nature neuroscience

Loss of mammalian *Sprouty2* leads to enteric neuronal hyperplasia and esophageal achalasia

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We report here that loss of the *Sprouty2* gene (also known as *Spry2*) in mice resulted in enteric nerve hyperplasia, which led to esophageal achalasia and intestinal pseudo-obstruction. Glial cell line–derived neurotrophic factor (GDNF) induced hyperactivation of ERK and Akt in enteric nerve cells. Anti-GDNF antibody administration corrected nerve hyperplasia in *Sprouty2*-deficient mice. We show Sprouty2 to be a negative regulator of GDNF for the neonatal development or survival of enteric nerve cells.

Sprouty and Spred family proteins are evolutionarily conserved inhibitors of tyrosine kinase signaling[1,2]. In *Drosophila melanogaster*, Sprouty has been genetically identified as an antagonist for fibroblast growth factor receptor (FGFR) in lung development and epidermal growth factor receptor (EGFR) in eye and wing development[1]. To define the physiological function of mammalian *Sprouty2*, we generated mice lacking the *Sprouty2* gene (**Supplementary Methods**, **Supplementary Fig. 1**). As expected, ERK activation was enhanced in response to FGF stimulation in *Sprouty2*$^{-/-}$ embryonic fibroblasts compared with wild-type embryonic fibroblasts (**Supplementary Fig. 1**).

Sprouty2-deficient offspring were born at the expected mendelian ratio from intercrosses of heterozygotes ($n = 30/125$; 24%). As reported recently[3], *Sprouty2*$^{-/-}$ mice have defects in hearing. In addition, about half of the *Sprouty2*$^{-/-}$ mice died within 6 weeks after birth. Many of the remaining homozygotes survived for at least 6 months, but they were significantly smaller than wild-type littermates (**Supplementary Fig. 2**). Dissection showed that the esophagus was dilated and clogged with saburra at >4 weeks of age (**Fig. 1a**), and the intestinal gauge was partly dilated and filled with gas in *Sprouty2*-deficient mice at >2 months of age (**Fig. 1b**, **Supplementary Fig. 2**). Oral administration of barium sulfate resulted in immediate transport to the stomach and small intestine in wild-type mice, whereas barium was retained in the esophagus in *Sprouty2*-deficient mice (**Fig. 1b**). These X-ray images resembled those of esophageal achalasia in humans.

The primary motor disorders in esophageal achalasia in humans are failure of the lower esophageal sphincter (LES) to relax and absence of peristalsis in the smooth muscle. Therefore, we measured the sphincteral contraction force of the esophagus and intestine[4], which are parasympathomimetically mediated by acetylcholine. The contraction force of LES of *Sprouty2*$^{-/-}$ mice in response to carbachol (CCh) was more than five times stronger than that of wild-type mice (**Fig. 1c**). However, nerve-independent contraction induced by a high-concentration potassium buffer was not very different between wild-type and *Sprouty2*$^{-/-}$ mice (**Fig. 1d**). These data suggest that strong nerve-dependent contraction of the LES causes the achalasia phenotype of *Sprouty2*$^{-/-}$ mice. In addition, the motility of the intestine in response to CCh was also abnormal in *Sprouty2*$^{-/-}$ mice (**Supplementary Fig. 3**).

The intestinal movement defects found in *Sprouty2*-deficient mice resemble Hirschsprung disease (HSCR) in humans or aganglionic megacolon in animals deficient of GDNF, endothelin and their receptors[5]. Therefore, we investigated disorder of the enteric nervous system (ENS) in *Sprouty2*$^{-/-}$ mice. Whole-mount immunostaining with an antibody against the neuronal marker protein gene product 9.5

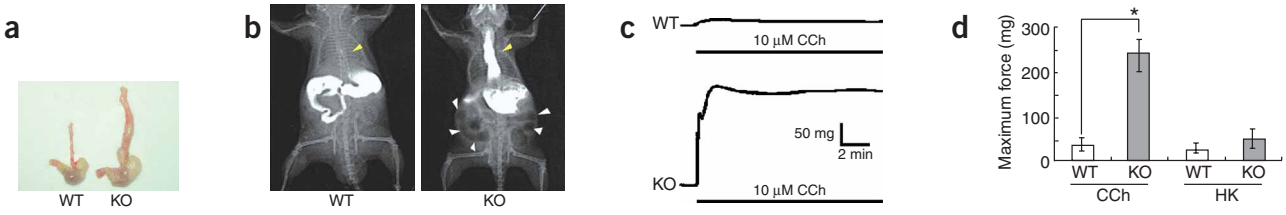

Figure 1 Esophageal achalasia and intestinal pseudo-obstruction in *Sprouty2*-deficient mice. (**a**) The esophagus and stomach from wild-type (WT) and *Sprouty2*$^{-/-}$ (KO) mice. (**b**) Soft X-ray 5 min after barium sulfate administration; 1.5×10^{-2} ml/g of barium sulfate was orally administrated using an animal feeding needle. Yellow arrowheads indicate esophagus, and white arrowheads indicate gas in the alimentary canal. (**c**) Recording of the contraction force of the LES in response to 10 μM carbachol (CCh). (**d**) Average values of maximum contraction force of the esophagus with 10 μM CCh and 60 mM K^+ (HK). $^*P < 0.005$ ($n = 13$).

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Published online 5 June 2005; doi:10.1038/nn1485

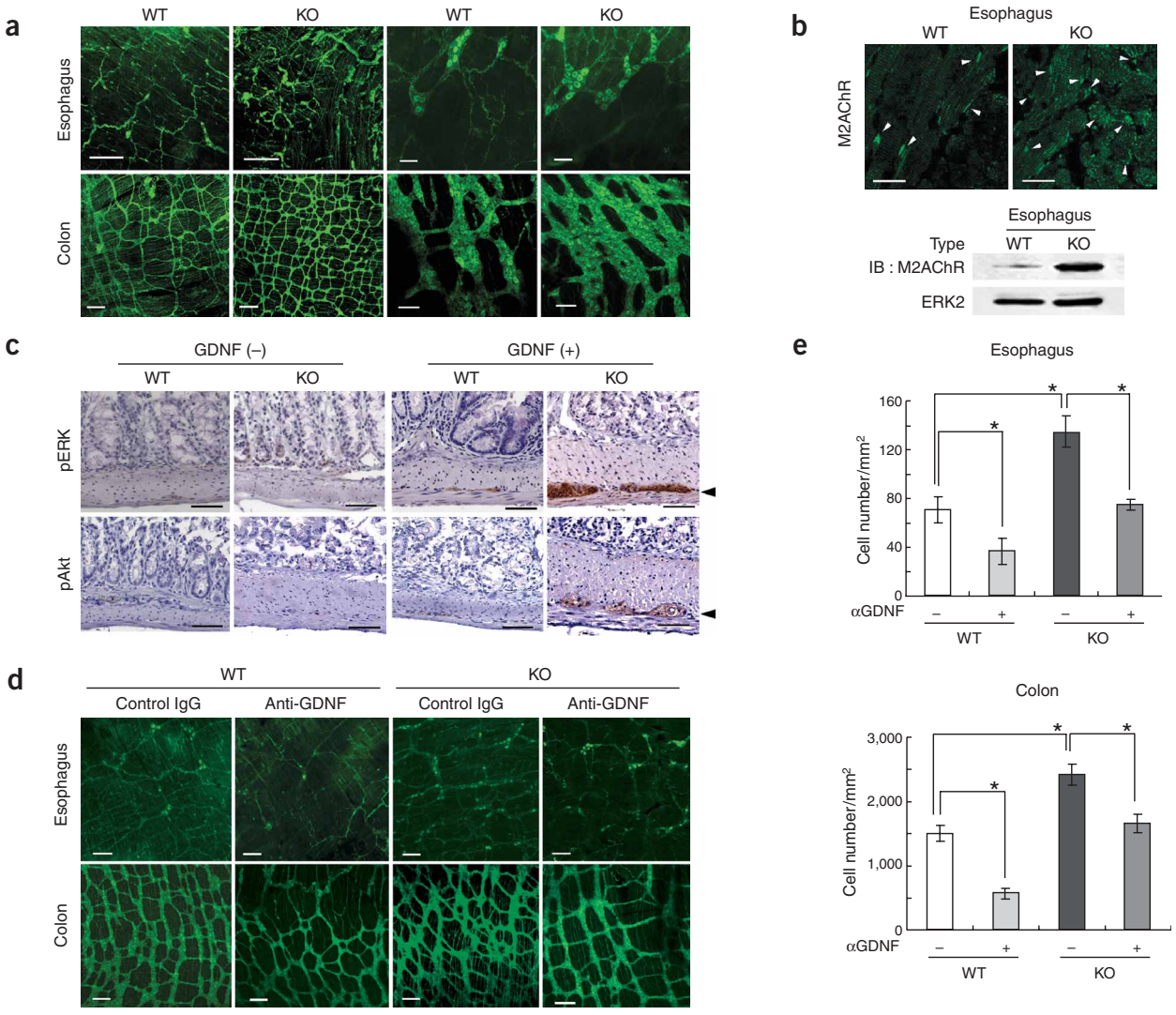

Figure 2 ENS hyperplasia in *Sprouty2*-deficient mice. (**a**) Whole-mount anti-PGP9.5 immunostaining of the esophagus and distal colon of wild-type (WT) or *Sprouty2*$^{-/-}$ (KO) mice at 8 weeks of age. Scale bars: 200 μm (two leftmost columns) and 50 μm (two rightmost columns). (**b**) Immunohistochemistry for M2AChR in the esophagus of wild-type and *Sprouty2*$^{-/-}$mice. The M2AChR seems to be present in clusters (arrows). Scale bars: 20 μm. Below: western blot analysis of M2AChR expression. (**c**) Immunohistochemical detection of phosphorylation of ERK and Akt in response to GDNF in the colon. Tissues were incubated in the presence or absence of 50 ng/ml GDNF for 30 min and then fixed. Transverse sections were stained with indicated antibodies. Arrowheads indicate enteric nerve ganglions. Scale bar: 50 μm. (**d**,**e**) Control IgG or monoclonal anti-GDNF antibody (R&D Systems) were intraperitoneally injected into two-week-old wild-type and *Sprouty2*$^{-/-}$ pups (0.125 mg/injection) twice a week until 4 weeks of age, and then the ENS was examined with whole-mount anti-PGP9.5 immunostaining (**d**). Scale bar: 100 μm. The numbers of PGP9.5-positive cells/mm^2 in the esophagus and distal colon were calculated (**e**). $^*P < 0.005$; three mice were used for each measurement.

(PGP9.5) demonstrated a marked hyperplasticity in the ENS plexus density in the esophagus and colon (**Fig. 2a**). We observed an increase in the neural networks and hypertrophy of ganglion strands in mutant mice compared with wild-type mice (**Fig. 2a**). Hyperganglionosis of ENS in *Sprouty2*$^{-/-}$ mice was confirmed by counting the number of ganglion cells and by immunoblotting stained with anti-PGP9.5 (**Supplementary Fig. 4**). The number and size distribution of dorsal root ganglia cells was not different between wild-type and *Sprouty2*-deficient mice (data not shown). Muscarinic2-acetylcholine receptor (M2AchR) was also expressed at extremely high levels in *Sprouty2*$^{-/-}$ mice and clustered at the neuromuscular junctions of the esophagus (**Fig. 2b**). These data suggested that hypercontraction of the LES of *Sprouty2*$^{-/-}$ mice was due to the increased number of M2AchRs.

GDNF and its receptor, c-Ret, are known to regulate the migration and colonization of neural crest cells and are also necessary for survival of ENS. A constitutively active mutation (M918T) in c-Ret is found in multiple endocrine neoplasia type 2B (MEN2B) disease, with which ENS ganglioneuromatosis and achalasia are often associated[6–8]. Thus, we examined GDNF-Ret signaling using immunohistochemistry with antibodies against phosphorylated ERK (pERK) and phosphorylated Akt (pAkt; **Fig. 2c**). We observed much stronger ERK and Akt activation in the ganglia of the colon in *Sprouty2*$^{-/-}$ mice than in wild-type mice. We did not observe activation of ERK and Akt in muscle and epithelial cells. A higher-magnification view suggested that ERK and Akt activation at the single–nerve cell level was also higher in *Sprouty2*$^{-/-}$ ENS than in wild-type ENS. Hypersensitivity of

Sprouty2-deficient ENS to GDNF was confirmed by immunoblotting of the ENS-enriched tissues with antibodies against pERK and pAkt (**Supplementary Fig. 5**). These data indicated that *Sprouty2*-deficient enteric nerve cells were hypersensitive to GDNF.

To confirm the involvement of GDNF in hyperganglionosis, we injected monoclonal antibodies against GDNF (anti-GDNF) into neonatal *Sprouty2*$^{-/-}$ mice. Anti-GDNF, but not control IgG, significantly corrected ENS hyperplasia (**Fig. 2d**,**e**; **Supplementary Fig. 6**) and esophagus dilation (**Supplementary Fig. 6**) observed in *Sprouty2*-deficient mice. Anti-GDNF antibodies also reduced the number of enteric nerve cells in wild-type mice (**Fig. 2d**). Furthermore, chimeric Ret-Fc recombinant protein, which is an antagonist of Ret[9], rescued the phenotype of enteric nerve hyperplasia of *Sprouty2*$^{-/-}$ mice (**Supplementary Fig. 7**). These data strongly suggest that the GDNF-Ret signaling system is important in the development and/or survival of neonatal ENS and that hyperresponsiveness of enteric neurons in *Sprouty2*-deficient mice to the GDNF-Ret system leads to ENS hyperganglionosis. Similarly, Sprouty1-deficient mice have developmental defects in kidney development because of GDNF-Ret hypersignaling[10], indicating that both Sprouty1 and Sprouty2 negatively regulate Ret signaling at specific organs.

Hyperganglionosis and hyperinnervation in ENS are known to cause megacolon or intestinal pseudo-obstruction in human[11]. The motor activity of the alimentary tract is controlled by the balance of activities between inhibitory and excitatory neurons in a complex manner. *Sprouty2*-deficient mice could represent a useful model for esophageal achalasia and intestinal motility disorders based on neuronal intestinal dysplasia, ganglioneuromatosis and hyperganglionosis.

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

ACKNOWLEDGMENTS

We thank T. Yoshioka, M. Ohtsu, E. Fujimoto and N. Kinoshita for technical assistance, M. Takahashi (Nagoya University) for discussion and comments and Y. Nishi for manuscript preparation. This work was supported by special grants-in-aid from the Ministry of Education, Science, Technology, Sports and Culture of Japan; the Haraguchi Memorial Foundation; the Yamanouchi Foundation for Research on Metabolic Disorders; the Takeda Science Foundation; the Kato Memorial Foundation and the Uehara Memorial Foundation.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 11 March; accepted 17 May 2005
Published online at http://www.nature.com/natureneuroscience/

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