In Vivo Regulation of TGF-β by R-Ras2 Revealed through Loss of the RasGAP Protein NF1

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Abstract

Ras superfamily proteins participate in TGF-β—mediated developmental pathways that promote either tumor suppression or progression. However, the specific Ras proteins, which integrate in vivo with TGF-β signaling pathways, are unknown. As a general approach to this question, we activated all Ras proteins in vivo by genetic deletion of the RasGAP protein NF1 and examined mice doubly deficient in a Ras protein to determine its requirement in formation of TGF-β—dependent neurofibromas that arise in NF1-deficient mice. Animals lacking Nf1 and the Ras-related protein R-Ras2/TC21 displayed a delay in formation of neurofibromas but an acceleration in formation of brain tumors and sarcomas. Loss of R-Ras2 was associated with elevated expression of TGF-β in Nf1-deficient Schwann cell precursors, blockade of a TGFβRII/AKT-dependent autocrine survival loop in tumor precursor cells, and decreased precursor cell numbers. Furthermore, the increase in size of sarcomas from xenografts doubly deficient in these genes was also found to be TGF-β—dependent, in this case resulting from cell nonautonomous effects on endothelial cells and myofibroblasts. Extending these findings in clinical specimens, we documented an increase in TGF-β ligands and an absence of TGF-β receptor II in malignant peripheral nerve sheath tumors, which correspond to tumors in the Nf1-deficient mouse model. Together, our findings reveal R-Ras2 as a critical regulator of TGF-β signaling in vivo. Cancer Res; 72(20): 5317–27. ©2012 AACR.
TGF-β onco genesis (16). Decreased or altered TGF-β responsiveness and increased expression or activation of TGF-β ligands is common as tumors progress. For example, TGF-β can promote tumorigenesis through sequestration of mutant p53 (18), and have nonautonomous effects on tumor stroma (19).

We identified TC21 as a regulator of TGF-β function in vivo. Previous studies examined cross talk between the Ras and TGF-β—signaling pathways, mainly in vitro (20–22). Ras/MAPK activates the TGF-β promoter (23, 24), and Ras/Erk signaling blocks SMAD translocation to the nucleus through phosphorylation of SMAD2/3 (20). In vivo, RAS/MAPK can phosphorylate p53, which then interacts with SMAD proteins (25).

Neurofibromas and MPNSTs derive from neural crest lineage cells, more mature Schwann cell precursors (SCP), and/or differentiated mature Schwann cells (26). To study development, we crossed TC21-deficient mice (TC21fl/fl; ref. 14) to Nf1fl/fl mice (27). To study tumorigenesis, we crossed TC21fl/fl; DhhCre mice that form neurofibromas, and Nf1fl/fl;Trp53−−/− (NPCis) mice that serve as a model of soft tissue sarcomas and brain tumors with histology of glioblastoma (28), and used a xenograft model of human MPNST.

We found that Nf1 mutation renders SCPs insensitive to TGF-β-mediated cell death and define a TGF-β autocrine survival loop, correlating with benign neurofibroma formation. Nf1 mutants lacking TC21 restore TGF-β sensitivity and benign tumorigenesis is delayed. Conversely, loss of TC21 increases TGF-β-induced malignancy in the NPCis model. In Nf1 MPNST xenografts, loss of TC21 accelerates tumor growth in a noncell autonomous manner. In summary, TC21 is a major regulator of TGF-β production that functions in development and tumorigenesis in vivo.

Materials and Methods

Mice

We housed mice in a temperature- and humidity-controlled vivarium on a 12-hour dark–light cycle with free access to food and water. TC21fl/fl and R-Hasfl/fl mice were obtained on C57BL/6/129 mixed background after 4 generations of backcross onto C57/B6 (14), then bred to homozygosity. We mated TC21fl/fl and R-Hasfl/fl mice to Nf1fl/fl C57/B6 mice (27) to obtain TC21−/−;Nf1−/− and R-Has−/−;Nf1−/− mice, which were intercrossed to obtain mutant embryos. TC21−/− mice were mated to Nf1fl/0;DhhCre mice (26) to obtain TC21fl/fl;DhhCre mice. F1 mice were intercrossed to obtain TC21−/−;Nf1fl/0;DhhCre mice and TC21−/−;Nf1fl/fl;DhhCre littermates. We bred TC21−/− mice to NPCis C57BL/6 mice (28) to obtain TC21−/−;NPCis mice. These mice were mated with TC21−/− to obtain TC21−/−;NPCis mice. TC21−/−;NPCis littermates were bred to C57BL/6 wild-type mice for parallel controls. For tumor experiments we analyzed male mice.

Cell culture

We dissociated dorsal root ganglia (DRG) from E12.5 embryos and plated cells in serum-free medium (29). We used cells direct from embryos for precursor numbers. At passage 2 to 3, 500 cells/well were plated then inhibitors, antibody or lentivirus added after 24 hours. Spheres were counted 3 days later.

Each experiment shown represents 3 or more independent experiments. Anti-TGF-β antibody, rhTGFβ1, and rabbit immunoglobulin G (IgG) were from R&D Systems, TGFβR1, MEK1 (Cayman Chemicals), Akt (Selleck Chemicals), p38MAPK, and ROCK (Calbiochem) inhibitors were dissolved in dimethyl sulfoxide.

MPNST cell lines included 26T, T265, 8814 WT for p53 (30) and S462TY [derived from the S462 cell line by 2 rounds of in vivo growth as xenografts (31)]. Cells were maintained in Dulbecco’s Modified Eagle’s Medium/10% FBS/1% penicillin/streptomycin.

Immunohistochemistry and histology

Six-micrometer formalin-fixed paraffin sections or 12-μm 4% paraformaldehyde-fixed frozen sections were stained with phospho-SMAD2/3, phospho-AKT (Cell Signaling), SMA (Dako), antineurofilament, and meca-32 (DSHB). Secondary incubations used host-appropriate secondary antibodies. Neurofibroma sections were submitted to the CCHMC Pathology Laboratory for hematoxylin and eosin stain (H&E), toluidine blue, and rabbit polyclonal anticow S100β (Dako).

Western blot analysis

We lysed cells, tissue, and tumor sections as described (32). We separated proteins on 4% to 20% TrisHCl acrylamide gels (Bioread), transferred to polyvinylidene difluoride membranes (Millipore), and probed membranes with: anti-TC21 (Abnova), phospho-AKT, phospho-SMAD2/3, phospho-42/44-MAPK, and β-actin (Cell Signaling). Detection used horseradish peroxidase-conjugated secondary antibodies (BioRad) and ECL Plus developing system (Amersham Biosciences).

qRT-PCR

We isolated total RNA with an RNaseasy kit (Qiagen) and carried out cDNA synthesis (Invitrogen Superscript III). We used triplicate reactions to conduct quantitative real-time PCR (qRT-PCR; ABI 7500-Sequence Detection System; 32). Values for genes of interest were normalized to glyceraldehyde-3-phosphate dehydrogenase (mouse samples) or β-actin (human samples) and fold change calculated by the ΔΔct method.

Lentiviral infection

We infected MPNST cells at 50% confluence with TRIPZ shTC21 or nontarget control (Open Biosystems). We incubated lentiviral particles with MPNST cells in the presence of polybrene (8 μg/mL; Sigma-Aldrich) daily for 3 days, followed by selection in puromycin (2.5 μg/mL; Sigma-Aldrich) then maintained cells in media containing puromycin; 2 μg/mL doxycycline (MP Biomedicals)-induced shRNA expression.

Mouse xenograft

We injected 2.3 × 106 S462TY cells in 150 μL with 30% matrigel (BD Biosciences) into flanks of 5- to 6-week-old female athymic nude (nu/nu) mice (Harlan). We maintained mice on 1.875 ppm doxycycline feed (Test Diet). Tumor volumes and weight were measured twice weekly. We sacrificed mice before tumor size reached 10% body weight. For anti-TGF-β...
Figure 1. Loss of TC21 extends survival of neurofibroma-bearing mice but decreases survival of NPCis mice. Kaplan-Meyer survival curves for Nf1fl/fl;DhhCre and Nf1fl/fl;DhhCre;TC21fl/fl mice (A; log-rank test, \( p < 0.0001 \)) and NPCis and NPCis;TC21fl/fl mice (B; log-rank test, \( p = 0.0001 \)).

To test for effects of treatment, we injected mice intraperitoneally with 3 mg/kg of IgG or anti-TGF-\( \beta \) antibody every 2 weeks. We dissected tumors; we fixed tumors at 4% paraformaldehyde or flash-froze and stored tumors at \(-80^\circ\)C.

Gene expression analysis

All samples except for normal nerves were previously described (33). Affymetrix probes were remapped to RefSeq genes (version 11.0.1). Comparisons and data visualization were conducted using GeneSpring GX v7.3.1 (Agilent Technologies).

Results

Loss of TC21 extends neurofibroma-bearing mouse survival but decreases NPCis mouse survival

To define roles for TC21, we used TC21\(^{-/-}\) mice (Supplementary Fig. S1A). TC21 mRNA was less than 10-fold and protein expression lost from WT (Supplementary Fig. S1B and S1C), confirming that the TC21 mutation is a null allele (14). Nf1\(^{+-}\) embryos die by embryonic day 12.5 (E12.5; 27), but 90\% of TC21\(^{+-}\);Nf1\(^{+-}\) embryos survived to E14.5 and 10\% survived to E16.5 (Supplementary Table S1). Partial rescue of embryo viability was not observed in R-Ras\(^{+-}\)/Nf1\(^{+-}\) embryos (Supplementary Table S1). Thus, TC21 plays a role in Nf1 embryonic development.

To determine TC21 relevance to tumorigenesis, we generated Nf1fl/fl;DhhCre;TC21fl/fl and NPCis;TC21fl/fl mice. Loss of TC21 significantly extended survival in Nf1fl/fl;DhhCre mice (Fig. 1A). Nf1fl/fl;DhhCre;TC21fl/fl (\( n = 15 \)) mice survived up to 20 months while littermate controls died by 15 months. Mice required sacrifice because of morbidity secondary to paralysis that correlated with neurofibroma formation and spinal cord compression. A second cohort of mice (\( n = 15 \)) showed identical results (not shown).

To test for effects of TC21 loss in malignancy, we generated NPCis;TC21fl/fl mice. NPCis;TC21fl/fl (\( n = 13 \)) mice died by 7 months whereas littermate controls (\( n = 7 \)) survived up to 13 months (Fig. 1B). Therefore, loss of TC21 in benign tumors extends survival, although paradoxically in a model of aggressive tumors, loss of TC21 decreases survival. In addition, NPCis;TC21fl/fl mice died early because of rapid formation of aggressive brain tumors (Supplementary Fig. S2).

A role for TC21 in tumor initiation

Grade 1 genetically engineered mice (GEM) neurofibromas from Nf1fl/fl;DhhCre mice with or without TC21 did not differ in histology on H&E staining, or anti-S100\beta staining to mark Schwann cells (34; Fig. 2A). Toluidine blue + metachromatic mast cells increased slightly in the absence of TC21 (not shown). At the time of sacrifice there was no difference in number or size (diameter) of neurofibromas in the 2 strains of mice (Fig. 2B and C).

To explain how loss of TC21 extends survival in Nf1fl/fl; DhhCre mice we postulated that TC21 diminishes numbers of neurofibroma-initiating or sustaining multipotent self-renewing cells (29). To test this, we used an in vitro model. SCPs from Nf1fl/fl DRG cells give rise to more spheres than do wild-type (WT) or Nf1fl/fl DRG cells, and Nf1fl/fl DRG sphere cells form neurofibroma-like lesions upon xenotransplantation (29). Nf1fl/fl DRGs formed significantly more primary spheres at clonal density than cells from WT DRG. Importantly, TC21fl/fl;Nf1fl/fl DRG cells formed WT levels of spheres (Fig. 2D) consistent with a role of TC21 regulating numbers of tumor-initiating cells early in Nf1 tumorigenesis.

A TGF-\( \beta \) autocrine loop in Nf1fl/fl SCPs

Because TGF-\( \beta \) proteins can act as tumor suppressors or tumor promoters and TC21 acts similarly (Fig. 1), and because TC21 and TGF-\( \beta \) have been linked in vitro (13), we examined TGF-\( \beta \) signaling in the context of Nf1 and TC21 null alleles. Cells from secondary spheres were plated at clonal density and tested for response to TGF-\( \beta \). WT SCP numbers were reduced by exposure to TGF-\( \beta \)1 in a dose-dependent manner. WT SCP cells died but Nf1fl/fl SCPs did not die when exposed to TGF-\( \beta \) (Supplementary Fig. S3A). Loss of TC21 restored sensitivity to TGF-\( \beta \)1 (Fig. 3A).

Nf1fl/fl SCPs had 20-fold higher levels of TGF-\( \beta \)1 mRNA and protein compared with WT precursors (Fig. 3B and C) and loss of TC21 rescued TGF-\( \beta \)1 levels in Nf1fl/fl spheres. TGF-\( \beta \) produced by Nf1fl/fl spheres was functional, as Nf1fl/fl SCPs plated at clonal density with function blocking anti-TGF-\( \beta \)

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antibody died but spheres in control IgG formed healthy spheres (Fig. 3D and E). In contrast, treatment of WT or TC21/–/– mice with anti-TGF-β antibody did not alter sphere numbers (Fig. 4A). Production of TGF-β and effects of function blocking antibody suggest that Nfi/–/– SCPs secrete TGF-β, enhancing their survival. The data support the hypothesis that Nfi/–/– SCPs are dependent on TGF-β for survival.

In contrast, TGF-β1 mRNA was only slightly elevated in mature Schwann cells in sciatic nerves from Nfi/–/–; DhhCre mice compared with WT sciatic nerve (2-fold change; Supplementary Fig. S3B). There was no significant difference in TGF-β1 levels between neurofibromas from Nfi/–/–; DhhCre (n = 8) and Nfi/–/–; DhhCre; TC21/–/– (n = 10) mice (Supplementary Fig. S3C). That elevation and rescue of TGF-β1 mRNA expression levels in the TC21/–/–; Nfi/–/– setting is most pronounced early in Schwann cell development suggests that TC21 activation plays roles in SCP viability and growth, not neurofibroma growth. The mechanism(s) by which TC21 loss enhances TGF-β expression may include alterations in signaling pathways that change in cell maturation and/or alterations because of as-yet-unidentified mutations in neurofibroma Schwann cells during tumor formation.

**Survival of Nfi/–/– SCPs requires TGF-β and AKT**

To determine how TGF-β affects survival of Nfi/–/– SCPs, we used specific inhibitors. Treatment of Nfi/–/– SCPs with a TGFβRI inhibitor blocked sphere formation but did not affect WT or TC21/–/–; Nfi/–/– spheres (Fig. 4B). PI3K/AKT has been implicated downstream of TGF-β signaling, and treatment with an AKT inhibitor significantly reduced Nfi/–/– sphere formation (Fig. 4C). In contrast, MEK1, ROCK, and p38SAPK inhibitors had no effect on Nfi/–/– sphere formation (Supplementary Fig. S4A). Efficacy of inhibitors was validated by Western blotting (not shown). Confirming a role for AKT signaling in a TGF-β autocrine loop, formation of Nfi/–/– but not WT or TC21/–/–; Nfi/–/– spheres was significantly reduced by shAKT (Fig. 4D). Thus, formation and survival of Nfi/–/– spheres in vitro are dependent on TC21, TGF-β, TGFβRI, and AKT.

We tested whether loss of TC21 affects signaling in vivo by immunochemistry. We stained E12.5 DRG tissue sections containing SCPs, the targets for Nfi loss in the Nfi/–/–; DhhCre model, with antiphospho-AKT. Nfi/–/– DRG SCPs showed elevated levels of phospho-AKT compared with WT and TC21/–/–; Nfi/–/– embryos (Fig. 4E and F). Nfi/–/– spheres from E12.5 DRG grown in vitro also contained increased phospho-AKT compared with WT and TC21/–/–; Nfi/–/– spheres (Supplementary Fig. S4B). P-AKT was similar in neurofibromas from Nfi/–/–; DhhCre and Nfi/–/–; DhhCre; TC21/–/– mice, consistent with TC21 affecting events shortly after Nfi loss (not shown). Phospho-SMAD2/3 staining, marking canonical TGF-β signaling was similar across genotypes (Supplementary Fig. S4C and S4D). Thus, losing TC21 in an Nfi/–/– background
TGF-β1 and TGF-β in NF1 Tumors

Figure 3. A TGF-β1 autocrine loop in NF1−/− SCPs. A, SCP spheres treated with TGF-β1 and then counted (p = 0.005 ANOVA). B, qRT-PCR mRNA expression of TGF-β1 in spheres compared with WT spheres (t test: NF1−/− vs. TC21−/− NF1−/−). C, ELISA for TGF-β1 protein. D and E, phase contrast micrographs of NF1−/− spheres after treatment. F, quantification of NF1−/− spheres after treatment.

Correlated with reduced expression of phospho-AKT in SCPs in vivo.

To test if TGF-β1 mRNA production in SCPs requires AKT, we examined levels of TGF-β1 mRNA in NF1−/− SCPs treated with the AKT inhibitor: the inhibitor decreased TGF-β1 mRNA (Fig. 4G). These data support an existence of an AKT, inactivation (Ki67) compared with controls (not shown). As TGF-β revealed no differences in morphology (H&E) or cell proliferation (Ki67) compared with those treated with IgG (Fig. 6D and E). We hypothesized that, as in neurofibromas, in the malignant setting with increased TGF-β expression we might detect increased phospho-AKT. Indeed, phospho-AKT was elevated in tumor cells expressing shTC21 confirmed sustained reduction of TC21 (not shown). Thus, loss of TC21 in NF1−/− MNPS cells increases tumor growth and requires TGF-β.

Loss of TC21 in MNPS cells increases tumor growth

In the NPC is model of GEM-sarcoma and GEM-glioblastoma multiforme, loss of TC21 caused early lethality (Fig. 1B). Brain tumors killed mice before the normal onset of sarcoma formation in the NPCis model. We showed that TC21 activity is high in human MNPS S462TY cells that are NF1−/− in comparison with NF1 WT 26T cells, using a Ras pull-down assay and blotting with anti-TC21 antibody (Supplementary Fig. S5A). To investigate the role of TC21 in sarcomas, we infected the S462TY cells with a stably expressing doxycycline-inducible TRIPZ lentivirus encoding shRNA targeting TC21 (shTC21), or control TRIPZ nontarget shRNA (NT). shTC21 cells were injected subcutaneously into the right flank of nu/nu mice and NT cells into the left flank and tumor growth measured. Tumors with shTC21 cells were larger than tumors with NT cells (Fig. 5A and B). Western blot analysis confirmed low TC21 expression in excised tumors (Fig. 5C). Thus, loss of TC21 in a human sarcoma xenograft increases tumor growth.

However, histologic analysis of shTC21 MNPS xenografts revealed no differences in morphology (H&E) or cell proliferation (Ki67) compared with controls (not shown). As TGF-β can induce blood vessel formation we analyzed tumor vasculature. The shTC21 tumors had more meca-32 vessels/mm³ than controls (Fig. 5D). Consistent with altered tumor stroma upon TC21 loss, an antibody against smooth muscle actin (SMA) detecting myofibroblasts revealed increased immunoreactivity in xenografts expressing shTC21 (Fig. 5E and F).

Increased MNPS growth due to loss of TC21 requires TGF-β

To investigate whether TGF-β plays a causal role in malignant tumor growth when TC21 is lost, we treated mice harboring MNPS xenografts with function-blocking anti-TGF-β1, 2, 3 in vivo (35). After tumors grew to approximately 250 mm³, rabbit IgG or anti-TGF-β antibody was administered by intraperitoneal injection. Tumors expressing shTC21 were larger than those with NT (Fig. 6A). Strikingly, mice injected with the anti-TGF-β antibody had tumors of similar size whether the cells were expressing shTC21 or control (Fig. 6B). Western blot analysis of tumor xenografts expressing shTC21 confirmed sustained reduction of TC21 (not shown). Thus, loss of TC21 in NF1−/− MNPS cells increases tumor growth and requires TGF-β.

To delineate signaling pathways modulated by the TGF-β antibody, we examined expression of phospho-SMAD in tumor sections. Phospho-SMAD was reduced in tumor cells and stroma from mice treated with the anti-TGF-β antibody compared with those treated with IgG (Fig. 6D and E). We hypothesized that, as in neurofibromas, in the malignant setting with increased TGF-β expression we might detect increased phospho-AKT. Indeed, phospho-AKT was elevated in tumor cells expressing shTC21. Phospho-AKT levels were subsequently reduced upon treatment with anti-TGF-β antibody (Fig. 6C and E).

The shTC21 tumors treated with anti-TGF-β also had significantly fewer meca-32 vessels than controls (Supplementary Fig. S5B). Together, these data suggest that in the NF1−/− setting, in transformed cells, loss of TC21 drives tumorigenesis in a TGF-β-dependent manner. TGF-β produced by MNPS cells acts in a nonautonomous fashion on stromal cells to increase blood vessels and promotes myofibroblasts formation.
MPNST cells express TGF-β and lose TGFβRII

We tested if TGF-β ligands or TGFβRs are altered in neurofibromas or MPNST. We measured relative abundance of TGF-β ligands and receptor mRNAs in a panel of human neurofibromas and MPNSTs compared with cultured neurofibroma Schwann cells, MPNST cell lines, normal human Schwann cells, and normal human nerves by Affymetrix gene expression analysis. The heat map (Fig. 7A) shows upregulation of TGF-β3 in most neurofibromas and MPNST, with TGF-β2 upregulation specifically in 50% of neurofibroma Schwann cell samples, 50% MPNST cell lines, and all human MPNST. TGFβRII was downregulated in all human MPNSTs, and in a subset (22/48) of neurofibroma Schwann cell samples (Fig. 7A). TGFβRI mRNA was slightly but not significantly upregulated. These data are consistent with findings that TGF-β ligands increase and receptors decrease in many tumor types during progression to malignancy (16).

To confirm increased expression of TGF-β in MPNST and test if TGF-β regulation is regulated by TC21 in sarcoma cells (as in SCPs), we examined TGF-β mRNAs and protein expressions in NFI human MPNST cells and in sarcomas from NPCis mice. In benign neurofibromas and SCPs, loss of TC21 reduced TGF-β1 mRNA levels in the NFI−/− setting. In NFI mutant mouse NPCis tumors TGF-β1 was elevated on loss of TC21, but TGF-β2 mRNA was not (Supplementary Fig. S5C and S5D). In NFI−/− human MPNST shTC21 xenotransplants, TGF-β2 mRNA levels were significantly elevated in tumors (Fig. 7B) whereas TGF-β1 and TGF-β3 levels were unchanged (Supplementary Fig. S5E and S5F). Thus in mouse and human, TGF-β levels are regulated by TC21, but the 2 species use different TGF-β ligands.

TGFβRs are frequently mutated or lost in malignancy (15). If TGFβRs show loss of function in NFI, then TGF-β effects would...
be unlikely to be cell autonomous. TGFβRII protein was undetectable by Western blotting in S462TY cells compared with an immortalized human Schwann cell line (Fig. 7D). In 2 of 3 additional human MPNST cell lines, TGFβRII protein was also reduced (Supplementary Fig. S5H). The absence of TGFβRII in S462TY cells predicted that the cells would not respond to TGF-β. Indeed, no effects of TGF-β on MPNST survival or migration were detected (not shown). To verify this finding, we examined expression of the TGF-β target genes, p21, p15, and c-myc (19). No significant change in mRNA expression of these genes was detected after addition of TGF-β to S462TY cells in serum-free medium at 30 minutes or 8 hours, in comparison with untreated cells (not shown).

Thus, when TC21 is lost in NFI-/- MPNST cells, TGF-β mRNA and secreted protein are increased (Fig. 7E). Because MPNST cells lack significant amounts of TGFβRII, TGF-β affects surrounding TGFβRII expressing endothelial and stromal fibroblasts, resulting in increased blood vessels, conversion of fibroblasts to myofibroblasts, and increased tumor growth.

Discussion

Using NFI models predicted to activate Ras proteins expressed in neurofibromin-dependent cells enabled the demonstration that the Ras-related protein TC21 critically regulates TGF-β in neurofibroma and MPNST. Loss of TC21 in the setting of NFI deficiency delayed formation of benign neurofibromas, accelerated formation of aggressive brain tumors and nerve sarcomas, and uniquely regulated expression of TGF-β. Our identification of a specific role for TC21 is consistent with recent studies showing differential localization of other Ras proteins (4), and different embryonic survival following knockout of the K-, H-, and N-Ras genes (5).

Our in vivo studies showed that TC21 functions as an oncogene in benign tumors and revealed a new role for TC21 as a tumor suppressor in nervous system malignancy. However, TC21 was identified as upregulated in some cancers (36, 37) and activated in lymphoma in vivo (8). Therefore, effects of TC21 activation are likely to be cell-type dependent. Mechanistically, we showed that PI3K/AKT is a critical effector of TC21. In all NF1 models tested, TC21 regulated TGF-β, and TGF-β acted as an oncogene.

Loss of TC21 in GEM neurofibroma extended mouse survival without affecting neurofibroma number or size, suggesting effects on early stages of tumor formation. Consistent with a critical role for TC21 early in neurofibroma formation, we identified an autocrine survival loop specific to NFI-/- SCPs, and dependent upon TGF-β, TGF-βRI, and the PI3K/AKT pathway.

Figure 5. Loss of TC21 in MPNST cells increases sarcoma growth. A, volume quantification of xenograft tumors over time. B, gross photographs of nude mice tumors. C, Western blot showing TC21 protein in S462TY xenograft tumors. D, quantification of anti-meca staining and measurement of numbers of vessels/high-power field. E and F, SMA immunostaining in xenograft sections (brown). Scale bar, 50 μm.
pathway. We have not excluded additional effects on more mature Schwann cells. TGF-β1 can kill mature WT Schwann cells as well as more immature cells (38, 39), and as in Nf1−/− SCPS, adult Nf1−/− nerves contained increased levels of TGF-β1 mRNA that depended on TC21. We failed to detect elevated levels of TGF-β1 mRNA in neurofibromas, but levels of TGF-β1 mRNA in Schwann cells might have been obscured by expression in neurofibroma mast cells or other cells in the tumor microenvironment (40).

Additional evidence supporting a role for TC21 early in neurofibroma formation comes from clonality assays. Numbers of Nf1−/− primary sphere-forming cells, after acute inactivation at E12.5 using the DhhCre allele, were reduced by loss of TC21. Nf1−/− primary sphere-forming cells were dependent on TGF-β they produced for their own survival. Secreted TGF-β is predicted to enhance numbers of developing Nf1−/− stem/progenitor cells and thus tumorigenic potential, leading to neurofibroma formation. Consistent with this idea, targeted loss of the TGFβRII in Schwann cells suppressed early Schwann cell death and proliferation (41). We conclude that TC21 controls TGF-β in vivo, reducing the growth and number of developing Schwann cells, and thereby acting as a brake on neurofibroma initiation/growth.

Precursor cell effects on neurofibroma initiation and/or growth through TC21 and TGF-β were not sufficient to prevent tumorigenesis. Other Ras proteins and/or Nf1 functions are likely necessary for neurofibroma growth. The idea that Nf1 regulates other Ras proteins—even in peripheral nerve cells—is supported by experiments in which farnesyl transferase inhibitors predominantly blocking H-Ras, blocks Nf1 mutant Schwann cell proliferation (42), and a report that N-Ras plays a major role in MPNST cells (43). Our finding that loss of TC21 delayed Nf1−/− embryonic lethality—but did not rescue embryos to birth—is also consistent with roles of multiple Ras proteins in development. How each Ras protein contributes to embryonic development and tumorigenesis downstream of Nf1 loss of function remains to be determined. In addition, other Ras proteins may regulate
TGF-β expression or signaling in non-NF1 tumor settings. The diverse regulation of TGF-β pathways in specific cell types may result in part because specific Ras proteins are cell-type specific (44).

We found that loss of TC21 enhances tumorigenesis in malignancy. In the NPCis mouse model, loss of TC21 dramatically accelerated formation of brain tumors. In a xenograft model, NF1−/− sarcoma cells showed accelerated tumor growth and increased levels of TGF-β when they expressed shTC21. Furthermore, inhibiting TGF-β blocked the elevated tumor growth caused by loss of TC21. The action of TGF-β as a growth promoter in malignancy correlated with increased TGF-β ligands in neurofibroma and MPNST and decreased expression of TGFβRII in human MPNST cell lines and tumors, as shown by gene expression and confirmed by protein analysis in cell lines. Genomic mutation or loss of one or more TGFβRs is common in many tumor types (15, 45). Despite absence of TGFβRII in S462TY cells, blocking TGF-β led to decreased expression of phospho-SMAD. This may result from use of a mutant p53 SMAD complex (18) and/or more complex activation of SMAD downstream of activin receptors. One possibility is that stromal cells secrete activins or bone morphogenetic protein that indirectly alter SMAD phosphorylation in tumor cells (16).

Our data are consistent with important noncell autonomous effects of MPNST cell produced TGF-β. This interpretation is consistent with increased blood vessels per area. SMA (myofibroblast) expression also increased upon downregulation of TC21, and both phenotypes were blocked by anti-TGF-β antibody. A noncell autonomous effect of TGF-β in malignancy has been documented previously in models of carcinoma, using genetic loss of TGFβRII (19).

In summary, TC21 has a dual effect: in developing Nf1 progenitor cells this Ras-related protein promotes cell survival...
by promoting TGF-β production and formation of an autocrine survival loop, so that loss of TC21 results in decreased precursors and delayed tumor formation. TC21 activation plays a role in SCP viability and growth rather than in neurofibroma growth per se. In MPNST cells loss of TC21 dramatically increases TGF-β production, increasing vascularization and tumor growth. Our results linking TC21 to regulation of TGF-β production are likely to be relevant to cancer in general, as NFI mutations are increasingly identified in sporadic cancers (46–48).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D.M. Patmore, T.P. Cripe, N. Ratner
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.M. Patmore, S. Welch, P.C. Fulkerson, J. Wu, N. Ratner
Analysis and interpretation of data (e.g., statistical analysis, biosstatistics, computational analysis): D.M. Patmore, S. Welch, P.C. Fulkerson, K. Choi, M.H. Collins, T.P. Cripe, N. Ratner

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