Several mouse models have been made that incorporate inactivating mutations at these phosphorylation sites, and their phenotypes are generally mild18,19.
Previously, we have characterised a knock in mouse model that carried a serine to alanine mutation on Ser312 (equivalent to human Ser315). Although these mice are healthy and have a normal lifespan, in response to ionising radiation they develop lymphomas and tumours in the liver, spleen, and kidney.

To determine the effect on tumour formation of both deleting E2F1 and preventing phosphorylation of p53 at Ser312, we crossed the p53Ser312Ala mice with E2F1 null mice and exposed them to a DNA alkylating agent, N-Methyl-N-nitrosourea (MNU). As was observed when these mice were exposed to ionising radiation, the p53Ser312Ala mice are more susceptible to tumour formation. Loss of E2F1 alone did not render these mice more susceptible to tumours, but loss of E2F1 alone had a slightly protective effect upon the tumour formation seen in p53Ser312Ala mice. Our study shows that in addition to protecting against tumours caused by ionising radiation, phosphorylation of p53 on Ser312 also contributes to the suppression of carcinogen-mediated tumorigenesis.

**Results**

We generated the mice for this study by intercrossing p53<sup>312A/-;</sup> E2F1<sup>+/−</sup> mice. The mice were born at the expected Mendelian frequency and showed no gross abnormalities (Table 1). Both males and females were born at close to the predicted numbers. None of the mice developed spontaneous tumours within the time frame examined.

We previously observed that p53<sup>312A/A</sup> mice are more susceptible to tumour formation than wild type mice following exposure to ionising radiation<sup>2</sup>. To determine if these mice are susceptible to other carcinogens, and whether the presence or absence of E2F1 affects this susceptibility, we gave 4–6 week old mice a single dose of the alkylating agent N-Methyl-N-nitrosourea (MNU). The study group consisted of 18 wild type (7 females and 11 males), 13 p53<sup>312A/A</sup> (9 females and 4 males), 13 E2F1<sup>+/−</sup> (6 females and 7 males) and 12 p53<sup>312A/A;</sup> E2F1<sup>+/−</sup> (6 females and 6 males). The mice were culled either when they showed signs of poor health or at 20 weeks post-injection when the study was terminated.

The mice began to develop tumours at 10–11 weeks post injection (Figure 1). Mice of all genotypes developed tumours, but p53<sup>312A/A</sup> mice were significantly more susceptible to tumours than both wild type and E2F1<sup>+/−</sup> mice, with p values of 0.0021 and 0.0146 respectively (Figure 1A and B). p53<sup>312A/A</sup> mice developed tumours within a shorter time frame than either wild type and E2F1<sup>+/−</sup> mice, and in the wild type mice in particular, the existence of a tumour was asymptomatic and only detected post-mortem at 20 weeks. Not all mice developed tumours within the time frame of this study, although only one p53<sup>312A/A;</sup> E2F1<sup>+/−</sup> mouse was tumour-free at 20 weeks. p53<sup>312A/A;</sup> E2F1<sup>+/−</sup> mice developed tumours earlier than wild type mice, although this was not significant (Figure 1C). Although fewer p53<sup>312A/A;</sup> E2F1<sup>+/−</sup> mice than p53<sup>312A/A</sup> mice developed tumours, again this difference was not significant (Figure 1D). There was little difference in tumour susceptibility between E2F1<sup>+/−</sup> and wild type mice (Figure 1E).

The predominant tumour type developed by mice of all genotypes was lymphoma, observable mainly as a grossly enlarged thymus that had lost its normal corticomedullary architecture, but also disseminated lymphomas that infiltrated organs such as the spleen, liver, kidney and lung (Figure 2A). This was observed as enlargement, pallor and mottling of the affected organs, with the thymus remaining unaffected. 8/19 (42.1%) wild type mice developed lymphomas, and 6/19 (31.6%) developed lung tumours which were almost always found at 20 weeks, with 5/19 (26.3%) showing no tumour development. One wild type mouse was found to have an abnormal liver at the end of the study. Of the p53<sup>312A/A</sup> cohort, 9/12 mice (75.0%) developed lymphomas, 2/12 (16.6%) had lung tumours and one mouse was tumour-free. 5/13 (38.5%) E2F1<sup>+/−</sup> mice developed lymphomas (including one mouse that had both thymic lymphoma and lung tumour), but 8/13 (61.5%) finished the study without developing tumours. 8/12 p53<sup>312A/A;</sup> E2F1<sup>+/−</sup> mice (66.7%) developed lymphomas, 2/12 (16.6%) developed lung tumours (again one mouse had both thymic lymphoma and a lung tumour) and 3/12 (25.0%) mice carried no tumours. Almost all the lung tumours (bronchioalveolar adenoma) were found at the end of the study and the incidence of lung tumours between the four groups was not significantly different (data not shown). Therefore, nearly all the p53<sup>312A/A</sup> mice developed tumours (91.6%), compared with 73.1% of wild type mice, 75% of p53<sup>312A/A;</sup> E2F1<sup>+/−</sup> mice and only 38.5% E2F1<sup>+/−</sup> mice.

Reflecting the trend of overall tumour incidence, p53<sup>312A/A</sup> mice were significantly more susceptible to lymphoma development than both wild type and E2F1<sup>+/−</sup> mice (p = 0.0106 and p = 0.0359 respectively) (Figures 2B and C). The median time of onset for lymphoma in p53<sup>312A/A</sup> mice was 112 days compared with over 140 days for wild type mice (fewer than 50% wild type mice had developed lymphomas at the end of the study). More p53<sup>312A/A;</sup> E2F1<sup>+/−</sup> mice developed lymphomas than wild type mice (Fig. 2A), although this was not significant (p = 0.194). All lymphomas were CD3 positive T cell lymphomas (Figure 2D): staining for the B cell marker CD45R/B220 was only found at a low level (data not shown).

In addition to thymic hyperplasia, lymphocytes were also frequently found in organs such as the kidneys, liver, lungs and spleen. Table 2 summarises according to genotype the organs where lymphocytes were found in mice carrying a thymic lymphoma. The extent of metastasis was similar for lymphomas from wild type, p53<sup>312A/A</sup> and p53<sup>312A/A;</sup> E2F1<sup>+/−</sup> mice. In addition to the organs listed in the table, lymphoma was found to have spread to the lymph nodes in two wild type mice and two p53<sup>312A/A</sup> mice.

The p53 cDNAs from 22 of the tumours isolated in this study were extracted and sequenced, and tumour 545 (E2F1<sup>+/−</sup>) was the only tumour found to have a mutation in the p53 open reading frame (GGA → GAA/Gly → Glu at codon 263) (Figure 3A). To analyse p53 levels in MNU-induced lymphomas, lysates were prepared from these tumours for analysis by immunoblotting (Figure 3B). p53 expression was retained in all the tumours analysed. The levels of total p53 were not especially high, except for tumour 545, which correlates with it carrying an inactivating mutation. Phosphorylation on p53 serine 312 could not be detected (data not shown), as found previously when trying to detect phosphoserine 312 in mouse embryonic fibroblasts<sup>9</sup>. E2F1 levels were also examined. E2F1 levels were low in normal thymus, but were elevated in some of the tumours derived from mice with wild type E2F1, indicating that E2F1 conferred a growth advantage in these circumstances, especially where p53 was mutant. Those tumours from the p53<sup>312A/A</sup> cohort that expressed high levels of E2F1 (e.g. numbers 467, 511 and 520) tended to die at an earlier time point and also with less lymphocyte spread to peripheral organs than those with lower levels of E2F1 (e.g. number 537) (Table 2). Other than where p53 was mutant (545), loss of E2F1 did not substantially elevate p53 levels.

| Table 1 | Birth ratios following intercross between p53<sup>312A/A;</sup> E2F1<sup>+/−</sup> mice. Figures in brackets represent predicted numbers based on Mendelian segregation |
| E2F1 | MALE | FEMALE | TOTAL |
|---|---|---|---|
| p53<sup>WT</sup> | WT | 10 (9) | 8 (9) | 18 (17) |
| | +/- | 24 (17) | 13 (18) | 37 (35) |
| | +/- | 6 (9) | 9 (9) | 15 (17) |
| p53<sup>312A/A</sup> | WT | 19 (17) | 20 (18) | 39 (35) |
| | +/- | 41 (35) | 35 (35) | 76 (70) |
| | +/- | 16 (17) | 10 (18) | 26 (35) |
| | +/- | 12 (9) | 15 (9) | 27 (17) |
| | +/- | 8 (17) | 18 (18) | 26 (35) |
| | +/- | 5 (9) | 10 (9) | 15 (17) |
| | 141 | 138 | 279 |
To analyse proliferation levels in this panel of tumours, phosphorylation of Histone H3 on serine 10 was used as a marker of mitosis (Figure 3B). There appeared to be little correlation between levels of histone phosphorylation and genotype, other than lower p53 levels correspond with higher levels of phosphorylation as would be predicted from p53’s role in preventing cell cycle progression.

Taken together, this data reinforces our previous finding that preventing phosphorylation of p53 on serine 312 reduces its efficacy as a tumour suppressor, as alteration of this residue to alanine both increases the proportion of mice that develop tumours in response to the carcinogen MNU and reduces time of onset.

Discussion

In this study we analysed the susceptibility to carcinogen-mediated tumour formation of mice lacking the ability to phosphorylate p53 on Ser312, and the impact upon this of E2F1 loss. We found that p53312A/A mice are significantly more susceptible to tumour development than both wild type and E2F1−/− mice. While p53312A/A mice are more susceptible than either wild type or E2F1−/− mice, this is not significant, and they are less vulnerable to tumours than p53312A/A mice.

MNU has been widely used in rodent tumour models and has been reported to cause tumours in a range of tissues including the gastrointestinal tract, lung, nervous system, pancreas and mammary glands. The tumours observed in this study were predominantly lymphomas. Lymphomas, especially of the thymus, are a common tumour type found in mice carrying mutations in the p53 pathway and in a previous study such lymphomas were found in p53312A/A mice exposed to ionising radiation. Lymphomas are also the predominant tumour type arising in p53+/− mice dosed with MNU. As in the present study, the lymphomas in p53+/− mice were CD3− positive and B220 negative. Although the predominant cause of death in p53+/− mice was lymphoma, other tumour types that were not seen in our study were also found, principally adenomas and adenocarcinomas of the intestine. Conversely, no lung neoplasms were observed in these other studies. This may be attributable to strain differences, as these studies were all carried out using mice with a C37BL/6 background, whereas the mice used in this study were on a mixed genetic background.
Also, the time of tumour onset for the p53<sup>+/–</sup> mice is comparable to that seen with the p53<sup>312A/A</sup> mice used in this study: the earliest onset with the p53<sup>+/–</sup> mice is between 56 days and 75 days<sup>22,23</sup> versus 74 days for the p53<sup>312A/A</sup> mice, and a median survival of 106 days<sup>24</sup> compared with 112 days with the p53<sup>312A/A</sup> mice. This is akin to the similarity in time of tumour onset for irradiated p53<sup>+/–</sup> and p53<sup>312A/A</sup> mice<sup>3,19</sup>. p53 is thought to suppress tumours in the thymus by inducing apoptosis, as p53 null mice develop spontaneous lymphomas, and thymocytes derived from these mice are resistant to apoptosis induced by DNA damage<sup>1,2,26,27</sup>. However, thymocytes derived from p53<sup>312A/A</sup> mice are not resistant to DNA damage and p53<sup>312A/A</sup> mice do not develop lymphomas spontaneously<sup>19</sup>. A previous study that exposed p53<sup>+/–</sup> mice to MNU found that many of the resulting tumours had lost the remaining p53 allele<sup>25</sup>, however all the tumours analysed in the current study retained p53 expression and p53 was only found to be mutated in one instance, a G263E mutation in the DNA binding domain (Figure 3). This suggests that p53Ser<sup>312</sup> phosphorylation is suppressing tumours by other means. Likewise, thymocytes from E2F1<sup>+/–</sup> mice are also sensitive to DNA damage-induced apoptosis<sup>28</sup>. However, thymocytes from p53<sup>312A/A</sup> mice do not develop lymphomas spontaneously<sup>19</sup>. A previous study that exposed p53<sup>+/–</sup> mice to MNU found that many of the resulting tumours had lost the remaining p53 allele<sup>25</sup>, however all the tumours analysed in the current study retained p53 expression and p53 was only found to be mutated in one instance, a G263E mutation in the DNA binding domain (Figure 3). This suggests that p53Ser<sup>312</sup> phosphorylation is suppressing tumours by other means. Whereas p53 phosphorylation mutants all retain some transcriptional activity<sup>19,31–34</sup>. Mice that have had inactivating tumour-derived mutations introduced e.g. p53R<sup>172H</sup> and p53R<sup>270H</sup> mice, spontaneously display a range of tumour types even when heterozygous<sup>35,36</sup>, reflecting the important contribution made by p53 mutations in the DNA binding domain towards tumour growth. Interestingly, the G263E mutation found in our study (equivalent to human G266E) is a deleterious mutation that has been found in tumours derived from tissues including lung and breast, although not at a high rate<sup>37</sup>. Deletion of E2F1 in the mice used in this study did not affect their susceptibility to tumour development in comparison with wild type mice (Figures 1E, 2C). p53<sup>312A/A</sup>; E2F1<sup>+/–</sup> mice developed more tumours than E2F1<sup>+/–</sup> mice, but fewer than p53<sup>312A/A</sup> mice (Figures 1D, 1F), although not to a significant degree. p53, when phosphorylated on Ser<sup>312</sup>, has been shown to compete with cyclin A in its interaction with E2F1<sup>9</sup>. In the wild type scenario, phosphorylation of p53 on Ser312 enables interaction with E2F1 which leads to increased nuclear retention of p53, cell death and tumour suppression<sup>10</sup>. In the absence of Ser312 phosphorylation in p53<sup>312A/A</sup> mice, this death pathway should fail to activate, facilitating cell survival and promoting tumour development. With E2F1<sup>+/–</sup> mice, wild type p53
is still able to cause apoptosis by E2F1-independent means, as the Ser312Ala mutation is only subtle and does not abrogate the majority of p53’s functions. However, while the p53-phosphoSer312-E2F1 death pathway cannot activate in p53312A/A; E2F12/2 mice, the loss of E2F1 may also restrict proliferation and counteract tumour formation when compared to p53312A/A mice, where E2F1 is present. This is indicated by the relationship between E2F1 levels and lymphocyte infiltration with or without E2F1 in p53312A/A mice. The primary symptom of thymic lymphoma that requires an animal to be sacrificed is laboured respiration due to constriction of the lungs by the tumour. As shown in Figure 3B and Table 2, p53312A/A mice with tumours with high E2F1 levels tended to have shorter lifespans and thymic lymphomas that did not infiltrate other organs (e.g. 511 and 520), perhaps suggesting that the tumours in these mice grew to a critical size within the thoracic cavity before lymphocyte migration to other organs was able to occur. In contrast p53312A/A mice with low E2F1 levels (e.g. 537) died at a later stage with increased lymphocyte infiltration. This is reflected in the double mutant p53312A/A; E2F12/2 mice, most of which also displayed extensive lymphocyte infiltration.

Data generated from studies that have crossed p53 null mice with E2F1 null mice have proved inconsistent. In one study, the deletion of E2F1 abolished the susceptibility of p532/2 mice to spontaneous thymic lymphomas, whereas another study found that the loss of E2F1 had no effect. One likely explanation is that two different E2F1 knockout mouse strains were used in these studies. The strain used by Wikonal et al. is also the strain used in the present study, so the partial rescue of the tumour susceptibility of p53312A/A mice by E2F1 deletion is consistent with their observations. Genetic background may also play a part, as another study crossing p532/2 mice with the E2F12/2 mice described by Field et al. but on a different strain background found that although overall survival was not affected in p532/2; E2F12/2 mice, the number of mice developing thymic lymphoma increased.

Table 2 | The extent of infiltration of peripheral organs by lymphocytes in mice that developed thymic lymphomas following exposure to MNU. An asterisk indicates sacrifice of the animal at the end of the study (20 weeks)

| Genotype     | Mouse | Time of tumour onset | Lung | Liver | Kidney | Spleen |
|--------------|-------|----------------------|------|-------|--------|--------|
| wild type    | 458   | 128                  | Y    | Y     | Y      | Y      |
|              | 483   | 100                  | -    | -     | -      | -      |
|              | 497   | 140                  | Y    | -     | -      | -      |
|              | 527   | 72                   | Y    | Y     | Y      | Y      |
|              | 555   | 140                  | Y    | Y     | Y      | Y      |
|              | 600   | 140                  | -    | -     | -      | -      |
|              | 616   | 140                  | Y    | Y     | Y      | Y      |
|              | 619   | 140                  | Y    | Y     | Y      | Y      |
|              | 670   | 109                  | Y    | Y     | Y      | Y      |
|              | 467   | 115                  | -    | Y     | -      | -      |
|              | 470   | 116                  | Y    | Y     | Y      | Y      |
|              | 489   | 108                  | Y    | Y     | Y      | Y      |
|              | 511   | 85                   | -    | -     | -      | -      |
|              | 520   | 97                   | -    | -     | -      | Y      |
|              | 537   | 131                  | Y    | Y     | Y      | Y      |
| E2F12/2     | 584   | 74                   | Y    | Y     | Y      | Y      |
|              | 479   | 80                   | Y    | Y     | Y      | Y      |
|              | 545   | 111                  | -    | -     | -      | -      |
|              | 562   | 140                  | -    | -     | -      | -      |
|              | 590   | 140                  | Y    | Y     | Y      | Y      |
|              | 456   | 137                  | -    | -     | -      | -      |
|              | 506   | 102                  | Y    | Y     | Y      | Y      |
|              | 521   | 75                   | Y    | Y     | Y      | Y      |
|              | 567   | 140                  | Y    | -     | -      | -      |
|              | 575   | 111                  | Y    | Y     | Y      | Y      |
|              | 580   | 124                  | Y    | Y     | Y      | Y      |
|              | 663   | 85                   | -    | Y     | -      | -      |

**Figure 3** | Variations in protein expression in thymic lymphomas derived from mice exposed to MNU. (A) Chromatogram showing the Gly to Glu point mutation found in tumour 545. (B) Immunoblots of tumour lysates derived from thymic lymphomas arising in mice exposed to MNU. Larger versions of these blots are shown in Supplementary Figure 1.
lymphomas was reduced with a corresponding increase in sarcomas and carcinomas.

In conclusion, we observed an increased susceptibility to lymphoma formation induced by MNU in p53 knock in mice carrying a Ser312 to Ala mutation. This strengthens our previous observation that these mice are susceptible to tumorigenesis after exposure to ionising radiation. Thus, phosphorylation on Ser312 enables p53 to fully function as a tumour suppressor.

**Methods**

**Mice, tumour induction and analysis.** E2F1 null mice described by Field et al. were obtained from The Jackson Laboratory. p53+/−/− knock in mice were described previously. The mice were on a mixed C57BL/6;129/Sv; FVB strain background. All animal breeding, maintenance and procedures were approved by the University of Oxford ethical committee and licensed by the U.K. Home Office.

4–6 week old mice were given a single intraperitoneal dose of N-Methyl-N-nitrosourea (MNU) (Sigma) in citrate buffered saline at 50 mg/kg. The mice were sacrificed either upon ill health or at 20 weeks post-injection. Following sacrifice, tissues were fixed and embedded in paraffin for histological analysis as described previously.

Immunohistochemistry and immunoblotting. Antibodies used were: anti-CD3 and β-tubulin (Abcam); anti-CD45R/B220 (BD Pharmingen); anti-p53 CM5 (Leica); anti-E2F1 and Histone H3 (Cell Signalling Technologies); Histone H3 phospho-Ser10 (Millipore).

Blocking Reagent (Roche) was used to block tissue sections and as antibody vehicle. Blocking and secondary antibodies were used for immunohistochemistry on paraffin sections; primary antibodies were either used directly as supplied or diluted in Blocking Reagent. The primary antibodies are listed in the Materials and methods section. Immunohistochemistry was performed as previously described.

**Data analysis and statistics.** Survival was determined using the Log-rank (Mantel-Cox) test and GraphPad Prism software. Statistical significance was determined using the Log-rank (Mantel-Cox) test and GraphPad Prism software.

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Author contributions

The study was designed by E.A.S. and X.L. E.A.S. performed the experiments and analysed the data. The manuscript was written by E.A.S.

Additional information

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