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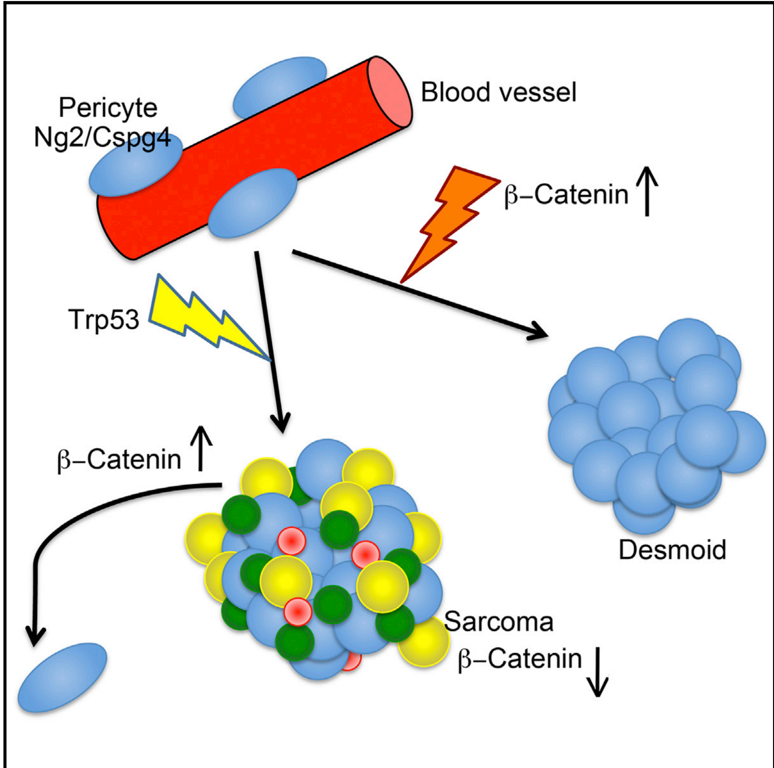
Report

Mesenchymal Tumors Can Derive from Ng2/Cspg4-Expressing Pericytes with β-Catenin Modulating the Neoplastic Phenotype

Graphical Abstract



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In Brief

Sato et al. use lineage-tracing studies in mice to show that bone and soft tissue sarcomas driven by the deletion of the *Trp53* tumor suppressor gene can derive from *Ng2/Cspg4*-expressing pericytes. Their data show that pericytes can be a cell of origin for mesenchymal tumors and that β-catenin plays a critical role in mesenchymal neoplasia.

Highlights

- Pericytes can be a cell of origin for benign and malignant mesenchymal neoplasms
- Malignant sarcomas show a decrease in β-catenin signaling compared to pericytes
- Benign desmoids show an increase in β-catenin signaling compared to pericytes

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Mesenchymal Tumors Can Derive from Ng2/Cspg4-Expressing Pericytes with β-Catenin Modulating the Neoplastic Phenotype

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SUMMARY

The cell of origin for most mesenchymal tumors is unclear. One cell type that contributes to this lineages is the pericyte, a cell expressing *Ng2/Cspg4*. Using lineage tracing, we demonstrated that bone and soft tissue sarcomas driven by the deletion of the *Trp53* tumor suppressor, or desmoid tumors driven by a mutation in *Apc*, can derive from cells expressing *Ng2/Cspg4*. Deletion of the *Trp53* tumor suppressor gene in these cells resulted in the bone and soft tissue sarcomas that closely resemble human sarcomas, while stabilizing β-catenin in this same cell type caused desmoid tumors. Comparing expression between *Ng2/Cspg4*-expressing pericytes lacking *Trp53* and sarcomas that arose from deletion of *Trp53* showed inhibition of β-catenin signaling in the sarcomas. Activation of β-catenin inhibited the formation and growth of sarcomas. Thus, pericytes can be a cell of origin for mesenchymal tumors, and β-catenin dysregulation plays an important role in the neoplastic phenotype.

INTRODUCTION

Tumors are initiated by mutations in specific cell types. Since progenitor cell populations can survive over longer periods of time, they may be more likely to accumulate mutations that cause neoplasia (Reya et al., 2001). Identifying the cell of origin of a tumor type can be used to identify critical events responsible for tumor formation, and driving oncogenesis in the cell of origin can be used to develop animal models that more accurately recapitulate human tumors (Visvader, 2011).

Sarcomas are malignancies found in the connective tissues, composed of cells with mesenchymal characteristics. There is a broad range of sarcoma types, including those that derive in bone, cartilage, fat, muscle, or vascular, tissues. Two of the most common sarcoma types are osteosarcoma and undifferentiated pleomorphic sarcomas, and yet much remains to be established about the critical steps required for tumor formation in these subtypes. Desmoid tumors are locally invasive mesenchymal tumors that do not metastasize. They are composed of fibroblast-like cells with a proliferative advantage, driven by somatic mutations activating β-catenin mediated signaling. Mutations in *Apc* or in β-catenin itself are identified in almost all cases of this tumor type (Alman et al., 1997a; Cheon et al., 2002). The precise cell of origin for these tumors is unknown. Since they have mesenchymal characteristics, it is likely that they derive from a mesenchymal lineage progenitor cell.

In addition to its role in desmoid tumors, β-catenin protein is also implicated in sarcomas. However, its role in sarcomas has been controversial. Some studies suggest that activated β-catenin signaling is important to drive the neoplastic phenotype, while others found an opposite effect (Cai et al., 2010, 2014; Du et al., 2014; Matushansky et al., 2007; Wan et al., 2014). In mesenchymal cell development, β-catenin is precisely regulated at different stages for normal differentiation, raising the possibility that either high or low β-catenin leads to pathology (Chen et al., 2007; Hoffman and Benoit, 2013; Li et al., 2008; Wan et al., 2013). Understanding the role of β-catenin-mediated signaling in neoplasia also has therapeutic implications, as β-catenin-modulating therapies are being developed for clinical use.

Pericytes are mesenchymal cells that surround endothelial cells in capillaries, venules, and small arterioles (Díaz-Flores et al., 2009; Hirschi and D'Amore, 1996). These cells express markers such as chondroitin sulfate proteoglycan 4 (CSPG4),

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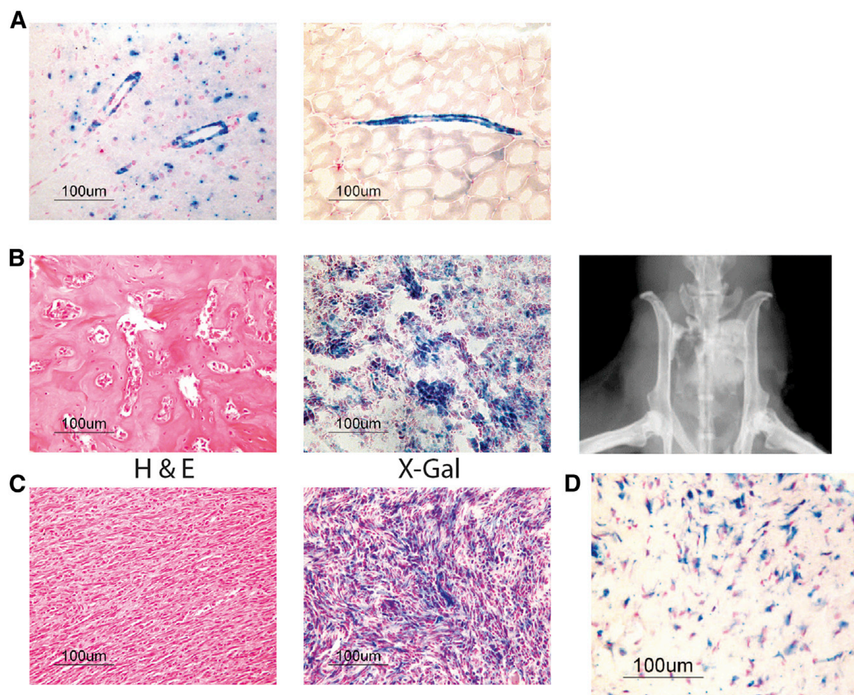

Figure 1. Mesenchymal Tumors Can Derive from *Ng2/Cspg4*-Expressing Cells

(A) X-gal staining in *Ng2/Cspg4-CreER; Rosa26R^{lacZ}* mice, showing blue staining (LacZ) in the brain (left) and in perivascular tissues in the skeletal muscle (right).

(B) Representative H&E, X-gal staining, and a radiograph from a mouse osteosarcoma that developed in a *Trp53*-deficient mouse, showing tumor cells that stained blue, indicating that they are derived from *Ng2/Cspg4*-expressing cells.

(C) Representative H&E and X-gal staining from a mouse soft tissue sarcoma that developed in a *Trp53*-deficient mouse showing histology consistent with undifferentiated pleomorphic sarcoma. Similar to the situation in osteosarcomas, tumor cells stained blue.

(D) Representative X-gal staining from a mouse desmoid tumor that developing in an *Apc1638N* mouse showing blue staining in the tumor cells. This shows that these benign mesenchymal tumors also derived from *Ng2/Cspg4*-expressing cells.

also termed neuron-glial antigen 2 (NG2), and CD146, also known as melanoma cell adhesion molecule (Bergers and Song, 2005; Covas et al., 2008; Crisan et al., 2008, 2012). This cell type is involved in the stability and contractility of blood vessels but also can be a progenitor for several mesenchymal cell types (Crisan et al., 2008, 2012; Dellavalle et al., 2007). Interestingly, human sarcomas are known to express genes that are characteristically expressed in pericytes (Benassi et al., 2009; Schiano et al., 2012). Thus, pericytes could be a cell of origin for some mesenchymal tumors.

Here, we addressed the role of *Ng2/Cspg4*-expressing cells and β-catenin in the origin of mesenchymal tumors. Lineage-tracing studies in murine sarcomas driven by the deletion of the *Trp53* tumor suppressor, or desmoid tumors driven by a mutation in *Apc*, were used to investigate *Ng2/Cspg4*-expressing cells as a cell of origin for mesenchymal tumors. We also determined the ability of *Trp53* deletion and/or stabilization of β-catenin in *Ng2/Cspg4*-expressing cells to result in tumor formation.

RESULTS

Mesenchymal Tumors Can Derive from *Ng2/Cspg4*-Expressing Cells

To determine if mesenchymal tumors might derive from *Ng2/Cspg4*-expressing cells, we undertook lineage-tracing studies in genetically modified mice that are known to develop mesenchymal tumors. We used *Trp53* deficient mice to study sarcomas. These mice are a model for Li-Fraumeni syndrome and develop malignancies, including lymphomas and sarcomas (Jacks et al., 1994). To study the origin of a benign tumor, we investigated desmoid tumors, which are benign locally invasive mesenchymal lesions driven by mutations activating β-catenin-mediated signaling. The *Apc1638N* mouse (Smits et al., 1998) harbors a mutation in *Apc* that results in the development of multiple desmoid tumors.

NG2/CSPG4 is a cell-surface proteoglycan expressed by pericytes, neural progenitor cells, chondrocytes, and hair follicles (Feng et al., 2010). To label *Ng2/Cspg4*-expressing cells, we crossed *Ng2/Cspg4-CreER* mice (Zhu et al., 2011) with *Rosa26R^{lacZ}* mice (Soriano, 1999). The transgene was activated by daily tamoxifen injections for 1 week after weaning (Madisen et al., 2010). β-Galactosidase (X-gal) staining was performed to identify the distribution of LacZ-positive cells, and this confirmed that LacZ was expressed in pericytes, neural cells, chondrocytes, and hair follicles (Figures 1A and S1A). In contrast, osteoblasts did not show expression of LacZ, a finding consistent with other studies using this animal (Feng et al., 2011), in which LacZ staining was only observed in bone during mesenchymal repair processes when the transgene was activated postnatally (Figure S1B). To verify which cells were expressing LacZ, we dissociated cells and sorted LacZ-positive and negative populations as in our previous publications (Amini-Nik et al., 2011, 2014). There was an increase in RNA expression of *Ng2/Cspg4* in the LacZ-positive population (Figure S1C). We next sorted NG2/CSPG4-positive and negative cells using a cell-surface antibody and analyzed the populations for expression of LacZ, finding that the NG2/CSP4-positive population expressed LacZ. We also analyzed the LacZ-positive and negative populations for the expression of CD146, a cell-surface marker expressed by pericytes (Wei et al., 2015), and found that the LacZ-expressing cells also expressed CD146 (Figure S1E). Taken together, these data show that LacZ effectively labels Ng2/Cspg4-expressing pericytes.

We next crossed *Ng2/Cspg4-CreER;Rosa26R^{lacZ}* mice with *Trp53*-deficient mice (Jacks et al., 1994) and injected them

Table 1. Tumor Distribution of *Ng2/Cspg4-Cre*-Mediated *Trp53* Conditional Knockout Mice

Genotype	Number of Mice	Bone Sarcoma (OS)	Soft Tissue Sarcoma (UPS)	Lymphoma	Other Malignancies	Average Latency of Sarcoma Development (days ± SD)
Ng2/Cspg4-Cre; Trp53$^{flox/flox}$	50	38 (76.0%)	8 (16.0%)	2 (4.0%)	0	299 ± 56
Male	19	10 (52.6%)	6 (31.6%)	0	0	337 ± 59
Female	31	28 (90.3%)	2 (9.7%)	2 (6.5%)	0	280 ± 44
Ng2/Cspg4-Cre; Trp53$^{flox/-}$	16	13 (81.2%)	3 (18.8%)	0	0	297 ± 61
Male	4	2 (50.0%)	2 (50.0%)	0	0	212 ± 49
Female	12	11 (91.7%)	1 (8.3%)	0	0	318 ± 43
Ng2/Cspg4-CreER; Trp53$^{flox/flox}$	30	20 (66.0%)	6 (20%)	0	0	361 ± 59
Male	10	6	2	0	0	324 ± 64
Female	20	14	4	0	0	380 ± 56
NG2-*Cre; Trp53*$^{flox/+}$	12	0	1 (8.3%)	0	0	407

with tamoxifen. 12 sarcomas developed in the mice; 5 were undifferentiated pleomorphic sarcomas, 6 were osteosarcomas, and 1 was an angiosarcoma. X-gal staining confirmed that the sarcomas derived from LacZ-expressing cells (Figures 1B and 1C). Given the expression pattern of LacZ in normal tissues, this suggests that the tumors derived from pericytes. The *Apc1638N* mouse (Smits et al., 1998) harbors a mutation in *Apc* that results in β-catenin activation and the development of multiple desmoid tumors. *Ng2/Cspg4-CreER; Rosa26R*^{lacZ} mice were crossed with *Apc1638N* mutant mice and injected them with tamoxifen. X-gal staining showed that the desmoid tumors that developed also express LacZ (Figure 1D).

Interestingly, not all cells stained blue. Solid tumors contain a subpopulation of non-tumoral cells, including normal stromal cells (Mao et al., 2013), but in mesenchymal tumors, there are no cytologic or cell-surface markers to distinguish stromal cells from neoplastic cells, making differentiation between tumoral and stromal cells problematic. We sorted LacZ-stained from non-stained cells in these tumors. Between 28% and 49% of cells in the tumors did not stain with LacZ. In desmoid tumors, we found a higher level of β-catenin in the LacZ-positive cells (Figure S2). Thus, there is a population of normal cells within bulk mesenchymal tumors that arise from non-*Ng2/Cspg4*-expressing cells.

Trp53 Deletion in *Ng2/Cspg4*-Expressing Cells Induces Bone and Soft Tissue Sarcomas

To determine whether mutations in *Ng2/Cspg4*-expressing cells could induce sarcomas, we crossed *Ng2/Cspg4-Cre* mice or *Ng2/Cspg4-CreER* mice with *Trp53*$^{flox/flox}$ mice (Marino et al., 2000) to generate *Ng2/Cspg4-Cre*-mediated Tr*p53* conditional knockout mice. In the case of mice expressing the *Ng2/Cspg4-CreER* allele, the conditional allele was activated by tamoxifen administration the week following weaning. In *Ng2/Cspg4-CreER;Trp53*$^{flox/flox}$ mice treated with tamoxifen, 66% developed bone sarcomas and 20% developed soft tissue sarcomas. In *Ng2/Cspg4-Cre;Trp53*$^{flox/flox}$ mice in which Cre is constitutively expressed in *Ng2/Cspg4*$^+$ cells, 76% developed bone sarcomas and 16.0% developed soft tissue sarcomas (Table 1). The mice succumbed to tumors by 14 months of age and had a survival that is better than for the *Trp53*$^{-/-}$ mice that we studied in our lineage-tracing analysis (Figure 2A). This is expected, since these mice only rarely developed tumors that were not sarcomas (Table 1) and as such succumbed to sarcoma-related mortality.

We then generated *Ng2/Cspg4-Cre;Trp53*$^{flox/+}$ and *Ng2/Cspg4-Cre;Trp53*$^{flox/-}$ mice. The mice expressing only the one conditional allele rarely developed tumors, while the mice also expressing a null allele developed tumors at a frequency equivalent to *Ng2/Cspg4-Cre;Trp53*$^{flox/flox}$ mice and had a nearly identical survival curve (Figure 2B), providing additional support to the concept that loss of Tr*p53* specifically in an *Ng2/Cspg4*-expressing cell predisposes to sarcoma formation.

The bone sarcomas displayed poorly marginated masses with osteoid formation, an appearance characteristic of osteosarcoma (Figures 2C and 2D). There was some heterogeneity in the cytology, with two tumors displaying chondroblastic-type osteosarcoma characteristics (Figure 2E). The osteosarcomas arose from multiple bones (Figure 2F). 20% of mice showed lung metastases (Figures 2G and 2H). Using X chromosome inactivation, as previously reported (Tsunashima et al., 1996), we found the same pattern of inactivation in the bone and lung lesions from female mice, suggesting that the lesions derived from the same initial tumor (Figure S3).

Soft tissue sarcomas were also detected in mice (Figure 2I). They arose from multiple tissues, including the cutaneous tissues, retroperitoneum, muscle, and in one case arose from the uterus. The soft tissue sarcomas were characterized by spindle-shaped cells forming rough bundles and fascicles with hyperchromatic nuclei and abundant atypical mitoses (Figure 2J). These sarcomas were consistent with undifferentiated pleomorphic sarcomas. Mice developing soft tissue sarcomas did not show distant metastasis.

Localized *Trp53* Deletion and Expression of *Kras*^{G12D} in *Ng2/Cspg4*-Expressing Cells Induces Soft Tissue Sarcomas

We then investigated a mouse in which soft tissue sarcomas can be generated using an inducible *Kras*^{G12D} mutation and *Trp53* deletion driven by Cre recombinase (Kirsch et al., 2007). These

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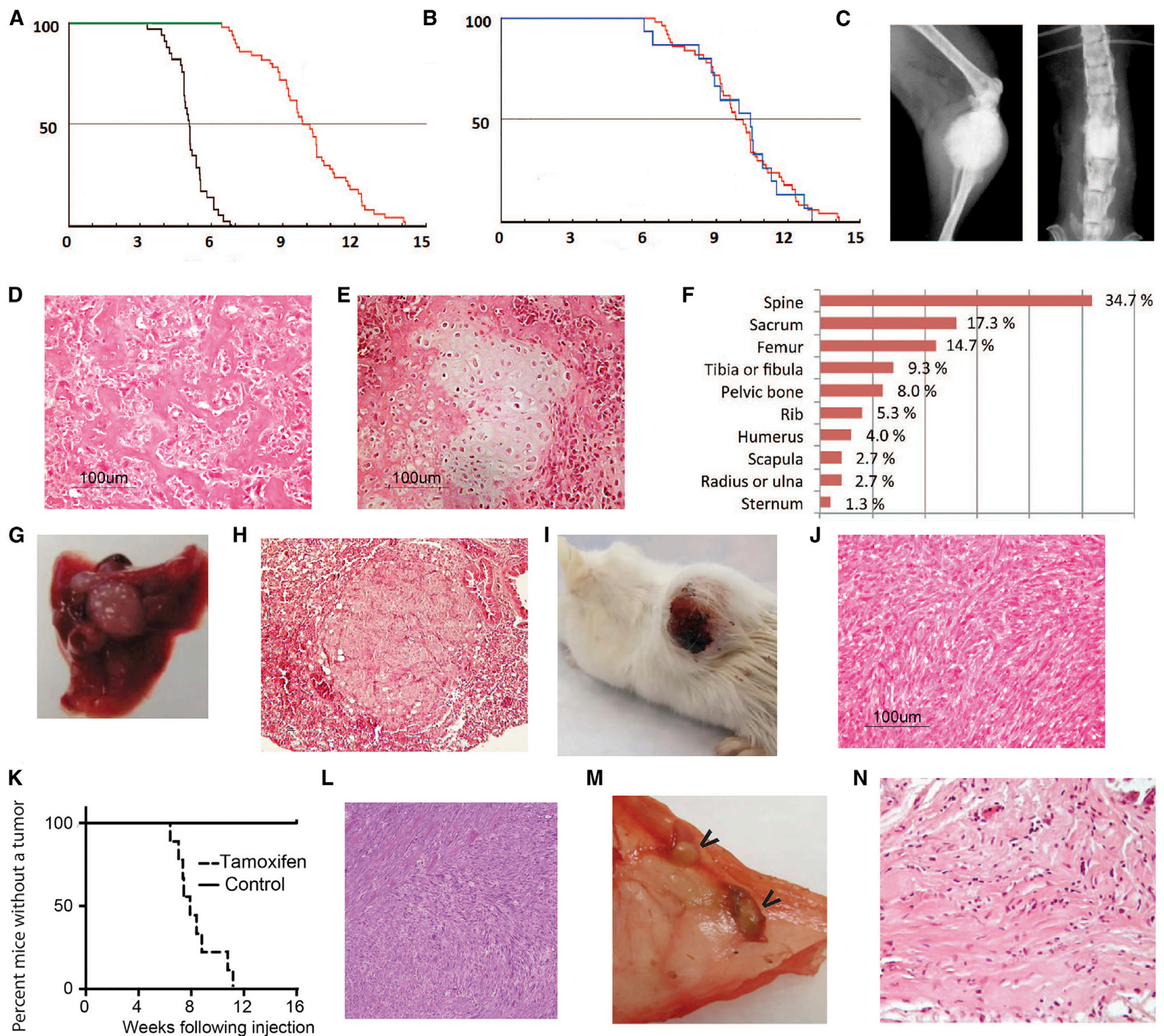


Figure 2. Deletion of *Trp53* in *Ng2/Cspg4*-Expressing Cells Causes Sarcomas, while Expression of a Stabilized Form of β-Catenin in *Ng2/Cspg4*-Expressing Cells Causes Desmoid Tumors

(A) Kaplan-Meier survival curves in months of survival for mice expressing conditional *Trp53* null alleles driven by *Ng2/Cspg4-Cre* (red curve) and mice expressing a germline deletion of *Trp53* in both alleles (green curve). There is a significantly better survival in mice expressing *Trp53* null alleles only in *Ng2/Cspg4*-expressing cells ($P < 0.01$).

(B) Kaplan-Meier survival curves in months of survival for *Ng2/Cspg4-Cre;Trp53*$^{flox/flox}$ (green curve) and *Ng2/Cspg4-Cre;Trp53*$^{flox/-}$ (red curve) showing little difference in survival.)

(C–H) Osteosarcomas developed in the mice lacking *Trp53* in *Ng2/Cspg4*-expressing cells. Radiographs (C), histology showing an osteoblastic (D) or a rare chondroblastic phenotype (E), anatomic location of the osteosarcomas (F), and lung metastasis that developed (G and H).

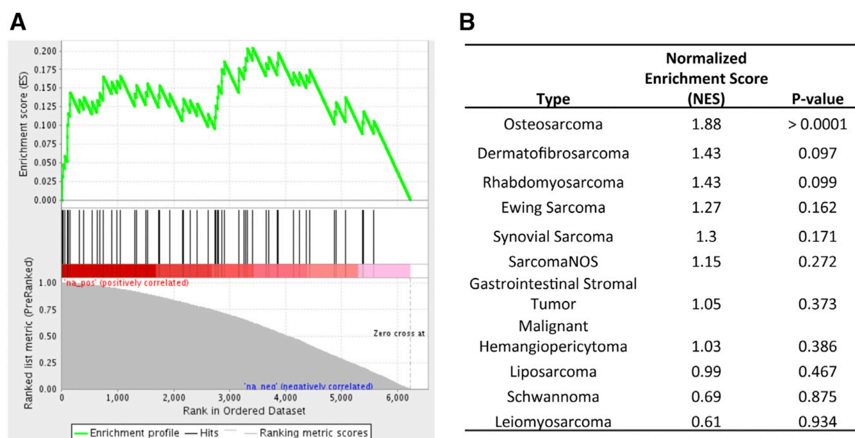
(I and J) Soft tissue sarcomas developed in mice lacking *Trp53* in *Ng2/Cspg4*-expressing cells. Gross (I) and histologic (J) view of a tumor, showing typical histology for an undifferentiated pleomorphic sarcoma.

(K) Kaplan-Meier curves in weeks following localized 4-hydroxy-tamoxifen intramuscular injection in *Ng2/Cspg4-CreER;Trp53*$^{flox/flox}$;*Kras*^{G12D} mice for the development of a palpable tumor.

(L) Typical histology of the soft tissue tumors that developed, an appearance consistent with undifferentiated pleomorphic sarcoma.

(M) Gross view of desmid tumors in the peritoneum of a mouse (arrows show tumors).

(N) Histology showing a typical appearance of a desmoid tumor that developed following tamoxifen regulated activation of the conditional stabilized β-catenin allele.



Type	Normalized Enrichment Score (NES)	P-value
Osteosarcoma	1.88	> 0.0001
Dermatofibrosarcoma	1.43	0.097
Rhabdomyosarcoma	1.43	0.099
Ewing Sarcoma	1.27	0.162
Synovial Sarcoma	1.3	0.171
SarcomaNOS	1.15	0.272
Gastrointestinal Stromal Tumor	1.05	0.373
Malignant Hemangiopericytoma	1.03	0.386
Liposarcoma	0.99	0.467
Schwannoma	0.69	0.875
Leiomyosarcoma	0.61	0.934

Figure 3. Comparative Expression between Mouse and Human Tumors Showing Similarities between Mouse and Human Osteosarcomas

(A) Gene set enrichment analysis demonstrating that genes differentially regulated in mouse osteosarcoma are significantly enriched in human osteosarcoma.

(B) Table showing gene set enrichment analysis statistics for comparison of genes differentially regulated in mouse osteosarcoma and various subtypes of human sarcoma.

mice develop localized sarcomas when injected with a virus expressing Cre recombinase into muscle. To determine if *Ng2/Cspg4*-expressing cells would drive soft tissue sarcomas in *LSL-Kras*^{G12D};*Trp53*$^{flox/flox}$ mice, we crossed them with *Ng2/Cspg4-CreER* mice, and drove expression of the conditional alleles using localized tamoxifen injection into muscle. In this way, recombination would occur in *Ng2/Cspg4*-expressing cells at the injection site. 12 mice were studied, and they developed a palpable soft tissue lesion 12 weeks following injection (Figure 2K), resulting in tumors with histology identical to that seen in the soft tissue sarcomas generated by *Trp53* deletion in the same cell types (Figure 2L).

β-Catenin Stabilization in *Ng2/Cspg4*-Expressing Cells Induces the Formation of Desmoid Tumors

To determine if stabilizing β-catenin mutations could induce desmoid tumors, we crossed *Ng2/Cspg4-CreER* mice with β-catenin conditionally stabilized *Ctnnb1*^{ex3} mice (Harada et al., 1999). The conditional β-catenin *Ctnnb1*^{ex3} allele lacks the phosphorylation sites in the amino terminal of β-catenin, preventing its ubiquitin-mediated degradation, thus activating β-catenin-mediated transcription. Mice were treated with tamoxifen and developed desmoid tumors with an histology identical to that seen in other murine desmoid tumors, including infiltration into local muscle tissues (Figures 2M and 2N).

Sarcomas from *Ng2/Cspg4-CreER;Trp53*$^{flox/flox}$ Mice Show Expression of Genes Similar to Those Seen in Human Tumors

A similar microarray platform as has been used in human tumors was used to compare mRNA from sarcomas that developed in *Ng2/Cspg4-CreER;Trp53*$^{flox/flox}$ mice with human tumors. The mouse sarcoma data were deposited in the GEO database (GEO: GSE63631). Gene expression data from a variety of human tumors were downloaded from the GEO (GEO: GSE2553). Differential expression was compiled as a gene set that was compared to expression data from various human cancer types and was called for each gene within each cancer type comparing it to the aggregate of all other cancer types using a moderated t-statistic. Gene set enrichment analysis (Mootha et al., 2003) was carried out to identify the significance of enrichment of the mouse genes with the most differentially expressed human genes that differentiate each cancer type. This showed the strongest similarities between the same mouse and human sarcoma subtypes (Figure 3). The expression pattern for the soft tissue sarcomas was nearly identical to that previously reported (Mito et al., 2009).

Mouse Sarcomas Express Genes that Are Distinct from *Trp53* Mutant Cells from which They Derive

Ng2/Cspg4-expressing cells were dissociated from non-cancerous skeletal muscle and sorted using an Ng2/Cspg4 antibody. RNA was extracted and RNA sequencing performed to determine gene expression differences between these cells and sarcomas that developed in the same mice. The data were deposited in the GEO database (GEO: GSE63679). Differentially expressed genes were analyzed using gene set enrichment analysis (Mootha et al., 2003), identifying the differential regulation of multiple genes associated with decreased β-catenin signaling in the tumors compared to the *Ng2/Cspg4*-expressing cells (Figures 4A and 4B). Using RT-PCR, we verified differential expression of several β-catenin transcriptional target genes (Figures 4C and 4D). While both activation and inactivation of β-catenin transcription in sarcomas have been reported (Dieudonné et al., 2010; Hoang et al., 2004; Iwao et al., 1999; Iwaya et al., 2003; Lin et al., 2013; Matushansky et al., 2007; Sakamoto et al., 2002; Wan et al., 2014), our data showed that β-catenin-mediated transcription was inactivated in both the bone and soft tissue sarcomas when compared to *Ng2/Cspg4*-expressing cells.

Activation of β-Catenin Suppresses Sarcoma Development and Growth

To determine the role of β-catenin stabilization in sarcoma formation, we generated *Ng2/Cspg4-Cre;Trp53*$^{flox/flox;}$;*Kras*^{G12D};*Ctnnb1*^{ex3} mice in which *Ng2/Cspg4*-expressing cells would harbor a mutation causing sarcomas and also express a stabilized form of β-catenin. When the conditional alleles were activated using tamoxifen, mice did not develop tumors. Since mice expressing the *Ctnnb1*^{ex3} allele would not form tumors, we analyzed cells from sarcomas induced by a localized injection of an adenovirus expressing Cre recombinase into *Trp53*$^{flox/flox;}$; *Kras*^{G12D} mice. Sarcoma cells were dissociated

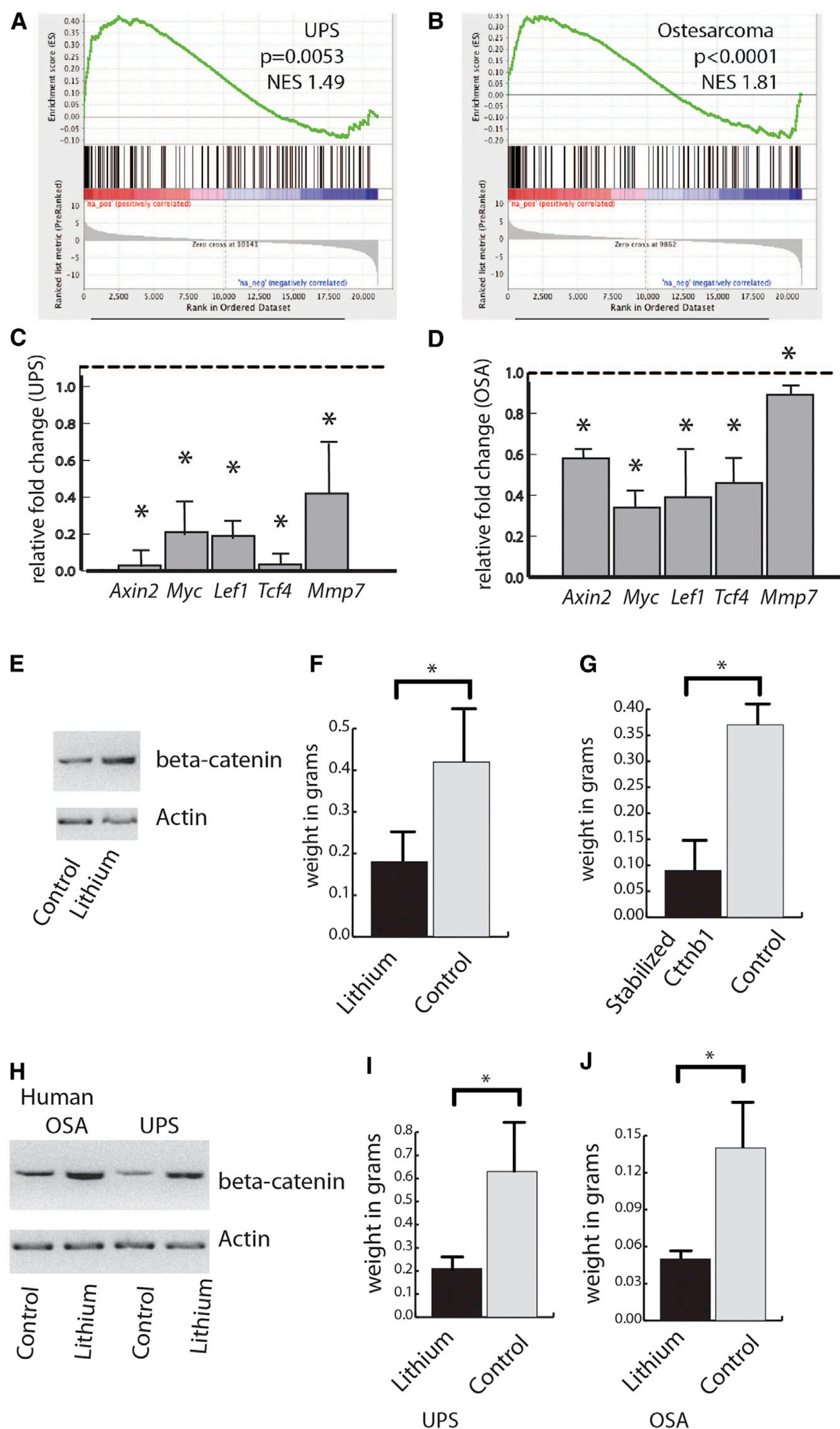


Figure 4. β-Catenin-Mediated Signaling Is Downregulated in Sarcoma, and Its Activation Inhibits Tumor Growth

(A and B) Gene set enrichment analysis from RNA-sequencing data comparing tumors to *Ng2/Cspg4*-expressing cells lacking *Trp53* showing inhibition of β-catenin-mediated signaling in both soft tissue sarcomas (A) and osteosarcomas (B).

(C and D) RT-PCR verifying downregulation of expression of β-catenin transcriptional target genes in murine tumors, soft tissue sarcomas (C), and osteosarcomas (D). Data are given as relative expression compared to *Ng2/Cspg4*-expressing cells lacking *Trp53*, which is arbitrarily defined as "1."

(E and F) Treatment of soft tissue sarcomas from *Trp53*$^{flox/flox}$;*Kras*^{G12D} mice grafted into immunodeficient mice treated with lithium results in a 5-fold increase in β-catenin protein level (E) and a significantly lower weight in grams (F) as compared to controls.

(G) Soft tissue sarcomas from *Trp53*$^{flox/flox}$;*Kras*^{G12D} mice expressing a stabilized version of β-catenin grafted into immunodeficient mice had a significantly lower weight in grams as compared to controls.

(H–J) Treatment of human undifferentiated pleomorphic sarcomas and osteosarcomas established as xenografts in immunodeficient mice with lithium results in a more than 5-fold increase β-catenin protein level in both tumor types (H) and a lower weight in grams in treated soft tissue sarcomas (I) and osteosarcomas (J). Means and 95% confidence intervals are shown for all data, with an asterisk indicating a decline with a $p < 0.05$.

and studied as grafts in immunodeficient NOD-*scid IL2r*γ^{null} mice (Wang et al., 2012). 10,000 cells were implanted subcutaneously, along with Matrigel. β-Catenin was activated by adding lithium to the drinking water at a known effective dose (Chen et al., 2007). As a second approach, the cell cultures were infected with a lentivirus expressing the ΔN89-β-catenin (a stabilized form of β-catenin that retains its signaling functions) construct, or an empty control, as previously reported (Fuerer and Nusse, 2010; Li et al., 1998). With lithium treatment (Figures 4E and 4F) or with expression of the stabilized form of β-catenin (Figure 4G), we observed significantly smaller tumor weights after 4 weeks.

Individual cells from ten primary human osteosarcomas or undifferentiated pleomorphic sarcomas were used to establish xenografts in immunodeficient NOD-*scid IL2r*γ^{null} mice as previously reported (Wang et al., 2012). Similar to the work in murine tumors, mice were treated with lithium (Chen et al., 2007). This resulted in a substantial increase in β-catenin protein levels in the tumor tissues (Figure 4H) and a substantial decrease in tumor volume (Figures 4I and 4J). These data are consistent with the notion that β-catenin transcriptional activity is lower in sarcomas than in the cells they arise from and that stabilization of β-catenin in sarcomas can suppress tumor growth.

DISCUSSION

Identifying the cell of origin of tumors is critical to determine the genetic events important in neoplastic progression and to develop models of cancers in mice that more accurately reflect human disease. However, for common sarcomas, the precise cellular origin is unclear. Since sarcomas have mesenchymal properties, mesenchymal stromal cells (MSCs) have been investigated as the cell of origin. Indeed, driving expression of oncogenes in this cell type can give rise to sarcomas (Mohseny et al., 2009; Rubio et al., 2013; Shimizu et al., 2010; Xiao et al., 2013). However, MSCs are a heterogeneous population of cells. Pericytes are mesenchymal cells that surround endothelial cells, have a multi-differentiation mesenchymal potential, and express genes that can be used as lineage markers in vivo (Covas et al., 2008; Crisan et al., 2008; Dellavalle et al., 2007). Our studies showed that both sarcomas and desmoid tumors can derive from *Ng2/Cspg4*-expressing cells, most likely from pericytes. By also using tamoxifen-inducible mice, our interpretation of the cell of origin for these mesenchymal tumors is not confounded by unanticipated expression of Cre during development, which has been shown for some constitutive Cre lines, such as *Myf6-Cre*.

Ng2/Cspg4 is expressed not only in pericytes but also in other cell types. As such, our lineage-tracing studies with *Ng2/Cspg4-Cre* mice cannot rule out the possibility that the mouse tumors are derived from other *Ng2/Cspg4*-expressing cells. However, our analysis of LacZ-labeled cells in the absence of tumors in the limbs shows that *Ng2/Cspg4*-expressing cells express high levels of the pericyte marker CD146. It is also possible that sarcoma cells in this model might activate *Ng2/Cspg4* expression during early tumorigenesis and thereby lineage-tag the tumor cells. While this possibility cannot be completely eliminated, the generation of sarcomas in *Ng2/Cspg4-CreER* mice following tamoxifen-inducible Cre suggests that the cell of origin of these mesenchymal tumors expresses *Ng2/Cspg4* at tumor initiation. Mice with *Trp53* mutations in *Ng2/Cspg4* developed both osteogenic and soft tissue sarcomas a finding consistent with the notion that pericytes can differentiate into a variety of mesenchymal cell types. The concept that a mesenchymal progenitor can form both bone and soft tissue sarcomas is in agreement with data from driving oncogenic mutations in MSCs, showing that the same mutation in MSCs can result in either bone or soft tissue sarcomas (Rubio et al., 2010, 2013). Furthermore, we found that different mutations in the same cell type can cause different mesenchymal tumors. Driving a stabilized form of β-catenin in *Ng2/Cspg4*-expressing cells results in desmoid tumors, while *Trp53* deletion causes sarcomas. Therefore, the same cell of origin can give rise to a variety of benign and malignant tumor types, with the type of mutation determining the tumor type that develops.

Tumors are intimately related to non-neoplastic stromal cells, but since sarcomas have mesenchymal characteristics, the identification of such cells in sarcomas has been problematic. In our lineage-tracing studies, we found that sarcomas contain a subpopulation of mesenchymal cells that do not stain for LacZ. These cells likely represent a population of reactive mesenchymal stromal cells within the sarcomas. The intermingling of reactive stromal cells within the neoplastic mesenchymal cells raises complexity in the interpretation of pathologic data in these tumor types, as it is difficult to distinguish these stromal cells from the neoplastic cells. Indeed, some of the controversy regarding roles in cell signaling activation and gene expression in these tumor types may be related to detecting expression or biologic findings from these normal cells. This is a notion supported by the finding of normal mesenchymal progenitor cells in human sarcomas (Morozov et al., 2010).

Other mouse osteosarcoma models have been developed based on the conditional deletion or mutation of *Trp53* (Berman et al., 2008; Lin et al., 2009; Walkley et al., 2008). However, driving deletion in a subset of cells and developing a sarcoma does not necessarily identify a specific cell of origin. In our work, a combination of lineage tracing and targeted deletion supports the pericyte as a cell of origin. The anatomic distribution of tumors in our mouse model closely mimics the situation in human sarcomas, the distal femur, proximal tibia, and proximal humerus. In contrast, driving deletion of *Trp53* in other cell types, such as osteoblasts, results in 80% lesions in axial skeletal sites (Berman et al., 2008; Lin et al., 2009; Walkley et al., 2008).

Desmoid tumors are a clonal proliferation of mesenchymal cells driven by mutations in *APC* or *CTNNB1* driving β-catenin protein stabilization (Alman et al., 1997a, 1997b; Tejpar et al., 1999). Interestingly, while a subset of desmoid tumors were previously thought to be mutation negative when analyzed by traditional Sanger sequencing, deep sequencing (Aitken et al., 2015) found that most of these mutation negative tumors do indeed harbor mutations. Similar to the finding in sarcomas, not all desmoid tumor cells in the mice stained for LacZ in the lineage-tracing studies, and these non-staining cells may be non-neoplastic stromal cells in the tumors. A high proportion of normal cells in a tumor mass can mask the detection of a mutation using traditional Sanger sequencing. Previous studies using mice that develop desmoid tumors (Cheon et al., 2002; Smits et al., 1998) to compare these tumors to normal cells are limited, as the most appropriate normal cell control is unknown. Here, we found a source of normal precursor cells for such analysis. The finding that these tumors derive from pericytes is consistent with data showing a correlation between numbers of mesenchymal progenitors and numbers of desmoid tumors that form in *Apc* mutant mice (Wu et al., 2010).

The role of β-catenin in sarcomas has been controversial, with both activation and inhibition reported. In addition, both activation and inhibition are suggested to increase tumor invasiveness (Chen et al., 2007; Hoffman and Benoit, 2013; Li et al., 2008; Wan et al., 2013). We found that β-catenin is inactivated in the sarcomas compared to the *Ng2/Cspg4*-expressing cell from which they derive. One possibility is that the undifferentiated pericytes maintain a high β-catenin level and this must be downregulated for differentiation into cells that become sarcomas. While our data cannot rule out this possibility, the finding that stabilization of β-catenin in these cells results in desmoid tumors, suggests that high β-catenin alone does not maintain the pericytes in a native undifferentiated state. Furthermore, driving β-catenin stabilization in sarcomas also suppressed tumor growth. Thus, similar to the situation in mesenchymal cell differentiation during

development and repair, the level of β-catenin protein is important in mesenchymal neoplasia, with higher or lower levels contributing to pathology. In this situation, its activation causes a benign locally invasive tumor, but its inhibition is required for sarcoma formation. This is a notion similar to that in colon cancer, where β-catenin needs to be maintained at the right level for cancer development (Albuquerque et al., 2002). The requirement for the precise regulation of β-catenin in mesenchymal neoplasia raises the possibility that modulating its level could be developed into a therapeutic approach for these tumor types.

EXPERIMENTAL PROCEDURES

Genetically Modified Mice

We used *Trp53* mutant (Jacks et al., 1994), *Apc1638N* (Smits et al., 1998), *Trp53*$^{flox/flox}$ conditional (Marino et al., 2000), LSL-Kras^{G12D}, *Catnb*^{ex3} (Harada et al., 1999), *Ng2/Cspg4-Cre* and *Ng2/Cspg4-CreER* transgenic (Feng et al., 2010; Zhu et al., 2008, 2011), and *Rosa26R*^{lacZ} reporter (Soriano, 1999) mice as previously reported. *Ng2/Cspg4-CreER;Rosa26R*^{lacZ} mice, *Ng2/Cspg4-CrERe;Rosa26R*^{lacZ}*;Trp53*$^{+/-}$ mice, *Ng2/Cspg4-CrERe;Rosa26R*^{lacZ}*;Trp53*$^{-/-}$ mice, and *Ng2/Cspg4-CreER;Rosa26R*^{lacZ}*;Apc1638N* mice were generated by crossing these mouse lines for lineage tracing studies. *Ng2/Cspg4-CreER;Trp53*$^{flox/flox}$, *Ng2/Cspg-CreER;Trp53*$^{flox/-}$, *Ng2/Cspg4-CreER;Trp53*$^{flox/+}$, *Ng2/Cspg4-CreER;Catnb*^{ex3}, *Ng2/Cspg4-Cre;Trp53*$^{flox/flox}$, *Ng2/Cspg4-Cre;Trp53*$^{flox/-}$, and *Ng2/Cspg4-Cre;Trp53*$^{flox/+}$ mice were generated by crossing the mice and used to determine if driving mutations in *Ng2/Cspg4*-expressing cells causes tumors. In addition, *Ng2/Cspg4-Cre;Trp53*$^{flox/flox}$*;Catnb*^{ex3} mice were generated. In the case of inducible Cre strains, the transgene was activated by daily intraperitoneal injection of tamoxifen for 1 week after weaning. *Trp53*$^{flox/flox;}$; *LSL-Kras*^{G12D} mice (Kirsch et al., 2007), were used in crosses with *Ng2/Cspg4-CreER* mice to generate soft tissue sarcomas, but tamoxifen was injected locally into the muscle. All of the comparisons from different genotypes were performed on littermates. An equal number of male and female mice were used in each study. The endpoint for the Kaplan-Meier survival curve was when a mouse was found dead or was sacrificed due to poor health. Mice that were sacrificed or found dead were investigated using a systematic autopsy to identify the exact tumor type and tumor location. Radiographs of the whole bodies of mice were obtained by using the Faxitron MX20 X-ray system (Faxitron Bioptics). All mouse protocols were approved by the animal care committee of the Toronto Center for Phenogenomics or the Institutional Animal Care and Use Committee committee of Duke University.

qPCR

Total RNA from mouse sarcomas and non-cancerous tissues was extracted using TRIzol reagent (Invitrogen). RNA was used to generate single-strand cDNA using SuperScript II reverse transcriptase (Invitrogen). To detect mRNA level, real-time qPCR was performed. TaqMan primers were used and the ΔΔCt method was used for the analysis of the data.

Microarray

For microarray, total RNAs were extracted from osteosarcomas (n = 4) and soft tissue sarcomas (n = 4) that developed in *Ng2/Cspg4-Cre;Trp53*$^{flox/flox}$ mice. Skeletal muscle tissues (n = 2) and bone marrow tissues (n = 2) were used as controls. Biotinylated cRNA was hybridized onto Mouse WG-6 v2.0 Expression BeadChips (Illumina). To identify gene signatures differentially expressed between sarcomas and non-cancerous tissues, linear models for microarray data were used. The false discovery rate was set at 0.01, and evaluated using Benjamini and Hochberg multiple testing procedures. Differential expression was compiled as a gene set that was compared to expression data from various human cancer types, with gene expression called for each gene within each cancer type comparing it to the aggregate of all other cancer types using a moderated t-statistic. Gene set enrichment analysis (Mootha et al., 2003) was carried out to identify the significance of enrichment of the mouse genes with the most differentially expressed human genes that differentiate each cancer type.

Cell Sorting

To isolate *Ng2/Cspg4*-expressing cells, tissues were harvested from mice, and dissociated into individual cells as previously reported (Wang et al., 2012; Wu et al., 2007). Flow cytometry was used to sort *Ng2/Cspg4*-expressing cells using an NG2 Ab (Abcam). Sorting for LacZ (Amini-Nik et al., 2011) and CD146 (Wei et al., 2015) was performed as we previously reported.

RNA Sequencing

For each sample, 10 ng total RNA was processed using the SMARTTM cDNA synthesis protocol including SMARTScribe Reverse Transcriptase. The amplified cDNA was subject to Illumina paired-end library construction. RNA-sequencing analysis was performed by the Genome Sciences Centre at the British Columbia Cancer Agency (Vancouver, Canada) using Illumina HiSeq 2000 sequencing at 75 base PET indexed lane, pooling two libraries per lane. Data were processed using the TrimGalore toolkit (Chen et al., 2014) to trim low-quality bases and Illumina sequencing adapters from the 3′ end of the reads. Only pairs where both reads were 35 nt or longer were kept for further analysis. Reads were mapped to the GRCm38.73 version of the mouse genome and transcriptome (Kersey et al., 2012) using the STAR RNA-sequencing alignment tool (Dobin et al., 2013). Gene counts were compiled using the HTSeq tool (http://www-huber.embl.de/users/anders/HTSeq). Normalization and differential expression were carried out using the DESeq2 (Love et al., 2014) Bioconductor (Gentleman et al., 2004) package with the R statistical programming environment (http://www.r-project.org). A negative binomial generalized linear model was employed to identify differentially expressed genes across sample types. Pathway analyses were performed using gene set enrichment analysis with parameters set to 2,000 gene set per mutations and gene sets size between 8 and 500 (Subramanian et al., 2005). Gene sets were obtained from the KEGG, MsigDB-c2, NCI, Biocarta, IOB, Netpath, Human Cyc, Reactome, and Gene Ontology (GO) databases (Kanehisa and Goto, 2000; Merico et al., 2010). An enrichment map was generated using Cytoscape with parameters set for a nominal p value of < 0.005, a false discovery rate < 0.25, and the Jaccard coefficient set to 0.5 (Saito et al., 2012).

Xenograft in Immunocompromised Mice

Primary sarcomas were dissociated into single cells (Wang et al., 2012). 10,000 dissociated cells were suspended with Matrigel (Becton Dickinson) and injected subcutaneously into 6- to 8-week-old NOD-*scid IL2r*γ^{null} (NSG) mice. After injection, the mice were observed for 3 weeks, and then lithium was added to the drinking water at a dose previously shown to increase β-catenin in mesenchymal tissues (Chen et al., 2007). The ΔN89-β-catenin construct, or empty control, was used as previously reported (Fuerer and Nusse, 2010; Li et al., 1998). Western analysis using an antibody to actin as a loading control (Tejpar et al., 1999) was used to determine β-catenin levels, and the tumors were weighed using an analytical balance, as previously reported (Wang et al., 2012).

ACCESSION NUMBERS

The accession number for the mouse sarcoma microarray data reported in this paper is GEO: GSE63631. The accession number for the RNA-sequencing data from *Ng2/Cspg4*-expressing cells and tumors reported in this paper is GEO: GSE63679.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.058.

AUTHOR CONTRIBUTIONS

Conceptualization, B.A.A., J.S.W., S.S., and Q.W.; Methodology, B.A.A., D.G.K., S.S., Y.J.T., and S.Y.W.; Validation, A.O. and S.T.; Investigation, S.S., Y.J.T., Q.W., M.H., A.W., I.H., H.W., and P.N.; Writing – Original Draft, S.S., J.S.W., and B.A.A.; Writing – Review & Editing, B.A.A., Y.J.T., and D.G.K.; Funding Acquisition, B.A.A., J.S.W., and D.G.K.; Supervision, J.S.W., D.G.K., and B.A.A.

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