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The pro-longevity gene *FoxO3* is a direct target of the p53 tumor suppressor

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Abstract

FoxO transcription factors play a conserved role in longevity and act as tissue-specific tumor suppressors in mammals. Several nodes of interaction have been identified between FoxO transcription factors and p53, a major tumor suppressor in humans and mice. However, the extent and importance of the functional interaction between FoxO and p53 have not been fully explored. Here, we show that p53 transactivates the expression of *FoxO3*, one of the four mammalian *FoxO* genes, in response to DNA damaging agents in both mouse embryonic fibroblasts and in thymocytes. We show that p53 transactivates *FoxO3* in cells by binding to a site in the second intron of the *FoxO3* gene, a genomic region recently found to be associated with extreme longevity in humans. While *FoxO3* is not necessary for p53-dependent cell cycle arrest, FoxO3 appears to modulate p53-dependent apoptosis. We also find that *FoxO3* loss does not interact with *p53* loss for tumor development *in vivo*, although the tumor spectrum of *p53* deficient mice may be affected by *FoxO3* loss. Our findings indicate that *FoxO3* is a p53 target gene, and suggest that

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Authors' contributions

V.M.R. designed, performed, and analyzed the lifespan and tumor spectrum of compound *FoxO3/p53* mice (Figure 8). P.U.T. completed Figure 1B, Figure 5B, Figure 6B, Figure 6E, Figure 7A and helped with Figure 2 and Figure 6D. K.L.H. completed Figure 5D, Figure 5E, Figure 6A, Figure 6B, and Figure 6C. J.L.W. completed Figure 2, Figure 6D, and helped with Figure 3 and Figure 7B. C.A.B. completed Figure 3 and Figure 7B. D.K.B. completed Figure 5C. O.S.V. initiated this project as her Honors Thesis and completed Figure 1A. T.M.J. completed Figure 4B. P.R.O. completed Figure 6F. Z.X. and E.E.S. identified p53 binding sites in *FoxO3* regulatory regions (Figure 5A) under the supervision of M.Q.Z. H.V. performed the tumor identification (Figure 8B). L.D.A. supervised C.A.B., D.K.B. and T.M.J. and provided ideas. A.B. supervised V.M.R., P.U.T., K.L.H., J.L.W. and O.S.V. The manuscript was written by A.B., V.M.R., and O.S.V., with input from L.D.A.

Conflict of interest

We declare that there are no competing financial interests in relation to the work described.

FoxO3 and p53 are part of a regulatory transcriptional network that may play an important role during aging and cancer.

Introduction

Aging and cancer are intimately linked. Many cancers have a striking age-dependent onset. Interventions that extend lifespan, such as dietary restriction, decrease the incidence of tumors (Hursting *et al.*, 2003; Masoro, 2005). The connection between aging and cancer raises the possibility that genes that extend lifespan may also be part of a molecular network that suppresses tumorigenesis. An example for such genes is provided by FoxO transcription factors, which play a pivotal role at the interface between longevity and tumor suppression (Greer and Brunet, 2005). In invertebrates, FoxO factors are necessary to extend lifespan downstream of the insulin pathway (Kenyon, 2005). In mammals, the four FoxO family members (FoxO1, FoxO3, FoxO4, and FoxO6) also function downstream of the insulin-signaling pathway (Greer and Brunet, 2005). Single nucleotide polymorphisms (SNPs) in the *FoxO3* gene have recently been found to be associated with extreme longevity in humans, suggesting a conserved function for FoxO3 in longevity (Anselmi *et al.*, 2009; Flachsbart *et al.*, 2009; Pawlikowska *et al.*, 2009; Willcox *et al.*, 2008). Interestingly, the FoxO family has also been found to act as a lineage-specific tumor suppressor in mammals (Paik *et al.*, 2007). Combined somatic deletion of FoxO1, FoxO3, and FoxO4 in mice leads to the development of tumors, particularly thymic lymphomas and hemangiomas (Paik *et al.*, 2007). *FoxO3*^{-/-} mice can also develop cancer, but at a lesser frequency and later in life than *FoxO1/FoxO3/FoxO4* compound mutant mice (Paik *et al.*, 2007). In humans, FoxO3 inactivation is correlated with poor prognosis of breast cancers (Hu *et al.*, 2004). Conversely, ectopic expression of FoxO3 in human cells is sufficient to delay tumor development in xenograft models (Hu *et al.*, 2004; Seoane *et al.*, 2004). Thus, FoxO3 may be an important part of a regulatory network that controls both aging and cancer.

FoxO3 is a potent transcriptional activator that triggers the expression of a program of genes involved in cell cycle arrest, DNA repair, hypoxia, and apoptosis (Bakker *et al.*, 2007; Brunet *et al.*, 1999; Medema *et al.*, 2000; Paik *et al.*, 2009; Renault *et al.*, 2009; Seoane *et al.*, 2004; Tothova *et al.*, 2007; Tran *et al.*, 2002; You *et al.*, 2006a). FoxO3 transcriptional activity is inhibited in response to insulin and growth factors via phosphorylation-dependent nuclear export (Brunet *et al.*, 1999; Brunet *et al.*, 2001; Brunet *et al.*, 2002). While the regulation of FoxO3 activity by post-translational modifications, such as phosphorylation, has been well studied (Calnan and Brunet, 2008; Van Der Heide *et al.*, 2004), the molecular mechanisms that regulate the expression of the *FoxO3* gene remain mostly unclear.

Given the connection between aging and cancer, it is interesting to note that there are a number of parallels between FoxO3 and the tumor suppressor protein p53. Like FoxO3, p53 induces cell cycle arrest, apoptosis, and DNA repair (Vousden and Lu, 2002). Several FoxO3 target genes such as *Gadd45*, *Wip1*, *p21^{Cip1}*, *Puma*, and *Sestrin1/PA26* are also regulated by p53 (el-Deiry *et al.*, 1993; Fiscella *et al.*, 1997; Kastan *et al.*, 1992; Nakano and Vousden, 2001; Velasco-Miguel *et al.*, 1999; Yu *et al.*, 2001). FoxO3 and p53 are extensively modified in response to stress stimuli, via phosphorylation and acetylation

(Calnan and Brunet, 2008; Vousden and Prives, 2009), and both p53 and FoxO3 bind to and are deacetylated by the Sirt1 deacetylase (Brunet *et al.*, 2004; Luo *et al.*, 2001; Motta *et al.*, 2004; Vaziri *et al.*, 2001). These extensive similarities between FoxO3 and p53 suggest that both transcription factors may be part of a common regulatory complex.

A number of direct and indirect links between FoxO3 and p53 have already been uncovered. First, FoxO3 directly binds to p53, at least in the context of overexpression (Nemoto *et al.*, 2004; Wang *et al.*, 2008). Second, FoxO3 leads to stabilization of the p53 protein and activation of p53-dependent apoptosis (You *et al.*, 2006b). FoxO3 also upregulates *p19^{ARF}*, a positive upstream regulator of p53 (Bouchard *et al.*, 2007). Conversely, p53 has been reported to inhibit FoxO3 function indirectly by inducing the protein kinase SGK, thereby resulting in the phosphorylation of FoxO3 and its sequestration in the cytoplasm (You *et al.*, 2004). In addition, p53 has been found to inhibit FoxO3 transcriptional activity under conditions of oxidative stress (Miyaguchi *et al.*, 2009) and to induce FoxO3 degradation (Fu *et al.*, 2009). Whether FoxO3 and p53 intersect in other ways, for example by regulating each other's transcription, remains largely unknown.

p53 is a potent tumor suppressor in humans, as underscored by the fact that nearly all human tumors have mutations or deletions in the *p53* gene itself or in the p53 pathway (Vogelstein *et al.*, 2000). Mutations in *p53* have been linked to poor prognosis in a variety of human cancers, including lung (Quinlan *et al.*, 1992), breast (Deng *et al.*, 1994), and gastric cancers (Scott *et al.*, 1991), as well as lymphomas (Gaidano *et al.*, 1991; Lo Coco *et al.*, 1993). Consistent with the prevalence of p53 loss in human tumors, *p53*^{-/-} mice are highly prone to cancer early in life (Donehower *et al.*, 1992; Harvey *et al.*, 1993a; Harvey *et al.*, 1993b; Jacks *et al.*, 1994). *p53*^{+/-} mice also develop tumors with high frequency (Donehower *et al.*, 1992; Harvey *et al.*, 1993a; Harvey *et al.*, 1993b; Jacks *et al.*, 1994). The connection between FoxO3 and p53 in cells raises the possibility that FoxO3 functionally interacts with p53 for tumor suppression. A dominant-negative form of FoxO factors has been shown to accelerate Myc-driven tumorigenesis by blocking p53-dependent apoptosis (Bouchard *et al.*, 2007). However, the genetic interaction between *FoxO3* and *p53* loss in cancer progression in the absence of oncogenic stimulation has never been tested.

Here, we explore the connections between FoxO3, a ubiquitously expressed FoxO family member, and p53 in cells and in mice. We find that p53 acts as a direct upstream transcriptional activator of the *FoxO3* gene in response to DNA damage in mouse embryonic fibroblasts and in lymphocytes. We show that p53 regulates the transcription of the *FoxO3* gene by binding to a site in the second intron of the *FoxO3* gene. Although FoxO3 is not necessary for p53-dependent cell cycle arrest, FoxO3 appears to play a role in p53-dependent apoptosis. We also find that while *FoxO3* loss does not synergize with *p53* loss for tumor development *in vivo*, tumor spectrum in *p53*-deficient mice may be affected by the loss of one or both *FoxO3* alleles. These results reveal a regulatory mechanism linking FoxO3 and p53, two critical molecules involved in the control of longevity and tumor suppression.

Results

DNA damage and Nutlin treatment increase FoxO3 protein levels in a p53-dependent manner in fibroblasts

To test if p53 regulates FoxO3 expression in mammalian cells, we compared FoxO3 protein levels in $p53^{+/+}$ and $p53^{-/-}$ primary mouse embryonic fibroblasts (MEFs) in the absence or presence of doxorubicin, a DNA damaging agent that activates endogenous p53. We found that doxorubicin treatment increased FoxO3 protein expression in $p53^{+/+}$ MEFs, but not in $p53^{-/-}$ MEFs (Figure 1A). Changes in FoxO3 protein levels were similar to those of $p21^{Cip1}$, a well-known target of p53 (Figure 1A). To activate p53 in a more specific manner, we used Nutlin, a chemical compound that inhibits binding of p53 to Mdm2, a ubiquitin ligase critical for p53 degradation (Vassilev *et al.*, 2004). Similar to what we observed for doxorubicin, Nutlin treatment increased FoxO3 protein in $p53^{+/+}$ MEFs, but not in $p53^{-/-}$ MEFs (Figure 1B). Together, these results indicate that p53 is necessary for FoxO3 protein accumulation in MEFs in response to DNA damage and Nutlin.

p53 is necessary for FoxO3 mRNA upregulation in response to DNA damage or Nutlin treatment in fibroblasts

To determine if the p53-dependent accumulation of FoxO3 protein is due to transcriptional or post-transcriptional changes, we compared *FoxO3* mRNA levels in $p53^{+/+}$ and $p53^{-/-}$ MEFs in response to Nutlin or to doxorubicin (Figure 2A). We found that Nutlin or doxorubicin led to an upregulation of *FoxO3* mRNA that was significantly attenuated in $p53^{-/-}$ MEFs (Figure 2A), similar to two known p53 targets, $p21^{Cip1}$ and *Mdm2* (Figure 2B–C). We noted that *FoxO3* mRNA expression at basal levels is lower in $p53^{-/-}$ MEFs than in $p53^{+/+}$ MEFs, whereas FoxO3 protein expression is similar in MEFs of both genotypes (see Figure 1), suggesting that there are additional levels of regulation of the FoxO3 protein by p53. In contrast to FoxO3, other FoxO family members (FoxO1, FoxO4, and FoxO6) did not show a p53-dependent increased mRNA expression in response to Nutlin and doxorubicin (Figure 2D–F). *FoxO6* mRNA was induced in response to doxorubicin, but in a p53-independent manner (Figure 2F), raising the possibility that other members of the p53 family (e.g. p73) may be responsible for FoxO6 regulation in response to DNA damage. Collectively, these observations indicate that p53 is necessary for the upregulation of *FoxO3* mRNA in MEFs in response to signals that activate p53.

p53 is necessary for FoxO3 mRNA upregulation in response to DNA damage in lymphocytes

We next asked if the induction of *FoxO3* mRNA by p53 was also observed in other cell types. We found that *FoxO3* mRNA was upregulated in response to γ irradiation in mouse thymocytes, but that this upregulation was no longer observed in $p53^{-/-}$ thymocytes (Figure 3A). The changes in *FoxO3* mRNA levels in thymocytes were similar to the changes observed for two well-known targets of p53, $p21^{Cip1}$ (Figure 3B) and *Mdm2* (Figure 3C). The expression of other FoxO family members (FoxO1, FoxO4, and FoxO6) was not strongly upregulated in response to γ irradiation in a p53-dependent manner (Figure 3D–F), although *FoxO1* mRNA was slightly affected by p53 (Figure 3D). These findings indicate

that the p53-dependent regulation of *FoxO3* mRNA by DNA damaging agents is relatively specific to this FoxO isoform, and is observed in multiple cell types.

p53 transcriptional activity is necessary and sufficient to regulate *FoxO3* mRNA in fibroblasts

To determine whether *FoxO3* mRNA upregulation in response to doxorubicin is mediated by p53's ability to act as a transcriptional activator, we examined *FoxO3* mRNA levels in knock-in MEFs expressing a transcriptionally-impaired mutant of p53 under the control of *p53* endogenous promoter (Figure 4A). This mutant p53 is transcriptionally deficient because leucine 25 and tryptophan 26, two key residues for p53 transcriptional activity, are replaced by glutamine and serine, respectively (Johnson *et al.*, 2005). In addition, the transcriptionally-impaired *p53* allele, as well as a control WT allele of p53, were rendered inducible by the presence of a transcriptional/translational STOP cassette flanked by *LoxP* sites upstream of the p53 coding region (*p53^{LSL-WT}* and *p53^{LSL-25,26}*) (Figure 4A) (Johnson *et al.*, 2005). In *p53^{LSL-WT}* and *p53^{LSL-25,26}* knock-in MEFs, introduction of Cre recombinase allows expression of the WT or transcriptionally-impaired p53 alleles (Johnson *et al.*, 2005). Northern-blot experiments revealed that the expression of *FoxO3* mRNA was higher in *p53^{LSL-WT}* MEFs in the presence of Cre (i.e. expressing WT p53) than in *p53^{LSL-WT}* MEFs in the absence of Cre (i.e. not expressing p53), similar to *p21^{Cip1}* mRNA, a classical p53 target. These results confirm that p53 is necessary for expression of the *FoxO3* mRNA (Figure 4B). In the presence of Cre, doxorubicin elicited a significant increase in *FoxO3* and *p21^{Cip1}* mRNA in *p53^{LSL-WT}* MEFs but not in *p53^{LSL-25,26}* MEFs (Figure 4B). There was a slight upregulation of *FoxO3* and *p21^{Cip1}* mRNA in *p53^{LSL-25,26}* MEFs in the presence of Cre and doxorubicin (Figure 4B), consistent with the fact that the *p53^{25,26}* mutant is not completely transcriptionally inactive (Johnson *et al.*, 2005). Together, these data indicate that p53 transcriptional activity is necessary for the DNA damage-dependent increase in *FoxO3* mRNA.

To test whether p53 transcriptional activation is sufficient to induce *FoxO3* mRNA, we used MEFs with a knock-in mutation of p53 fused to the transactivation domain of the Herpes virus transactivator VP16 (*p53^{LSL-VP16}*, Figure 4A), thus rendering p53 maximally active (Johnson *et al.*, 2005; Johnson *et al.*, 2008). We observed that similar to *p21^{Cip1}* mRNA, *FoxO3* mRNA was potently induced in Cre-treated *p53^{LSL-VP16}* MEFs, even in the absence of doxorubicin (Figure 4B). These findings indicate that p53 transactivation activity is sufficient for *FoxO3* mRNA upregulation. Taken together, these results indicate that *FoxO3* gene expression is transcriptionally regulated by p53.

p53 directly binds and transactivates regulatory regions in the *FoxO3* gene

To determine if *FoxO3* is a direct target gene of p53, we searched the 5kb upstream regulatory region as well as all introns of the mouse *FoxO3* gene for the presence of putative p53 binding sites, based on the known p53 consensus binding site (el-Deiry *et al.*, 1992). This analysis identified four potential p53 binding elements: three (p53-1, p53-2, and p53-3) in the *FoxO3* promoter; and one (p53-4) in the second intron of the *FoxO3* gene (Figure 5A). p53-4 has a near perfect match to the p53 consensus binding site and only one base pair of spacing between the two half-sites (Figure 5A), which is a characteristic of optimal p53

binding sites (el-Deiry *et al.*, 1992). In addition, while the region containing p53-4 is not perfectly conserved in human *FoxO3*, there is also an optimal p53 binding site in the second intron of the human *FoxO3* gene, suggesting that the presence of p53 binding site in the second intron may be a conserved feature of the *FoxO3* gene. Interestingly, the second intron of the human *FoxO3* gene contains SNPs associated with extreme human longevity (Anselmi *et al.*, 2009; Flachsbart *et al.*, 2009; Willcox *et al.*, 2008).

To assess if p53 binds to these two p53 binding sites in MEFs, we performed chromatin immunoprecipitation (ChIP) assays with antibodies to p53 in chromatin extracts from MEFs that had been treated with doxorubicin. The recruitment of p53 to the regulatory regions of the *FoxO3* gene was assessed by quantitative PCR using primers surrounding the p53-1 or p53-4 binding sites (Figure 5B). We found that endogenous p53 occupied the p53-4 binding site in the intron of the *FoxO3* gene, but not the p53-1 binding site in the *FoxO3* promoter, in doxorubicin-treated MEFs (Figure 5B). Endogenous p53 was bound to the p53-4 binding site in the intron of the *FoxO3* gene even in the absence of doxorubicin, but the recruitment of p53 to that site was slightly increased in response to a short treatment with doxorubicin (Figure 5C). p53 occupancy at the p53 binding site in *FoxO3* second intron was similar to that in the *p21^{Cip1}* promoter, although p53 recruitment to the *p21^{Cip1}* promoter in response to short treatment with doxorubicin was more robust (Figure 5C). Taken together, these results indicate that p53 directly binds to a site within the second intron of the mouse *FoxO3* gene *in vivo*.

p53 is necessary for FoxO3 transactivation

To determine if p53 can transactivate the *FoxO3* gene by binding to the p53-4 binding site, we generated reporter constructs in which luciferase expression is driven by an SV40 minimal promoter fused to a 500 bp region surrounding either p53-1, p53-2, p53-3, or p53-4 and analyzed the luciferase activity of these reporter constructs in *p53^{+/+}* and *p53^{-/-}* MEFs (Figure 5D and data not shown). The region surrounding the p53-4 site in the *FoxO3* second intron induced transcriptional activation of the luciferase reporter gene in *p53^{+/+}* MEFs, but not in *p53^{-/-}* MEFs (Figure 5D). In contrast, the transcriptional activity of the p53-1, p53-2, and p53-3 regions was low, regardless of the presence of p53 (Figure 5D, data not shown). Note that region surrounding the p53-4 binding site induced transcriptional activation of the luciferase reporter gene in a p53-dependent manner even in the absence of doxorubicin, perhaps because transfection itself triggered a stress to the cells or because basal *FoxO3* mRNA levels are also regulated by p53 (see Figure 2A). Together, these findings indicate that the region surrounding p53-4 in the second intron of the *FoxO3* gene can be transactivated by p53.

To examine if the transcriptional activity of the region containing p53-4 was indeed due to this p53 binding site, we created a luciferase reporter construct with a mutated version of p53-4 (p53-4m) that can no longer be bound by p53 because it is missing the critical bases necessary for p53 binding (Figure 5A). Mutating the p53-4 site abolished the transcriptional activity of the luciferase reporter gene (Figure 5E). These experiments indicate that p53 binding to p53-4 in *FoxO3* second intron is pivotal for the regulation of the *FoxO3* gene by p53.

p53-induced FoxO3 does not play a role in cell cycle arrest in MEFs

p53 is necessary for cell cycle arrest in response to double-strand breaks in MEFs (Kastan *et al.*, 1992). FoxO3 can also promote cell cycle arrest when overexpressed in cells (Medema *et al.*, 2000), although FoxO3 is not necessary for cell cycle arrest in response to DNA damage in MEFs (Castrillon *et al.*, 2003). To test if the upregulation of *FoxO3* by p53 mediates part of the cell cycle arrest controlled by p53, we isolated MEFs from *FoxO3^{+/+}p53^{-/-}* (+/+), *FoxO3^{-/-}*, and *p53^{-/-}* mice and assessed BrdU incorporation in these cells in response to doxorubicin (Figure 6A) and Nutlin (Figure 6B). We found that *FoxO3^{-/-}* MEFs underwent cell cycle arrest to the same extent as +/+ MEFs in response to doxorubicin (Figure 6A) and Nutlin (Figure 6B). In contrast, *p53^{-/-}* MEFs were partially resistant to cell cycle arrest caused by doxorubicin (Figure 6A) and completely resistant to cell cycle arrest induced by Nutlin (Figure 6B). These results indicate that unlike p53, FoxO3 is not necessary for DNA damage- and p53-dependent cell cycle arrest in MEFs or that there is compensation by another factor, perhaps another FoxO isoform (see below). Figure 6C shows that FoxO3 was dispensable for cell cycle arrest induced by long-term treatment by chronic oxidative stress (H₂O₂) and by hydroxyurea (HU), which both lead to p53-dependent cellular senescence (Marusyk *et al.*, 2007) (T.M.J. and L.D.A., unpublished). However, *FoxO3^{-/-}* MEFs displayed significant cell cycle arrest compared to +/+ MEFs in basal long-term culture conditions (Figure 6C), suggesting that *FoxO3* loss may itself induce cell cycle arrest over several cellular passages. Consistent with the absence of role for FoxO3 in p53-dependent cell cycle arrest, the expression of *p27^{Kip1}*, a well-known target of FoxO3 involved in cell cycle arrest (Kops *et al.*, 2002), did not change in response to Nutlin or doxorubicin in *p53^{+/+}* MEFs and was even upregulated in *p53^{-/-}* MEFs (Figure 6D). These observations indicate that the p53-dependent upregulation of *FoxO3* mRNA and protein is not accompanied by an increase in FoxO3 transcriptional activity toward *p27^{Kip1}*, which is likely due to the fact that the FoxO3 protein is known to be exported to the cytoplasm in response to DNA damage in different cell types (A.B., data not shown) (You *et al.*, 2004).

The role of FoxO3 in p53-dependent cell cycle arrest may be masked by compensation by other FoxO family members

FoxO3 loss has been found to be compensated by other FoxO family members (Bouchard *et al.*, 2007; Paik *et al.*, 2007). Even though other FoxO family members are not regulated by p53 to the same extent as FoxO3 in MEFs and thymocytes (see Figures 2 and 3), we determined whether interfering with more than one FoxO family member had a more pronounced impact on cell cycle arrest than interfering with FoxO3 alone. We found that Nutlin-induced cell cycle arrest was attenuated in MEFs infected with lentiviruses expressing an shRNA hairpin directed to several FoxO family members (“pan FoxO”) (Hribal *et al.*, 2003) (Figure 6E–F), although Nutlin still caused some cell cycle arrest in these cells. These findings suggest that the FoxO family partially contributes to p53-dependent cell cycle arrest and that the role of FoxO3 in p53-dependent cell cycle arrest may be masked by compensation by other FoxO isoforms.

p53-induced FoxO3 appears to play some role in apoptosis

FoxO has been shown to be important for p53-dependent apoptosis in the context of Myc oncogene-transformed cells (Bouchard *et al.*, 2007). We asked if FoxO3 played a role in p53-dependent apoptosis in MEFs. MEFs do not usually respond to p53 activation by undergoing apoptosis, unless they have been transformed with an oncogene, including the E1A protein of the virus (Lowe *et al.*, 1993). We found that Nutlin treatment combined with serum starvation slightly enhanced the percent of cells undergoing apoptosis in E1A-transformed wildtype MEFs (Figure 7A). *FoxO3*^{-/-} E1A-transformed MEFs appeared to be slightly impaired in their ability to undergo apoptosis in response to Nutlin and serum starvation, although this effect was more modest than that observed in *p53*^{-/-} E1A-transformed MEFs (Figure 7A). We also found that *Bim*, a well-known FoxO3 target involved in apoptosis (Dijkers *et al.*, 2000), was upregulated in response to γ -irradiation in thymocytes and that this upregulation was dependent on p53 (Figure 7B). Taken together, these results are consistent with the notion that FoxO3 contributes, at least in part, to p53-mediated apoptosis.

FoxO3 loss does not cooperate with p53 loss for tumor suppression in mice, but may have an impact on tumor spectrum

Our study and published findings indicate that FoxO3 and p53 interact in many different ways in cells. While the relevance of the interaction between FoxO3 and p53 *in vivo* has been tested in a mouse lymphoma model (Bouchard *et al.*, 2007), it has not been assessed in the absence of oncogenic stimulation. Loss of one or both *p53* alleles in mice results in predisposition to cancer and death at an early age (Donehower *et al.*, 1992; Jacks *et al.*, 1994). In contrast, *FoxO3*^{-/-} mice only develop tumors at a very low frequency, and tumor development in *FoxO3*^{-/-} mice manifests late in life (Paik *et al.*, 2007), probably due to the redundancy of FoxO family members. To test if FoxO3 and p53 interacted *in vivo* for overall survival and tumor survival, we generated a cohort of compound *FoxO3/p53* mutant mice in a mixed FVB/N-129Sv/J background and monitored overall survival in these mice (Figure 8). Loss of one or both alleles of *FoxO3* did not accelerate the mortality rate of *p53*^{-/-} mice in this genetic background (Figure 8A). Similarly, loss of one or both alleles of FoxO3 did not significantly affect the survival rate of *p53*^{+/-} mice (Figure 8B), indicating that *FoxO3* and *p53* loss do not cooperate to diminish overall survival *in vivo*. This result further suggests that a model lacking an activated oncogene may not be sufficient to reveal FoxO3 interaction with p53 for tumor suppression. Alternatively, the absence of synergy between *FoxO3* and *p53* loss for tumor development could be due to the fact that both molecules are in the same genetic pathway. Histopathological analysis on a subset of *FoxO3/p53* compound mutant mice revealed that the loss of *FoxO3* in the context of *p53*^{-/-} mice resulted in the appearance of tumors such as adenocarcinomas and angioliipomas (Figure 8C–D). As these tumor types are rarely seen in *p53*^{-/-} mice in the same genetic background (Sharpless *et al.*, 2002), this result is consistent with the notion that *FoxO3* loss may impact the tumor spectrum of p53-deficient mice. Collectively, these findings suggest that FoxO3 and p53 are part of a common transcriptional network that may affect cellular and organismal responses that are important to counter aging and cancer.

Discussion

Our results indicate that p53 transactivates the expression of the *FoxO3* gene by binding to a site located in the second intron of the *FoxO3* gene. The regulation of *FoxO3* gene expression is likely an important mechanism for the generation of a pool of FoxO3 molecules that could be made active or inactive by post-translational modifications. Our data indicates that DNA damage upregulates the expression of *FoxO3* mRNA. *FoxO3* gene expression has been shown to be upregulated in response to a number of other environmental stimuli, including nutrient deprivation, growth factor deprivation, and hypoxia (Bakker *et al.*, 2007; Essagher *et al.*, 2009; Furuyama *et al.*, 2002; Imae *et al.*, 2003), raising the possibility that p53 might transduce the expression of FoxO3 in response to some of these stimuli. The induction of *FoxO3* mRNA by hypoxia in MEFs is dependent on hypoxia inducible factor 1 (HIF1 alpha) (Bakker *et al.*, 2007), although whether HIF1 directly binds to *FoxO3* regulatory regions is not known. In addition, E2F1, a transcription factor involved in cell cycle progression and apoptosis, has been shown to upregulate *FoxO3* mRNA in human neuroblastoma cell lines by binding to two conserved sites in the promoter of the human *FoxO3* gene (Nowak *et al.*, 2007). These observations raise the possibility that p53, HIF1, and E2F1 may all interact in controlling the expression of the *FoxO3* gene.

The regulation of *FoxO3* gene expression by p53 may be specific to a subset of tissues or cell types. FoxO3 and p53 are both expressed relatively ubiquitously, but may function more prominently in some tissues/cells versus others. A cell-type specific regulation has indeed been observed for the *FoxO3* gene. For example, *FoxO3* mRNA is upregulated by E2F1 in neuroblastoma cell lines and in U2OS cells, but not in HeLa cells, human diploid foreskin fibroblasts, or PC12 cells (Nowak *et al.*, 2007). Similarly, *FoxO3* mRNA expression is downregulated by growth factors in human AG01518 fibroblasts, but not in BJ-hTert fibroblasts (Essagher *et al.*, 2009). Whether the regulation of *FoxO3* by p53 that we have identified in MEFs and thymocytes is observed in all cell types will be interesting to test. While our study was being completed, another study reported that in normal adult mouse liver, p53 could bind a response element 4 kb upstream of the *FoxO3* transcriptional start site and transactivate *FoxO3* mRNA expression (Kurinna *et al.*, 2010). During hepatic regeneration, the binding of p53 to the binding site upstream of the *FoxO3* gene was disrupted, leading to a decrease in *FoxO3* mRNA expression. Consistent with our findings, p53-dependent activation of FoxO3 was also observed in MEFs and in mouse hepatoma cells overexpressing p53 (Kurinna *et al.*, 2010). Interestingly, another member of the p53 family, p73, also binds to the same regulatory region in the promoter of the *FoxO3* gene (Kurinna *et al.*, 2010). Together with our study, these findings indicate that the p53 family of transcription factors regulates FoxO3 in a number of different cell types, by binding to at least two different binding sites in the *FoxO3* gene, one 4kb upstream of FoxO3 transcriptional start site and one in the second intron of the *FoxO3* gene (our study). It is possible that p53 occupancy at different sites is dependent on cell-type or specific environmental conditions.

p53 and FoxO3 interact at many levels: 1) p53 and FoxO3 proteins physically interact (Nemoto *et al.*, 2004; Wang *et al.*, 2008); 2) p53 and FoxO3 share common target genes (Jacobs *et al.*, 2007; Riley *et al.*, 2008; Tran *et al.*, 2002; Vousden and Lu, 2002; Zhao *et al.*,

2000); 3) FoxO3 stabilizes p53 protein (You *et al.*, 2006b); 4) FoxO3 indirectly activates p53 by upregulating p19ARF, which inhibits the p53 ubiquitin ligase Mdm2 (Bouchard *et al.*, 2007); 5) p53 indirectly inhibits FoxO3 activity by inducing SGK (You *et al.*, 2004), or more directly, by inhibiting FoxO3 transcriptional activity (Miyaguchi *et al.*, 2009) and by inducing FoxO3 degradation via Mdm2 (Fu *et al.*, 2009); and 6) *FoxO3* is a p53 target gene (this study and (Kurinna *et al.*, 2010)). Thus, p53 and FoxO3 likely form a regulatory network that elicits appropriate cellular responses in response to stress stimuli. Negative and positive feedback loops within this network could be beneficial for triggering a finely tuned response to cellular stresses. The observation that p53 upregulates *FoxO3* mRNA but also indirectly inhibits FoxO3 protein activity (Fu *et al.*, 2009; Miyaguchi *et al.*, 2009; You *et al.*, 2004) suggests that co-incident signals may be needed to activate the FoxO3 molecules generated by p53-dependent transcription. Careful analysis of the kinetics of *FoxO3* mRNA upregulation and FoxO3 protein activation in response to co-occurring signals will be required to tease apart the molecular links between p53 and FoxO3.

The presence of a binding site for p53 in the second intron of the *FoxO3* gene is a feature that is conserved in the human genome. Mining the genome-wide chromatin immunoprecipitation data available for p53 (Wei *et al.*, 2006) revealed that p53 binds to *FoxO3* second intron in the human genome in human HCT116 cells. These observations suggest that the binding of p53 in the second intron of the *FoxO3* gene may be crucial for the regulation of *FoxO3* gene expression in mammals. Binding sites in introns have been reported previously for a number of p53 target genes, including TRAIL (Takimoto and El-Deiry, 2000), GADD45 (Kastan *et al.*, 1992; Smith *et al.*, 1994) and Mdm2 (Juven *et al.*, 1993; Wu *et al.*, 1993). Thus, p53 binding sites in introns may act as enhancers. Particularly notable are the recent findings that the second intron of the human *FoxO3* gene contains single nucleotide polymorphisms (SNPs) associated with extreme longevity in human centenarians from Japanese, German, and Italian descent (Anselmi *et al.*, 2009; Flachsbart *et al.*, 2009; Willcox *et al.*, 2008). Although the causative SNP associated with longevity in the *FoxO3* gene has not been located yet, these observations raise the intriguing possibility that sequence variations in the second intron of the *FoxO3* gene may be important for human longevity, perhaps by leading to subtle differences in transcription factor binding in this region, ultimately affecting *FoxO3* mRNA levels.

Given that FoxO3 and p53 share so many common functions, it is surprising to note that these two molecules have been reported to have antagonistic roles on organismal lifespan. Indeed, FoxO factors extend lifespan in invertebrates (Giannakou *et al.*, 2004; Henderson and Johnson, 2001; Hwangbo *et al.*, 2004), and mutants of the insulin/IGF-1 receptor – which lead to FoxO activation – also display an extended lifespan in mice (Bluhner *et al.*, 2003; Holzenberger *et al.*, 2003). In contrast, p53 activity appears to promote aging in worms and flies (Arum and Johnson, 2007; Bauer *et al.*, 2005). Interestingly, ectopic expression of p53 decreased lifespan in male flies, but increased lifespan in female flies (Shen and Tower, 2010). In a FoxO-null background, p53 no longer shortened the lifespan of males, but still extended the lifespan of females, suggesting that FoxO plays a differential role in males and females downstream of p53 (Shen and Tower, 2010). Increased p53 activity has also been shown to elicit premature aging in mice (Maier *et al.*, 2004; Tyner *et*

et al., 2002), although p53 can actually prolong mouse lifespan in the context of p19ARF expression (normally regulated by p53?) (Matheu *et al.*, 2007). The molecular bases for the differences between FoxO3 and p53 in regulating lifespan are not known, but understanding of the connections between these two molecules should give crucial insights into the mechanisms that regulate longevity.

Materials and methods

Constructs

A bacterial artificial chromosome (BAC) containing the full-length mouse *FoxO3* gene was purchased from BACPAC. The 500 bp regions surrounding the four putative p53 binding sites in the FoxO3 regulatory region were amplified by PCR and were sub-cloned into the pGL3 vector with the minimal SV40 promoter (SV40-pGL3). The mutated p53-4 binding site was generated by site-directed mutagenesis (Quickchange) using the following primers: Forward 5'-GGAGGGTCCTGGGTAAATTTGGGTATACCCAGATGAGTAG-3' and Reverse 5'-CTACTCATCTGGGTATACCCAAAATTTACCCAGGACCCTCC-3'. The mutated fragment was entirely sequenced and sub-cloned into SV40-pGL3. The E1A plasmid was described previously (Lowe and Ruley, 1993). The "Pan" FoxO shRNA construct was generated by subcloning the following primers (Hribal *et al.*, 2003) into the pSicoR (PSR) lentiviral expression vector between the HpaI and XhoI sites (Ventura *et al.*, 2004): Forward 5'-TGGATAAGGGCGACAGCAACTTCAAGAGAGTTGCTGTCGCCCTTATCCTTTTTC-3' and Reverse 5'-TCGAGAAAAAAGGATAAGGGCGACAGCAACTCTCTTGAAGTTGCTGTCGCCCTTATCCA-3'

Antibodies

Antibodies to human FoxO1, FoxO4, FoxO4, and mouse FoxO6 were generated by immunizing rabbits with a fusion protein between GST and each FoxO family member and purified by affinity (Quality Controlled Biochemicals). The FoxO3 antibody was used previously (Greer *et al.*, 2007; Renault *et al.*, 2009). The FoxO6 antibody was used previously (de la Torre-Ubieta *et al.*, 2010). The antibody to Mek1 was described previously (Lenormand *et al.*, 1993). Antibodies to p53 were obtained from Oncogene Science (Ab1), from Novocastra Laboratories or from Vector Labs (CM5), and from Santa Cruz (DO-1, SC-126X; pAb 1801, S-98X). Antibodies to p21^{Cip1} and β -actin were purchased from Santa Cruz and Novus Biological, respectively. Antibodies to cleaved caspase 3 and to BrdU were purchased from Cell Signaling and Technology and from AbD Serotec, respectively.

Thymocyte extraction

Thymocytes were extracted from 6–10 week-old mice of pure 129/Sv or mixed 129/Sv x C57/Bl6 background as described (Ihrie *et al.*, 2003). The following genotypes were compared: *p53*^{+/+} versus littermate *p53*^{-/-} or *p53*^{LSL-WT} mice, which result in complete *p53*-deficiency. Briefly, mice were euthanized and the thymi of the mice were removed and placed in PBS on ice. Each thymus was passed through a 40 μ m nylon cell strainer (BD Falcon) and divided into 2 plates (1 treated and 1 untreated). Thymocytes were then γ -

irradiated with 10 Gy using a ^{137}Cs irradiator and RNA was isolated 3 hours later using Trizol (Invitrogen).

Real-time PCR

One μg of total RNA was reverse transcribed with Random hexamers using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol or using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). Real-time PCR was performed on a Bio-rad iCycler using iQ SYBR green (Bio-rad) with the following forward and reverse primers:

FoxO3 F: AGT GGA TGG TGC GCT GTG T

R: CTG TGC AGG GAC AGG TTG T

Mdm2 F: AGC GCA AAA CGA CAC TTA CA

R: ACA CAA TGT GCT GCT GCT TC

p21^{Cip1} F: CAC AGC TCA GTG GAC TGG AA

R: ACC CTA GAC CCA CAA TGC AG

FoxO1 F: ACG AGT GGA TGG TGA AGA GC

R: TGC TGT GAA GGG ACA GAT TG

FoxO4 F: GGT GCC CTA CTT CAA GGA CA

R: GGT TCA GCA TCC ACC AAG AG

FoxO6 F: TGC CCT ACT TCA AGG ATA AAG G

R: CAG CTG CTT CTT GCT CG

p27^{Kip1} F: GGA CCA AAT GCC TGA CTC GT

R: CGC TTC CTC ATC CCT GGA C

Bim F: TCC TGT GCA ATC CGT ATC TCC

R: CGC AAG CTT CCA TAC GAC AGT

Gapdh F: TGT GTC CGT CGT GGA TCT GA

R: TTG CTG TTG AAG TCG CAG GAG

The experiments were conducted in triplicate and the results were expressed as $2^{-(\text{Gene of interest number of cycles} - \beta \text{ actin number of cycles})}$.

Northern-blot

MEFs were isolated at embryonic day 13.5 from mice of different genotypes (*p53^{LSL-WT}*, *p53^{LSL-25,26}*, and *p53^{LSL-VP16}*). MEFs were infected by adenovirus expressing the Cre recombinase (University of Iowa) at a multiplicity of infection (MOI) of 100 for 24 hours, as described (Johnson *et al.*, 2005). RNA was isolated using the TRIZOL protocol. For Northern-blot experiments, 15 μg of RNA was resolved on a denaturing agarose gel and

transferred onto a nylon membrane. Prehybridization and hybridization were performed using ExpressHyb hybridization solution (Clontech). Probes were prepared using a Prime-It II Random Primer Labeling Kit (Stratagene). A KpnI-NotI fragment of the mouse *FoxO3* cDNA was used as a probe. The probe for *p21^{Cip1}* was described (Attardi *et al.*, 2000).

Western-blot

Cells were lysed in lysis buffer (Tris HCl pH 8.0 (50 mM), NaCl (100 mM), EGTA (2 mM), NaF (10 mM), β -glycerophosphate (40 mM), Triton X-100 (0.4%), aprotinin (10 μ g/ml), phenylmethylsulfonyl fluoride (PMSF, 1 mM)). Fifty μ g of protein extract was resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies and the primary antibody was visualized using HRP-conjugated anti-mouse or anti-rabbit secondary antibodies and enhanced chemiluminescence (ECL, Amersham).

Chromatin immunoprecipitation with p53 antibodies

MEFs were seeded in 15-cm dishes at a density of 10^5 to 4×10^5 cells per ml. Twelve to twenty-four hours after plating, cells were stimulated with doxorubicin (0.2 μ g/ml) for 18–20 hours (Figure 4B) or 6 hours (Figure 4C). Cells were cross-linked with formaldehyde (1%) for 10 minutes and incubated with glycine (0.125M) for 5 minutes. Cells were washed with PBS and lysed in the Farnham lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% NP-40, protease inhibitor cocktail (#11697498001, Roche Laboratory)). Nuclear extracts were collected by centrifuging at 2000 rpm for 5 minutes. The cell nuclei were resuspended in RIPA buffer (1 x PBS, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS supplemented with Roche protease inhibitor cocktail). Chromatin was sonicated 5–8 times, for 30 s each time, with a Sonics VirCell 130 sonicator equipped with a stepped microtip. The chromatin was flash-frozen in liquid nitrogen and an aliquot was used to verify that sonication was effective. Antibodies to p53 (Figure 4B: DO-1 and pAb 1801 from Santa Cruz, 2.5 μ g each; Figure 4C: CM5 from Novocastra, 25 μ l per reaction) or IgG control (Zymed Laboratory or Sigma) were coupled to rabbit secondary antibody coated Dynal Magnetic beads (Invitrogen) in PBS + 5 mg/ml BSA overnight at 4°C. Chromatin extracts were pre-cleared on beads and then incubated with the beads coupled to the p53 antibody overnight at 4°C. The beads were washed twice in 1 ml low-salt ChIP buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), three times in 1 ml high-salt ChIP buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), four times with 1 ml LiCl ChIP buffer (0.25M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris, pH 8.1), and once or twice in 1 ml TE buffer. The chromatin complex was eluted in 200–300 μ l of IP elution buffer (1% SDS, 0.1 M NaHCO₃) at 65°C overnight. The DNA was purified by phenol/chloroform/isoamylalcohol and by PCR purification columns (Qiagen). Quantitative PCR was performed in triplicate using SYBR green (Bio-rad or SA-Biosciences) and a 7900HT Fast Real-Time PCR machine (Applied Biosystems) or a Bio-rad iCycler on 2.5 μ l of eluted DNA using the following sets of primers:

Mouse negative control forward primer: GGG GGA TAA TGA TTG CAA AA

Mouse negative control reverse primer: GCG TGG ACA GAG ATC TAG GC

p53-1 forward primer: CCT AAT GCC ACA GCA GAA CTC ATC

p53-1 reverse primer: TGG GAA TGG AAC TCA GTC AGT GC

p53-4 forward primer: GGG TGG GGG ATT CTT TTC ACT C

p53-4 reverse primer: CGA GGT AAG CCA GCA CAT ACA AAT G

p21 forward primer: GAGACCAGCAGCAAAATCG

p21 reverse primer: CAGCCCCACCTCTTCAATTC

For each primer set, a standard curve was established using a 5 to 10-fold dilution series.

Luciferase assays

MEFs were cultivated in DMEM supplemented with 10% FBS (Invitrogen) and antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine). For luciferase assays, MEFs were seeded at 35,000 cells/well in 24 well plates. One hundred and twenty-five ng of vector encoding the luciferase reporter under different promoters was transfected together with 62.5 ng of a vector expressing Renilla Luciferase to control for variation in transfection efficiency. Two days after transfection, cells were lysed and firefly luciferase and Renilla luciferase were measured according to the Promega protocol.

Lentiviral infection of MEFs

Lentiviruses were produced by transfection of 293T cells with the PSR lentiviral vectors and the helper plasmids 8.2 and VSVg. MEFs were plated at 10^5 cells/ml in 10 cm plates and 0.45 µm filtered 293T supernatant was applied 24 hours later and the infection was repeated 2–3 times in the presence of Polybrene (8 µg/ml). Puromycin (5 µg/ml) was added to select MEFs that were infected with the virus. On day 5 post-infection, MEFs were used for BrdU or western-blot assays.

BrdU incorporation assays

FoxO3^{+/+}p53^{+/+}, *p53^{-/-}*, or *FoxO3^{-/-}* MEFs were seeded in coverchambers at 300,000 cells/ml. Cells were stimulated with doxorubicin (0.2 µg/ml) or Nutlin (10 µM) for 24 hours, unless otherwise noted. Bromodeoxyuridine (BrdU) was added for the last 16 hours and the cells were fixed in 4% formaldehyde for 10 minutes and permeabilized in 0.4 % Triton for 30 minutes. Coverslips were incubated with 2 N HCl for 30 minutes, and washed extensively with PBS. Non-specific antibody binding sites were blocked by incubation with PBS containing 10% goat serum and 7.5% BSA. Coverslips were then incubated with primary antibodies (rat anti-BrdU, 1:500) for 2 hours, and washed five times with PBS. Cells were then incubated for 1 hour with a secondary antibody (goat anti-rat Alexa 488, 1:400). Coverslips were mounted in Vectashield containing DAPI and examined under epifluorescent illumination using a Zeiss microscope digital camera with AxioVision 4 software. For quantification, at least 250–400 cells per coverslip were counted in a blinded manner. The ratio of BrdU-positive nuclei over the total number of nuclei was calculated.

Apoptosis assays in E1A-transformed MEFs

Phoenix cells were plated at 200,000 cells per ml in 10 cm plates and transfected with 10 μ g of the E1A-encoding plasmid with the calcium phosphate method. Phoenix cell supernatant was filtered through 0.45 μ m filters onto MEFs (70,000 cells per ml in 10 cm plates) in the presence of Polybrene (8 μ g/ml) and the infection was repeated once. Infected MEFs were selected 24 hours after the last infection with selection media containing 250 μ g/ml Hygromycin. E1A-transformed MEFs were seeded on coverslips at 200,000 cells per well. 24 hours later, MEFs were stimulated with Nutlin (10 μ M) for 8 hours in the presence or absence of serum in the medium. Cells were fixed in 4% formaldehyde for 10 minutes and permeabilized in 0.4% Triton for 30 minutes. Non-specific antibody binding sites were blocked by incubation with PBS containing 10% goat serum and 7.5% BSA. Coverslips were then incubated with primary antibodies (rat anti-cleaved caspase 3, 1:500) for 2 hours, and washed five times with PBS. Cells were then incubated for 1 hour with a secondary antibody (goat anti rat Alexa 555, 1:400). Coverslips were mounted in Vectashield containing DAPI and examined under epifluorescent illumination using a Zeiss microscope digital camera with AxioVision 4 software. For quantification, at least 250–400 cells per coverslip were counted in a blinded manner. The proportion of cleaved caspase 3-positive nuclei over the total number of nuclei was calculated.

Mouse crosses and survival curves

Aging cohorts were produced by three different mating strategies: i) *FoxO3*^{-/-}*p53*^{+/-} (male) and *FoxO3*^{+/-}*p53*^{-/-} (female), ii) *FoxO3*^{+/-}*p53*^{+/-} and *FoxO3*^{+/-}*p53*^{+/-} and iii) *FoxO3*^{+/-}*p53*^{+/+} and *FoxO3*^{+/+}*p53*^{+/-}. Twenty-one *FoxO3*^{-/-}*p53*^{-/-}, 29 *FoxO3*^{+/-}*p53*^{-/-} and 8 *FoxO3*^{+/-}*p53*^{-/-} mice were generated. Twenty-one *FoxO3*^{-/-}*p53*^{+/-}, 62 *FoxO3*^{+/-}*p53*^{+/-} and 21 *FoxO3*^{+/+}*p53*^{+/-} mice were generated. The cohorts were on a mixed FVB/N and 129Sv/J background. Animals were genotyped by PCR and allowed to age at a maximum of 5 mice per cage with standard chow and water *ad libitum* in a standard light-day cycle. Mice were monitored once to twice a week and were sacrificed by CO₂ asphyxiation and scored as a death in survival analysis when moribund or if external tumors exceeded 1 cm in diameter. Survival analysis was performed using Kaplan-Meier curves with the logrank test.

Histopathology

All tissues, except skin and bone marrow, were fixed in 10% formaldehyde for 24 to 48 hours. Bones from the head, legs and rib cage were fixed in Bouin's fixative for 1 week before being decalcified for 48 hours. Tissues were embedded in paraffin, sectioned in 5- μ m sections, dewaxed and stained with hematoxylin and eosin at the Comparative Medicine Department, Stanford Medical School. Tumors were identified in a blinded manner.

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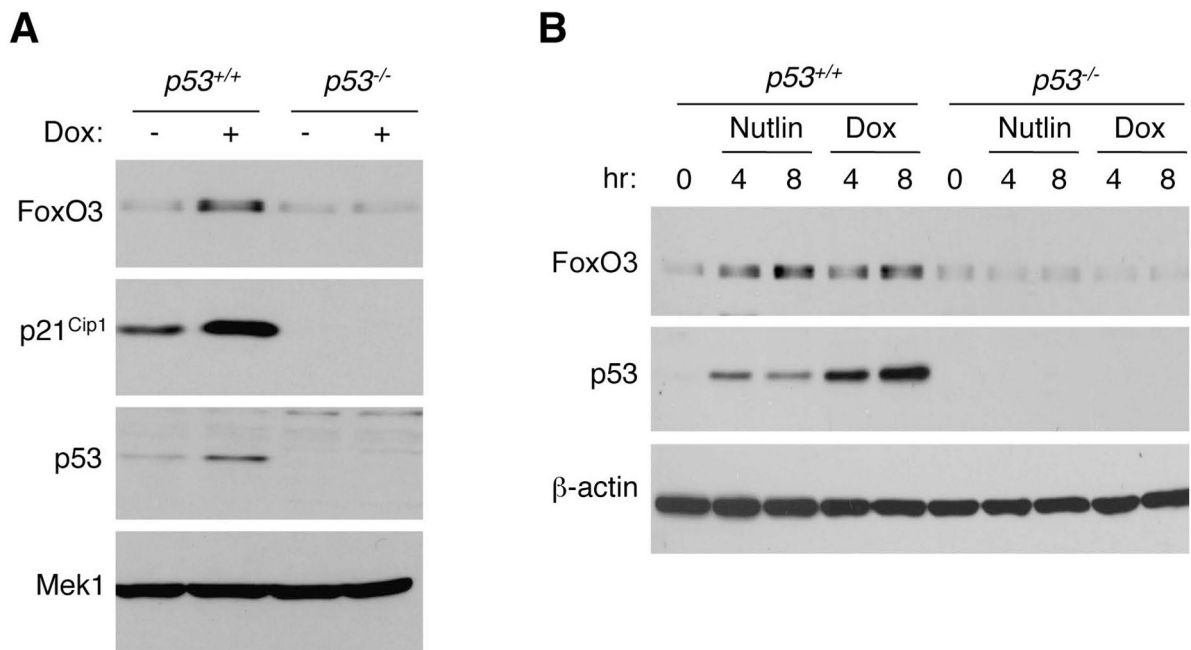


Figure 1. Doxorubicin and Nutlin elicit an increase in FoxO3 protein expression that is p53-dependent in MEFs

(A) Western-blot of protein extracts from *p53*^{+/+} and *p53*^{-/-} MEFs incubated in the absence (-) or presence (+) of doxorubicin (Dox, 0.2 μg/ml) for 8 hours, using antibodies to FoxO3, p21^{Cip1} (a well-known target of p53), p53, and Mek1 (loading control). (B) Western-blot of protein extracts from *p53*^{+/+} and *p53*^{-/-} MEFs incubated with Nutlin (10 μM), a p53 activator, or Doxorubicin (Dox, 0.2 μg/ml) for 0, 4, and 8 hours, using antibodies to FoxO3, p53, and β-actin (loading control). Western-blots are representative of at least two independent experiments, conducted on independent cultures of MEFs.

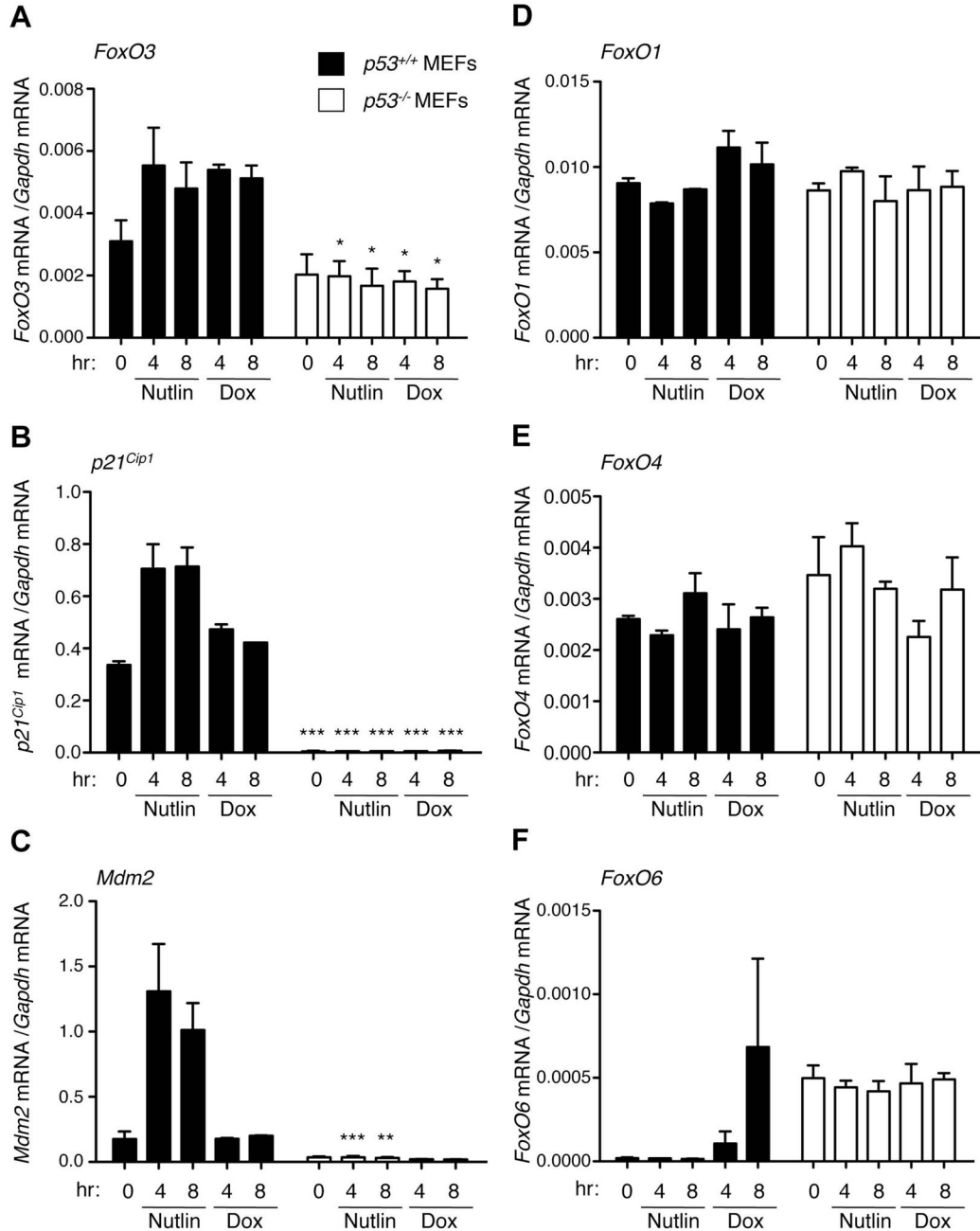


Figure 2. p53 is necessary for FoxO3 mRNA upregulation in response to doxorubicin or Nutlin in MEFs

Real time quantitative PCR analysis of *FoxO3* (A), *p21^{Cip1}* (B), *Mdm2* (C), *FoxO1* (D), *FoxO4* (E), and *FoxO6* (F) mRNA levels in *p53*^{+/+} and *p53*^{-/-} MEFs in response to 4 and 8 hours of treatment with Nutlin (10 μ M) or Doxorubicin (Dox, 0.2 μ g/ml). Mean \pm SEM of two independent experiments conducted in triplicate. * p < 0.05, ** p < 0.01, *** p < 0.001 between *p53*^{+/+} and *p53*^{-/-} MEFs at a given time point, two-way ANOVA with Bonferroni post-test.

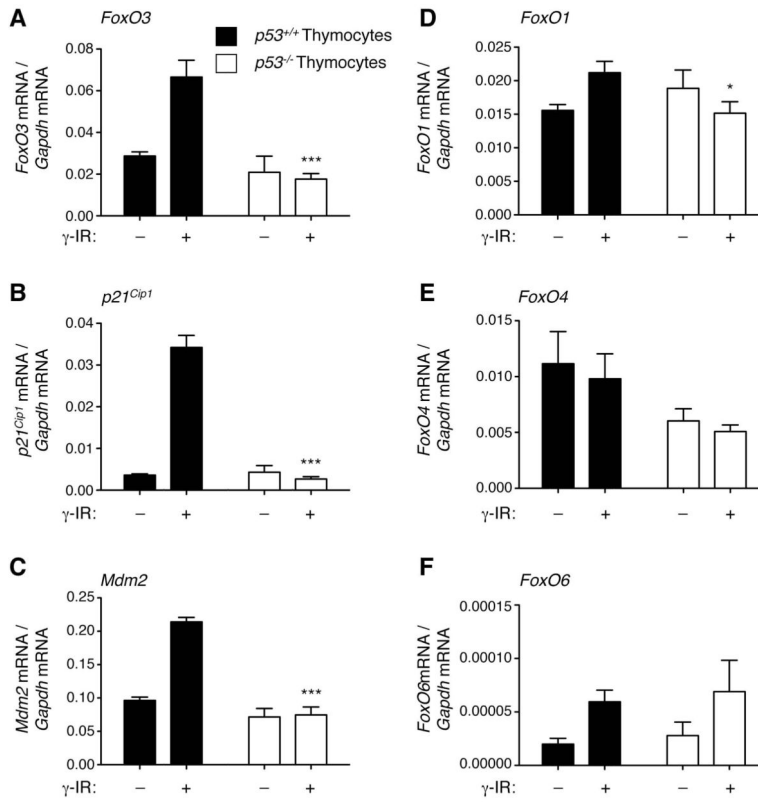


Figure 3. p53 is necessary for FoxO3 mRNA upregulation in response to γ irradiation in thymocytes
 Real time quantitative PCR analysis of *FoxO3* (A), *p21^{Cip1}* (B), *Mdm2* (C), *FoxO1* (D), *FoxO4* (E), and *FoxO6* (F) mRNA levels in *p53*^{+/+} and *p53*^{-/-} thymocytes, 3 hours after γ irradiation (γ IR, 10 Gy). Mean \pm SEM of two independent experiments conducted in triplicate on samples from 3–5 mice per genotype. * p < 0.05, ***p < 0.001 between *p53*^{+/+} and *p53*^{-/-} thymocytes at a given time point, two-way ANOVA with Bonferroni post-test.

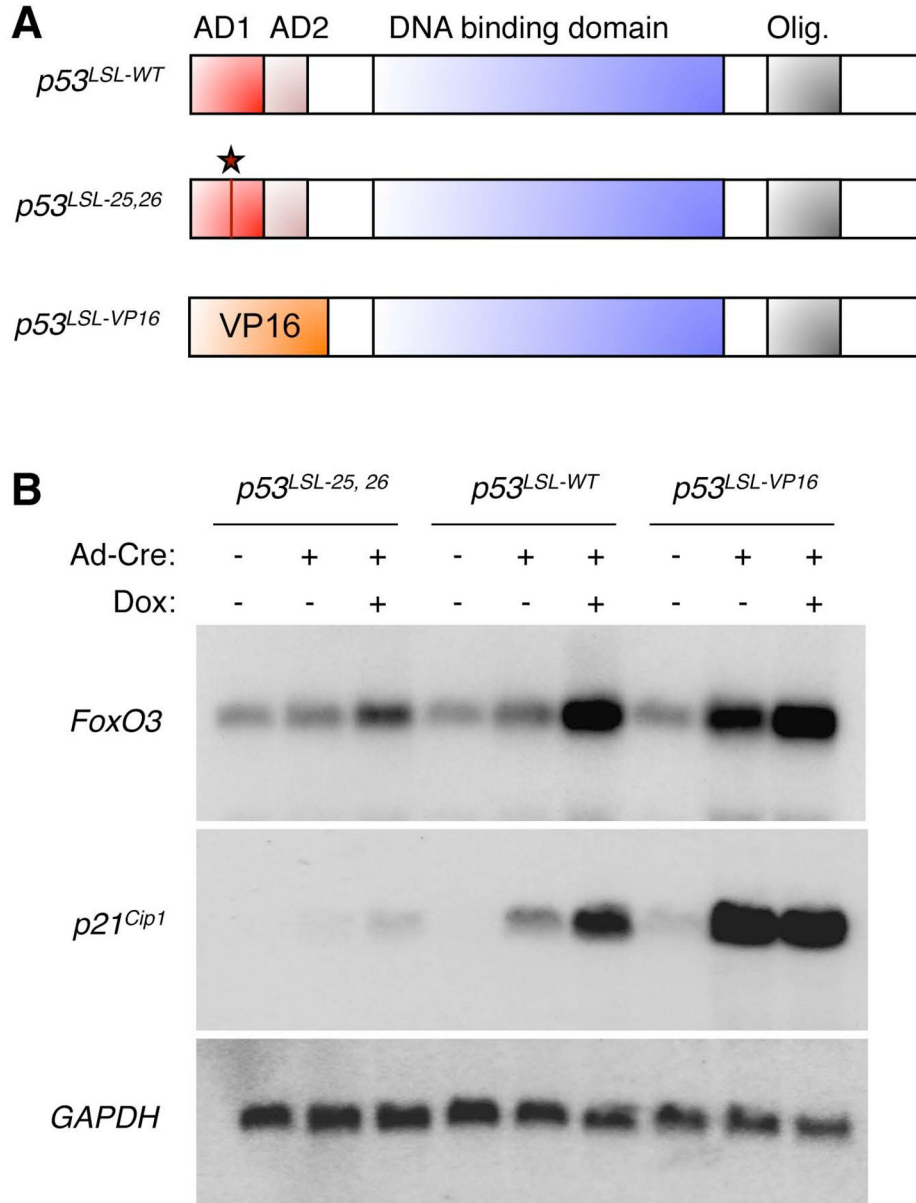


Figure 4. p53 transcriptional activity is necessary and sufficient for *FoxO3* mRNA upregulation (A) Schematic of the p53 knock-in alleles used. *p53^{LSL-WT}*: inducible allele encoding a form of wildtype p53. *p53^{LSL-25,26}*: inducible allele encoding a transcriptionally-impaired mutant of p53 in which leucine 25 is replaced by a glutamine and tryptophan 26 is replaced by a serine. *p53^{LSL-VP16}*: inducible allele encoding a mutant of p53 in which the transactivation domains (AD1 and AD2) are replaced by the transactivation domain of VP16. AD: activation domain; Olig.: oligomerization domain. The star indicates the location of the 25,26 mutation. (B) Northern-blot analysis of MEFs in which the endogenous allele of p53 has been replaced by an allele encoding inducible forms of WT p53 (*p53^{LSL-WT}*), a transcription-deficient mutant (*p53^{LSL-25,26}*), or a mutant of p53 in which the transactivation domains of p53 were replaced by that of VP16 (*p53^{LSL-VP16}*). The addition of an adenovirus

containing Cre recombinase (Ad-Cre) allows the deletion of the *Lox-STOP-Lox (LSL)* cassette upstream of each allele and allows the expression of each p53 variant. Cells were exposed to 8 hours of doxorubicin (Dox, 0.2 µg/ml). Northern-blots were analyzed with a probe to *FoxO3*, *p21^{Cip1}* (a known target of p53), and *GAPDH* (loading control).

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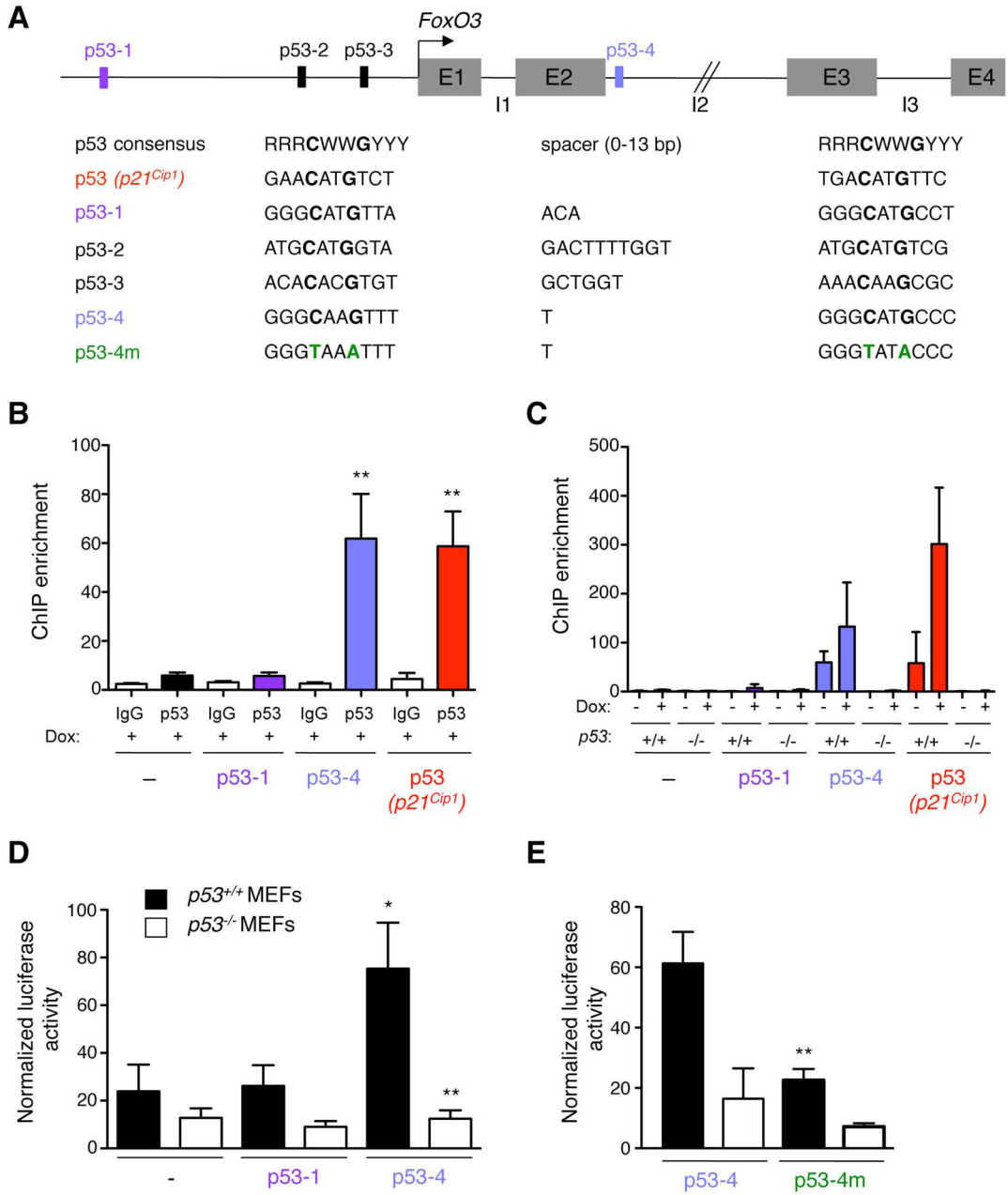


Figure 5. p53 is recruited to a binding site in the second intron of the *FoxO3* gene

(A) Location and sequence of the p53 binding sites (p53-1, p53-2, p53-3, and p53-4) in the promoter and second intron of the mouse *FoxO3* gene. R: G or A; W: T or A; Y: C or T; E: exon; I: intron. Also depicted are the consensus for p53 binding sites, the p53 binding site in *p21^{Cip1}* promoter, and the mutant of critical bases in p53-4 (p53-4m). (B) ChIP on MEFs treated with Doxorubicin (Dox, 0.2 μ g/ml) for 16–20 hours, using antibodies to p53 (colored bars) or control IgG (white bars). The chromatin bound to p53 or to the control IgG was analyzed by quantitative-PCR with primers surrounding a region that did not contain p53 binding sites (–), the distal p53 binding site in *FoxO3* promoter (p53-1), the p53-4 binding site in *FoxO3* intron 2 (p53-4), and the p53 binding site in the *p21^{Cip1}* promoter (p53

p21^{Cip1}). The fold enrichment over the IgG control is represented. Mean \pm SEM of three independent experiments. **: $p < 0.01$, one-way ANOVA. **(C)** ChIP on *p53^{+/+}* and *p53^{-/-}* MEFs in the absence or presence of Doxorubicin (Dox, 0.2 μ g/ml) for 6 hours, using antibodies to p53 or control IgG. The chromatin bound to p53 or to the control IgG was analyzed by quantitative-PCR with primers surrounding a region that did not contain p53 binding sites (-), the distal p53 binding site in *FoxO3* promoter (p53-1), the p53-4 binding site in *FoxO3* intron 2 (p53-4) and the p53 binding site in the *p21^{Cip1}* promoter (p53 *p21^{Cip1}*). The fold enrichment over the IgG control is represented. Mean \pm SD from triplicates of one experiment. **(D)** Normalized activity of luciferase reporter constructs driven by 500bp surrounding the p53 binding sites p53-1 or p53-4 in *p53^{+/+}* (black) and *p53^{-/-}* (white) MEFs. Mean \pm SEM of four independent experiments conducted in triplicate. *: $p < 0.05$ between p53-4 and control in *p53^{+/+}* MEFs, **: $p < 0.01$ between *p53^{+/+}* and *p53^{-/-}* MEFs for p53-4, one-way ANOVA. **(E)** Normalized activity of luciferase reporter constructs driven by the region surrounding the p53-4 binding site or by the region surrounding the p53-4 binding site in which the p53 binding site was mutated (p53-4m) in *p53^{+/+}* (black) and *p53^{-/-}* (white) MEFs. Mean \pm SEM of three independent experiments conducted in triplicate. **: $p < 0.01$ between p53-4m and p53-4 in *p53^{+/+}* MEFs, one-way ANOVA.

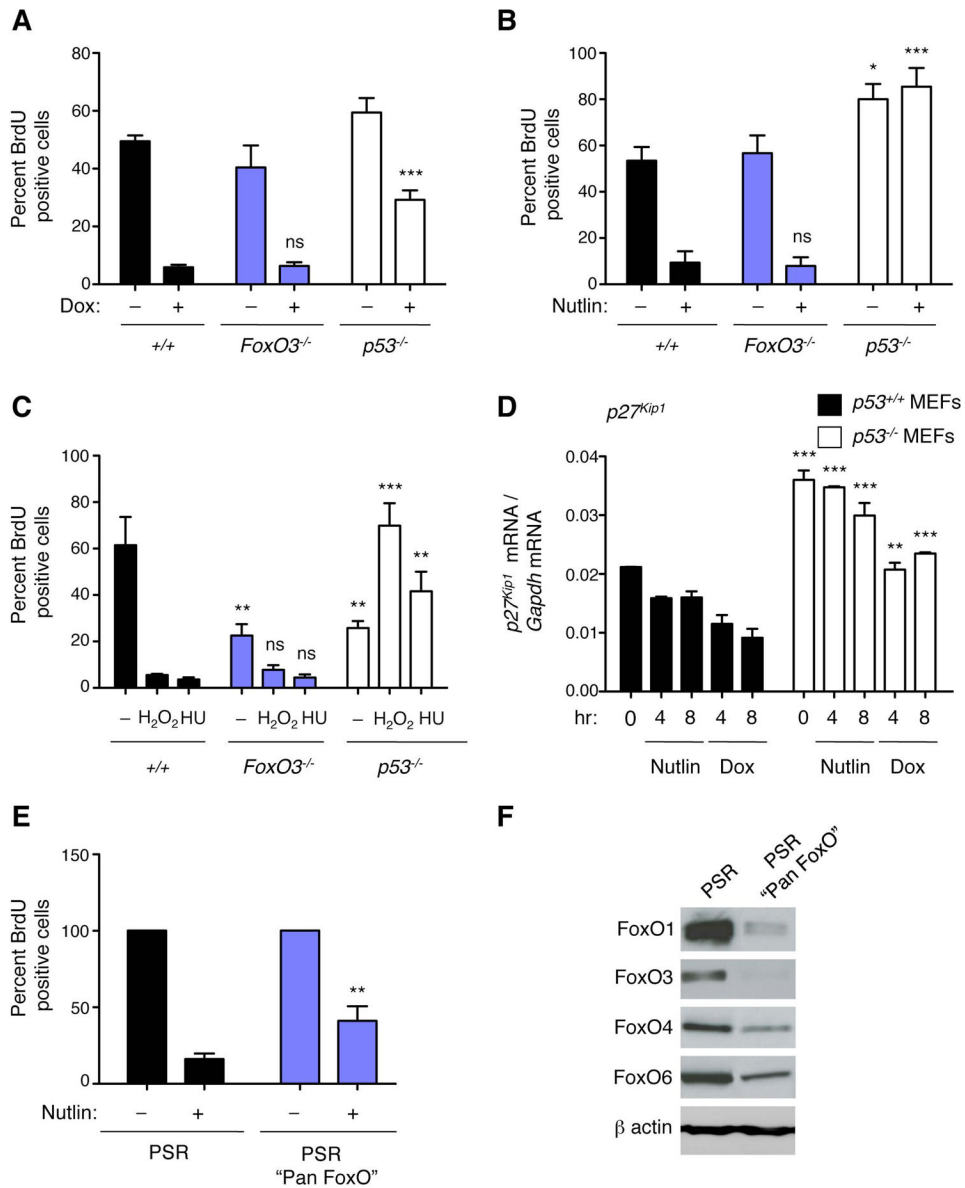


Figure 6. FoxO3 is not necessary for p53-dependent cell cycle arrest in MEFs, but other FoxO family members may compensate for FoxO3 loss

(A) Percent BrdU-positive cells in *FoxO3*^{+/+}*p53*^{+/+} (+/+), *FoxO3*^{-/-} and *p53*^{-/-} MEFs in the presence or absence of doxorubicin (Dox, 0.2 μg/ml) for 24 hours. Mean ± SEM of three independent experiments, two of which were conducted with independent MEF cultures from distinct animals. ***p<0.001 between +/+ and *p53*^{-/-} MEFs for the same treatment; ns: non significant between +/+ and *FoxO3*^{-/-} MEFs for the same treatment, two-way ANOVA with Bonferroni post-test. (B) Percent BrdU-positive cells in *FoxO3*^{+/+}*p53*^{+/+} (+/+), *FoxO3*^{-/-} and *p53*^{-/-} MEFs in the presence or absence of Nutlin (10 μM) for 24-36 hours. Mean ± SEM of three independent experiments. *p<0.05, ***p<0.001 between +/+ and *p53*^{-/-} MEFs for the same treatment; ns: non significant between +/+ and *FoxO3*^{-/-} MEFs for the same treatment, two-way ANOVA with Bonferroni post-test. (C) Percent BrdU-positive cells in *FoxO3*^{+/+}*p53*^{+/+} (+/+), *FoxO3*^{-/-}

and $p53^{-/-}$ MEFs in the presence or absence of chronic treatment of H_2O_2 or hydroxyurea (HU). Mean \pm SEM of two independent experiments with different lines of MEFs. ** $p < 0.01$, *** $p < 0.001$ between $+/+$ and $FoxO3^{-/-}$ or $p53^{-/-}$ MEFs for the same treatment; ns: non significant between $+/+$ and $FoxO3^{-/-}$ MEFs for the same treatment, two-way ANOVA with Bonferroni post-test. **(D)** Real time quantitative PCR analysis of $p27^{Kip1}$ mRNA levels in $p53^{+/+}$ and $p53^{-/-}$ MEFs in response to 4 and 8 hours of treatment with Nutlin (10 μ M) or Doxorubicin (Dox, 0.2 μ g/ml). Mean \pm SEM of two independent experiments conducted in triplicate. *** $p < 0.001$ between $p53^{+/+}$ and $p53^{-/-}$ MEFs at a given time point, two-way ANOVA with Bonferroni post-test. **(E)** Percent BrdU-positive cells in MEFs infected with control lentiviruses (PSR) or with lentiviruses expressing an shRNA to FoxO family members (PSR “pan FoxO”) in the presence or absence of Nutlin (Nutlin, 10 μ M) for 24 hours. The data are expressed as fold decrease, respective to the value obtained in the absence of Nutlin. Mean \pm SEM of two independent experiments. ** $p < 0.01$, between PSR and PSR “pan” FoxO-infected MEFs in the presence of Nutlin, two-way ANOVA with Bonferroni post-test. **(F)** Western-blot of protein extracts from MEFs infected with control lentiviruses (PSR) or with lentiviruses expressing an shRNA to FoxO family members (PSR “pan FoxO”), using antibodies to FoxO1, FoxO3, FoxO4, FoxO6, and β actin.

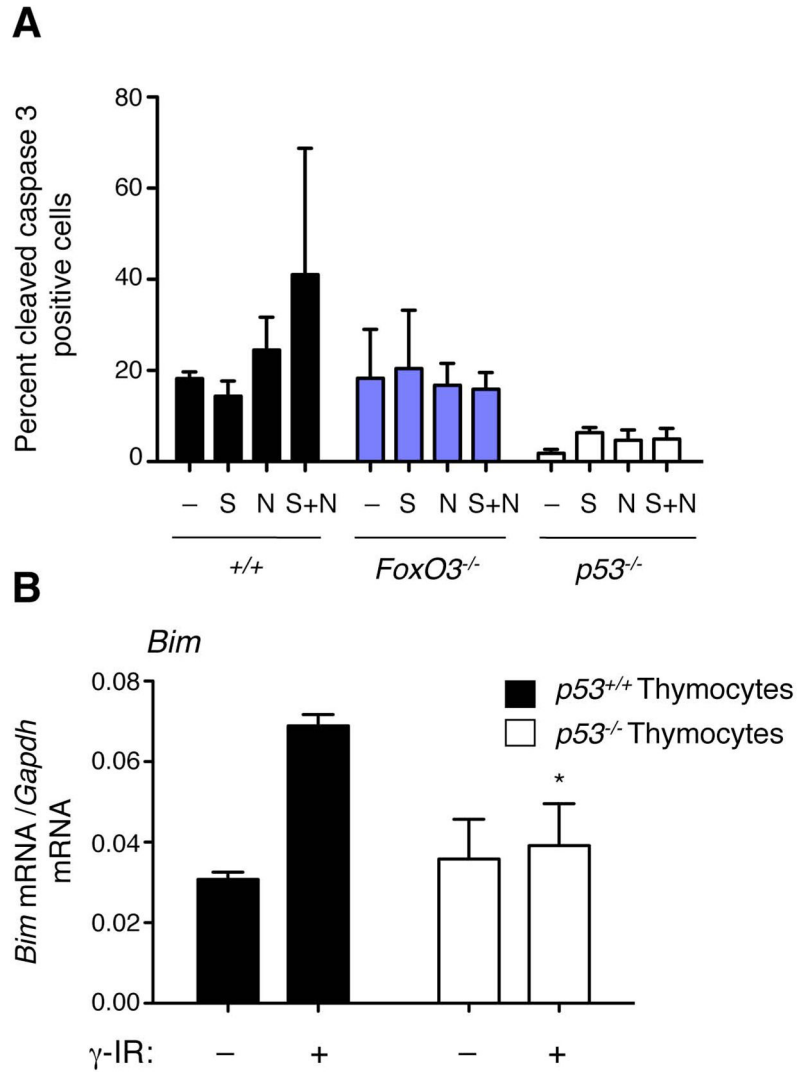


Figure 7. FoxO3 plays a role in p53-dependent apoptosis

(A) Percent cleaved caspase 3-positive cells in E1A-transformed MEFs ($FoxO3^{+/+}p53^{+/+}$ (+/+), $FoxO3^{-/-}$, and $p53^{-/-}$) in the absence of treatment (-) or in response to Nutlin (N), serum starvation (S), and Nutlin + serum starvation (N+S). Mean \pm SEM of three independent experiments. (B) Real time quantitative PCR analysis of *Bim* mRNA levels in $p53^{+/+}$ and $p53^{-/-}$ thymocytes, 3 hours after γ irradiation (γ IR, 10 Gy). Mean \pm SEM of two independent experiments conducted in triplicate on samples from 3–5 mice per genotype. * $p < 0.05$ between $p53^{+/+}$ and $p53^{-/-}$ thymocytes at a given time point, two-way ANOVA with Bonferroni post-test.

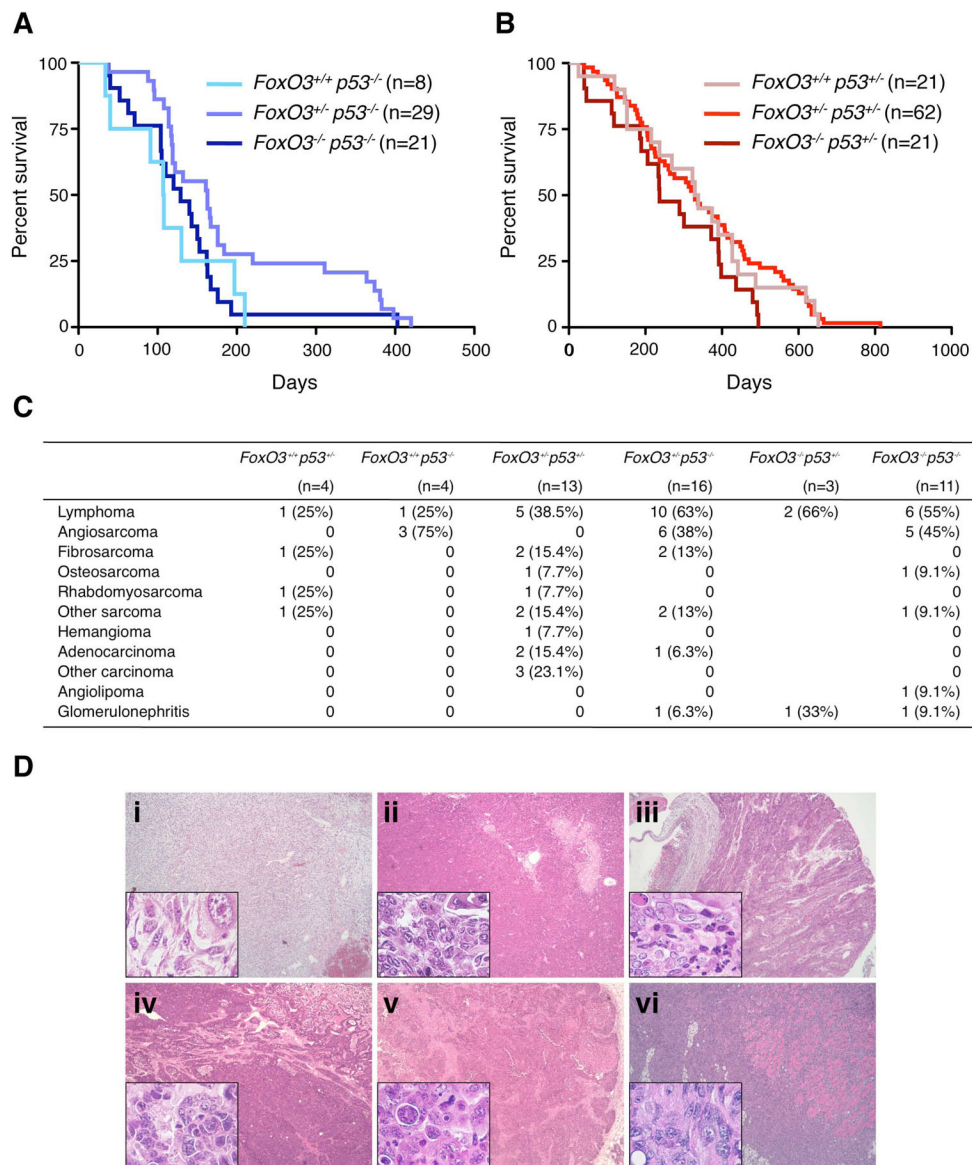


Figure 8. *FoxO3* loss does not affect survival in mice that are lacking one or both *p53* alleles, but may alter tumor spectrum

(A). Percent survival of mice with different alleles of *FoxO3* in the *p53*^{-/-} background as a function of time. Kaplan-Meier survival curves with the number of mice indicated for each genotype. $p = 0.10$, logrank test. (B) Percent survival of mice with different alleles of *FoxO3* in the *p53*^{+/-} background as a function of time. Kaplan-Meier survival curves with the number of mice indicated for each genotype. $p = 0.13$, logrank test. (C) Tumor types and glomerulonephritis in mice with different alleles of *FoxO3* in the *p53*^{+/-} and *p53*^{-/-} background. The number of mice is indicated for each genotype. (D) Examples of sarcomas and carcinomas in compound *FoxO3/p53* mutant mice. Main panels: 50x. Insets: 630x. i: subcutaneous fibrosarcoma in a *FoxO3*^{+/-}*p53*^{-/-} mouse; ii: osteosarcoma in the leg of a *FoxO3*^{-/-}*p53*^{-/-} mouse; iii: colon carcinoma in a *FoxO3*^{+/-}*p53*^{-/-} mouse; iv: uterine

carcinoma in a *FoxO3^{+/-}p53^{+/-}* mouse; v: breast carcinoma in a *FoxO3^{+/-}p53^{+/-}* mouse;
vi: muscle carcinoma in the arm of a *FoxO3^{+/-}p53^{+/-}* mouse.

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