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FANCD2 Activates Transcription of TAp63 and Suppresses Tumorigenesis

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SUMMARY

Fanconi Anemia (FA) is a rare genetic disorder characterized by an increased susceptibility to squamous cell cancers. Fifteen FA genes are known, and the encoded proteins cooperate in a common DNA repair pathway. A critical step is the monoubiquitination of the FANCD2 protein, and cells from most FA patients are deficient in this step. How monoubiquitinated FANCD2 suppresses squamous cell cancers is unknown. Here we show that *Fancd2*-deficient mice are prone to Ras oncogene-driven skin carcinogenesis, while *Usp1*-deficient mice, expressing elevated cellular levels of Fancd2-Ub, are resistant to skin tumors. Moreover, Fancd2-Ub activates the transcription of the tumor suppressor TAp63, thereby promoting cellular senescence and blocking skin tumorigenesis. For FA patients, the reduction of FANCD2-Ub and TAp63 protein levels may account for their susceptibility to squamous cell neoplasia. Taken together, *Usp1* inhibition may be a useful strategy for upregulating TAp63 and preventing or treating squamous cell cancers in the general non-FA population.

Keywords

Fanconi Anemia; Deubiquitinating Enzymes; Senescence; Ras; USP1

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AUTHOR CONTRIBUTIONS

E.P and A.D.D conceived and designed the study. E.P. performed all experiments and analyzed the data. J.M.K. was involved in the establishment of mouse models and animal experiments. H.K. contributed to the xenograft study and B.P. provided technical assistance during animal experiments. Y.X. and B.P. contributed to the elucidation of the transcriptional activity of the FANCD2-Ub protein. E.P., J.M.K., H.K., A.M, and A.D.D. participated in discussions and interpretations of the experiments. E.P., J.M.K., and A.D.D. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests

Accession Numbers

Chi-seq data have been deposited in the GEO database and the NCBI Sequence Read Archive database under accession numbers GSE46902.

INTRODUCTION

Squamous cell carcinoma (SCC) of the skin is one of the most common forms of cancer in humans, and its rising incidence may reflect increased sun exposure and a diminished ozone layer (Alam and Ratner, 2001). One of the most common molecular abnormalities observed in SCC is the overexpression of the p53 family member, p63 (Hu et al., 2002). Like p53, p63 is a transcriptional regulator, and it is expressed as two primary isoforms, the tumor suppressive TAp63 isoform (Koster and Roop, 2004) and the oncogenic ΔNp63 isoform (Rocco and Ellisen, 2006; Rocco et al., 2006). Moreover, expression of p63 is required for normal skin development (Mills et al., 1999) (Yang et al., 1999). The TAp63 isoform is primarily expressed in the dermis, and ΔNp63 is primarily expressed in the epidermis (Koster and Roop, 2004). Acquired mutations in the p63 gene derived from human squamous cell tumors are predicted to disrupt the expression of the tumor suppressive TAp63 isoform, suggesting that this isoform plays a critical role in suppressing the progression of SCC in the general (non-FA) population (Stransky et al., 2011).

Fanconi Anemia (FA) is a rare autosomal recessive or X-linked recessive disease characterized by multiple congenital abnormalities, bone marrow failure, and an increased susceptibility to squamous cell cancers, including skin, oral, and esophageal carcinomas (Alter et al., 2003). Primary cells isolated from FA patients exhibit hypersensitivity to DNA interstrand crosslinking agents, such as mitomycin C and diepoxybutane, resulting from an underlying defect in DNA repair (Auerbach, 2009). The fifteen known FA proteins cooperate in a common DNA repair pathway (reviewed in (Kee and D'Andrea, 2010). Eight of the FA proteins (A, B, C, E, F, G, L, M) are assembled into a nuclear protein complex, the FA core complex, which binds to DNA, primarily at stalled replication forks containing an interstrand crosslink (ICL). The FA core complex is a multisubunit E3 ubiquitin ligase which subsequently monoubiquitinates two additional FA proteins (FANCD2 and FANCI) (Garcia-Higuera et al., 2001) (Smogorzewska et al., 2007). The monoubiquitinated FANCD2 protein (FANCD2-Ub) then functions as a landing pad, recruiting an additional downstream FA protein, FANCP/SLX4 (Yamamoto et al., 2011) as well as the FAN1 nuclease (O'Donnell and Durocher, 2010). This regulated recruitment of endonucleases is required for the excision of the DNA crosslink. Downstream FA proteins (D1, N, O, and J) are further required for additional downstream repair events. Whether monoubiquitinated FANCD2 protein, the critical intermediate in the FA pathway, has additional cellular functions beyond DNA repair is unknown.

Recent studies have suggested that monoubiquitinated FANCD2 (FANCD2-Ub) may have an additional role as a tumor suppressor. *Fancd2*-deficient mice have an increase in spontaneous epithelial cancers (Houghtaling et al., 2003), and the incidence of these cancers is further increased in the setting of *p53* deficiency (Houghtaling et al., 2005). Moreover, FANCD2-Ub is expressed at high levels in tissues which are prone to malignant transformation, such as the squamous cell epithelia of the head and neck region and the uterine cervix, suggesting that the protein may function as a tumor suppressor in these tissues (Holzel et al., 2003). Whether FANCD2-Ub suppresses squamous cell cancers by regulating the level of other known suppressor proteins, such as the TAp63 protein, is unknown.

In the current study, we demonstrate that FANCD2-Ub and its binding partner FANCP/SLX4 directly suppress squamous cell cancer development by transcriptionally upregulating the cellular level of TAp63. *Fancd2*-deficient mice have an increased incidence of *Ras* oncogene-induced squamous cell cancers, compared to normal sibling control mice. Conversely, mice deficient in Usp1, the protease which deubiquitinates Fancd2 (Nijman et

al., 2005) (Kim et al., 2009), have elevated cellular levels of Fancd2-Ub and are resistant to Ras-induced squamous cell cancers. Fancd2-Ub promotes normal Ras-induced cellular senescence, and absence of Fancd2-Ub results in increased cellular transformation. Moreover, Fancd2-Ub binds to the promoter of the TAp63 gene and promotes its transcription. Taken together, our results indicate that the FA pathway suppresses squamous cell tumor development in a TAp63-dependent mechanism, accounting, at least in part, for the specific predisposition of FA patients to this class of cancer.

RESULTS

Fancd2-deficient mice exhibit increased squamous cell tumorigenesis

Previous studies have indicated that *Fancd2* (-/-) mice have elevated levels of spontaneous epithelial carcinomas (Houghtaling et al., 2003). We initially tested whether topical treatment with DMBA (dimethylbenz(a)anthracene), followed by a 28 week course of TPA (12-O-tetradecanoylphorbol-13-acetate) (Park et al., 2007) (Figure 1A), would further enhance squamous cell carcinogenesis in a strain of *Fancd2*-deficient mice developed in our laboratory (Parmar et al., 2010). As expected, *Fancd2* (+/-) mice exhibited an increased incidence and number of tumors following DMBA/TPA exposure (Figure 1B). All of the *Fancd2* (+/-) mice developed tumors by 21 weeks of DMBA/TPA treatment. These tumors did not exhibit loss of heterozygosity at the *Fancd2* locus (data not shown). In contrast, only 60% of the sibling control *Fancd2* (+/+) mice developed tumors by 28 weeks of DMBA/TPA treatment. *Fancd2* (+/-) mice developed more papillomas than *Fancd2* (+/+) mice (mean 4.5 vs 2.3 tumors per mouse, $P<0.01$) (Figure 1C). Moreover, the papillomas from the *Fancd2* (+/-) mice had a six times higher rate of progression to malignant carcinoma than the papillomas from the *Fancd2* (+/+) mice (Figure S1), which rarely developed invasive cancers (data not shown). Skin papillomas were excised from *Fancd2* (+/+) and *Fancd2* (+/-) mice exposed to the DMBA/TPA treatment and further analyzed. As expected, activating Ras mutations resulting from the exposure to carcinogen were found in tumors derived from the treated mice, regardless of genotype. Specifically, the H-RasV61 mutation was detected in most tumors by PCR. H-Ras mutations were not detected in the skin surrounding the tumors.

FANCD2 monoubiquitination is required for Ras oncogene induced senescence

We next examined the effect of TPA application alone on the epidermis of *Fancd2*-deficient mice (Figure 1D). Interestingly, *Fancd2* (-/-) and *Fancd2* (+/-) mice exhibited increased epidermal thickening following TPA exposure, compared to sibling control wild-type mice, consistent with the role of Fancd2 as a suppressor of cell proliferation. Furthermore, we applied TPA to the skin of *Fancg*-deficient mice, which also have a cellular defect in the synthesis of monoubiquitinated Fancd2. Like *Fancd2*-deficient mice, the *Fancg* (-/-) mice also exhibited enhanced epidermal thickening following TPA treatment (Figure S2A).

This increase in skin thickening suggested that *Fancd2* (-/-) cells may have a defect in Ras oncogene-induced senescence (OIS), a normal cellular process which limits cell proliferation after oncogene activation (Serrano et al., 1997) (Keyes et al., 2005). To test this hypothesis, we examined the level of senescence-associated (SA)-β galactosidase staining, a measure of *in vivo* senescence (Lin et al., 2010), in skin extracted from the TPA exposed animals (Figure 1E). Consistent with the reduced proliferation observed in TPA treated *Fancd2* (+/+) epithelial cells, we detected a higher accumulation of SA-β galactosidase staining in this tissue, compared to *Fancd2* (+/-) epithelium.

To further quantify cellular senescence, we measured OIS in *Fancd2* (-/-) primary murine embryo fibroblasts (MEFs) (Figure 2). *Fancd2* (-/-) MEFs showed decreased OIS (Figure

2A), as measured by RAS oncogene-induced SA-β galactosidase staining, and exhibited an increased number of transformed foci in soft agar (Figure 2B). Interestingly, complementation with the cDNA encoding the wild-type human FANCD2, but not with the cDNA encoding the FANCD2-K561R mutant protein, which fails to undergo monoubiquitination (Garcia-Higuera et al., 2001) (Figure 2C), restored OIS and suppressed cell proliferation. Consistent with these results, primary bone marrow stromal cells derived from a *Fancd2* (-/-) mouse were also defective in Fancd2-monoubiquitination and RAS-induced senescence (data not shown). Thus, monoubiquitination of FANCD2 is critical for oncogene-induced senescence and its loss may account for the increased TPA-inducible epithelial thickening in *Fancd2* (-/-) and *Fancg* (-/-) mice.

The failure of *Fancd2*-deficient MEFs to undergo OIS suggested that these cells may also be deficient in the expression or activation of a tumor suppressor protein. The TAp63 protein is a tumor suppressor known to be required for Ras oncogene-induced senescence (Guo et al., 2009). The *Fancd2*-deficient MEFs had reduced expression of DNA damage-inducible TAp63 mRNA (Figure 2D and Figure S2B) and protein (Figure 2E, lanes 3 and 4), compared to *Fancd2* (+/+) MEFs, suggesting a possible molecular mechanism for the loss of OIS in the *Fancd2*-deficient cells. Specifically, the reduced expression of TAp63 in Fancd2-deficient cells may result in reduced expression of TAp63-inducible genes. Such genes encode proteins known to mediate DNA repair and to suppress cell growth (Lin et al, 2009). Like *Fancd2*-deficient MEFs, human FANCD2-deficient keratinocytes (HaCat cells) also exhibited decreased OIS (Figure S2 C, D), suggesting that Fancd2-Ub may play a broader role in activating senescence, limiting cell proliferation, and suppressing tumorigenesis.

Usp1-deficient mice are resistant to Ras-driven skin carcinogenesis

We reasoned that mice with elevated levels of Fancd2-Ub may therefore have elevated cellular senescence and may be resistant to DMBA/TPA induced tumors. The protease Usp1 normally deubiquitinates Fancd2-Ub (Nijman et al., 2005) (Cohn et al., 2007), and *Usp1* (-/-) mice have elevated cellular levels of Fancd2-Ub (Kim et al., 2009). *Usp1* (-/-) mice are born at sub-Mendelian frequency and exhibit small size, infertility, bone marrow defects, and cellular hypersensitivity to DNA interstrand crosslinking agents. Further analysis revealed that *Usp1* (-/-) mice exhibit abnormal ectodermal phenotypes such as malformed eyes (data not shown) and a thin epidermal layer (Figure 3A), compared to *Usp1* (+/+) sibling control mice. In contrast to *Fancd2*-deficient mice, treatment of the skin of *Usp1* (-/-) mice with TPA resulted in decreased epidermal cellularity and thickening (Figure 3B), compared to wild-type sibling control mice.

Furthermore, *Usp1* (-/-) and *Usp1* (+/-) mice were resistant to DMBA/TPA-induced tumorigenesis, compared to wild-type sibling controls (Figure 3C) and developed skin tumors later than the wild-type animals ($P<0.05$). Tumor multiplicity was not statistically significantly different between *Usp1* (+/+) and *Usp1* (+/-) mice. The delayed tumorigenesis of the *Usp1* (+/-) mice, compared to Usp1 (+/+) mice, was not a result of decreased H-RasV61 mutations or tumor initiation (Figure S1A), but instead was a result of delayed malignant progression (Kemp et al., 1993), presumably due to increased Fancd2-Ub mediated senescence. Interestingly, the Usp1(-/-) mice did not develop detectable papillomas.

The papillomas excised from the Usp1(+/-) mice exhibited a benign, differentiated phenotype (data not shown). In contrast, the papillomas excised from *Fancd2* (+/+) mice ranged in histology from benign to malignant (Figure S1B, C). In general, the benign, more differentiated papillomas expressed high levels of Fancd2-Ub and low levels of Usp1, while the less differentiated papillomas or skin carcinomas expressed low levels of Fancd2-Ub and high levels of Usp1. The papillomas excised from the *Fancd2* (+/-) mice more often

displayed the less differentiated cellular phenotype (Figure S1D). Taken together, these results suggest that low levels of Usp1 and the corresponding high levels of Fancd2-Ub may promote OIS, thereby providing a barrier to oncogene-induced tumorigenesis, as previously described (Bartkova et al., 2006).

Enhanced Ras oncogene-induced senescence due to Usp1 depletion requires TAp63 and FANCD2-Ub

The p63 isoforms are abundantly expressed in human keratinocytes, and *p63* (-/-) mice exhibit a severe defect in skin development and differentiation (Mills et al., 1999; Yang et al., 1999), similar to the ectodermal phenotype of the *Usp1* (-/-) mice. Since TAp63 suppresses epithelial cancers by promoting cellular senescence (Guo et al., 2009), we reasoned that the *Usp1* (+/-) mice may exhibit elevated TAp63 expression. The severe ectodermal phenotypes of the *Usp1* (-/-) newborn mice further suggested a possible defect in p63 expression or function. Therefore, we examined the skin of *Usp1* (-/-) newborn mice for defects in the expression of the various p63 isoforms (Figure 4). Indeed, skin extracted from *Usp1* (-/-) newborn mice exhibited increased TAp63 expression (Figure 4A). Other epithelial tissues from the newborn *Usp1* (-/-) mice, including mammary tissue, ovarian tissue, and cultured keratinocytes also exhibited increased TAp63 expression (data not shown). TAp63 was identified by its immunoreactivity with an anti-p63 antibody and by its characteristic gel migration which contrasts with other p63 isoforms (Figure S3). The level of p21 was also elevated in the skin from *Usp1* (-/-) mice. The p53 level, in contrast, was comparable to the level in skin extracted from a wild-type control mouse (Figure 4A). Elevated TAp63 protein resulted, at least in part, from an increase in the TAp63 mRNA transcript level, as observed in non-damaged *Usp1* (-/-) MEFs (Figure 4B). An increase in TAp63 mRNA expression was also observed in wild-type MEFs following DNA damage (Figure S5E).

We reasoned that the elevated TAp63 levels may result in elevated oncogene-induced cellular senescence, accounting for the decreased tumorigenesis of *Usp1*-deficient mice. We therefore tested MEFs derived from *Usp1* (+/+), *Usp1* (+/-), and *Usp1* (-/-) mice for RAS oncogene-induced senescence (OIS) (Figure 4C, Figure S4). Infection with a retrovirus encoding oncogenic H-RAS^{V12} strongly induced senescence in *Usp1* (-/-) MEFs, as shown by elevated SA-β-galactosidase staining. Moreover, *Usp1* depletion induced senescence in wild-type MEFs (Figure 4D). To further test the requirement of TAp63 in OIS promoted by *Usp1* depletion, we depleted Usp1 in *TAp63* (-/-) MEFs using shRNA and examined OIS (Figure 4 D-F) (Guo et al., 2009). Usp1 depletion failed to induce senescence in the absence of TAp63, whereas Usp1 depletion in wild-type MEFs enhanced cellular senescence. Taken together, these results indicate that the increased OIS, resulting from Usp1 depletion and Fancd2-Ub elevation, requires TAp63 activity.

In wild-type MEFs, Usp1 depletion increased the levels of Fancd2-Ub, TAp63, and p21 (Figure 4G, lanes 1, 2) and enhanced OIS (Figure 4H). In contrast, Usp1 depletion failed to induce TAp63 or p21 expression levels and only slightly induced senescence in *Fancd2* (-/-) MEFs. Taken together, these results further indicate that elevated Fancd2-Ub is required for TAp63 induction and for Ras-induced senescence.

Monoubiquitinated FANCD2 promotes transcription of TAp63 after DNA damage

Although FANCD2-Ub has well known functions as a DNA repair protein, little is known regarding its possible function as a transcription factor. We reasoned that FANCD2-Ub may play a direct role in transcriptionally activating TAp63 expression. Consequently, we performed chromatin immunoprecipitation (ChIP) assays to determine whether FANCD2-Ub could directly bind to the promoter region of TAp63 (Figure 5). The promoter regions of

the p63 gene have previously been analyzed (Buckley et al., 2011) (Waltermann et al., 2003). We initially divided the promoter of TAp63 into six regions (A1-A6), shown schematically in Figure 5A. The nucleotide sequence of the promoter is shown in Figure S5A. FANCD2 binds specifically to the A3 region of the TAp63 promoter in a DNA damage-inducible manner (Figure 5B). The same pattern of inducible FANCD2-Ub binding was obtained following cellular exposure to other DNA damaging agents (MMC, IR) (Figure S5H).

We next determined whether monoubiquitination of FANCD2 is required for promoter binding. Interestingly, the wild-type FANCD2 protein, but not the unubiquitinated FANCD2-K561R mutant protein, interacted with the A3 sequence of the TAp63 promoter following DNA damage (Figure 5C), demonstrating that FANCD2 monobubiquitination is critical for promoter binding. USP1 depletion, which also causes an increase in FANCD2-Ub, even in the absence of DNA damage, increased promoter binding of FANCD2-Ub (Figure 5D). We also examined FANCA-deficient cells (FA-A cells) which have a defect in the FA core ubiquitin E3 ligase complex (Kee and D'Andrea, 2010) and are therefore unable to monoubiquitinate FANCD2. These cells also exhibited decreased FANCD2-Ub binding to the A3 promoter sequence (Figure 5E), further indicating that FANCD2 monoubiquitination is required for its targeting to the TAp63 promoter.

FANCP/SLX4 was recently identified as another downstream protein in the FA pathway (Crossan et al., 2011) (Stoepker et al., 2011) (Kim et al., 2011). FANCP/SLX4 has structural features consistent with other direct transcriptional activators, including a SAP domain and a BTB domain (Fekairi et al., 2009; Kim et al., 2013; Svendsen et al., 2009). FANCP/SLX4 also has two ubiquitin binding domains (UBZ4), and it interacts with the ubiquitin moiety of FANCD2-Ub (Yamamoto et al., 2011). We next tested whether FANCP/SLX4 can also bind to the A3 region of the TAp63 gene (Figure 5 F, G, H). Interestingly, FANCP/SLX4 interacted with the A3 region following DNA damage with UV light (Figure 5F) or following MMC treatment (data not shown). Knockdown of SLX4 using siRNA reduced the UV-inducible binding of FANCD2-Ub to the A3 region, suggesting that FANCD2-Ub and FANCP/SLX4 cooperate in the transcriptional activation of TAp63 (Figure 5G). Consistent with this hypothesis, *Slx4* (-/-) MEFs have reduced levels of TAp63 mRNA and protein (data not shown). Moreover, FANCD2-Ub was not required for the FANCP/SLX4 interaction with the A3 sequence (Figure 5H), suggesting that FANCP/SLX4 binds to the A3 promoter region independently and functions, at least in part, to recruit FANCD2-Ub to this site. In contrast, another downstream FA protein, FANCD1/BRCA2, was not required for damage-inducible localization of FANCD2-Ub to the A3 site (data not shown). Taken together, these data suggest that a complex of FANCD2-Ub and FANCP/SLX4 may cooperate in the transcriptional activation of the TAp63 tumor suppressor gene and perhaps other genes.

In order to identify other genes which are transcriptionally regulated by FANCD2-Ub, we next performed a genome-wide ChIP-Seq analysis (Figure S5B-G). Human 293T cells were treated with or without UV radiation, and an anti-FANCD2 antibody was used to immunoprecipitate chromatin fragments. DNA damage activated a 163-fold increase in the number of annotated peaks of FANCD2 protein binding. We hypothesized that some of these FANCD2-Ub binding sites were non-specific sites of DNA damage while other sites were bonafide sites of transcriptional activation. The genome-wide distribution of inferred DNA binding sites is shown in Figure S5B. To determine whether the DNA damage-induced binding sequences map to genes with distinct functional annotations, we used the USCS genome browser. Interestingly, FANCD2-Ub-induced binding sequences were highly associated with genes that are essential for epithelial development and morphogenesis (Figure S5C). Representative genes in this epithelium-related ontology, which exhibited

FANCD2-Ub binding after DNA damage, were further identified (Table S2). Examination of the ChIP-seq tracks indicated specific genes which have DNA damage-inducible FANCD2-Ub binding (Figure S5D-G). The sequence preferences for FANCD2-Ub are shown in Table S1. To confirm that these binding sites participate in the transcriptional activation of the genes, we performed a quantitative PCR (qPCR) validation of the targets (Figure S5E-G). Indeed, DNA damage activated the Fancd2-Ub dependent transcription of the *TAp63*, *ΔNp63*, and *Brca2* mRNAs. The level of induction varied, depending, at least in part, on the kind of genotoxic stress used. For instance, while IR and UV yielded relatively strong induction of these transcripts, MMC was a weaker stimulant. IR and UV may be more potent inducers since they are delivered during a short time interval. In contrast, MMC is delivered overnight and must be taken up by cells before it crosslinks the DNA. This delay may account for its reduced transcriptional induction. Damage by IR, MMC, or UV failed to upregulate transcription of these genes in Fancd2-deficient MEFs. The FANCD2-Ub mediated upregulation of both p63 isoforms is surprising, since TAp63 and ΔNp63 appear to exhibit antagonist functions (Koster and Roop, 2004).

The ChIP results demonstrate that FANCD2-Ub can bind to relevant cis-acting regulatory sequences of the TAp63 gene, leading to enhanced TAp63 expression and enhanced cellular senescence. To further determine the functional role of FANCD2-Ub in transcription, these regulatory regions were used to construct luciferase reporters (Figure 5I schematic). The constructs were transfected into FA cells and corrected FA cells. FANCD2-Ub, but not the K561R (unubiquitinated) mutant form of FANCD2, enhanced the damage-inducible transcription of the TAp63 promoter (enhancer) constructs (Figure 5J). Taken together, these results demonstrate that monoubiquitinated FANCD2 localizes to the promoter of TAp63 and contributes to its DNA damage-inducible transcription.

Usp1 knockdown promotes senescence and reduces tumor growth *in vivo*

These results suggest that Usp1 has a growth-promoting activity while its substrate, Fancd2-Ub, promotes senescence and opposes cell growth by inducing TAp63 mRNA and protein expression. Indeed, recent studies have indicated that Usp1 acts as an oncogene, and its overexpression can transform 3T3 fibroblasts (Williams et al.). To further test this hypothesis, we depleted USP1 in a RAS-driven human lung cancer cell line, A549, and tested the resulting clones in a mouse xenograft model (Figure 6). As predicted, A549 cells expressing USP1 shRNA showed an increase in the level of FANCD2-Ub (Figure 6A). The reduction of USP1 expression by shRNA, and the increased FANCD2-Ub expression, inhibited the colony formation of A549 cells in soft agar (Figure 6B) and inhibited the tumor xenograft growth in nude mice (Figure 6 C, D). Consistent with these results, doxycycline induction of the shRNA to FANCD2 in A549 cells resulted in increased tumor growth in a xenograft model (Figure 6 E, F). Taken together, these data further indicate that depletion of USP1 results in increased cellular levels of FANCD2-Ub, causing increased senescence and decreased epithelial tumor growth *in vivo*.

Depletion of Usp1 decreases epidermal proliferation in *Fancd2* (+/-) mice

We next examined the effect of knocking down Usp1 on epidermal proliferation in a *Fancd2*-deficient genetic background (Figure 7). For these studies, we used a double heterozygous mouse strain, *Fancd2* (+/-)/ *Usp1* (+/-), which was previously described (Kim et al., 2009). Topical TPA treatment increased epidermal proliferation in *Fancd2* (+/-) mice. Interestingly, this hyperproliferation was reduced in *Fancd2* (+/-)/ *Usp1* (+/-) double heterozygous mice (Figure 7A, B). The increased proliferation in the *Fancd2* (+/-) mice correlated with increased Ki67 staining (Figure 7A) and decreased senescence (data not shown). *Usp1* heterozygosity resulted in elevated Fancd2-Ub levels in the *Fancd2* (+/-) heterozygote background (Figure 7C, lane 3), correlating with the reduced epidermal

proliferation, and consistent with the function of Fancd2-Ub as a tumor suppressor. These data further indicate that a decrease in Usp1 expression causes an increase in Fancd2-Ub levels, and that this increase may account for the decreased epidermal cell proliferation in the double heterozygote animals.

DISCUSSION

Our results indicate that FANCD2-Ub, a critical downstream product of the FA pathway, acts as a tumor suppressor in squamous epithelium. FANCD2-Ub accumulates in epithelial cells in response to DNA damage or oncogenic stress, or following USP1 knockdown, resulting in enhanced cellular senescence (Figure 7D). In addition to promoting DNA repair via the canonical FA pathway (Kee and D'Andrea, 2010), FANCD2-Ub, along with its binding partner FANCP/SLX4, is also a transcriptional regulator of TAp63. TAp63 may in turn activate cellular senescence (Guo et al., 2009) by transcriptionally upregulating other tumor suppressor proteins, such as RAD51 and BRCA2 (Lin et al., 2009). Indeed, *Fancd2* (-/-) and *TAp63* (-/-) MEFs have reduced levels of *Brca2* mRNA and protein, compared to wild-type MEFs (data not shown). Taken together, our results suggest that the squamous cell-specific tumorigenesis in FA patients results from loss of this tumor suppressor pathway. Somatic disruption of the pathway may also account for the development of SCC in the general (non-FA) population.

Recent studies suggest that FANCD2-Ub has transcriptional activity. For example, FANCD2-Ub appears to bind to a concensus sequence for NF-κB on the TNFα promoter and to repress transcription of the TNFα gene (Matsushita et al., 2011). Although FANCD2-Ub binds to a region of the TAp63 promoter (Region A3), which also contains two NF-κB binding motifs (Figure S5A), our ChIP-Seq study did not reveal a statistically significant sequence preference of FANCD2-Ub for these motif (Table S1). Other recent studies indicate that FANCD2-Ub forms a protein complex with BRG1 and localizes to the promoters of anti-oxidant genes (Du et al., 2012). Taken together, our study further supports a direct role of FANCD2-Ub in transcription of the TAp63 gene and provides a mechanism for the tumor suppressor activity of the FA pathway.

Fancd2-Ub appears to exert its anti-tumor activity through both cell autonomous and cell extrinsic mechanisms. First, Fancd2-Ub functions as a tumor suppressor in a cell autonomous manner. Indeed, Fancd2-Ub is a DNA damage response (DDR) protein which directly promotes TAp63-mediated cellular senescence and inhibits the growth of tumor xenografts (Figure 6). In this regard, the Fancd2-Ub tumor suppressor activity is similar to the pro-senescence tumor suppressor activity observed for other DDR proteins, such as ATM (Bartkova et al., 2006) and CHK2 (Di Micco et al., 2006). The decrease in epidermal tumors observed in the DMBA/TPA treated *Usp1* (+/-) mice likely results directly from the tumor suppressor activity of Fancd2-Ub generated by the FA pathway in H-Ras mutant epidermal cells. Second, Fancd2-Ub may also suppress squamous cell tumorigensis indirectly, through a cell extrinsic mechanism. For instance, for *Usp1* (-/-) newborn mice, the Fancd2-Ub-mediated senescence of dermal fibroblasts may account for their thin overlying epidermal layer. The thin epidermis of the *Usp1*-deficient mice contains fewer total epidermal target cells, thus providing another mechanism for the decreased incidence of DMBA/TPA induced tumors. Other functions of FANCD2-Ub in the underlying stromal cells, such as its ability to limit vascularization, may further suppress tumor growth of the overlying epithelium.

Our study specifically examined the mechanism by which Usp1 disruption and FANCD2-Ub elevation can suppress the development of H-Ras-driven tumors of the epidermis. It will be interesting to determine whether *Usp1* knockout mice are also resistant to other Ras

driven tumors, such as K-Ras driven lung cancers or pancreatic cancers. *Usp1* knockout has recently been shown to confer an anti-cancer activity by promoting the differentiation of mesenchymal cells (Williams et al., 2011), suggesting that *Usp1*-deficient mice may be resistant to mesenchymal tumors, such as sarcomas and leukemias. Thus, FANCD2-Ub may have a more general anti-tumor function in other tissues, although the suppression of SCCs appears to be especially pronounced, as demonstrated by the specific increased incidence of this tumor class in FA patients.

Our model (Figure 7D) predicts that somatic disruption of FA genes or of TAp63 may contribute to squamous cell carcinogenesis in the general (non-FA) population. A mutational screen of head and neck cancers has recently shown that some SCCs have mutations in the *p63* gene. These mutations are predicted to result in the loss of the TAp63 isoform and the upregulation of the oncogenic ΔNp63 isoform (Stransky et al., 2011). Somatic mutations in FA genes were also identified in sporadic SCCs (Romick-Rosendale et al., 2013).

TAp63 also plays a critical role in maintaining the female germline (Suh et al., 2006). Recent studies have shown that TAp63 is the predominant p63 isoform in primordial follicle oocytes, and it regulates DNA damage inducible apoptosis of oocytes *in vivo* (Kerr et al., 2012). *Fancd2*-deficient mice (Houghtaling et al., 2003) and other FA pathway deficient mice (Parmar et al., 2009) also have dysplastic ovaries and reduced fertility, and exhibit an increased incidence of ovarian cancer (Bakker et al., 2012; Wong et al., 2003). Taken together, these findings further support a model in which activation of TAp63 expression by the FA pathway is required, not only for suppression of SCC, but also for normal ovarian development.

Finally, our results suggest a possible therapeutic strategy for human SCCs by inducing tumor cell senescence. Small molecule inhibitors of Usp1 may enhance FANCD2-Ub levels and TAp63 levels and perhaps promote cellular senescence. USP1 inhibitors may therefore exert both chemosensitization and tumor cell senescence activities.

METHODS

Animals and DMBA/TPA-induced skin carcinogenesis

Fancd2-, *Fancg*, and *Usp1*-deficient mice were maintained on a C57BL/6 background and genotyped as previously described (Kim et al., 2009) (Parmar et al., 2010). All animal studies were carried out under an Animal Care and Use Committee-approved protocol. For mouse skin carcinogenesis, the back skin of each female mouse (6-8 weeks) was shaved 1 day before topical treatment, with 7,12-dimethylbenz(*a*)anthracene (DMBA; D3254, Sigma-Aldrich, Inc, St. Louis, MO), 100 μg in 200 μL of acetone. One week later, mice were topically treated with 2.5 μg of 12-*O*-tetradecanoylphorbol-13-actate (TPA; p-1680, LC Laboratories, Woburn, MA) in 200 μL of acetone, 5 times a week for 28 weeks. Onset, number and size of tumors were monitored once a week. Paraffin-embedded sections of tumor tissue and skin were prepared and fixed with 4% paraformaldehyde, and H&E staining was done in our Histology and Tissue Core.

Cell proliferation assay

Primary MEFs were seeded in 6-well plates (10^3 cells per well) in triplicate. After 2 weeks, cells were fixed with methanol and stained with 0.05 % crystal violets. For *in vivo* cell proliferation assays, mice were topically treated with TPA (2.5 μg) for four weeks. Skin samples were fixed with formalin and paraffin-embedded sections of skin were stained with H&E and Ki67.

SA-β-Galactosidase Staining

Staining for SA-β-galactosidase activity in cultured cells was carried out using a staining kit (Cell signaling catalog, #9860). Briefly, cells were seeded in 6-well plates (10^4 cells per well) in triplicate. After 10 days, the cells were fixed with β-Gal Fixative and stained with complete β-Gal Stain Solution followed by addition of β-Gal Holding Solution. For *in vivo* senescence staining, frozen sections of skin were prepared with optimal cutting temperature (O.C.T.) compound and followed by staining. The sections were counterstained with Eosin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Monoubiquitinated FANCD2 is a tumor suppressor protein
- *Fancd2*-deficient mice have an increased incidence of DMBA/TPA-induced skin cancers
- *Usp1*-deficient mice have elevated Fancd2-Ub and are resistant to skin cancers
- FANCD2-Ub transcriptionally increases TAp63 and promotes cellular senescence

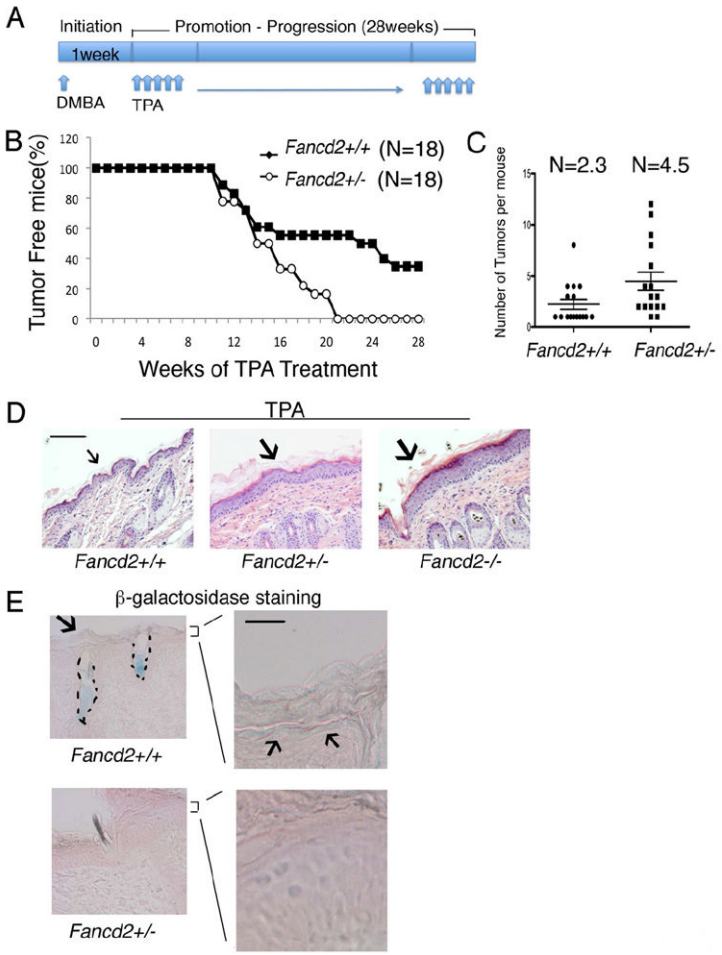

Figure 1. *Fancd2*-deficient mice exhibit increased squamous cell tumorigenesis

(A) Schematic diagram shows a DMBA/TPA two-stage tumor induction procedure. (B) Tumor incidence in *Fancd2* (+/+) and *Fancd2* (+/-) mice treated with DMBA/TPA. Increased skin tumor formation in *Fancd2* (+/-) mice versus wild-type *Fancd2* (+/+) mice (*P<0.01*). For each group, n = 18 mice (See also Figure S1, lane 1-4). (C) The distribution of total number of tumors per mouse in *Fancd2* (+/-) mice versus *Fancd2* (+/+) mice 28 weeks after TPA treatment (*P<0.01*). (D) Loss of Fancd2 enhances skin proliferation after 4 weeks of TPA treatment. TPA-induced epithelial cell proliferation was evaluated in each indicated group (four mice per group). The photomicrograph shows skin from a representative mouse at 100X magnification. Arrows indicate the epidermis. Scale bar =100 μm. (E) *In vivo* senescence detected by β-gal staining. Skin from mice of the indicated genotype was stained. The *Fancd2* (+/+) skin shows enhanced blue senescent staining in the base of the hair follicles (lower magnification) and in the interfollicular epidermis (arrows, higher magnification). Scale bar=25μm. The blue senescent staining was reduced in the skin from the *Fancd2* (-/-) mouse.

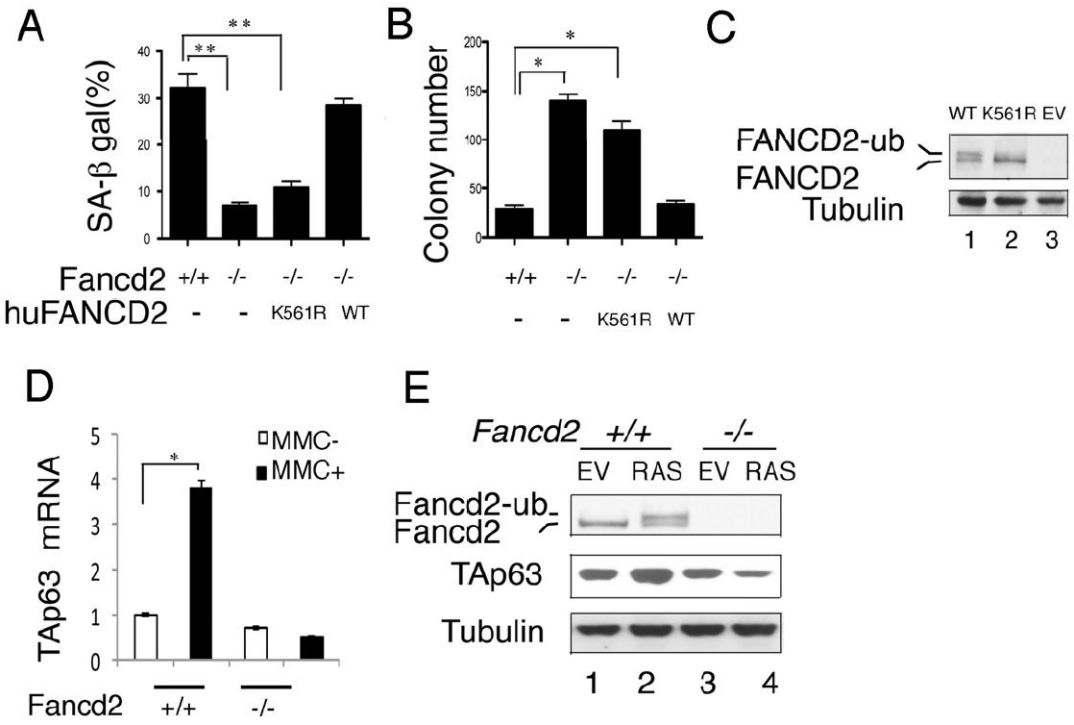

Figure 2. FANCD2 monoubiquitination is required for Ras oncogene-induced senescence and for TAp63 induction after DNA damage

(A-C)Primary *Fancd2* (-/-) MEFs (passage 1) were infected with either control retrovirus (EV) or a retrovirus carrying wild type FANCD2 (WT) or mutant FANCD2 (K561R). After puromycin selection, cells were infected with H-Ras^{V12} or control virus. Data shown are mean ± standard errors of three independent experiments, at passage 2 of the MEFs. Each experiment was performed in triplicate plates for three sibling MEFs of each genotype. (A) Cells were stained for SA-β-gal expression after 2 weeks from infection. (B) Cell proliferation was assayed by crystal violet staining. (C) Immunoblot confirming expression of FANCD2 in retrovirus-infected cells. The FANCD2 (K561R) protein is not monoubiquitinated. (D) *TAp63* mRNA was measured by Real-Time PCR in primary *Fancd2* (+/+) and *Fancd2* (-/-) MEFs after MMC treatment (500 nM). Data shown are mean ± standard errors of triplicate plates for three sibling MEFs of each genotype. Data shown are representative of three independent experiments. (E) Immunoblot showing expression of Fancd2 and TAp63 protein in primary MEFs infected with retrovirus. *, P<0.001; **, P<0.005. See also Figure S2.

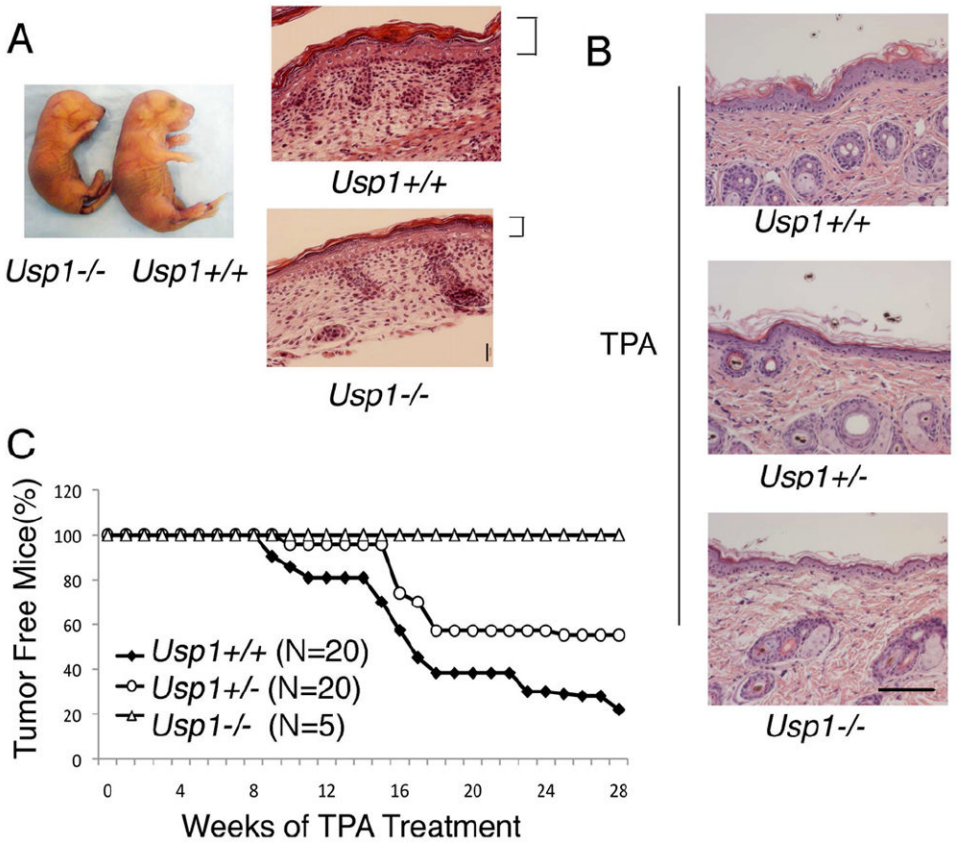

Figure 3. *Usp1*-deficient mice are resistant to Ras-driven skin carcinogenesis

(A) Histology of epidermis and dermis of *Usp1* (+/+) versus *Usp1* (-/-) newborn mice. The epidermis is indicated with a bracket. (B) Loss of Usp1 decreases skin proliferation after TPA treatment. *Usp1* (+/+), *Usp1* (+/-) and *Usp1* (-/-) mice were exposed to topical TPA for four weeks (four mice per group). Skin thickness was examined by microscopy at 100x magnification. Scale bar =100μm. (C) Delayed tumor formation in *Usp1* (-/-) and *Usp1* (+/-) mice following DMBA/TPA treatment ($P<0.05$). Tumor multiplicity was not statistically different between *Usp1* (+/+) and *Usp1* (+/-) mice. These data are representative of two independent experiments.

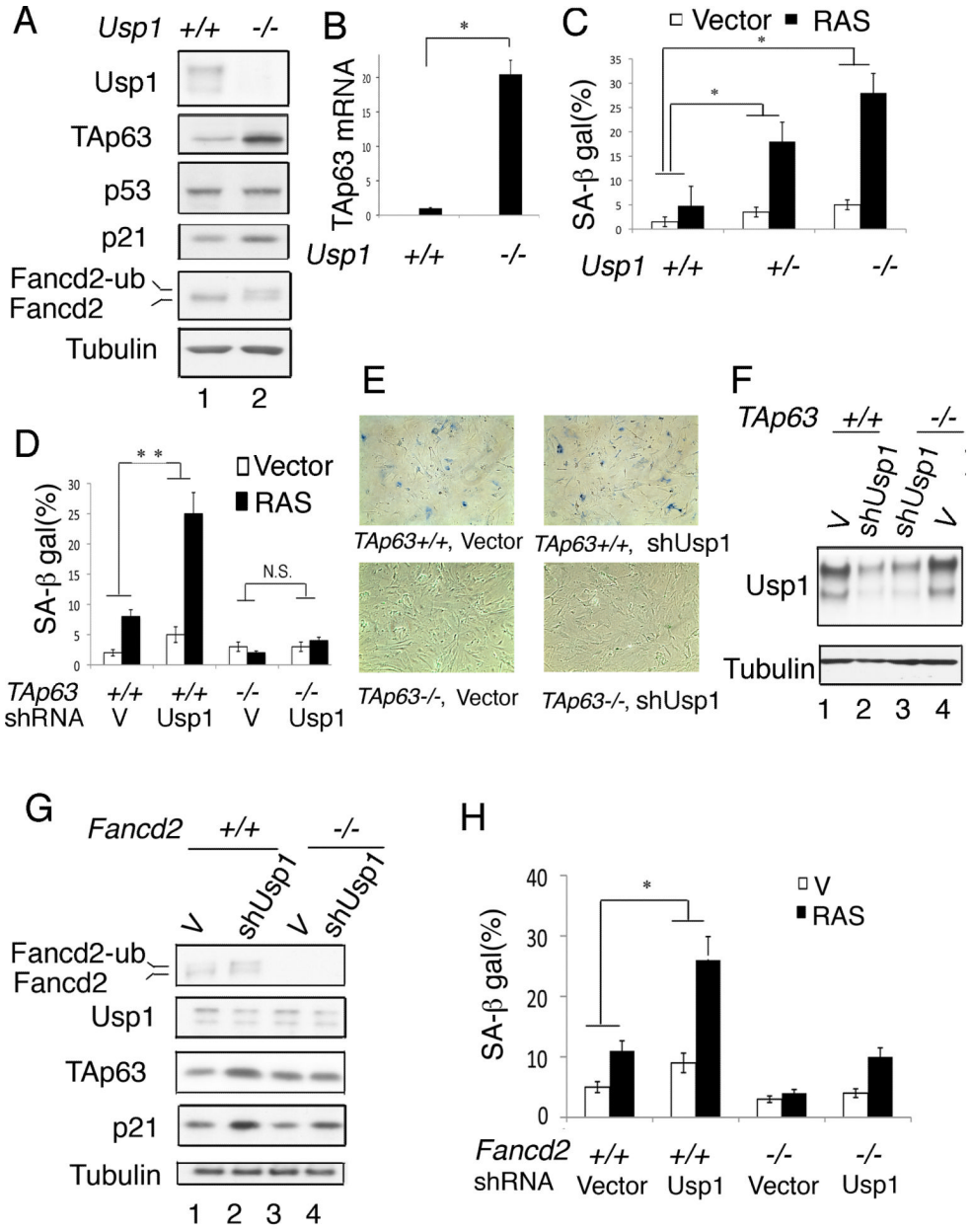

Figure 4. Enhanced Ras oncogene-induced senescence by Usp1 depletion requires TAp63 and FANCD2-Ub

(A) Skin was obtained from a *Usp1* (+/+) or *Usp1* (-/-) newborn mouse, and tissue lysates were analyzed by immunoblotting with the indicated antibodies (See Figure S3). (B) *TAp63* mRNA expression was measured by Real-Time PCR and found to be elevated in *Usp1* (-/-) primary MEFs, as indicated. *, P<0.001. Data shown are mean ± standard errors (S. E.) of triplicate plates for three sibling MEFs of each genotype. Data shown are representative of three independent experiments. (C) *Usp1* (+/+), *Usp1* (+/-) or *Usp1* (-/-) primary MEFs (passage 1) were infected with a retrovirus expressing H-Ras^{V12} or vector only. H-Ras^{V12} -expressing *Usp1* (-/-) and *Usp1* (+/-) MEFs exhibited significantly increased senescence (i.e., SA-ß-galactosidase staining) after 2 weeks. Each experiment was performed in triplicate plates for three sibling MEFs of each genotype. Data shown are mean ± standard errors of three independent experiments, at passage 2 of the MEFs. (D-F) *TAp63* (+/+) or

TAp63 (-/-) MEFs (passage 2) were infected with lentivirus expressing murine Usp1 shRNA or vector only. After puromycin selection, cells were infected with H-RAS ^{V12} retrovirus or vector only. (D) The quantification of SA-β-gal staining is shown. Data shown are mean ± standard errors of triplicate plates for three sibling MEFs of each genotype, representatively of the three independent experiments. (E) Primary MEFs were stained for SA-β-gal expression 2 weeks after retrovirus infection, and the cells were photographed at 100x magnification. (F) The efficiency of Usp1 depletion was shown by immunoblotting. (G-H) *Fancd2* (+/+) and *Fancd2* (-/-) primary MEFs (passage 2) were infected with a lentivirus expressing Usp1 shRNA or vector only. After puromycin selection, cells were infected with H-Ras^{V12} or control retrovirus. (G) Cell lysates were collected 2 days after retrovirus infection and immunoblotted with the indicated antibodies. (H) Indicated cells were stained for SA-β-gal expression 10 days after retrovirus infection. Data shown are mean ± standard errors of triplicate plates for three sibling MEFs of each genotype, representatively of the three independent experiments. *, P<0.001 (paired t-Test); **, P<0.005. See also Figure S3 and S4.

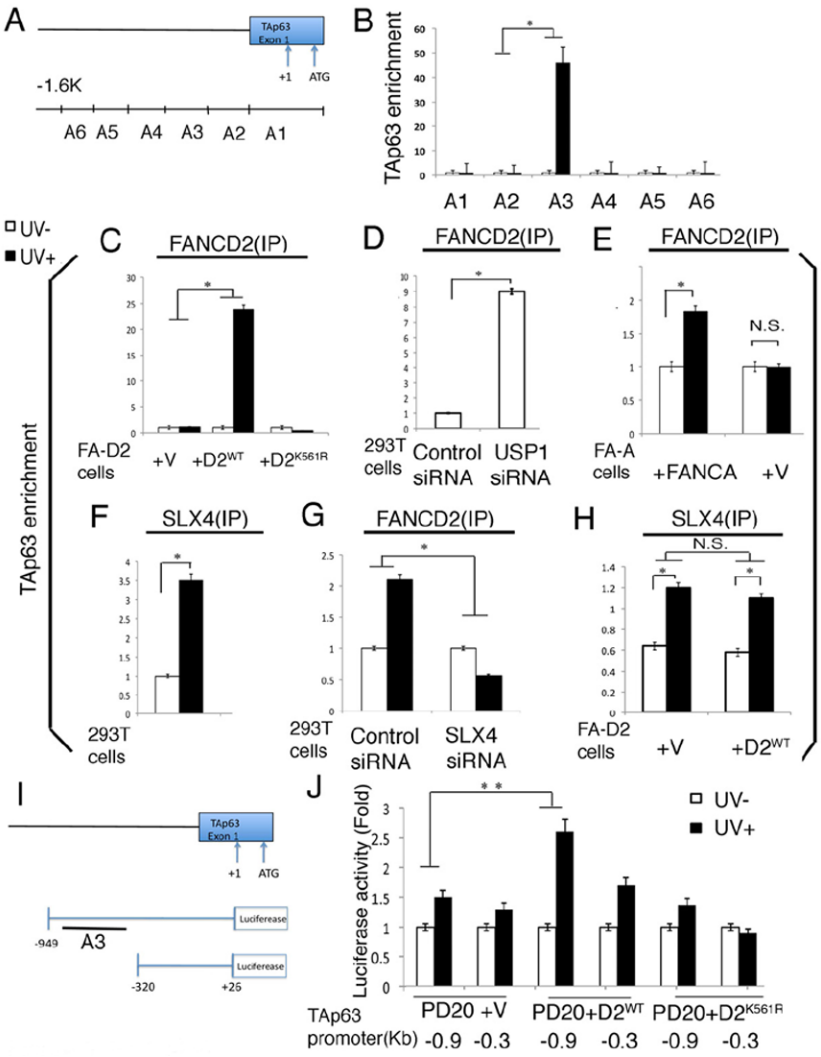

Figure 5. Monoubiquitinated FANCD2 promotes transcription of TAp63

(A) Schematic diagram of primer sets for the *TAp63* promoter. (A1: +99 bp to -20 bp of *TAp63* promoter, A2: -21 to -320, A3: -321 to -801, A4: -802 to -980, A5: -981 to -1220, A6: -1221 to -1601). (B) 293T cells were damaged with UVC (30 J/m^2) or left untreated. After 3 hours, cells were cross-linked with formaldehyde, pellets were harvested, and cross-linked chromatin was sonicated, followed by immunoprecipitation with an anti-FANCD2 antibody. Genomic DNA was purified from the immunoprecipitates and subjected to Real-Time PCR using the indicated primer sets (A1 to A6). ChIP data were calculated as FANCD2 IP/Input (%). IgG (negative control) had no signal. For B-H, the ChIP experiments shown were performed three times. Data are mean ± standard errors of triplicate plates. Data shown are representative of three independent experime nts. (C) Binding of FANCD2-Ub to the *TAp63* promoter detected by ChIP assay with an anti-FANCD2 antibody, followed by PCR using the A3 primer set for the promoter region of *TAp63*. PD20 cells are FANCD2-deficient human fibroblasts. PD20+ V, PD20+D2^{WT}, and PD20+D2^{K561R} cells were treated with UVC (30 J/m^2), cross-linked with formaldehyde after 3 hours, and harvested pellets. Cells were used in ChIP assays, using an anti-FANCD2 antibody, followed by Real-Time PCR with the indicated primer set. ChIP data are represented as FANCD2 IP/Input (%). (D) 293T cells were transfected with siRNA against USP1 and were cross-linked with formaldehyde 48 hours after transfection. ChIP assay was

performed with the anti-FANCD2 antibody. (E) FA-A cells (FANCA-deficient GM6914 fibroblasts) transduced with empty vector (V) or corrected with the cDNA encoding FANCA were treated with UVC (30 J/m^2) or left untreated, followed by formaldehyde fixation. Cells were analyzed by ChIP with anti-FANCD2 antibody and subjected to Real-Time PCR using the indicated primer sets. (F)ChIP assay was performed with an anti-FANCP/SLX4 antibody in 293T cells. (G)ChIP assay was performed with an anti-FANCD2 antibody in 293T cells, as indicated. (H)ChIP assay was performed with an anti-FANCP/SLX4 antibody in PD20 (FANCD2-deficient) cells. (I) Schematic representation of the *TAp63* promoter and luciferase reporter gene constructs. (J) PD20 (FANCD2-deficient) cells were transiently co-transfected with luciferease reporter constructs bearing various lengths of the human *TAp63* promoter region (100 ng) and *Renilla* luciferease reporter plasmid. TAp63 luciferase activity of UVC treated (30 J/m^2) cells was normalized to untreated cells and represented as fold induction from individual duplicate experiments. Data shown are mean ± standard errors of three independent experiments. *, P<0.001 (t-Test); **, P<0.005. See also Figure S5, Table S1 and Table S2.

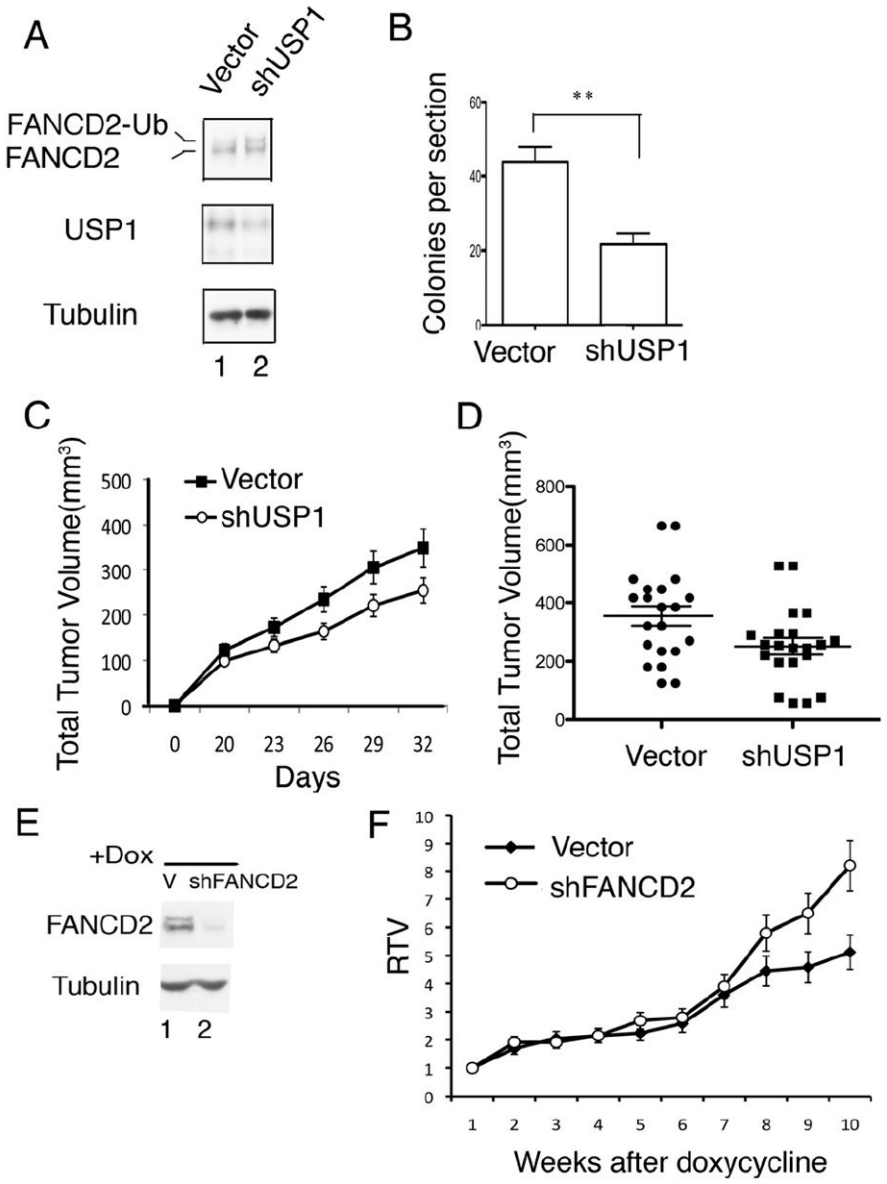

Figure 6. Usp1 knockdown promotes senescence and reduces tumor growth *in vivo*

(A-B) The USP1 shRNA or a control vector were stably expressed in the Ras driven human lung adenocarcinoma epithelial cell line, A549. (A) Cell lysates were immunoblotted with the indicated antibodies. (B) A549 cells were plated in soft agar and transformed foci were counted at 100x magnification 2 weeks later. Data shown are mean ± standard errors of three independent experiments in cell lines. Each experiment was performed triplicated plates. **, P<0.005, t-Test. (C-D)Comparison of tumorigenicity of USP1 shRNA or control shRNA transfected A549 cells. A total of 2×10^6 A549 cells were injected as xenografts into nude mice. Data shown are mean ± standard errors from obtained all tumors. (C) Every 3 days, tumor volume (mm) was measured for the indicated time period. The data represent the average tumor size of 10 mice in each group ($P < 0.05$). (D) Quantification of tumor volume of the xenografts at day32. (E-F) A549 cells expressing an inducible shRNA targeting FANCD2 or control vector, following doxycycline treatment. (E) Immunoblotting showing expression of FANCD2 in A549 cells grown in doxycycline for 3 days. (F) Growth

of A549 cells with shRNA targeting FANCD2 tumor xenografts. In each group, 10 mice were given doxycycline (200 μg/ml) by drinking water when the tumor reached 200 mm^3. RTV is relative tumor volume based on 200 mm^3 for individual tumors. Data shown are mean ± standard errors from obtained all tumors.

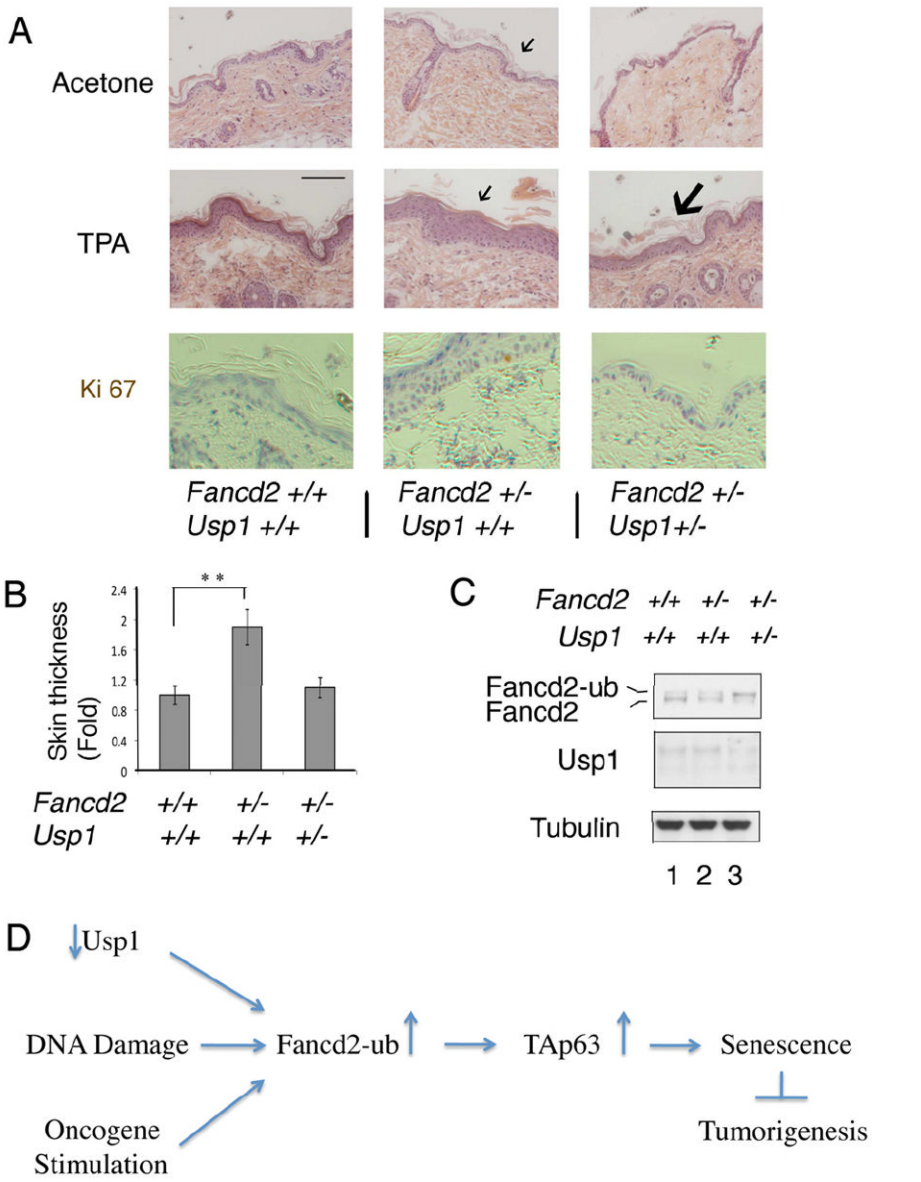

Figure 7. Usp1 depletion decreases epidermal proliferation in *Fancd2* (+/-) mice

(A) Mice from the indicated genotypes were exposed topically to TPA or acetone alone for four weeks. Mice were sacrificed, and skin histology was evaluated by microscopy. Arrows indicate the sites of epidermal proliferation. Scale bar=100 μm. Ki67 staining was performed to evaluate cellular proliferation. (B) Bar graphs showing skin thickness of the three mouse cohorts after TPA exposure (n = 3 female mice per cohort). **, P<0.005 (t-Test). Data shown are mean ± standard errors of triplicate plates for three sibling MEFs of each genotype. Data shown are representative of three independent experiments. (C) TPA-treated skin was collected, and tissue lysates were immunoblotted with the indicated antibodies. The double heterozygote has increased Fancd2-Ub expression (lane 3), consistent with the decreased epithelial cell proliferation observed. (D) Proposed role of Fancd2 monoubiquitination in tumor suppression.