Priority Report

Cancer Research

Smurf2 Regulates the Senescence Response and Suppresses Tumorigenesis in Mice

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Abstract

The E3 ubiquitin ligase Smurf2 mediates ubiquitination and degradation of several protein targets involved in tumorigenesis and induces senescence in human cells. However, the functional role of Smurf2 in tumorigenesis has not been fully evaluated. In this study, we generated a mouse model of Smurf2 deficiency to characterize the function of this E3 ligase in tumorigenesis. Smurf2 deficiency attenuated p16 expression and impaired the senescence response of primary mouse embryonic fibroblasts. In support of a functional role in controlling cancer, Smurf2 deficiency increased the susceptibility of mice to spontaneous tumorigenesis, most notably B-cell lymphoma. At a premalignant stage of tumorigenesis, we documented a defective senescence response in the spleens of Smurf2-deficient mice, consistent with a mechanistic link between impaired senescence regulation and increased tumorigenesis. Taken together, our findings offer the genetic evidence of an important tumor suppressor function for Smurf2. *Cancer Res; 72(11); 2714–9. ©2012 AACR.*

Introduction

The E3 ubiquitin ligase Smurf2 has been shown to mediate ubiquitination of several proteins, including components of the TGF-β and Wnt signaling, Runx2, Rap1B, Smurf1, and Id1 (1–11). These diverse substrates suggest that Smurf2 is involved in many biologic processes. We have previously shown that Smurf2 expression increases in response to telomere shortening in senescent cells (12). Furthermore, we have discovered that Smurf2 recruits both the p53 and pRb pathways to activate senescence (13), suggesting that Smurf2 is a critical regulator of senescence. As an important tumor suppressor mechanism, senescence restricts the proliferation of cells at risk of malignant transformation (14, 15). Consistent with this model, we have found that upregulation of Smurf2 leads to growth arrest in various tumor cells (16).

Despite the advance in our understanding of the molecular function of Smurf2, the precise role of Smurf2 in tumorigenesis has not been established. In this study, we generated a mouse model of Smurf2 deficiency and showed that Smurf2 deficiency leads to an impaired senescence response in primary cells. Consistent with its function in senescence regulation, Smurf2-deficient mice exhibited increased spontaneous tumorigenesis, indicating that Smurf2 functions as a tumor suppressor.

Materials and Methods

Smurf2-deficient mice

Mouse embryonic stem (ES) cell line RRA098 was purchased from Mutant Mouse Regional Resource Center at University of California-Davis (Davis, CA). ES cells were injected into C57BL/6 blastocysts to obtain chimeric mice, which were bred to generate Smurf2-deficient mice. Mouse cohorts on a mixed 129OlaxC57BL/6 background were monitored, and moribund mice were sacrificed for necropsy. Paraffin-embedded tissue sections were stained for hematoxylin and eosin and B220 (Abcam). Frozen sections were fixed, stained for senescence-associated β-galactosidase (SA-β-gal) activity (13), and counterstained with eosin. Mouse studies were approved by the Institutional Animal Care and Use Committee of University of Massachusetts Medical School (Worcester, MA).

Assays of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEF) were prepared from E12.5 embryos and cultured as described (17). To analyze cell proliferation, MEFs were seeded in 6-well plates, harvested in triplicate, and counted daily using a Particle Counter (Beckman Coulter). In senescence assay, MEFs were plated in 10-cm dish and subcultured every 3 days before they reached high cell density. In 3T9 immortalization assay, MEFs were plated at 3×10^6 per 10-cm dish in triplicate and passaged every 3 days.

MEFs were infected with lentivirus-expressing Smurf2 or GFP as previously described (16) and stained with crystal violet or SA-β-gal activity (13) after selection with puromycin. To analyze DNA damage response, MEFs were treated with 1

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Note: While the manuscript was in revision, Smurf2 as a tumor suppressor was independently reported (Blank and colleagues, ref. 20).

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μmol/L doxorubicin (Sigma), and cell lysates were collected for Western blotting.

Southern blotting

Genomic DNA was extracted using genomic DNA isolation kit (Lamda Biotech), digested with restriction enzymes (New England Biolabs), electrophoresed in agarose gel, and transferred to Hybond nylon membrane (GE Healthcare). Probe labeling, hybridization, and detection were carried out using DIG-High Prime DNA Labeling and Detection Starter Kit (Roche).

Quantitative PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen), and reverse-transcribed using Superscript II (Invitrogen). Real-time PCR was carried out using SYBR Green PCR Kit (Bio-Rad) with the following primers: Smurf2 (5′-ATGAAGTCATTCCCCAGCAC-3′; 5′-AACCGTGCTCGTCTCTCTTC-3′), p16 (5′-CGAACTCTTTCGGTCGTACCC-3′; 5′-CGAATCTGCACCGTAGTTGAG-3′), or β-actin (5′-GCTCTTTTCCAGCCTTCCTT-3′; 5′-GTGCTAGGAGCCAGAGCAGT-3′).

Genomic DNA was used in real-time PCR to determine the relative abundance of the *Smurf2* alleles with the following primers: wild-type *Smurf2* (5′-GAGGGTGTTGGTGAGAGGAA-3′; 5′-TTACACAGCATCTGGCAAGG-3′), trapped *Smurf2* (5′-GAGGGTGTTGGTGAGAGGAA-3′; 5′-GACAAGTAGATCCCGGCGCTC-3′), or *GAPDH* (5′-AGCCTTAAAAGCCCTTGAGC-3′; 5′-CTAGGAAGAGGGGGAGAGGA-3′).

Western blotting

Western blotting was carried out as described (11) with the following antibodies: Smurf2 (Epitomics); p53 (Calbiochem); α-tubulin (Sigma); and p16, p19, p21, and GAPDH (Santa Cruz Biotechnology).

Statistical analyses

Kaplan–Meier survival curves were plotted and analyzed with the log-rank test. The Student *t* test (2-tailed and unpaired) was used for pairwise comparisons.

Results

A mouse model of Smurf2 deficiency

We used a mouse ES cell line RRA098 with a gene-trapping vector (pGT1lxf) inserted into intron 1 of *Smurf2* (Fig. 1A) to generate a mouse model of Smurf2 deficiency. A single integration of the trapping vector in RRA098 genome was observed in Southern blot analysis (Fig. 1B) and further determined by sequencing analysis (Supplementary Fig. S1A and Table S1). *Smurf2* transcripts were increased in ES cells after Cre-mediated excision of *loxP*-flanked splicing acceptor (SA; Fig. 1C), indicating that Smurf2 expression is compromised in RRA098 cells, and removal of SA restores its expression.

Mice heterozygous for the trapped allele ($Smurf2^{+/T}$) were viable and developmentally normal. Intercrossing of $Smurf2^{+/T}$ mice generated $Smurf2^{T/T}$ mice with expected Mendelian frequency (36:72:35 for $Smurf2^{+/+}$:$Smurf2^{+/T}$:$Smurf2^{T/T}$) and

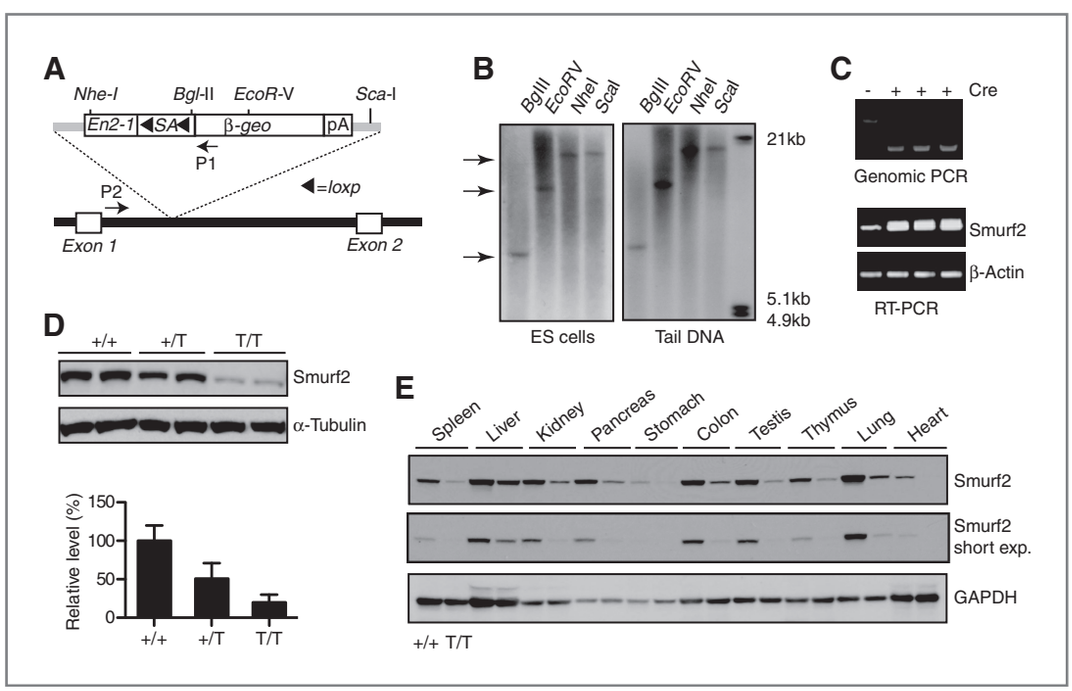

Figure 1. Characterization of a mouse model of Smurf2 deficiency. A, schematics of the trapped *Smurf2* allele. *En2-1*, mouse *En2* intron 1; *SA*, splicing acceptor; *pA*, SV40 polyadenylation signal. B, Southern blot analysis of genomic DNA of RRA098 cells or $Smurf2^{+/T}$ mouse tail using *Neo* as a probe. Specific bands are indicated by arrows at left with DNA ladder at right. C, elevated Smurf2 expression in Cre-expressing ES clones, which were identified by genomic PCR with primers P1 and P2 shown in A. D, Smurf2 expression in wild-type (+/+), $Smurf2^{+/T}$ (+/T), and $Smurf2^{T/T}$ (T/T) MEFs analyzed by Western blotting and quantitative real-time PCR (RT-PCR). Smurf2 expression in wild-type was set as 100% after normalization with β-actin. Error bars were SDs of 3 independent experiments. E, Smurf2 expression in tissues of 2-month-old wild-type and $Smurf2^{T/T}$ littermates.

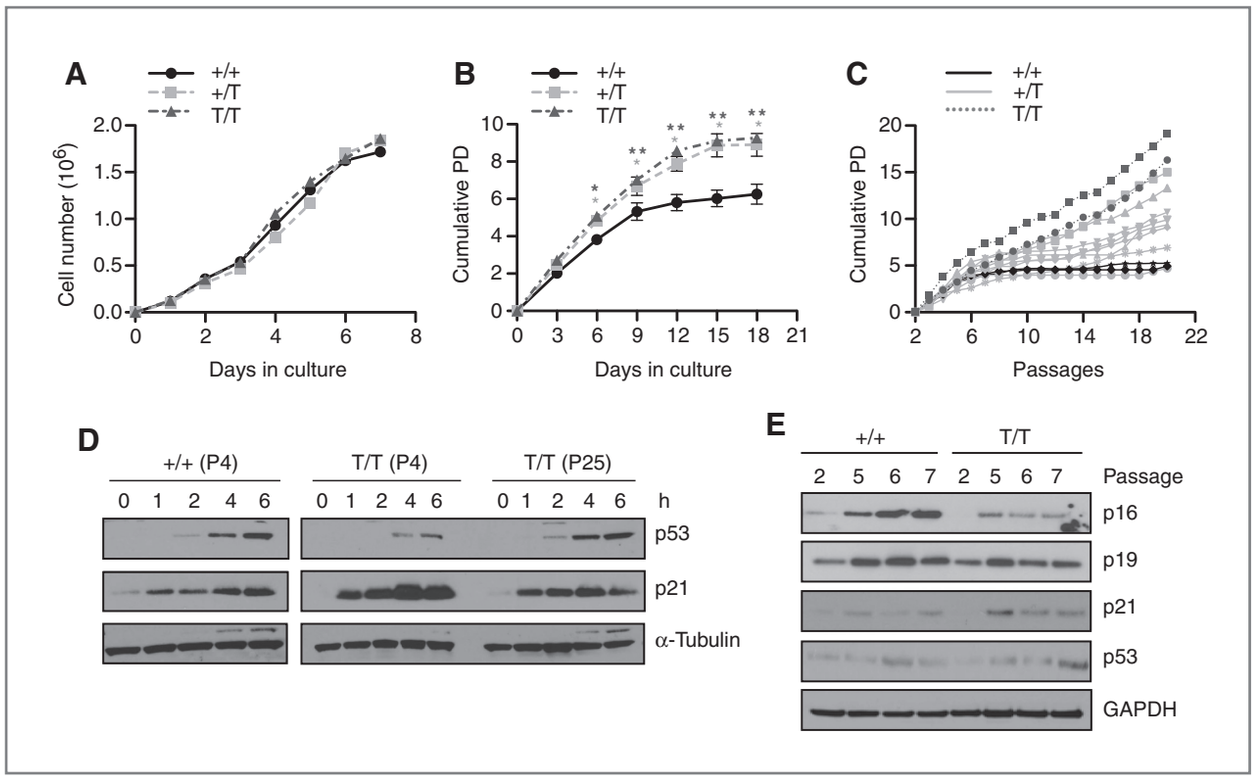

Figure 2. Impaired senescence response in Smurf2-deficient MEFs. Cell proliferation (A) and senescence analyses (B) in early-passage (P2) wild-type (+/+), *Smurf2*$^{+/T}$ (+/T), and *Smurf2*$^{T/T}$ (T/T) MEFs. The Student *t* test was used in pairwise comparison with statistical significance indicated as: *, $P < 0.05$; **, $P < 0.01$. C, immortalization assay of MEFs starting from passage 2 with population doubling (PD) set as 0. D, Western blot analysis of p53 and p21 in MEFs following doxorubicin treatment. E, Western blot analysis of senescence regulators in MEFs passaged in B.

grossly normal development (Supplementary Fig. S2). Smurf2 expression in *Smurf2*$^{+/T}$ MEFs or embryos was reduced to approximately 50% of wild-type, whereas Smurf2 (~20%) was still detected in *Smurf2*$^{T/T}$ MEFs or embryos (Fig. 1D; Supplementary Fig. S1C), suggesting that this trapped allele is hypomorphic. Consistent with this notion, Smurf2 expression was reduced but not absent in many tissues of *Smurf2*$^{T/T}$ mice compared with wild-type littermates (Fig. 1E). The *Smurf2*$^{T/T}$ mouse thus serves as a model of Smurf2 deficiency.

Impaired senescence response in Smurf2-deficient MEFs

At early passage, no obvious difference in cell proliferation was noted among MEFs of different genotypes (Fig. 2A). However, senescence entry was delayed in *Smurf2*$^{T/T}$ and *Smurf2*$^{+/T}$ MEFs compared with wild-type (Fig. 2B). We next passaged MEFs with a 3T9 protocol starting from passage 2. Wild-type MEFs entered senescence around passage 7. In contrast, *Smurf2*$^{T/T}$ and even some *Smurf2*$^{+/T}$ MEFs showed a tendency to become immortalized and continued to proliferate beyond passage 20 (Fig. 2C), indicating that Smurf2 deficiency promotes spontaneous immortalization of primary fibroblasts.

To assess whether immortalization of *Smurf2*$^{T/T}$ MEFs is the result of Smurf2 deficiency or caused by secondary mutations in other genes, we tested whether Smurf2 can induce senescence in these MEFs. Ectopic expression of Smurf2 or a ligase mutant C716A (13) induced senescence in early and late passage already immortalized *Smurf2*$^{T/T}$ MEFs (Supplementary Fig. S3), suggesting that Smurf2 is essential for senescence. Furthermore, we found that p53 and p21$^{CIP1/WAF1}$ (p21) increased in early-passage and immortalized *Smurf2*$^{T/T}$ MEFs after doxorubicin treatment (Fig. 2D), indicating that Smurf2-deficient MEFs have a functional DNA damage response, and immortalization of *Smurf2*$^{T/T}$ MEFs is independent of p53 loss.

To further investigate the mechanism underlying the impaired senescence response in Smurf2-deficient MEFs, we examined the expression of known senescence regulators during serial passage. No obvious change in the expression of p19^{Arf} (p19), p21, or p53 was observed between wild-type and *Smurf2*$^{T/T}$ MEFs (Fig. 2E). Consistent with our recent finding that Smurf2 regulates p16^{INK4a} (p16) expression through ubiquitination of Id1 (11), p16 expression in early-passage *Smurf2*$^{T/T}$ MEFs was decreased compared with wild-type MEFs. While p16 expression was elevated in late-passage wild-type MEFs, this p16 elevation was significantly attenuated in *Smurf2*$^{T/T}$ MEFs (Fig. 2E), suggesting a mechanistic link between inhibition of p16 expression and delayed senescence in Smurf2-deficient MEFs.

Increased spontaneous tumorigenesis in Smurf2-deficient mice

Smurf2$^{T/T}$ (30.6%) and *Smurf2*$^{+/T}$ (23.8%) mice developed tumors spontaneously within 20 months, whereas no tumor

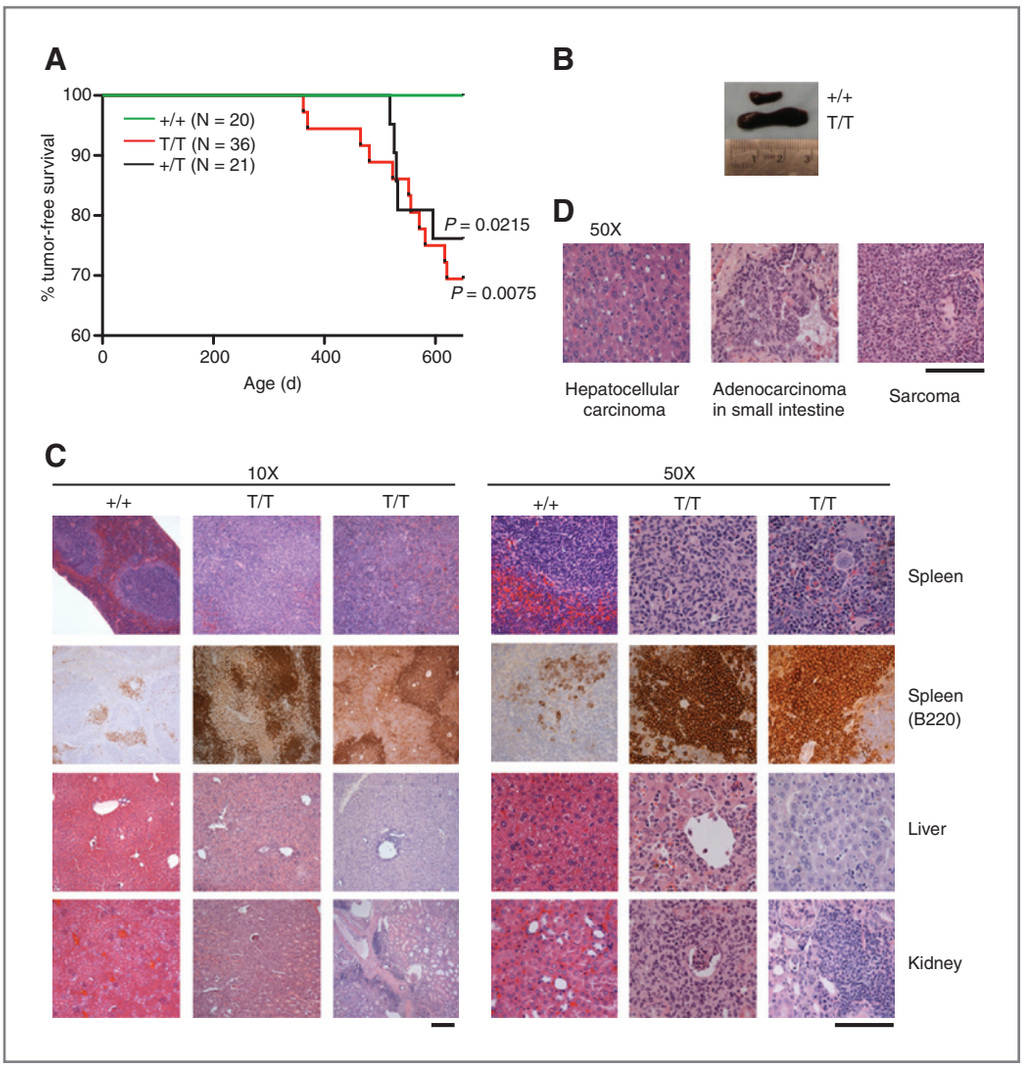

Figure 3. Increased spontaneous tumorigenesis in Smurf2-deficient mice. A, Kaplan–Meier curves of tumor-free survival of wild-type (+/+), *Smurf2*$^{+/T}$ (+/T), and *Smurf2*$^{T/T}$ (T/T) mice. Statistical significance was analyzed by the log-rank test. B, representative spleens of a moribund *Smurf2*$^{T/T}$ mouse and an age-matched wild-type mouse. C, representative hematoxylin and eosin and B220 staining of lymphomas in spleen, liver, and kidney of *Smurf2*$^{T/T}$ mice, in comparison with sections of wild-type mice. D, representative H&E staining of other types of tumors found in Smurf2-deficient mice. Scale bars for ×10 and ×50 images are 200 and 100 μm, respectively.

was found in wild-type mice within the same period (Fig. 3A). All affected Smurf2-deficient mice had enlarged spleens (Fig. 3B) and developed lymphomas. A majority of lymphomas (82.4%) stained strongly for B220 (Fig. 3C; Supplementary Fig. S4), indicating a B-cell origin. The lymphomas often spread to other organs: 47.1% and 23.5% of mice with lymphoma in spleen were found to have lymphoma in kidney and liver, respectively (Fig. 4C; Supplementary Fig. S5). In addition to lymphoma (72.7% of tumors), we also found other types of tumors, including hepatocellular carcinoma (13.6%), adenocarcinoma in small intestine (4.5%), and sarcoma (9.1%; Fig. 3D). Collectively, these results indicate that Smurf2 deficiency leads to increased spontaneous tumorigenesis in mice and suggest that Smurf2 functions as a tumor suppressor.

Increased spontaneous tumorigenesis was also observed in *Smurf2*$^{+/T}$ mice, which had approximately 50% of Smurf2 protein. Interestingly, 3 of the 4 lymphoma samples from *Smurf2*$^{+/T}$ mice showed lower level of Smurf2 protein than normal spleen of *Smurf2*$^{+/T}$ mice (Supplementary Fig. S6A). To test whether LOH occurred during development of these lymphomas, we used quantitative PCR to compare the abundance of the wild-type and trapped *Smurf2* alleles with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in tail and spleen (Supplementary Fig. S6). Three lymphoma samples from *Smurf2*$^{+/T}$ mice with decreased Smurf2 protein levels showed a reduction in the abundance of the wild-type *Smurf2* allele compared with unaffected corresponding tail DNA (Supplementary Fig. S6D), whereas the relative abundance of the trapped *Smurf2* allele was either unchanged or increased

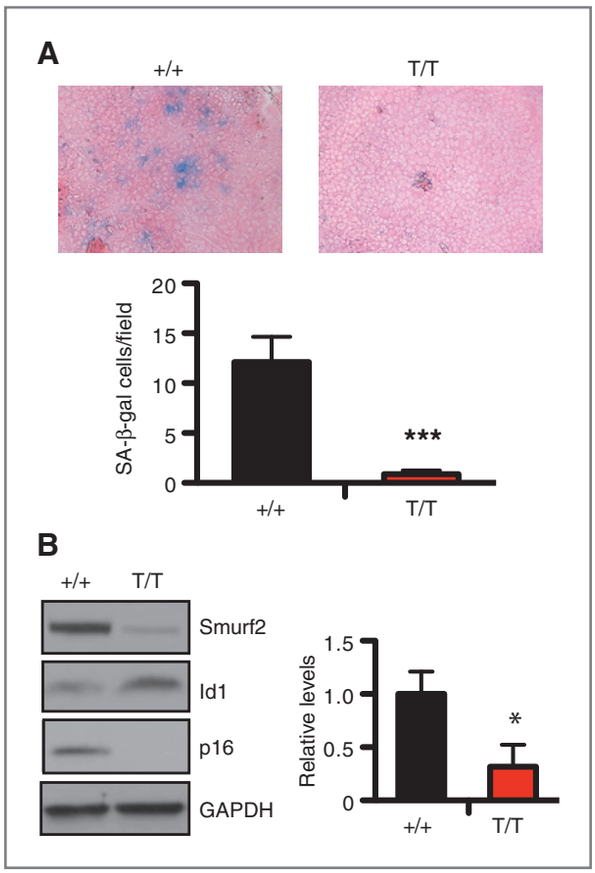

Figure 4. Impaired senescence response in spleens of aged Smurf2-deficient mice. A, representative staining for SA-β-gal activity and quantitation (10 randomly selected fields) in spleens of 12-month-old premalignant wild-type (+/+) and *Smurf2*$^{T/T}$ (T/T) mice. B, Western blot and quantitative real-time PCR (RT-PCR) analyses of p16 expression in spleens of 12-month-old mice. The relative expression of p16 in wild-type was set to be 1 after normalization with β-actin. Error bars were SDs of 3 independent experiments. The Student *t* test was used in statistical analysis (*, $P < 0.05$; ***, $P < 0.001$).

(Supplementary Fig. S6E). These results suggest a loss of the wild-type *Smurf2* allele in these lymphomas and confirm that loss of Smurf2 results in the formation of tumors in mice.

Senescence regulation in spleens of Smurf2-deficient mice

As the majority of tumors found in Smurf2-deficient mice were B-cell lymphomas in spleen, we investigated whether senescence response was impaired in spleens of Smurf2-deficient mice. In 12-month-old premalignant mice, we found positive staining for SA-β-gal activity in spleens of wild-type mice, whereas such positive staining was largely absent in spleens of *Smurf2*$^{T/T}$ mice (Fig. 4A), indicating an impaired senescence response in spleens of Smurf2-deficient mice. Consistent with our recent finding that Smurf2 regulates p16 expression through ubiquitination and degradation of Id1 during senescence (11), we found that Id1 was elevated, whereas p16 expression was decreased in spleens of 12-month-old premalignant *Smurf2*$^{T/T}$ mice compared with age-matched wild-type littermates (Fig. 4B). These results suggest that the Smurf2/Id1/p16 axis regulates senescence *in vivo*. As senescence acts as an important tumor suppressor to restrict the proliferation of cells at risk of malignant transformation (14, 15), an impaired senescence response in Smurf2-deficient cells provides an underlying mechanism of increased spontaneous tumorigenesis in Smurf2-deficient mice.

Discussion

In this study, we found that Smurf2-deficient mice exhibited an increased susceptibility to spontaneous tumorigenesis, indicating that Smurf2 has a tumor suppression function. Further support for Smurf2 as a tumor suppressor is the observation of increased spontaneous tumorigenesis in *Smurf2*$^{+/T}$ mice accompanied by frequent loss of the wild-type *Smurf2* allele in these tumors. Consistent with this notion, *Smurf2* mutations have been found in human melanoma (2 of 8) and lung carcinoma (1 of 145) samples in COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic). The identified nonsense mutations (1222C>T, R408* or 1774C>T, R592*) would result in truncated Smurf2 proteins lacking a functional HECT domain, thus disrupting its function as an E3 ubiquitin ligase and its ability to induce senescence (13). Collectively, these observations support the notion that Smurf2 is a tumor suppressor.

Increased tumorigenesis was preceded by an impaired senescence response in spleens and primary fibroblasts of Smurf2-deficient mice. Meanwhile, apoptosis as determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was not altered in Smurf2-deficient lymphomas or precancerous spleens compared with wild-type (data not shown). Consistent with our recent finding that Smurf2 ubiquitinates Id1 to regulate p16 expression during senescence in culture (11), we found elevation of Id1 and corresponding decrease in p16 expression in spleens of premalignant Smurf2-deficient mice, suggesting that the Smurf2/Id1/p16 axis regulates senescence response *in vivo*. Emerging evidence indicates that senescence is an important tumor suppressor mechanism by restricting proliferation of cells at risk of neoplastic transformation (14, 15). Our current study provides a mechanistic link between Smurf2-mediated senescence regulation and its role as a tumor suppressor *in vivo*.

Smurf2 is suggested to target Smads and TGF-β receptor for ubiquitination and degradation (1–3). However, these cell culture studies are challenged by recent studies of Smurf2 knockout mice, which indicate that Smurf2 does not regulate protein stability of Smads and TGF-β receptor under physiologic conditions *in vivo* (9, 18). Smurf2 has also been implicated to ubiquitinate both negative (Axin and GSK-3β) and positive (β-catenin) regulators of the canonical Wnt signaling pathway (4, 8, 10). Studies of Smurf1/2 double knockout mice reveal that Smurf2 instead regulates the noncanonical Wnt signaling through ubiquitination of Prickle1 (9). We have recently discovered that Smurf2 ubiquitinates Id1 (11), which is involved in diverse cellular processes including proliferation, differentiation, and angiogenesis (19). It will be interesting to

determine whether Smurf2-mediated ubiquitination of any of these or other yet unidentified protein substrates, in addition to senescence regulation, plays an important role in its function as a tumor suppressor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Ramkumar, H. Zhang
Development of methodology: C. Ramkumar, R.M. Gerstein, H. Zhang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Ramkumar, Y. Kong, H. Cui, S.N. Jones, H. Zhang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Ramkumar, Y. Kong, H. Cui, S. Hao, R.M. Gerstein, H. Zhang
Writing, review, and/or revision of the manuscript: C. Ramkumar, S.N. Jones, R.M. Gerstein, H. Zhang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Zhang
Study supervision: H. Zhang

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