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ATF3 Deficiency Promotes Genome Instability and Spontaneous Tumorigenesis in Mice

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Abstract

Mice lacking genes involving in the DNA damage response (DDR) are often tumor prone owing to genome instability caused by oncogenic challenges. Previous studies demonstrate that activating transcription factor 3 (ATF3), a common stress sensor, can activate the tumor suppressor p53 and regulate expression of p53 target genes upon DNA damage. However, whether ATF3 contributes to the maintenance of genome stability and tumor suppression remains unknown. Here we report that *Atf3*-deficient ($Atf3^{-/-}$) mice developed spontaneous tumors, and died significantly earlier than wild-type ($Atf3^{+/+}$) mice. Consistent with these results, $Atf3^{-/-}$ mouse embryonic fibroblasts (MEFs) had more aberrant chromosomes and micronuclei, and were genetically unstable. Whereas we demonstrated that ATF3 activated p53 and promoted its pro-apoptotic activity in mouse thymi and small intestines, the chromosomal instability caused by *Atf3* deficiency was largely dependent on the regulation of p53 by ATF3. Interestingly, loss of *Atf3* also promoted spontaneous

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Author Contributions: ZY, YH, and HY bred the mice and carried out histopathological examinations of tumor sections supervised by RK, WD, and CY. LL isolated MEFs and prepared slides for cytogenetic analysis. ZY and YH carried out immunohistochemical and immunofluorescence staining with the help of JZ and HD. TH provided the $ATF3^{-/-}$ mice. BJ, JZ, and HD edited the manuscript. CY and WD conceived the study and analyzed the data. CY wrote the manuscript.

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tumorigenesis in *Trp53*$^{+/-}$ mice, but did not affect tumor formation in *Trp53*$^{-/-}$ mice. Our results thus provide the first genetic evidence linking ATF3 to the suppression of the early development of cancer, and underscore the importance of ATF3 in the maintenance of genome integrity.

Keywords

ATF3; genome stability; tumorigenesis; knock-out mouse

Introduction

Genome instability is a hallmark of cancer, and often caused by genetic defects in the DNA-damage response (DDR) pathway that is mediated by the tumor suppressor p53 and regulated by many p53 network proteins (*e.g.*, the histone acetyltransferase Tip60) [1]. p53 guards genome integrity mainly through inducing cell cycle arrest for DNA repair, or triggering apoptosis when the damage is irreparable [2]. Indeed, whereas the p53 gene is mutated in almost half of human cancers, p53-deficient (*Trp53*$^{-/-}$) mice are highly susceptible to tumorigenesis, and develop spontaneous tumors (mainly thymic lymphomas) at an average age of 4.5 months [3-5]. Mouse embryonic fibroblasts (MEFs) derived from these mice are genetically unstable, exhibiting a high degree of aneuploidy with wide-spread chromosomal aberrations [6]. p53 heterozygous (*Trp53*$^{+/-}$) mice also succumb to spontaneous tumorigenesis, albeit with long latency [4], indicating that a mere decrease in p53 activity can increase tumor susceptibility in mice as well. Mice carrying defects in other DDR genes (*e.g.*, 53BP1 and H2AX) are also tumor prone [7,8], and loss of these DDR genes often synergize with p53 deficiency to increase genome instability and promote tumorigenesis [8-10].

Widely-known for its induction by a large variety of cellular stresses [11], activating transcription factor 3 (ATF3) is a member of the ATF/CREB transcription factors. ATF3 can regulate diverse cellular functions through binding to the ATF/CREB *cis*-regulatory element [11], or interacting with other proteins (*e.g.*, p53, and NF-κB) [12,13]. As ATF3 expression can be rapidly induced by DNA damage caused by γ-irradiation (IR), UV, chemotherapeutic agents, as well as oncogene expression [14-16], it has been long hypothesized that ATF3 plays an important role in regulating DDR. Indeed, recent studies have yielded evidence supporting that ATF3 likely engages in cell-fate decisions in response to UV and IR. For instance, it has been shown that ATF3 can promote UV-induced cell death by inducing Hif-2α expression [17], or by regulating Tip60 [18]. However, ATF3 can also promote the repair of UV-caused DNA lesions thereby protecting cells from UV-induced apoptosis by inducing p15^{PAF} expression [19], or through facilitating the recruitment of DNA-repair proteins to damaged DNA sites [18]. Whereas it appears that p53 functionality in cells determines whether ATF3 protects cells from, or promotes, UV-caused death [18], we have demonstrated that ATF3 can block p53 ubiquitination and activate the tumor suppressor in response to DNA damage [12,20]. We also found that ATF3 co-localizes with p53 at a large number of genomic sites and thus cooperates with p53 to regulate gene expression upon DNA damage [21]. Moreover, we showed that ATF3 can stabilize Tip60 to activate the ATM-mediated checkpoint signaling for p53 activation and the repair of double-strand breaks (DSBs)

induced by IR [22]. Accordingly, knockdown/knockout of ATF3 expression by short hairpin RNA (shRNA) or single-guided RNA (sgRNA) impairs DSB repair and sensitizes cells to γ-irradiation [22]. These results strongly argue for a notion that ATF3, like other DDR genes, likely contributes to the maintenance of genome stability thereby suppressing tumorigenesis. Whereas this notion is partly supported by the observations that *ATF3* expression is frequently down-regulated in human cancers [23-26] and that *ATF3* deficiency promotes cellular transformation induced by oncogenic Ras [12], genetic evidence linking ATF3 to the suppression of the onset of tumorigenesis is currently lacking.

Here, we report that *Atf3*-deficient ($Atf3^{-/-}$) mice were susceptible to spontaneous tumorigenesis and died significantly earlier than the wild-type mice. Consistent with these *in vivo* data, $Atf3^{-/-}$ MEFs exhibited increased genome/chromosomal instability. Whereas these effects appeared largely dependent on ATF3-mediated regulation of p53, *Atf3* deficiency also promoted tumorigenesis in $Trp53^{+/-}$ mice. Our results thus underscore the importance of ATF3 in the maintenance of genome stability.

Results

Atf3 deficiency increases tumor susceptibility in mice

In light of the emerging evidence supporting the role of ATF3 in DDR [17-22] and the fact that DDR is an early anti-cancer barrier [27], we sought to explore whether *Atf3* deficiency confers mice with genome instability and tumor susceptibility. $Atf3^{-/-}$ mice were developmentally normal [28]. We thus housed cohorts of $Atf3^{-/-}$ and wild-type ($Atf3^{+/+}$) mice in a SPF suite, and inspected them periodically for tumor growth until they were moribund or died. While the median tumor-free survival of the wild-type mice was 113 weeks, loss of *Atf3* significantly decreased the survival time to 100 weeks (p=0.0017) (Fig 1a), suggesting that $Atf3^{-/-}$ mice were tumor prone. Indeed, among those 13 $Atf3^{-/-}$ mice that were autopsied and examined, 9 mice (69.2%) were found to develop tumors (Fig 1b and 1c). In contrast, only 4 out of 17 (23.5%) $Atf3^{+/+}$ mice had tumors (Fig 1c). While the aged wild-type mice grew lymphomas and liver tumors as expected [29], $Atf3^{-/-}$ mice, like $Trp53^{+/-}$ mice [4,5], had a broader tumor spectrum. Not only sarcomas, but mesenteric and subcutaneous carcinomas, and carcinomas in lungs and kidneys were found in $Atf3^{-/-}$ mice (Fig 1b, 1d, and Table 1). Moreover, $Atf3^{-/-}$ mice often develop multiple tumors in different tissues. For instance, splenic lymphoma, renal carcinoma, and a tail sarcoma were found in the same mouse ID#2. Our results thus argue for a notion that *Atf3* deficiency promoted spontaneous tumorigenesis in mice.

ATF3 deficiency increases genome instability in mouse embryonic fibroblasts

DDR proteins can suppress tumorigenesis through guarding genome stability upon oncogenic stress [30]. To elucidate the mechanism by which *Atf3* deficiency increased tumor susceptibility, we analyzed karyotypes of early passages (passage 2 or 3) of $Atf3^{-/-}$ and $Atf3^{+/+}$ MEFs (Fig 2a) to determine whether *Atf3* deficiency causes genome instability in mouse cells. Whereas the normal mouse karyotype has 40 chromosomes, a large number of $Atf3^{+/+}$ MEFs contained atypical numbers of chromosomes as reported [7, 31] (Fig 2b), indicating that the rapid growth of MEFs generated replication stress and oxidative stress

that could challenge the integrity of the genome. Importantly, we found that *Atf3* deficiency dramatically increased the chromosomal/genome instability in MEFs (Fig 2c). Not only the number of diploid cells was largely decreased (Fig 2d), but the chromosomes of *Atf3*$^{-/-}$ MEFs had significantly more aberrations (Fig 2e), such as ring/radial chromosomes, chromatid/chromosome breakage, and telomere fusions (Fig 2b, arrows). We further examined chromosomal instability by counting micronuclei, and found that the number of *Atf3*$^{-/-}$ cells carrying micronuclei was significantly increased (Fig 2f). Together these results indicate that *Atf3* deficiency likely increased genome instability, thereby promoting spontaneous tumorigenesis in mice.

Atf3$^{-/-}$ MEFs had defects in DNA repair and ATM activation in response to IR

As it has been shown that ATF3 plays important roles in DDR [17-22], the increased genome instability in *Atf3*$^{-/-}$ cells might be caused by a defect in DDR. Indeed, DDR was impaired in *Atf3*$^{-/-}$ MEFs, as these cells had significantly more IR-induced γH2AX foci (indicative of DNA lesions) than the wild-type MEFs 8 h and 24 h post irradiation (Fig 3a and 3b). These results are reminiscence of our previous finding that ATF3 down-regulation leads to accumulation of damaged DNA in irradiated human cells [22], suggesting that the activation of ATM - the major kinase triggering signaling for efficient DSB repair - might be impaired in *Atf3*$^{-/-}$ MEFs. In line with this notion, phosphorylation of ATM and p53 upon IR were suppressed in *Atf3*-deficient MEFs (Fig 3c). To explore a possibility that the suppression of ATM activation in *Atf3*$^{-/-}$ cells contributes to the defect in DNA repair, we treated the cells with an ATM-specific inhibitor, KU-55933, and counted γH2AX foci 24 h post IR. As expected, ATM inhibition resulted in accumulation of damaged DNA in both *Atf3*$^{+/+}$ and *Atf3*$^{-/-}$ cells (Fig 3d). Importantly, ATM inhibition by KU-55933 decreased the extent of DNA-repair defect caused by *Atf3* deficiency (Fig 3e), arguing for a notion that the effects of ATF3 on DNA repair and genome maintenance partly attributes to its capability to promote ATM activation upon DNA damage [22]. As ATM contributes to the repair of fewer than 15% of DSBs induced by IR [32], our results also suggest that ATF3 might regulate DNA repair through additional mechanism(s) independent of ATM activation.

Loss of ATF3 impairs p53 activation in mice

Previously, we demonstrated that ATF3 can also regulate DDR by activating p53 [12]. In MEFs, we found that *Atf3* deficiency impaired p53 activation and expression of its target genes (*p21* and *PUMA*) induced by the DNA-damaging agent doxorubicin as well (Supplementary Fig S1). To explore a possibility that the increased genome instability and tumor susceptibility in *Atf3*$^{-/-}$ mice was also a consequence of impaired p53 activation, we determined the p53 activity in *Atf3*$^{-/-}$ mice after irradiating the mice with 8 Gy of IR. We first isolated thymocytes from irradiated mice, and subjected the cells to Western blotting to measure expression levels of p53 and its well-characterized target p21. While IR concurrently induced ATF3 expression and p53 activation (evidenced by induction of p21 expression) in the wild-type cells, IR-induced p53 activation was largely impaired in *Atf3*$^{-/-}$ thymocytes (Fig 4a), suggesting that ATF3 could also activate p53 *in vivo*. As IR-induced p53 activation can promote thymocytes and intestinal crypt cells to undergo apoptosis [33-35], we next stained thymocytes and small intestines for expression of cleaved caspase 3 to determine the apoptosis-promoting activity of p53 in mice. In lined with impaired p53

activation (Fig 4a), IR-induced apoptosis was significantly suppressed in *Atf3*$^{-/-}$ thymocytes (Fig 4b and 4c). Similarly, the number of apoptotic crypt cells induced by IR (Fig 4d, arrows) was also dramatically decreased in irradiated *Atf3*$^{-/-}$ mice as compared to *Atf3*$^{+/+}$ mice (Fig 3d and 3e). Of note, IR failed to induce apoptosis in intestinal crypts of *Trp53*$^{-/-}$ mice (Fig 4f), confirming that this apoptosis-inducing activity was dependent on p53 (ref 35). These results thus demonstrated that *Atf3*$^{-/-}$ mice had decreased p53 activity that might lead to tumorigenesis.

Loss of p53 compromises the effects of Atf3 deficiency on genome stability

To test whether decreased p53 activity in *Atf3*$^{-/-}$ mice contributes to increased genome instability, we crossed *Atf3*$^{-/-}$ mice with *Trp53*$^{-/-}$ mice, and isolated *Atf3*$^{+/+}$*;Trp53*$^{-/-}$ (p53-KO) and double-knockout (*Atf3*$^{-/-}$*;Trp53*$^{-/-}$; DKO) MEFs (Fig 5a) for genome stability analysis. As reported [6], p53-KO cells were mostly aneuploid (Fig 5b and 5c), and often carried micronuclei (Fig 5d). Further knockout of *Atf3* expression did not increase the number of aneuploid cells (Fig 5b and 5c), or micronucleus-carrying cells (Fig 5d), indicating that *Atf3* deficiency failed to further increase genome instability in the absence of p53. These results argue for that ATF3 maintained genome stability largely by regulating p53 in mouse cells.

Atf3 deficiency promotes tumorigenesis in Trp53$^{+/-}$ mice

Trp53$^{+/-}$ mice are also tumor prone, but have latency longer than 10 months [4]. As *Atf3*$^{-/-}$ mice had decreased p53 activity (Fig 4), we hypothesized that *Atf3* deficiency could further decrease p53 activity thereby promoting tumorigenesis in *Trp53*$^{+/-}$ mice. To test this, we generated cohorts of *Atf3*$^{+/+}$ and *Atf3*$^{-/-}$ mice in different *Trp53* backgrounds (Fig 6a), and monitored them for tumor growth. Consistent with the previous results (Fig 1), *Atf3*$^{-/-}$ mice carrying the wild-type *Trp53* gene (*Atf3*$^{-/-}$*;Trp53*$^{+/+}$) died significantly earlier than wild-type mice (*Atf3*$^{+/+}$*;Trp53*$^{+/+}$) (median survival 89.5 vs. 110 weeks, p=0.0047) (Fig 6b), and also developed tumors at a higher incidence (66.7% vs. 21.7%, $p < 0.01$) (Fig 5c). These results confirm that *Atf3*$^{-/-}$ mice were more susceptible to tumorigenesis. Loss of one *Trp53* allele decreased the tumor-free survival time to 75 weeks while increasing the tumor incidence to 70.4% (Fig 6c). As previously reported [4,36], more than 50% of *Trp53*$^{+/-}$ mice developed sarcomas (Table 2). Knockout of *Atf3* in the *Trp53*$^{+/-}$ background further decreased the tumor-free survival time to 61 weeks (p=0.0067) (Fig 6b). Although the tumor incidence was not changed (Fig 6c), *Atf3*$^{-/-}$*;Trp53*$^{+/-}$ mice developed more lymphomas than sarcomas (Table 2), and thus they were more pheonotypically similar to *Trp53*$^{-/-}$ mice [5]. These results were consistent with a lower p53 activity in the *Atf3*$^{-/-}$*;Trp53*$^{+/-}$ mice as compared to the *Atf3*$^{+/+}$*;Trp53*$^{+/-}$ mice. Indeed, while the tumor incidence was increased to 81.2% (Fig 5c), the *Trp53*$^{-/-}$ mice (*Atf3*$^{+/+}$) predominantly carried thymic lymphomas (Table 2), and died at an average 22 weeks of age (Fig 6b). However, neither the tumor incidence, nor the median survival time, nor the tumor spectrum, was significantly altered when *Atf3* was depleted from the *Trp53*$^{-/-}$ mice (Fig 6c, 6d, and Table 2). These results thus demonstrated that *Atf3* deficiency promoted tumorigenesis in *Trp53*$^{+/-}$ mice. The failure of *Atf3* deficiency to promote tumorigenesis in *Trp53*$^{-/-}$ mice was in line with the inability of ATF3 to alter genome stability in p53-deficient cells (Fig 5c and 5d), arguing for the notion that ATF3 suppressed tumorigenesis, at least in part, through regulating p53. It is important to note that

we also found an ovarian carcinoma and skin squamous cell carcinomas in *Atf3*$^{-/-}$ mice (Fig 6d and 6e). As these tumors have not been found/reported in *Trp53*$^{+/-}$ or *Trp53*$^{-/-}$ mice [5], *Atf3* deficiency might also promoted tumorigenesis via a mechanism independent of p53.

Discussion

As a common stress sensor, ATF3 has been reported to involve in many important human diseases including atherosclerosis [37], infections [38], cardiac hypertrophy [39], and hypospadias [40]. In this report, we showed that *Atf3*$^{-/-}$ mice were predisposed to tumorigenesis likely owing to increased genome instability. This study thus provides the first genetic evidence supporting a role that ATF3 plays in the suppression of the onset of cancer, and accordingly adds a new function to the growing list of the anti-cancer effects of this important stress-responsive gene. Indeed, although it was first identified as a metastasis-promoting gene in a mouse model of melanoma [41], ATF3 has recently been shown to suppress growth and/or progression of colon [42], bladder [43], esophageal [25], prostate [44], and lung cancers [24,45] through diverse mechanisms including inhibition of Akt and cytoskeleton remodeling. It was also frequently reported that ATF3 mediates cytotoxic effects of therapeutic agents such as curcumin [46], cisplatin [47], and pracinostat [48]. More recently, ATF3 was shown to sensitize glioma stem cells to proteasome inhibitors [49] – a result supporting the notion that ATF3 can serve as a biomarker for predicting outcomes of targeted anti-cancer therapies.

Our finding that *Atf3* deficiency increased tumor susceptibility suggests that ATF3 is likely a putative tumor suppressor. Whereas this notion is supported by the previous reports that *ATF3* expression is frequently down-regulated in human cancers[23-26], a web-based database (Gene Expression across Normal and Tumor Tissues, or GENT) [50] that contains gene-expression data of 24,300 clinical samples also reveals that *ATF3* expression was commonly down-regulated in human cancers as compared to their corresponding normal tissues (Supplementary Fig S2). Although the mutation rate of the *ATF3* gene is low (COSMIC, cancer.sanger.uk), *ATF3* expression might be down-regulated by epigenetic mechanisms (*e.g.*, histone acetylation) in human cancer [51]. Surprisingly, 19% of breast cancers have *ATF3* copy gains [52]. Whereas the amplified *ATF3* gene might express ATF3 variants (e.g., ATF3ΔZip) that can function as nature antagonists of the full-length ATF3 protein [53], a recent report suggests that ATF3 expressed in stromal cells can promote breast cancer metastasis in mouse models [54]. However, ATF3 was also shown to promote untransformed mammary epithelial cells to undergo apoptosis, and thus, ATF3 could suppress the early development of breast cancer [55]. The latter results are consistent with our previous finding that *Atf3*$^{-/-}$ MEFs can bypass oncogene-induced senescence and subsequently be transformed by oncogenic Ras [12]. Therefore, although the role of ATF3 in cancer progression appears to be context-dependent [56], the genetic evidence presented in this study strongly argues for the notion that ATF3 can suppress tumorigenesis in the initial step of cancer development.

Previously, we showed that ATF3 is a DDR factor that can interact with p53 and activate p53 by blocking MDM2-mediated ubiquitination and proteasomal degradation [12]. Whereas the ATF3-mediated tumor suppression activity is well in line with its crucial role in DDR, here we have extended our previous finding and demonstrated that ATF3 could activate p53 *in*

vivo as well. Not only the p53 transcriptional activity in thymocytes, but its activity to induce apoptosis in intestinal crypts, was impaired in *Atf3*$^{-/-}$ mice when the mice were subjected to whole-body exposure to IR (Fig 4). Although this lethal dose of IR induces a large amount of DNA lesions that do not faithfully model oncogenic challenges faced by cells, it is a reasonable extrapolation that p53 activation induced by oncogenic stress would be impaired in *Atf3*$^{-/-}$ mice. Indeed, in an earlier study investigating the role of ATF3 in prostate tumorigenesis induced by *Pten* loss, we found that the oncogenic stress caused by the loss of the tumor suppressor failed to induce p53 in *Atf3*$^{-/-}$ mice [44].Well defined as the guardian of the genome, p53 can maintain genome stability through diverse mechanisms, including the induction of cell-cycle arrest, apoptosis, senescence, and ferropotosis [1,57]. Although the exact mechanism by which p53 suppresses tumorigenesis remains elusive [58], the p53-activation defect in the *Atf3*$^{-/-}$ mice would result in loss of genome stability, leading to early onset of tumorigenesis as we observed in this study. Interestingly, it appears that *Atf3* deficiency could further decrease p53 activity in *Trp53*$^{+/-}$ mice as loss of *Atf3* promoted spontaneous tumorigenesis in these p53 heterozygous mice (Fig 6b). However, unlike other DDR genes (e.g., H2AX and 53BP1) known to mediate DDR independent of p53, loss of *Atf3* did not appear to promote tumorigenesis in *Trp53*$^{-/-}$ mice. Whereas these results support the notion that ATF3 activates p53 for tumor suppression, the dramatic tumorigenic phenotype of homozygous depletion of *Trp53* might obscure any activity that ATF3 harbors to suppress tumorigenesis independent of p53. Indeed, ATF3 is known to protect cells from reactive oxygen species that are often carcinogenic owing to their abilities to damage DNA [38,59]. Alternatively, it might be that ATF3 regulates Tip60 (ref 22), a histone acetyltransferase which can promote DNA repair and suppress oncogene-induced tumorigenesis through diverse mechanism including the activation of ATM [60, 61]. We have shown that the ATM signaling and the ATM-mediated DNA repair were indeed suppressed in *Atf3-/-* MEFs (Fig 3).

ATF3 and p53 can mutually regulate each other [12,62]. Whereas several studies including ours have shown that ATF3 can interact with p53 and promote its activity [12,21,63,64], it is worth noting that ATF3 was also reported to inhibit the p53 activity by directly down-regulating the p53 mRNA level [65,66], or repressing the p53 transactivation activity [67]. However, the latter results were mainly obtained using cultured cells ectopically over-expressing ATF3, and thus might not be relevant to physiological settings. Moreover, it has been well established that the p53 protein level is tightly controlled by ubiquitin-mediated proteasomal degradation, rather than its mRNA abundance [68, 69]. As we found that ATF3 was required for maximal p53 activity upon DNA damage in mouse thymocytes and intestines (Fig 3), it is more likely that ATF3 acts a p53 activator in DDR.

Materials and Methods

Animals and genotyping

Animal experiments were carried out in accordance with a protocol approved by the Institutional Committee of Animal Care and Use (ICACU) of Augusta University and the Albany Medical College. *ATF3* knockout (*ATF3*$^{-/-}$) mice were described previously [28]. The knockout and wild-type mice (all in C57BL/6 background, roughly half males and half

females) were housed in a SPF suite, and monitored periodically for tumor growth until they were moribund or died. Moribund mice were sacrificed and subjected to autopsy to examine tumor development. All available tumors were fixed in 4% of formalin overnight, and then embedded in paraffin for sectioning, H&E staining, and histopathological examinations. *Trp53*$^{-/-}$ mice (B6.129S2-*Trp53*^{tm1Tyj}/J) were generated by backcrossing the *Trp53*^{tm1Tyj} line at least five times to C57BL/6J inbred mice, and purchased from the Jackson Laboratory. To generate compound mutants, *ATF3*$^{-/-}$ mice were first crossed with *Trp53*$^{-/-}$ mice, and then back-crossed with *Atf3*$^{+/+}$ and *Atf3*$^{-/-}$ mice, yielding *Atf3*$^{+/+}$;*Trp53*$^{+/-}$ and *Atf3*$^{-/-}$;*Trp53*$^{+/-}$ mice. Self-cross of the latter two strains generated *Atf3*$^{+/+}$;*Trp53*$^{+/+}$, *Atf3*$^{+/+}$;*Trp53*$^{+/-}$, *Atf3*$^{+/+}$;*Trp53*$^{-/-}$, *Atf3*$^{-/-}$;*Trp53*$^{+/+}$, *Atf3*$^{-/-}$;*Trp53*$^{+/-}$, and *Atf3*$^{-/-}$;*Trp53*$^{-/-}$ mice for the tumorigenesis experiments. For genotyping, mouse tails were lysed in PBND buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl2, 0.45% NP-40, 0.45% Tween-20) supplemented with 0.2 μg/ml Proteinase K at 55°C overnight, and lysates were subjected to PCR following a protocol provided by the Jackson Laboratory.

Apoptosis, immunohistochemistry (IHC), and immunofluorescence staining

To determine p53 activation *in vivo*, mice at the age of 5-6 weeks were exposure to 8 Gy of γ-irradiation. Thymocytes were then isolated by gently pressing thymi through cell strainers (40 μm) using syringe plungers, and then subjected to Western blotting for gene expression, or stained with an antibody against cleaved caspase 3 (1:800, Cell Signaling #9661) for flow cytometry analysis. Small intestines from irradiated and control mice were also fixed in formalin, and sectioned after paraffin embedding. After antigen retrieval in hot citrate buffer, sections were blocked in 5% of normal horse serum and 1% of normal goat serum, and subjected to immunohistochemical staining using the ABC Elite Kit and the DAB Kit (Vector) according to the manufacturers' recommendations. For immunofluorescence staining, cells were fixed in 4% of paraformaldehyde, blocked, and subsequently stained with the γ-H2AX antibody (1:800, #613401, BioLegend) and the DyLight 594-conjugated anti-mouse IgG antibody (1:500, ab96881, Abcam).

Isolation of mouse embryonic fibroblasts and cytogenetical analysis

MEFs were isolated from mouse embryos (d13.5) using a standard protocol. Briefly, embryo tissues were minced and digested in 0.05% trypsin/EDTA. After washes, cells were re-suspended in DMEM containing 10% of FBS and cultured in dishes coated with 0.2% gelatin for genotyping and propagation. For metaphase analysis, cells were treated with 0.5 μM of nocodazole for 3 h, and then suspended in 0.075 M of KCl after trypsinization. MEFs were then washed and fixed with fresh Carnoy's Fixative (75% methal and 25% acetic acid), and dropped onto slides to prepare chromosome spreads. After air dry, slides were stained with the Giemsa Stain (Sigma, GS500) and observed under a microscope. At least 100 metaphases were counted for each slide.

Western blotting

These were carried out as described previously [12]. Briefly, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche)), and subjected to SDS-polyacrylamide electrophoresis for Western blotting. The

following antibodies were used: ATF3 (1:1,000, sc-188) and p53 (1:1,000, sc-6243) from Santa Cruz, p21 (1:1,000, #556432) from BD Pharmingen, mouse ATM (1:1,000, NB100-104) and ATM(pSer1981) (1:1,000, AF1655-SP) from Novus Biologicals, and β-actin (1:10,000, A5441) from Sigma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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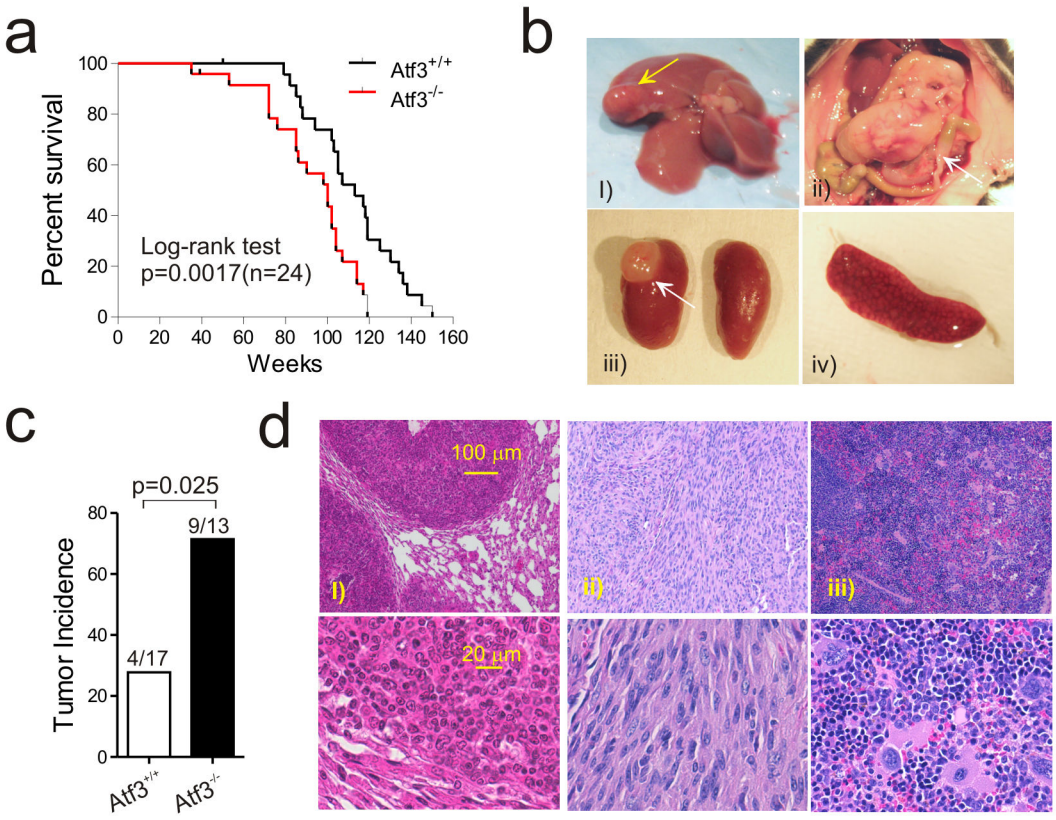

Fig 1. Loss of *Atf3* promotes spontaneous tumorigenesis in mice

(**a**) Tumor-free survival curves of *Atf3*$^{+/+}$ and *Atf3*$^{-/-}$ mice. 24 mice were analyzed for each genotype. (**b**) Representative tumors found in *Atf3*$^{-/-}$ mice. i) liver tumor; ii) mesenteric tumor; iii) a kidney tumor (left); iv) marked splenomegaly. Arrows indicate tumors. (**c**) Tumor incidences of *Atf3*$^{+/+}$ and *Atf3*$^{-/-}$ mice. Fisher's exact test. (**d**) H&E staining of representative tumors found in *Atf3*$^{-/-}$ mice (upper, 10×; lower, 40×). i) lung tumors; ii) sarcoma found in the tail of a mouse; iii) splenic lymphoma.

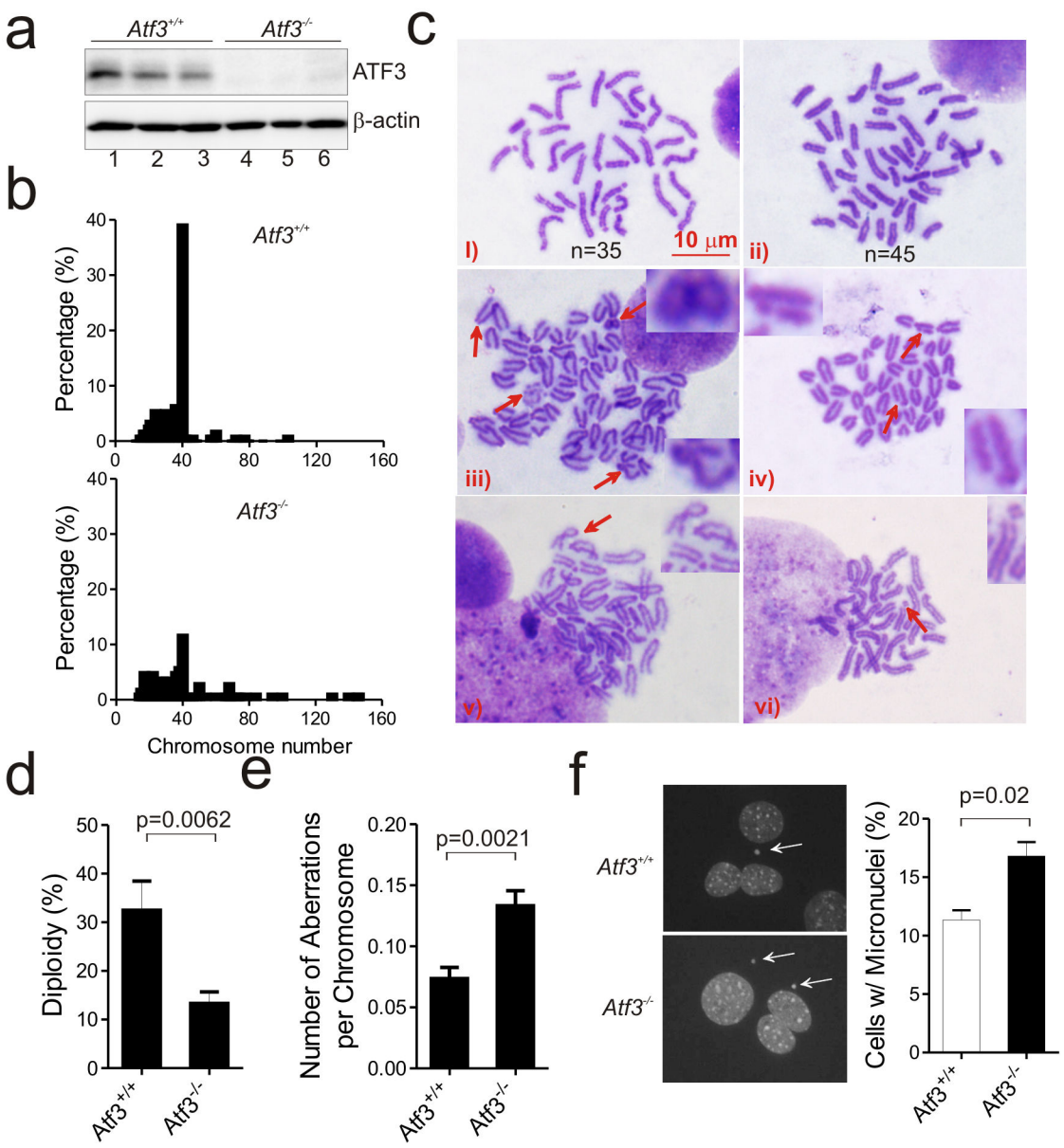

Fig 2. *Atf3* deficiency increases chromosomal instability of MEFs

(**a**) *Atf3*$^{+/+}$ and *Atf3*$^{-/-}$ MEFs (each prepared from 3 embryos) were subjected to Western blotting for ATF3 expression. (**b**) Representative distribution of the chromosome number of indicated MEF lines. Chromosomes in at least 100 metaphases were counted for each cell line. (**c**) Representative metaphase images of *Atf3*$^{-/-}$ MEFs. Arrows indicate chromosomal abnormalities. (**d**) Percentages of *Atf3*$^{+/+}$ and *Atf3*$^{-/-}$ MEFs with normal diploid chromosomes (n=40). 3 independent MEF lines were analyzed for each genotype. At least 100 metaphases were counted for each line. (**e**) The numbers of aberrations per chromosome of indicated MEFs. 1,000 chromosomes were examined for each MEF line; 3 lines for each genotype. (**f**) *Atf3*$^{+/+}$ and *Atf3*$^{-/-}$ MEFs were stained with DAPI and observed under a fluorescence microscope. Arrows indicate micronuclei. The plot shows percentages of cells carrying micronucleus/micronuclei. 300 cells were counted for each MEF line. 3 MEF lines

were analyzed for each genotype. The data in d, e, and f are presented as mean ± SD, Student's t-tests.

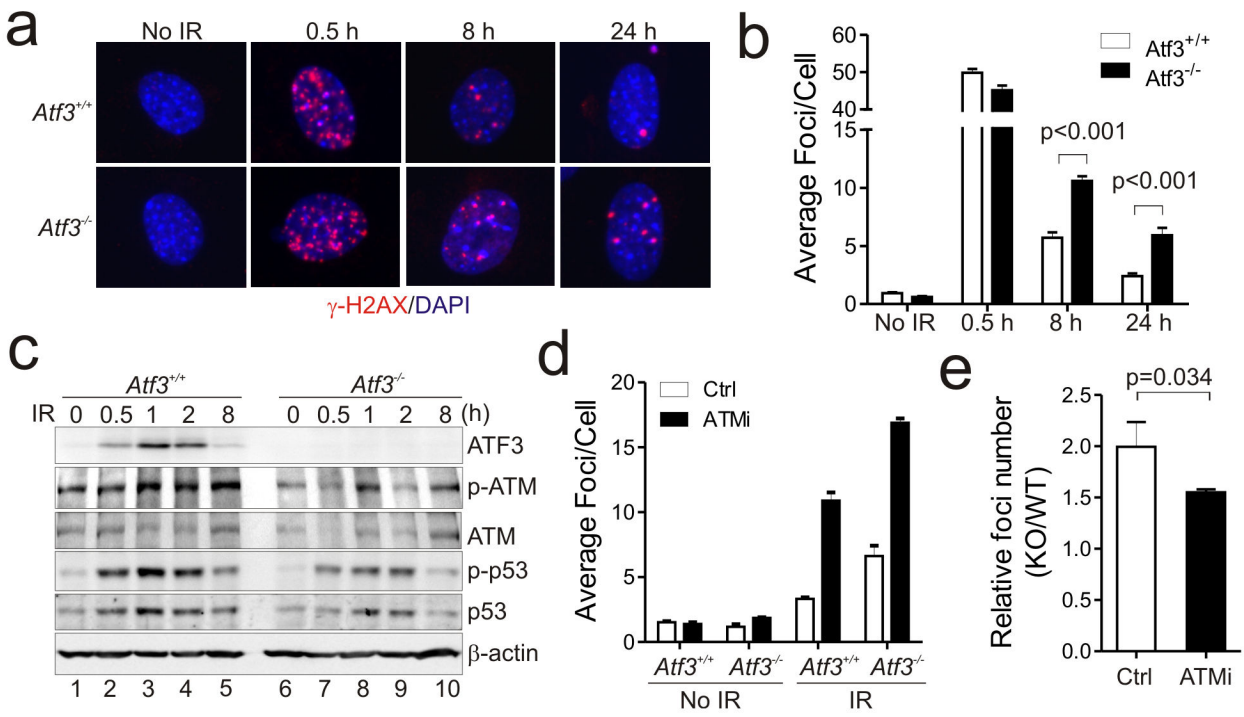

Fig 3. *Atf3*$^{-/-}$ MEFs had a defect in DNA repair

(**a, b**) MEFs were irradiated with 2 Gy of γ-rays and stained for γ-H2AX foci. γ-H2AX foci in 50 cells from each group were counted, and average foci numbers were calculated. (**c**) MEFs subjected to 10 Gy of IR were lysed for Western blotting. (**d, e**) Indicated cells were pre-treated with 10 μM of KU-55933 for 1 h, and then subjected to 2 Gy of IR. Cells were fixed 24 h after IR, and stained for γ-H2AX foci for counting. The data in b, d, and e are presented as mean ± SD, Student's t-tests.

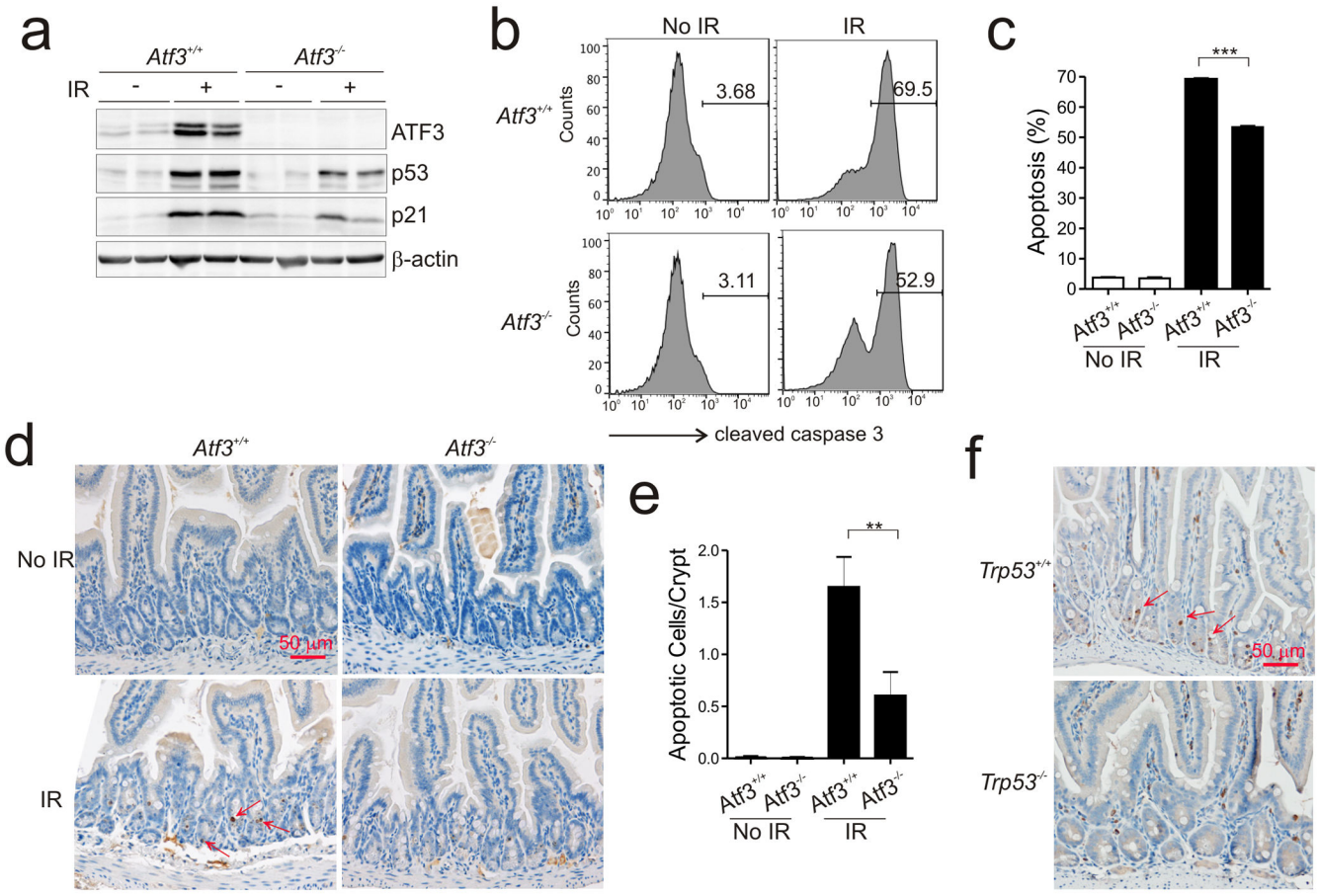

Fig 4. Loss of *Atf3* impairs p53 activation in mice

(**a**) Indicated mice (2 for each group) were subjected to 8 Gy of IR. 16 h later, thymocytes were isolated and subjected to Western blotting for p53 and p21 expression. (**b, c**) Thymocytes from control and irradiated mice (3 for each group) were stained for cleaved caspase 3 and subjected to flow cytometry to quantitate apoptotic cells (positive for cleaved caspase 3 staining). Representative flow cytometry plots were shown in (b). (**d, e**) Intestine sections from control and irradiated mice (3 for each group) were stained for cleaved caspase 3 expression. Arrows indicate apoptotic crypt cells. The data in c and e are presented as mean ± SD. **, $p<0.01$; ***, $p<0.001$, Student's t-test. (**f**) Sections were stained for cleaved caspase 3 expression (indicated by arrows).

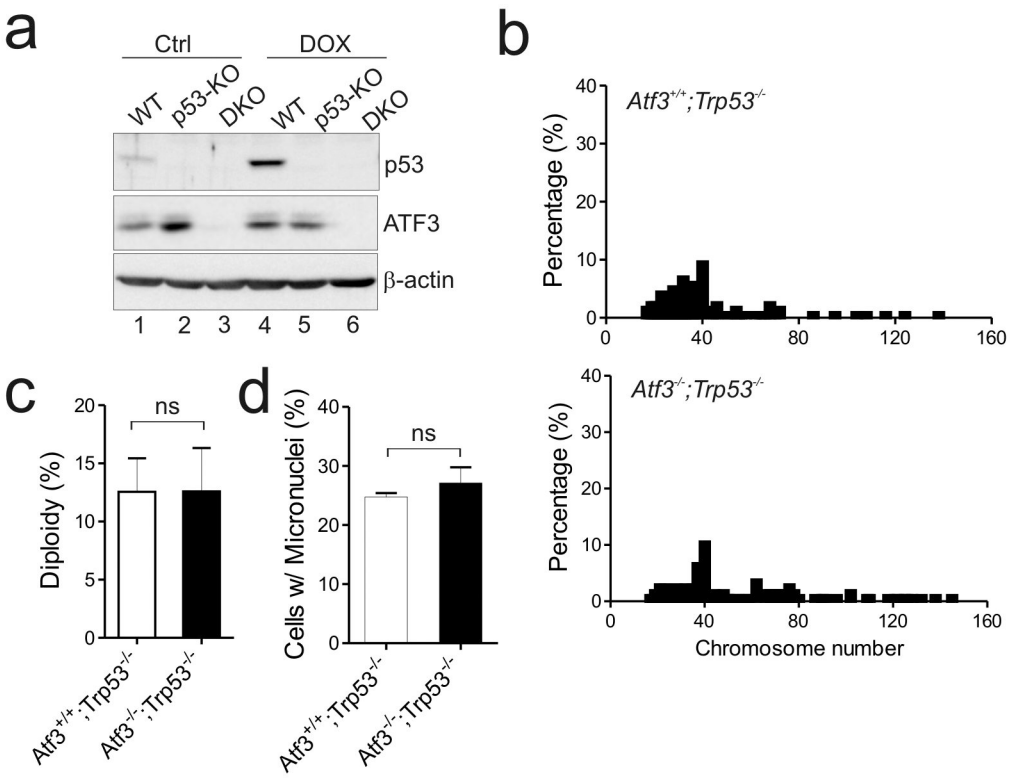

Fig 5. The effects of ATF3 on chromosomal instability were dependent on p53

(**a**) The wild-type (WT), *Trp53*$^{-/-}$ (p53-KO), and *Atf3*$^{-/-}$;*Trp53*$^{-/-}$ (DKO) MEFs were treated with/without 0.2 μg/ml doxorubicin, and subjected to Western blotting for ATF3 and p53 expression. (**b)** Representative distribution of chromosome numbers of indicated MEFs. (c) Percentages of MEFs with normal diploid chromosomes. 3 independent MEF lines were analyzed for each genotype. At least 100 metaphases were counted for each line. (**d**) The numbers of aberrations per chromosome of indicated MEFs. 1,000 chromosomes were examined for each MEF line; 3 line for each genotype. The data are presented as mean ± SD; ns, not significance, Student's t-test.

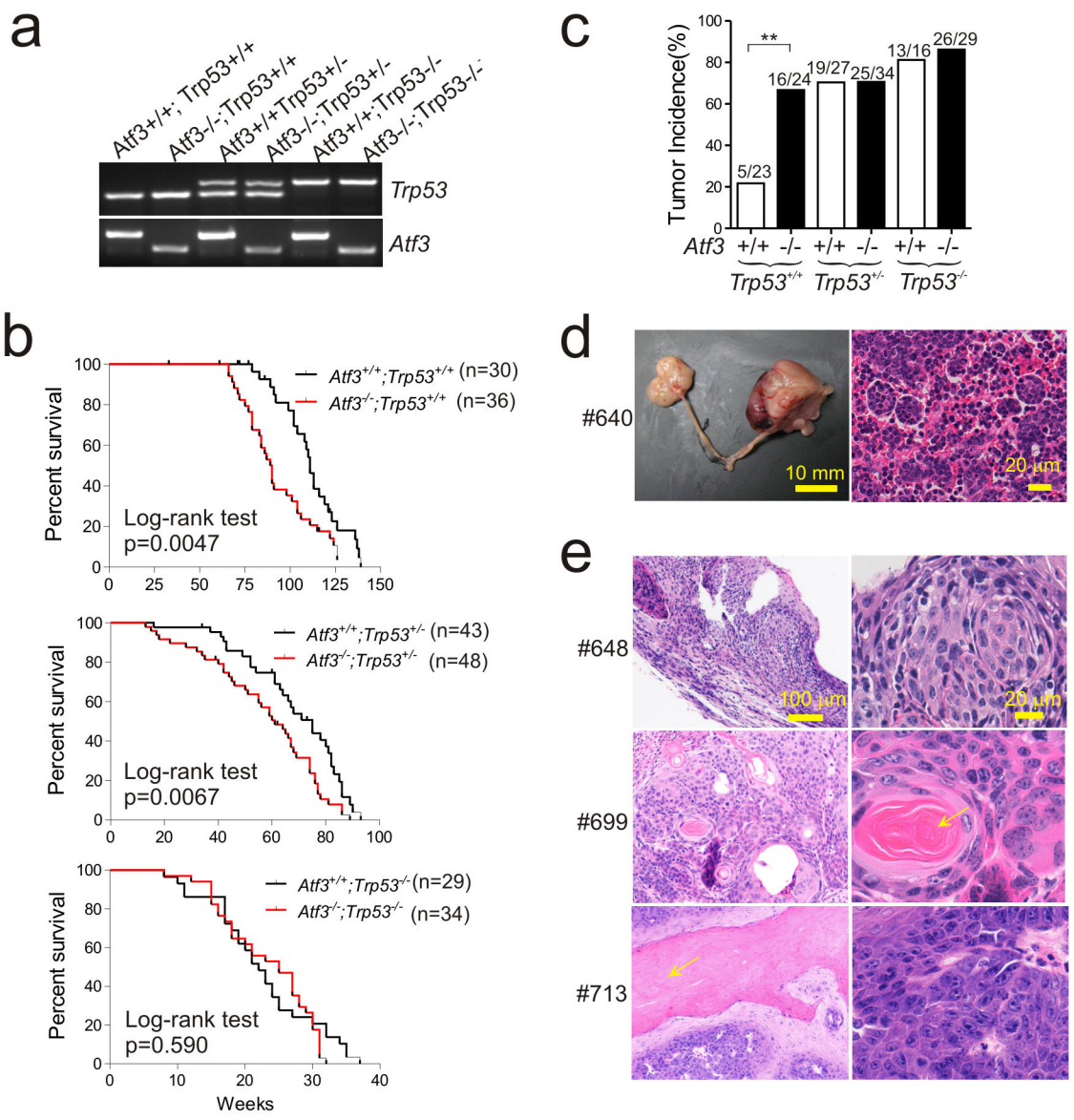

Fig 6. *Atf3* deficiency promotes tumorigenesis in *Trp53*$^{+/-}$ mice

(**a**) Representative genotyping results showing mice with desired genotypes. (**b**) Tumor-free survival curves of *Atf3*$^{+/+}$ and *Atf3*$^{-/-}$ mice in different *Trp53* backgrounds. Upper, *Trp53*$^{+/+}$; middle, *Trp53*$^{+/-}$; lower, *Trp53*$^{-/-}$. (**c**) Tumor incidences of indicated mice. **, p<0.01, Fisher's exact test. (**d**) A tumor was found in the right ovary of a *Atf3*$^{-/-}$;*Trp53*$^{+/-}$ mouse (ID#640). (**e**) Squamous cell carcinomas were found in a *Atf3*$^{-/-}$;*Trp53*$^{+/+}$ (ID#648) and two *Atf3*$^{-/-}$;*Trp53*$^{+/-}$ (ID#699 and #713) mice. Arrows indicate keratin pearls.

Table 1

The tumor spectra of *Atf3*$^{+/+}$ and *Atf3*$^{-/-}$ mice

	Atf3(+/+)	*Atf3(-/-)*
Lymphoma (%)	2 (50.0)	5 (29.4)
Sarcoma (%)		1 (5.9)
Carcinoma in		
Liver (%)	2 (50.0)	4 (23.5)
Mesenteric (%)		2 (11.8)
Lung (%)		2 (11.8)
Kidney (%)		1 (5.9)
Subcutaneous		2 (11.8)
No. of Mouse Examined	17	13
No. of Mouse w/ tumor (%)	4 (23.5)	9 (69.2)

Table 2

The tumor spectra of *Atf3*-*Trp53* compound-mutant mice

	Trp53(+/+)		*Trp53(+/-)*		*Trp53(-/-)*	
	Atf3(+/+)	*Atf3(-/-)*	*Atf3(+/+)*	*Atf3(-/-)*	*Atf3(+/+)*	*Atf3(-/-)*
Splenic Lymphoma (%)	2 (28.6)	6 (26.1)	5 (21.7)	9 (36.0)	3 (18.8)	2 (6.9)
Thymic Lymphoma (%)				2 (8.0)	8 (50.0)	20 (69.0)
Sarcoma (%)		1 (4.3)	13 (56.5)	4 (16.0)	3 (18.8)	4 (13.8)
Carcinoma in						
Liver (%)	3 (42.8)	5 (21.7)	2 (8.7)	3 (12.0)	1 (6.2)	2 (6.9)
Mesenteric (%)	2 (28.6)	8 (34.8)	1 (4.3)	2 (8.0)	1 (6.2)	
Lung (%)		1 (4.3)	1 (4.3)	1 (4.0)		1 (3.4)
Ovary (%)		1 (4.3)				
Skin (%)		1 (4.3)		2 (8.0)		
Subcutaneous (%)			1 (4.3)	2 (8.0)		
No. of Mouse examined	23	24	27	34	16	29
No. of Mouse w/ tumor (%)	5 (21.7)	16 (66.7)	19 (70.4)	25 (70.6)	13 (81.2)	26 (86.2)