A tumor suppressor function of Smurf2 associated with controlling chromatin landscape and genome stability through RNF20

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In addition to allelic mutations, cancers are known to harbor alterations in their chromatin landscape. Here we show that genomic ablation of Smad ubiquitin regulatory factor 2 (Smurf2), a HECT-domain E3 ubiquitin ligase, results in dysregulation of both the DNA damage response and genomic stability, culminating in increased susceptibility to various types of cancers in aged mice. We show that Smurf2 regulates the monoubiquitination of histone H2B as well as the trimethylation of histone H3 at Lys4 and Lys79 by targeting ring finger protein 20 (RNF20) for proteasomal degradation in both mouse and human cells. We also show that Smurf2 and RNF20 are colocalized at the γH2AX foci of double-stranded DNA breaks in the nucleus. Thus, Smurf2 has a tumor suppression function that normally maintains genomic stability by controlling the epigenetic landscape of histone modifications through RNF20.

RESULTS
Aged Smurf2−/− mice show a wide spectrum of tumor phenotypes
Smurf2−/− mice are relatively normal in their early lives19, however, in our study, as they aged, an unusually large number of these mice developed tumors of some sort. Eighty weeks after birth, Smurf2−/− mice had a significantly higher rate of tumor incidence than wild-type (WT) control mice (Fig. 1a). At 120 weeks after birth, 44.1% of Smurf2−/− mice grew tumors spontaneously compared to only 15.7% of WT control mice (Fig. 1a). A histopathologic examination revealed a wide spectrum of tumor types in the liver, blood, lung, pituitary and Harderian gland in Smurf2−/− mice (Fig. 1b,c). Occasionally, we also detected tumors in the skin, mammary gland and testis of Smurf2−/− mice (Fig. 1b,c).

To investigate the underlying genetic and molecular causes of the tumor phenotype, we isolated mouse embryonic fibroblasts (MEFs) from Smurf2−/− or WT littermate control embryos that had been bred into either a mixed 129/SvJ × NIH black swiss (BL) background or a pure C57BL/6 (B6) genetic background and cultured them in successive passages until immortalization using a modified 3T3 protocol20. Although indistinguishable in both morphology and proliferation rate from WT cells in the early passages (passages 4–6), Smurf2−/− cells from either the BL or B6 genetic background became notably smaller in size and grew much faster when immortalized after 21–26 passages (Fig. 1d and Supplementary Fig. 1), which is consistent with the tumor burden difference in aged Smurf2−/− and WT mice. Concordant with the greater proliferation of Smurf2−/− cells than WT cells, global gene expression patterns were markedly altered in Smurf2−/− cells compared to WT cells (Supplementary Fig. 2).

Ubiquitin modification controls a wide array of cellular functions by tagging proteins for proteasomal degradation or incorporation into other regulatory complexes1. Central to this system are the E3 ubiquitin ligases that function in a chain of reactions resulting in the attachment of ubiquitin moieties to target proteins. Smurf2 was initially recognized as a negative regulator of TGF-β signaling by targeting Smads and the type I receptor2–4. Subsequent studies broadened the repertoire of the substrates of Smurf2 and extended its known functions to controlling neuronal polarity and planar cell polarity7–9. In human cells, Smurf2 upregulation was linked to telomere attrition, and forced expression of Smurf2 was sufficient to induce senescence in fibroblasts10. Abnormal expression of Smurf2 was also reported in subsets of esophageal squamous cell carcinomas and breast carcinomas11,12, but whether dysregulation of Smurf2 leads to tumorigenesis is not clear. In an attempt to address the physiological function of Smurf2, we found that genomic ablation of Smurf2 leads to global changes in histone modifications and a predisposition to a wide spectrum of tumors. It is well established that in addition to allelic mutations, cancer cells harbor epigenetic alterations in their patterns of histone and DNA modification as well as in their chromatin structure13. Here we present evidence for a tumor-suppressor function of Smurf2 that controls the chromatin landscape by targeting RNF20, the major E3 ligase responsible for the monoubiquitin modification of histone H2B (ubH2B)14,15; ubH2B is actively engaged in transcription16 and is also involved in DNA damage repair in the nuclear foci of DNA double-stranded breaks17,18.

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Loss of Smurf2 leads to increased tumorigenicity. (a) Spontaneous tumor incidence in WT (n = 35) and Smurf2\(^{-/-}\) (n = 50) mice. The statistical difference in tumor incidence between Smurf2\(^{-/-}\) and WT mice was assessed by Gehan-Breslow-Wilcoxon test. (b) Distribution of tumor types in Smurf2\(^{-/-}\) mice. Some animals developed multiple primary tumors. (c) H&E staining of histological sections of representative malignancies in aged Smurf2\(^{-/-}\) mice. Scale bar, 100 \(\mu\)m. (d) Growth curves of WT and Smurf2\(^{-/-}\) (KO) MEFs at passages 4 (p.4)–6 (p.6) or passages 21 (p.21)–26 (p.26) that were derived from either a mixed 129/SvJ x BL or B6 genetic background. Data are presented as means ± s.d (n = 3). Scale bar, 1 cm. (f) Enlarged images of crystal-violet-stained transformed foci. WT cells were grown in a monolayer only. Scale bar, 100 \(\mu\)m. (g) Allograft tumor formation assay. WT or Smurf2\(^{-/-}\) MEFs at passage 57 from the C57BL/6 background were injected subcutaneously into nude mice (n = 10). Tumor lumps (arrows) and control injection sites 30 d after the injection are shown (left). Scale bar, 1 cm. Tumor incidence (middle) and average tumor volumes (right) were calculated. Data are presented as means ± s.d.

Different in WT and Smurf2\(^{-/-}\) cells in the late passages (passages 28–33) (Supplementary Fig. 2 and Supplementary Tables 1–3). The difference in growth and gene expression in these two cell types is randomly associated with the disruption of p53 or p16 function as a result of immortalization\(^{21,22}\) (Supplementary Fig. 3a,b), indicating that the observed growth advantage is specific to the loss of Smurf2. Notably, although the passage-dependent increase in cell proliferation was a consequence of Smurf2 loss, reintroducing Smurf2 back into the immortalized Smurf2\(^{-/-}\) cells did not reverse this trend (Supplementary Fig. 3c).

In the colony formation assay\(^{23}\), Smurf2\(^{-/-}\) cells of passage 57 gave rise to many large foci that stained intensely with crystal violet, were very dense and lacked contact inhibition (Fig. 1e,f). In the allograft tumor formation assay, Smurf2\(^{-/-}\) cells of passage 57 began to develop tumors as early as 8 d after subcutaneous injection into nude mice (Fig. 1g). The average volume of these tumors increased exponentially from day 12 to day 30 after injection to about 500 mm\(^3\) (Fig. 1g). A histopathological examination revealed that all the tumor-bearing mice developed neoplasm with severe-to-moderate multifocal muscle invasion (Supplementary Fig. 3d). These findings are notable because immortalization without further oncogenic activation is not sufficient to cause transformation\(^{21,22}\), which is in agreement with what we observed in WT cells of passage 57 that only gave rise to a few lightly stained foci in the plates (Fig. 1e,f) and did not generate any tumor when injected into nude mice (Fig. 1g). Thus, after 57 passages, Smurf2\(^{-/-}\) cells have already gone through the oncogenic transformation.

Smurf2 regulates DNA damage response and genomic stability

The greater tumor burden in aged Smurf2\(^{-/-}\) mice than aged WT mice and the accelerated Smurf2\(^{-/-}\) cell growth in the late passages described above suggest that loss of Smurf2 could initiate a series of cascading events that confer a growth advantage to Smurf2\(^{-/-}\) cells and ultimately lead to tumorigenesis. One possible scenario for this process would require Smurf2 to have a role in regulating both the DNA damage response and genomic stability; this function of Smurf2 or the lack thereof would not impinge directly on the control of cell growth, but, rather, it would reduce the threshold to additional genetic lesions. To test whether Smurf2 has this function, we challenged Smurf2\(^{-/-}\) MEFs with etoposide, a topoisomerase II inhibitor that
Smurf2 controls the DNA damage response. (a) Western blot analysis of γ-H2AX in chromatin fractions of immortalized WT and Smurf2−/− MEFs. The quantification of the bands is shown on the right. (b) Immunofluorescence staining of γ-H2AX in immortalized WT and Smurf2−/− MEFs after being treated with etoposide for 1 h. (c) γ-H2AX immunostaining of early passage MEFs (passage 6), as in b. The quantification is shown on the right. (d) Western blot analysis of γ-H2AX and Smurf2 in early passage MEFs, as in a. (e) Colocalization of MYC-tagged Smurf2 (MYC-Smurf2) with γ-H2AX in U2OS cells treated with etoposide for 1 h. Scale bars, 10 µm. (f) Colocalization of GFP-tagged Smurf2 (GFP-Smurf2) with 53BP1 in U2OS cells, as in e. (g) Colocalization of endogenous Smurf2 with γ-H2AX in U2OS cells, as in e. Inset shows an enlargement (5.6x) of the merged Smurf2 and γ-H2AX staining.

Induces DNA double-stranded breaks24. Compared to WT cells, the etoposide treatment of late passage Smurf2−/− cells led to a more rapid and robust phosphorylation of the chromatin-bound histone H2AX (γ-H2AX) (Fig. 2a), which is a quantitative marker for the DNA damage response at the site of double-strand breaks25. We next examined the status of several key cell cycle regulators, among which the DNA damage-response transducer Chk1 was upregulated and became rapidly activated after exposure to etoposide (Supplementary Fig. 4a,b). Immunofluorescence staining also showed a greater number of γ-H2AX–positive foci in the nuclei of Smurf2−/− cells than in those of WT cells (Fig. 2b). Notably, we also observed enhancement of the γ-H2AX response to etoposide in early passage Smurf2−/− cells (Fig. 2c,d), implying the direct involvement of Smurf2 in the DNA damage response. The colocalization of transfected Smurf2 with either γ-H2AX or tumor protein p53 binding protein 1 (53BP1) nuclear foci in human U2OS (osteocarcinoma) cells (Fig. 2e,f and Supplementary Fig. 4c) after the cells were challenged with etoposide corroborated this previously unrecognized role of Smurf2 in the DNA damage response, as did the colocalization of endogenous Smurf2 with γ-H2AX foci in U2OS cells after challenge with etoposide (Fig. 2g). Thus, Smurf2 is directly involved in the regulation of the DNA damage response.

The increase in the number of etoposide-induced γ-H2AX–positive foci in Smurf2−/− cells hints at a defect in the DNA damage checkpoint control, which would allow the cells to continue to grow in the presence of unrepaired DNA damage, leading to the accumulation of genetic lesions and genomic instability. Smurf2−/− cells indeed showed greater viability after facing a spectrum of etoposide or ultraviolet light subtype C insults compared to WT cells (Supplementary Fig. 4d), and Smurf2−/− cells underwent less apoptosis than WT cells when challenged with 20 µM etoposide (Supplementary Fig. 4e). On mitotic chromosome spreads, Smurf2−/− and WT cells at passage 6 did not

Figure 2
Figure 3 Smurf2 controls both chromatin compaction and patterns of histone modification. (a) MNase digestion of whole nuclei prepared from immortalized WT and Smurf2−/− MEFs. N1, N2 and N3 indicate the length of the DNA wrapped around 1, 2 or 3 nucleosomes, respectively. (b) Quantification of N1, N2 and N3 in a. OD, optical density. (c) MNase digestion of whole nuclei isolated from Smurf2−/− cells stably transfected with Smurf2 or a control vector. (d) Quantification of N1 in c. (e) Western blot analyses of ubH2B and ubH2A in immortalized WT and Smurf2−/− cells from the BL and B6 backgrounds. The arrow points to ubH2A. (f) Western blot analyses of various forms of histone methylation in immortalized WT and Smurf2−/− cells from the BL and B6 backgrounds. (g) Quantification of the histone trimethylation in f. (h) Western blot analyses of histone modifications in the spleens derived from WT and Smurf2−/− mice. Each sample is a mixture of three spleens from three different mice.

have a substantial difference in chromosome abnormality, which is consistent with the lack of difference in growth seen in these two types of cells at this early passage. At passage 37, both Smurf2−/− and WT cells showed hyperplosity (data not shown), which is a typical trait of immortalization; however, Smurf2−/− cells at this passage contained much higher numbers of Robertsonian translocations, undefined translocations and marker chromosomes than their WT counterparts (Supplementary Fig. 4f). These results indicate that the loss of Smurf2 caused a defect in the maintenance of genomic stability that was exacerbated in immortalized MEFs after moving through the mitotic crisis, and they suggest that this compromised control of genomic stability may render Smurf2−/− mice more likely to develop tumors under the stress of aging than WT mice. The failure of exogenous Smurf2 to suppress the elevated growth of immortalized Smurf2−/− MEFs (Supplementary Fig. 3c) corroborates the results suggesting genomic instability as the root cause of this elevated tumorigenic tendency.

Smurf2 regulates the global chromatin landscape

Both the increased differential gene expression activities and the increased DNA damage response associated with Smurf2 loss point to possible alterations in the chromatin structure and in patterns of histone modifications, which collectively constitute the epigenetic landscape that regulates transcription and the responses to DNA damage. To assess whether such alterations occur as a result of Smurf2 loss, we examined the effect of Smurf2 hyperexpression on Smurf2−/− and WT cells using micrococcal nuclease (MNase) digestion, which cuts linker DNA that connects the nucleosomes. Whole nuclei that we isolated from immortalized Smurf2−/− cells (Fig. 3a,b) or that we freshly prepared from Smurf2−/− dermal fibroblasts (Supplementary Fig. 5a,b) were more thoroughly digested than their WT counterparts within 2–10 min of treatment with MNase, suggesting that loss of Smurf2 rendered the chromatin more loosely compacted. To determine whether Smurf2 loss alone is responsible for loose chromatin compaction, we restored Smurf2 expression in Smurf2−/− MEFs and found that the ability of MNase to release mononucleosomes under such a condition was indeed impeded (Fig. 3c,d).

The post-translational modification of histones regulates various chromatin functions, including chromatin condensation, DNA damage repair and transcription. Because Smurf2 is an E3 ligase, we examined the ubiquitin modification of acid-extracted histones using a western blot analysis. This analysis revealed that the amount of ubH2B, but not ubH2A, was markedly higher in Smurf2−/− cells relative to WT cells in both the BL and B6 backgrounds (Fig. 3e). Concurrent with the higher amount of ubH2B seen in Smurf2−/− cells, histone H3 trimethylation on Lys4 and Lys79 (K4Me3 and K79Me3, respectively), which is consequentially associated with ubH2B, was significantly higher in Smurf2−/− cells than WT cells (Fig. 3f,g). Dimethylation of the histone H3 at Lys9 and Lys36, which is closely linked to transcriptional elongation, was slightly higher, whereas other forms of modifications of H3, including trimethylation at Lys27 and Lys9 and dimethylation at Lys4, Lys79 and Lys27, as well as the acetylation of core histones (H2A, H2B, H3 and H4), were the same in Smurf2−/− and WT cells (Fig. 3f and Supplementary Fig. 5c). To determine the
temporal origin of the changes in the chromatin landscape, we isolated spleen tissues from Smurf2−/− mice and WT mice and found that the amounts of ubH2B and histone H3 K4Me3 and K79Me3 were all greater in Smurf2−/− mice than WT mice (Fig. 3h). Thus, the changes in the histone modification patterns were a direct consequence of Smurf2 loss. It is possible that in the absence of Smurf2, the chromatin landscape is reshaped in such a way that it alters global gene expression patterns and weakens the control of genomic stability, which ultimately propels tumorigenesis in aged Smurf2−/− mice.

Smurf2 regulates the stability of the ubH2B E3 ligase RNF20
To investigate how loss of Smurf2 leads to the described changes in the chromatin landscape, we turned our attention to RNF20 (also known as hBre1B), the major ubH2B-specific E3 ligase4,13. In Smurf2−/− cells, the amount of endogenous RNF20 was significantly upregulated relative to that seen in WT cells in both early and late passages, whereas the amounts of a closely related RNF40 (also known as hBre1B) and the ubH2B deubiquitinase USP22 were comparable in the two cell types (Fig. 4a and Supplementary Fig. 6a). We also observed upregulation of RNF20 in Smurf2−/− nuclei after immunofluorescence staining (Fig. 4b and Supplementary Fig. 6b), and this upregulation was probably a post-transcriptional event, as we detected no difference in RNF20 mRNA level between Smurf2−/− and WT cells using RT-PCR (Supplementary Fig. 6c). Treating cells with the proteasome inhibitor MG132 increased the accumulation of RNF20 in WT cells but did not further increase the already high accumulation of RNF20 in Smurf2−/− cells (Fig. 4c). In addition, blocking protein synthesis with cycloheximide showed that RNF20 had a slower turn-over rate in Smurf2−/− than in WT cells (Supplementary Fig. 6d).

In tissue sections prepared from Smurf2−/− mice, we confirmed upregulation of RNF20 in the thymus, lymph node and lung using immunohistochemical (IHC) staining (Fig. 4d) and in the spleen using IHC staining and western blot analysis (Fig. 4e). We saw moderate upregulation of RNF20 in the salivary gland and the liver sections from Smurf2−/− mice using IHC staining (Fig. 4d). The distribution of high RNF20 expression in a panorama of mouse tissues is consistent with the wide spectrum of tumors seen in Smurf2−/− mice (Fig. 4b,c). This distribution of high RNF20 expression in mouse tissues also correlates with the distribution of tissue origins of human tumors highly expressing RNF20 that are documented in the Human Protein Atlas (http://www.proteinatlas.org/ENSG00000155827)35. Notably, IHC staining showed that the tumors from Smurf2−/− mice bore the same hallmark of high RNF20 expression as the surrounding normal tissues did (Supplementary Fig. 6e and data not shown), suggesting that these tumors are probably clonal descendants of cells highly expressing RNF20.

To determine whether the relaxed chromatin compaction in Smurf2−/− cells is a direct consequence of increased RNF20 expression, we knocked down RNF20 using RNF20-specific siRNA (siRNF20),...
RNF20 is a direct target of Smurf2 for proteasome-dependent degradation. (a) Coimmunoprecipitation (IP) analysis of the interaction of endogenous Smurf2 and RNF20 in WT and Smurf2−/− MEFs. WCL, whole cell lysate. (b) The GST pulldown experiment showing a direct interaction between purified GST-Smurf2 and Flag-RNF20. Coomassie blue staining shows purified GST and GST-Smurf2. (c) Western blot analysis of the polyubiquitination of RNF20 by Smurf2 in vivo. HA-Ub, HA-ubiquitin. (d) Western blot analysis of the polyubiquitination of RNF20 translated in vitro by recombinant GST-Smurf2 in the presence of E1, E2 and HA-Ub. GST-Smurf2CA was used as a control. F, Flag tagged; E, endogenous RNF20. (e) Overexpression of MYC-Smurf2 leads to a reduction in the amount of ubH2B (Fig. 5f). Under this condition, the number of mononucleosomes released by MNase digestion in siRNF20-transfected Smurf2−/− cells decreased substantially (Fig. 4g). To determine whether altering RNF20 expression affects cell growth, we generated stable WT MEFs overexpressing RNF20 which grew much faster than control cells overexpressing a vector (Fig. 5a). Conversely, stable WT MEFs overexpressing RNF20 grew much faster than control cells overexpressing a vector (Fig. 4j). These data strongly support a role for Smurf2 in the maintenance of the chromatin structure through RNF20.

**RNF20 is a substrate of Smurf2-mediated ubiquitination**

We conducted several lines of experiments to determine whether RNF20 is a direct substrate of Smurf2-mediated ubiquitination and proteasome degradation. First, we showed an interaction between Smurf2 and RNF20 using coimmunoprecipitation experiments with the endogenous proteins (Fig. 5a) and the transfected proteins produced in HEK293 cells (Supplementary Fig. 7a), as well as by using a GST pulldown experiment with purified Flag-RNF20 and recombinant GST-Smurf2 (Fig. 5b). Second, we showed that coexpression of Flag-RNF20, hemagglutinin (HA)-ubiquitin and MYC-Smurf2 in HEK293 cells could enhance the background level of the polyubiquitination of RNF20, which underwent autoubiquitination as a result of its intrinsic E3 ligase activity; however, we did not see this enhancement in the E3-ligase–defective Smurf2 mutant containing a C716G substitution (Smurf2CG)4, implying specificity of our assay condition (Fig. 5c). We further reconstituted the ubiquitination of RNF20 in vitro using ubiquitin-activating enzyme (E1), ubiquitin conjugase (E2), HA-ubiquitin, purified Flag-RNF20 from in vitro translation and bacterially expressed GST-Smurf2, but not the mutant GST-Smurf2 containing a C716A substitution (GST-Smurf2CA) (Fig. 5d). Third, coexpression of Flag-RNF20 with MYC-Smurf2, but not with the mutant MYC-Smurf2CG, decreased the amount of RNF20 protein (Fig. 5e), and this Smurf2-induced degradation could be blocked by treatment with MG132 (Fig. 5f). Fourth, in transfected U2OS cells, we found GFP-Smurf2 to colocalize with endogenous RNF20 and reduce the amount of endogenous RNF20 accordingly (Fig. 5g and Supplementary Fig. 7b,c). In addition, when we challenged the transfected U2OS cells with etoposide, both GFP-Smurf2 and RNF20 colocalized with the γ-H2AX foci (Fig. 5h). Taken together, these results show that RNF20 is a direct substrate of Smurf2 and that Smurf2 is required for maintaining the histone modification pattern over the general chromatin landscape and for regulating the acute DNA damage response at sites of DNA double-stranded breaks by fine tuning the amount of RNF20.

Smurf2 regulates RNF20 stability in human cancers

The RNF20-mediated alteration of the chromatin landscape offers an attractive model to account for the difference of tumor burden in aged Smurf2−/− and aged WT mice. To address whether this cause of tumorgenesis also applies to human tumors, we analyzed a number of established (immortalized) human tumor cell lines. In all cases, knockdown...
of Smurf2 with siRNA in OVCAR8 (ovarian carcinoma), A549 (lung carcinoma), HCT116 (colon carcinoma) and U2OS cells markedly increased RNF20 expression (Fig. 6a). We also extracted chromatin from HCT116 and U2OS cells after Smurf2 knockdown and found that the amount of ubH2B was also increased (Fig. 6a). However, knockdown of RNF20 in U2OS or HCT116 cells reduced the extent of digestion of isolated whole nuclei by MNase, resulting in increased chromatin compaction in these cells (Supplementary Fig. 5a, b). Thus, decreasing RNF20 expression causes chromatin to be more tightly compacted in human cancer cells. These results are in agreement with our observation that increasing RNF20 protein level in Smurf2-deficient cells relaxed their chromatin structure (Fig. 3a, b and Supplementary Fig. 5a, b).

Previously, we showed a switch in the distribution of Smurf2 expression from high in the nucleus in normal tissues to high in the cytoplasm in breast cancer cells in an array of 40 human breast cancer and matching normal tissues, despite the fact that the levels of Smurf2 expression are mixed in these tissues (12). (Fig. 6b). It is possible that the nuclear function of Smurf2 was partially impaired in these cancer cells. In this same array and using a scoring matrix that takes into account both the intensity of IHC staining in an RNF20-positive cell and the percentage of RNF20-positive cells in a given field, we found a strong correlation between high RNF20 expression and only 5 showing low RNF20 expression compared to the nuclear staining of Smurf2 in the corresponding normal breast tissues (Fig. 6b, d and Supplementary Table 5). Thus, the chromatin landscape in human tumors is sensitive to fluctuations in RNF20 expression, and loss of Smurf2 is probably one of the contributing events that cause genome instability in certain human cancers.

**DISCUSSION**

Like the classical ‘caretaker’ tumor suppressor genes MSH2 and MLH1 of the DNA repair pathway, (36) our data show that altering Smurf2 expression has no immediate impact on cell growth, which is reflected by a few sporadic reports describing the roles of Smurf2 in tumorigenesis in the literature (10–12, 37). Through long-term observation of tumor phenotypes in aged mice and close examination of growth and oncogenic transformation properties of immortalized MEFs, we linked loss of Smurf2 to tumorigenesis. Our data indicate that the tumorigenic role of Smurf2 is associated with the defective DNA damage response events that cause genome instability in certain human cancers.
of Smurf2, because RNF20 is the major ubiquitin ligase that controls histone ubiquitination and consequentially histone methylation. Although there are conflicting reports about the role of RNF20 in tumorigenesis, we firmly established the oncogenic role of RNF20 by showing that altering RNF20 expression with RNF20-specific siRNA or complementary DNA can change the patterns of histone modification as well as cell growth and oncogenic transformation properties. Moreover, we showed a causal relationship between impairment of the nuclear function of Smurf2 and the increase in RNF20 expression in human tumors, implying a role of loss of Smurf2 function to human cancers. On the basis of these data, we postulate that loss of Smurf2 could promote tumorigenesis by altering the pattern of histone modification and the extent of chromatin compaction, which consequentially introduces instability into the genome and changes gene expression profile (Supplementary Fig. 9); ultimately, this leads to cancer.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

M.T. and Y.T. maintained mouse colonies and generated primary MEFs and mouse dermal fibroblasts. M.Y., S.Y.C. and Y.E.Z. observed and analyzed the spontaneous tumor formation in mice. S.S.B. performed karyotyping analyses. Y.E.Z. analyzed microarray data. M.B. performed all other experiments described in the manuscript. M.B. and Y.E.Z. conceived of the study, analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS

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**ONLINE METHODS**

**Mice and histopathology.** For the investigation of spontaneous tumor development, Smurf2−/− mice in a mixed 129/SvJ x BL and in a pure C57BL/6 background19 were monitored twice weekly, and moribund mice or mice with obvious tumors were killed and subjected to necropsy. After 28 month of age, all mice were killed and necropsied.

To assess the tumorigenicity of immortalized MEFs in vivo, 4- to 6-week-old female athymic nude mice were subcutaneously injected with immortalized WT or Smurf2−/− MEFs in two hind flanks (2 x 10^6 cells per flank).

Tumor histology was read by a board-certified veterinary pathologist in the National Cancer Institute core facility. All mice were maintained and handled according to protocols approved by the Animal Care and Use Committee of the US National Cancer Institute.

**Cell culture and transfections.** Primary MEFs and mouse dermal fibroblasts were derived as described19,39. To establish immortalized MEFs, MEFs derived from three different Smurf2−/− embryos and two different WT embryos in a 129/SvJ x BL background were mixed at passage 4 according their genotype to derive BL-Smurf2−/− and BL-WT cells, respectively. These cells were then passaged on a 3T3 protocol20 to generate immortalized cells. Simultaneously, MEFs derived from a Smurf2−/− and a sibling WT embryo in a C57BL/6 background were also passaged according to the above 3T3 protocol to generate immortalized B6-Smurf2−/− and B6-WT cells, respectively. Human U2OS, HeLa and HEK293 cells were obtained from the American Type Culture Collection. HCT116 and A549 cells were from the Division of Cancer Treatment and Diagnosis Tumor Repository of the US National Cancer Institute. FuGENE 6 (Roche), Lipofectamine 2000 and oligofectamine (Invitrogen) reagents were used according to the manufacturer’s instructions for transfection and siRNA transfection experiments. Smurf2-specific siRNA and control siRNA were described previously12. The target siRNA sequences to transfect plasmid DNA and siRNA. Smurf2-specific siRNA and control non-silencing siRNA were described previously12. The target siRNA sequences used for RNF20 were: 5′-CTTGCGTAACATGCGTAAA-3′ (mouse)39; and 5′-CGGTATCATCCTTGAAGTT-3′ (human).

For reconstitution of Smurf2 in Smurf2−/− cells, immortalized MEFs were infected with retroviral particles containing pBabe-Flag-Smurf2-puro vector12. For generation of WT MEFs overexpressing RNF20, cells were infected with retroviruses containing pBabe-Flag-RNF20-puro vector.

**Immunofluorescence, immunohistochemistry and immunoprecipitation.** Immunofluorescence was conducted as described30,41. Immunohistochemistry of tissue sections with antibodies to RNF20 and Smurf2 (H-50) was performed using enzynatic Avidin-Biotin Complex (ABC)-diaminobenzidine (DAB) staining. Nuclei were counterstained with hematoxylin.

All comparative images were obtained using identical microscope and camera settings. The breast cancer tissue array 801 were from US Biomax. Immunohistochemistry of the array was analyzed and quantified by a board certified pathologist in the US National Cancer Institute.

For the immunoprecipitation experiments, cells were lysed either in NP-40 (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetra-acetic acid (EGTA), 0.5% NP-40) or in freeze-thaw lysis buffer (600 mM KCl, 20 mM Tris-HCl, pH 7.8; 20% glycerol, EDTA-free protease and phosphatase inhibitors) after repetitive freezing-thawing cycles and were subsequently treated with 250 U benzonase (Novagen) as described42. The lysates were then clarified by centrifugation (10,000g for 20 min at 4 °C) and diluted four times with freeze-thaw dilution buffer (45 mM Tris-HCl, pH 7.6, 2.25 mM EDTA, 0.1% NP-40). All immunoprecipitations were conducted overnight at 4 °C. Soluble chromatin fractions were prepared as described43. Nucleosome core histones were extracted from the cells using a modified acid-based extraction protocol44.

**Micrococcal nuclease digestion of chromatin.** The assay was performed as described45, with a few modifications. In brief, cell nuclei were isolated using hypotonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM diethiothreitol (DTT)) supplemented with EDTA-free protease, phosphatase inhibitors and 0.1% Triton-X. After washing in the hypotonic buffer without Triton-X, an aliquot of the nuclei was resuspended in 1 N NaOH and normalized to a DNA concentration of ~1.5 mg ml−1 in the reaction buffer (15 mM Tris-HCl, pH 7.4, 60 mM KCl, 0.25 mM sucrose, 1 mM CaCl2, 0.5 mM DTT). The digestion was carried out with MNase (Roche) at 5 U MNase per 250 µl of reaction buffer at 25 °C for the indicated period of time. The reaction was terminated by adding an equal volume of 2x TNE buffer (20 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 2 mM EDTA, 2% SDS) with freshly added protease K (0.2 mg ml−1). The samples were then incubated overnight at 37 °C, and genomic DNA was purified as described46.

**GST fusion protein, pulldown assays and in vitro ubiquitination assays.** GST fusion proteins were prepared from Escherichia coli using glutathione-Sepharose beads (Amersham Pharmacia Biotech). In vitro translated (TNT kit, Promega) Flag-RNF20 in rabbit reticulocyte lysate was immunoprecipitated using Flag-M2 beads and was eluted from the beads using Flag peptide (Sigma). The purified Flag-RNF20 was incubated with GST or GST-Smurf2 fusion proteins for 1 h at 4 °C in the binding buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2 mM EDTA, 0.1% NP-40). RNF20 proteins that bound to the GST-fusion proteins were retrieved on glutathione-Sepharose beads and subjected to immunoblot analyses. The purified Flag-RNF20 and GST-Smurf2 fusion proteins were also used in the in vitro ubiquitination assay as described46.

**Additional methods.** Detailed methodology is described in the Supplementary Methods.