Science 3 May 2002:
vol. 296 no. 5569 pp. 920-922
DOI: 10.1126/science.1068452

Neurofibromas in NF1: Schwann Cell Origin and Role of Tumor Environment

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Supplementary Material

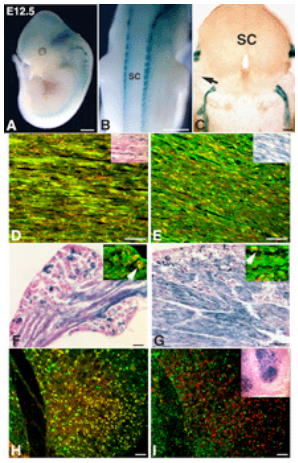
Control and conditional mutant mice. The control mice used in this study are a pool of phenotypically indistinguishable mice with four genotypes: NF1+/−;Krox20-cre, NF1flox/+;Krox20-cre, NF1+/−, and NF1flox/flox mice. Mice with NF1flox/−;Krox20-cre genotype at the age of 10-12 months (n = 12) along with their control littermates (n = 12) were subjected to complete necropsy. In addition, six month old NF1flox/−;Krox20-cre mice (n = 3) along with their control littermates (n = 3) were subjected to complete necropsy. Mice with NF1flox/flox;Krox20-cre genotype (n = 2) at the age of 12 months along with their control littermates were also subjected to complete necropsy. Due to sickness of mutant mice, only one NF1flox/−;Krox20-cre mutant and one NF1flox/flox;Krox20-cre mutant at the age of 15 months were kept for analysis. All NF1flox/−;Krox20-cre vs. NF1flox/flox;Krox20-cre mice in this study were littermates.

LacZ staining and double immuofluorescence. E12.5 embryos or cryostat sections from adult tissues were prepared and subjected to LacZ staining as described previously (1). Adjacent sections were subjected to double immunofluorescence with anti-LacZ (rabbit, 1:200, 5' and 3') and anti-NeuN (mouse, 1:200, Chemicon) or anti-S100 (mouse, 1:1000, Sigma) as described (2). Peritoneal cells were collected as described previously (3) and smears were prepared by cytospin, which were subjected to LacZ staining and counterstained with nuclear fast red. The identity of mast cells was determined by either a modified Romanowsky stain (Wescor) or Giemsa stain (Sigma diagnostics). Perineurial fibroblasts were isolated from a modified explant approach (4) (see below).

Tissue culture. Sciatic nerves from P0.5 pups or adult mice were dissected under microscope and cut into 2-4 mm long explants. Explants were washed with L15 media and plated into a 35 mm culture dish. Two to four days later, fibroblasts were allowed to migrate from the explants for further analysis. Next, all explants were collected and dissociated with a mixture of collagenase and trypsin as described (5). Residual fibroblasts were removed by complement-mediated lysis and Schwann cells were plated in the presence of 100 μg/ml of glial growth factor (GGF) and 2 μM of forskolin. Schwann cells were confirmed by S100 immunocytochemistry.

Histology and tumor analysis. Mutant and control littermates were perfused and post-fixed with 4% paraformaldehyde overnight at 4°C. Trigeminal and sciatic nerves were dissected and processed for paraffin embedding. Internal organs including lung, kidney, liver, small intestine, colon and stomach were removed and processed for paraffin-embedded sections. Heads, spinal columns and limbs were further decalcified and cut into 1 mm pieces followed by processing for paraffin-embedded sections. Sections were cut at 5 u and stained with hematoxylin and eosin (H&E), Leder staining (Sigma diagnostics), or subjected to immunohistochemistry as described (2). The dilutions of primary antibodies used in this study were: S100 (anti-rabbit, 1:1000, Signet), GFAP (anti-rabbit, 1:2000, DAKO), NF160 (anti-rabbit, 1:500, Chemicon), NCAM (anti-mouse, 1:100, Sigma), p75NGFR (anti-rabbit, 1:200, Chemicon), EMA (epithelial membrane antigen) (anti-mouse, 1:100, DAKO). For quantification of neurofibromas, heads and spinal columns from NF1flox/−;Krox20-cre (n = 7) and NF1flox/flox;Krox20-cre (n = 3) were completely sectioned and analyzed via microscopy by YZ and DKB. The number of neurofibromas in the NF1flox/−;Krox20-cre and hyperplastic lesions in the NF1flox/flox;Krox20-cre were quantified and the data were subjected to statistic analysis using paired t test.

Supplemental Figure 1. Krox20-cre-mediated recombination during development and in adulthood. E12.5 embryos from the Krox20-cre;Rosa26-LacZ cross were subjected to LacZ staining as described previously (*2*). Cre-mediated LacZ activation is confined to derivatives of rhombomeres 3 and 5 including cranial neural crest (**A**) and boundary cap cells (a Schwann cell lineage) within the spinal roots (**B**). (**C**) Transverse sections of LacZ stained embryos show that LacZ staining is restricted to spinal roots, but not the spinal cord (SC) and DRG (arrow). In adult Cre/LacZ double transgenic (Tg) mice (n = 3), double-labeling immunoanalysis with anti-S100 (green) and anti-LacZ (red) antibodies for spinal nerves (**D**) and cranial nerves (**E**) indicates that most of S100 positive Schwann cells have undergone Cre-mediated recombination in peripheral nerves. Inset, LacZ staining for adjacent sections of (C) and (D). LacZ staining indicates that only a small number of adult DRG neurons (**F**) and trigeminal neurons (**G**) have Cre activity, which is confirmed by double-staining with a neuronal marker, anti-NeuN (green)/anti-LacZ (red) (insets). Double-labeling of the adult brainstem with anti-NeuN (green)/anti-LacZ (red) (**H**) and with anti-S100 (green)/anti-LacZ (red) (**I**) demonstrates that, in contrast to spinal and cranial nerves, Cre-mediated recombination is primarily found in neurons of the brainstem. Inset, LacZ staining for the adjacent section of (H) and (I). Scale bar: (A) and (B), 1 mm; (C) to (I), 100u.



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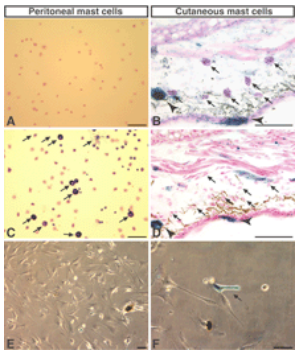
Supplemental Figure 2. Krox20-cre is not expressed in mast cells and fibroblasts. Peritoneal mast cells from adult Krox20-Cre/LacZ double Tg mice were isolated by peritoneal lavage (*3*), from which cytospinned smears were prepared either for LacZ staining followed by counterstaining with nuclear fast red (**A**), or for a modified Romanowsky stain (**C**) to identify mast cells (arrows). No LacZ positive cells were found in the smears. Adjacent sections from ear skins were prepared for LacZ staining followed by counterstaining with either Giemsa stain (**B**) to label cutaneous mast cells (arrows), or nuclear fast red (**D**). Arrows in (D) point to the mast cell nuclei according to the position as compared to its adjacent section (B). Arrowheads in (B) and (D) indicate LacZ positive hair follicle cells that serve as a positive control. Of note, no LacZ activity was detected in cutaneous mast cells. Perineurial fibroblasts were isolated by a modified explant approach (*4*) (see methods) and subjected to LacZ staining (**E**). No evidence of Cre-mediated LacZ activation was found. (**F**) A contaminating LacZ positive Schwann cell (arrow) from the same culture (E) is indicated as a positive control. Scale bar, 100u.



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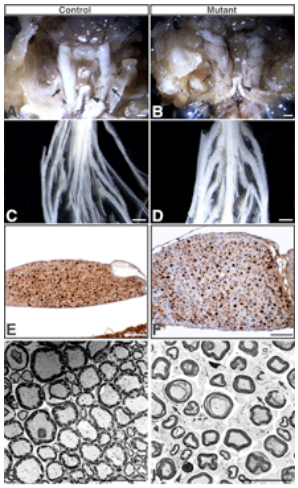
Supplemental Figure 3. NF1flox/−;Krox20-cre mice have enlarged cranial and spinal nerves. Twelve-month-old mutant and control mice were perfused and post-fixed. Mutant trigeminal nerves (arrows) are enlarged compared to controls while optic nerves (arrowhead) are unaffected. Spinal cords with attached spinal nerves and sensory ganglia from control (**C**) and mutant (**D**) mice demonstrate enlargement of mutant nerves. Transverse sections from control (**E**) and mutant (**F**) spinal nerves were subjected to immunohistochemistry using an antibody against neurofilament 160 (NF160), to label axons. Electron microscopy (EM) of mutant nerves (H) demonstrates expansion of endoneurial space compared to controls (**G**), with separation of myelinated fibers by collegan, Schwann cell cytoplasmic processes (arrows), mast cells (arrowhead), and occasional unmyelinated axons. Scale bar, (**A** to **D**), 1 mm; (E) and (F), 50 u; (G and **H**), 10 u.



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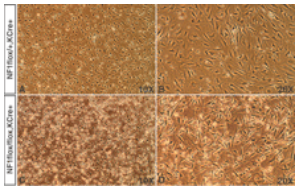
Supplemental Figure 4. Schwann cells from newborn NF1flox/flox;Krox20-cre sciatic nerves appear transformed and are morphologically indistinguishable from NF1−/− Schwann cells (5). Schwann cells were isolated from sciatic nerves of P0.5 pups. (**A**) Low and (**B**) high magnification view of Schwann cells from NF1flox/+;Krox20-cre sciatic nerves (n = 3). (**C**) Low and (**D**) high magnification view of Schwann cells from NF1flox/flox;Krox20-cre sciatic nerves (n = 8). Objective magnification, (A) and (C), 10×; (B) and (D), 20×.



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Supplemental Table 1. Expression of neural crest, Schwann cell precursor, Schwann cell, neuronal, and perineurial cell markers in the NF1flox/−;Krox20 neurofibromas. The score for immunoreactivity is based upon the number of positive cells within tumors as well as the intensity of immuonreactivity.

Mouse Number	Tumors	S100	GFAP	NF160	NCAM	P75NGFR	EMA
Y212	1	++++	++++	+++	++++	++++	+++
	2	++++	++++	+++	++++	++++	+++
Y207	1	++++	+++	++	++++	++	++
	2	++++	+++	++	++++	++	+++
	3	++	-	+++	+++	++	++
	4	+++	-	+++	+++	+++	+++
Y206	1	+++	ND	++	++	ND	ND
Y188	2	++++	+	++	++++	+++	+++
Y205	1	+/-	+/-	+	+++	+	+++
	2	+++	++	+++	+++	++	ND
Y191	1	++	++	++	++	++	+++
	2	+++	++	+++	+++	++	+++

++++ Very strong immunostaining +++ Strong immunostaining
++ Moderate immunostaining + Weak immunostaining
- No detectable immunostaining ND Not determined

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