

# A Novel Pathogenesis of Megacolon in *Ncx/Hox11L.1* Deficient Mice

Masahiko Hatano,\* Taito Aoki,\* Mari Dezawa,§ Seiichi Yusa,\* Yoshinori Iitsuka,\* Haruhiko Koseki,‡ Masaru Taniguchi,‡ and Takeshi Tokuhisa\*

\*Division of Developmental Genetics, †Division of Molecular Immunology, Center for Biomedical Science, §Department of Anatomy, Chiba University School of Medicine, Chiba 260, Japan

## Abstract

The *Ncx/Hox11L.1* gene, a member of the *Hox11* homeobox gene family, is mainly expressed in neural crest-derived tissues. To elucidate the role of *Ncx/Hox11L.1*, the gene has been inactivated in embryonic stem cells by homologous recombination. The homozygous mutant mice were viable. These mice developed megacolon with enteric ganglia by age 3–5 wk. Histochemical analysis of the ganglia revealed that the enteric neurons hyperinnervated in the narrow segment of megacolon. Some of these neuronal cells degenerated and neuronal cell death occurred in later stages. We propose that *Ncx/Hox11L.1* is required for maintenance of proper functions of the enteric nervous system. These mutant mice can be used to elucidate a novel pathogenesis for human neuronal intestinal dysplasia. (*J. Clin. Invest.* 1997; 100:795–801.) **Key words:** gene targeting • homeobox • enteric ganglia • NADPH diaphorase • hyperinnervation

## Introduction

Vertebrate homeobox genes are classified into several gene families depending on their homology of homeobox sequence and their location on chromosomes. There are 38 genes organized into four different chromosomal clusters: Hox A, B, C, and D (1). Members of the clusters specify position along axes in an embryo and limbs (1). Besides these clustered homeobox genes, certain of the unclustered and more divergent homeobox genes have been assigned roles in growth control, organogenesis, or establishment of cellular phenotypes (2–5). Indeed, one of the unclustered homeobox gene, *HOX11* (6–9), controls the genesis of spleen (10, 11). The *Ncx/Hox11L.1* gene, belonging to the *Hox11* gene family (12, 13), is also mapped outside the clustered homeobox gene on mouse chromosome (14). It is expressed in a subset of neural crest-derived tissues such as dorsal root ganglia, cranial nerve ganglia, sympathetic ganglia, and enteric nerve ganglia in embryos

between days 9.5 (E9.5) and 13.5 (E13.5) (15). However, a function of the *Ncx/Hox11L.1* gene in those tissues is unknown.

Neural crest cells develop at the dorsal part of neural tube in embryos at E8.5 and start to migrate into peripheral regions at E9.5 (16). Those cells generate into most of the peripheral nervous system, skin melanocytes, and mesodermal derivatives such as smooth muscle cells, bone, and cartilage (17). The migration and colonization of neural crest cells is mainly controlled by signals through the *c-ret* protooncogene (18, 19) and the endothelin-B receptor gene (20, 21). Mice lacking one of those gene products develop megacolon with the absence of enteric ganglia by perturbation of the migration (18, 21). Human homologues of those genes are also responsible for the human congenital megacolon known as Hirschsprung's disease (22–24).

The *Ncx/Hox11L.1* gene is also expressed in enteric nerve ganglia and adrenal glands in adult mice. To elucidate the function in development of those tissues, we have disrupted the gene in embryonic stem cells by homologous recombination. Mice rendered deficient in *Ncx/Hox11L.1* (*Ncx*<sup>-/-</sup>)<sup>1</sup> were born without any major morphological disorder. *Ncx*<sup>-/-</sup> mice developed megacolon after 3–5 wk of age. Histochemical analysis revealed that the enteric neurons hyperinnervated in the proximal part of colon. We discuss a novel pathogenesis of megacolon.

## Methods

**Materials.** Mice were purchased from Japan SLC (Hamamatsu, Japan). Restriction enzymes were purchased from New England Biolabs, Inc. (Beverly, MA), or Takara Shuzo Co., Ltd. (Otsu, Japan). Antibodies against substance P and neuropeptide Y were from Amersham International (Buckinghamshire, United Kingdom) and Peninsula Laboratories Inc. (Belmont, CA), respectively. FITC-conjugated antibody to rabbit IgG was from Organon Teknika Corp. (Durham, NC).  $\beta$ -NADPH and nitroblue tetrazolium were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

**Targeted disruption of the *Ncx/Hox11L.1* gene.** A murine *Ncx/Hox11L.1* genomic clone was isolated from a 129/Sv genomic library. A targeting vector was constructed by replacing a 0.9-kb Xho1-Not1 fragment containing the 5' flanking region and a part of exon 1 of the *Ncx/Hox11L.1* gene with the neo resistant cassette (pMC1-Neo) (Fig. 1A). The herpes simplex thymidine kinase gene was inserted downstream of the short arm. The linearized targeting vector was transfected into R1 embryonic stem cells by electroporation. Homologous recombination in the G418 and gancyclovir selected clones was screened by Southern blot. Approximately 200 clones were examined and the homologous recombination was detected in 6 clones. Two in-

Address correspondence to Dr. Masahiko Hatano, Division of Developmental Genetics, Center for Biomedical Science, Chiba University School of Medicine, Inohana 1-8-1, Chuo-ku, Chiba 260, Japan. Phone: 81-43-226-2181; FAX: 81-43-226-2183; E-mail: hatano@med.m.chiba-u.ac.jp

Received for publication 18 March 1997 and accepted in revised form 7 July 1997.

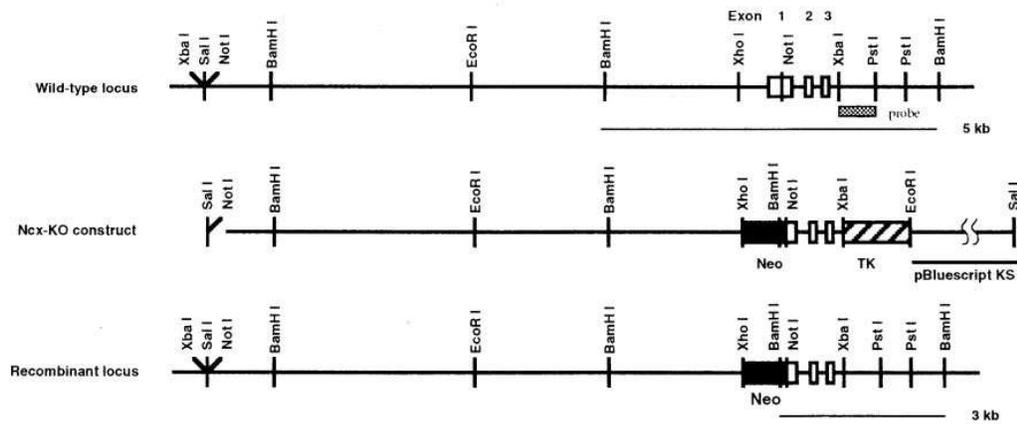
J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.  
0021-9738/97/08/0795/07 \$2.00

Volume 100, Number 4, August 1997, 795–801  
<http://www.jci.org>

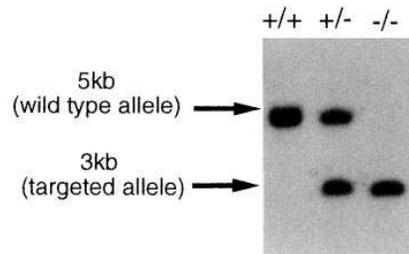
1. **Abbreviations used in this paper:** DIG, digoxigenin; *Ncx*<sup>-/-</sup>, *Ncx* homozygous mutant; *Ncx*<sup>+/-</sup>, *Ncx* heterozygous mutant.

A



**Figure 1.** Targeted disruption of the *Ncx/Hox11L.1* gene. (A) Restriction map of the murine *Ncx/Hox11L.1* gene (top), the targeting vector (middle), and the disrupted *Ncx/Hox11L.1* locus (bottom). Closed boxes and a hatched box indicate the neo resistant cassette and the herpes simplex thymidine kinase gene, respectively. (B) Southern blot analysis of BamHI-digested genomic DNA from F1 offspring by interbreeding of heterozygous mutant mice.

B



dependent targeted clones were used to generate chimeric mice by the aggregation method (25) with slight modification. Heterozygous mutant mice were interbred to obtain homozygous mutant mice.

**Southern blot analysis.** Genomic DNA isolated from the mutant offspring was digested with BamHI, separated on a 1% agarose gel, and transferred to a nylon membrane. The filter was hybridized with a digoxigenin (DIG) labeled probe. The probe was detected by the enhanced chemiluminescent detection system with sheep anti-DIG antibody labeled with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany). For probe, a 0.75-kb XbaI-PstI fragment which is external to the targeting vector was subcloned into a pGEM vector (Promega, Madison, WI) and labeled with DIG (Boehringer Mannheim) by PCR using SP6 and T7 primers. The probe detected the wild-type allele as a 5.0-kb fragment and the mutant allele as a 3.0-kb fragment (Fig. 1 B).

**Histological analysis.** Animals were perfused with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Organs were dissected from mice and postfixed with 4% paraformaldehyde for 12 h. The tissues were equilibrated with 20% sucrose, sectioned at 10  $\mu$ m on a cryostat. Hematoxylin and eosin staining was done using a standard protocol. For immunohistochemistry, sections were incubated for overnight at 4°C with rabbit antibodies against substance P or neuropeptide Y followed by FITC-conjugated antibody to rabbit IgG for 2 h at room temperature. The sections were washed in PBS and inspected in a confocal laser scanning microscopy (Bio-Rad Lab., Hercules, CA). The in situ TdT-mediated dUTP-biotin nick end labeling method was performed using cell death detection kits (Boehringer Mannheim).

**NADPH diaphorase histochemistry.** Whole mount tissues or sections were incubated with 0.1 M phosphate buffer (pH 7.4) containing 1.0 mg/ml  $\beta$ -NADPH, 0.1 mg/ml nitroblue tetrazolium, and 0.3% Triton X-100 for 30 min for 1 h at 37°C. The mean  $\pm$  SD of NADPH diaphorase positive neurons was calculated by numbers in eight seg-

ments of each section from three *Ncx*<sup>-/-</sup> mice and three wild-type littermates.

**Electron microscopy.** Animals were perfused with a mixture of 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep ether anesthesia. The specimen was dissected out and postfixed in phosphate-buffered 1% osmium tetroxide (pH 7.4) for 2 h, dehydrated in an ascending series of ethanol solutions, passed through propylene oxide, and embedded in EPON 812. Thin sections were stained in uranyl and lead salt solutions.

## Results

**Megacolon in *Ncx/Hox11L.1* deficient mice.** A part of the first exon including the ATG translation start codon and the 5' flanking region of the *Ncx/Hox11L.1* gene were replaced by the neomycin resistant gene in embryonic stem cells (Fig. 1 A). Heterozygous mutant (*Ncx*<sup>+/-</sup>) mice showed no abnormality up to 2 yr of age. *Ncx*<sup>+/-</sup> mice were interbred and their progeny were genotyped by Southern blot analysis (Fig. 1 B). Homozygous mutant (*Ncx*<sup>-/-</sup>) mice were born in the expected Mendelian frequency and appeared normal at birth. Although *Ncx/Hox11L.1* is expressed in neural crest-derived tissues such as trigeminal ganglia, dorsal root ganglia, and adrenal medulla in embryos (15), histological analysis of these tissues from *Ncx*<sup>-/-</sup> neonates revealed no abnormality (data not shown). However,  $\sim$  50% (34/69) of *Ncx*<sup>-/-</sup> mice became sick with distended abdomen and died by age 3–5 wk, while the rest of the deficient mice were indistinguishable from the wild-type mice and survived for > 1 yr. Gross anatomical analysis of all these dead mice indicated megacolon (Fig. 2 A). Ap-

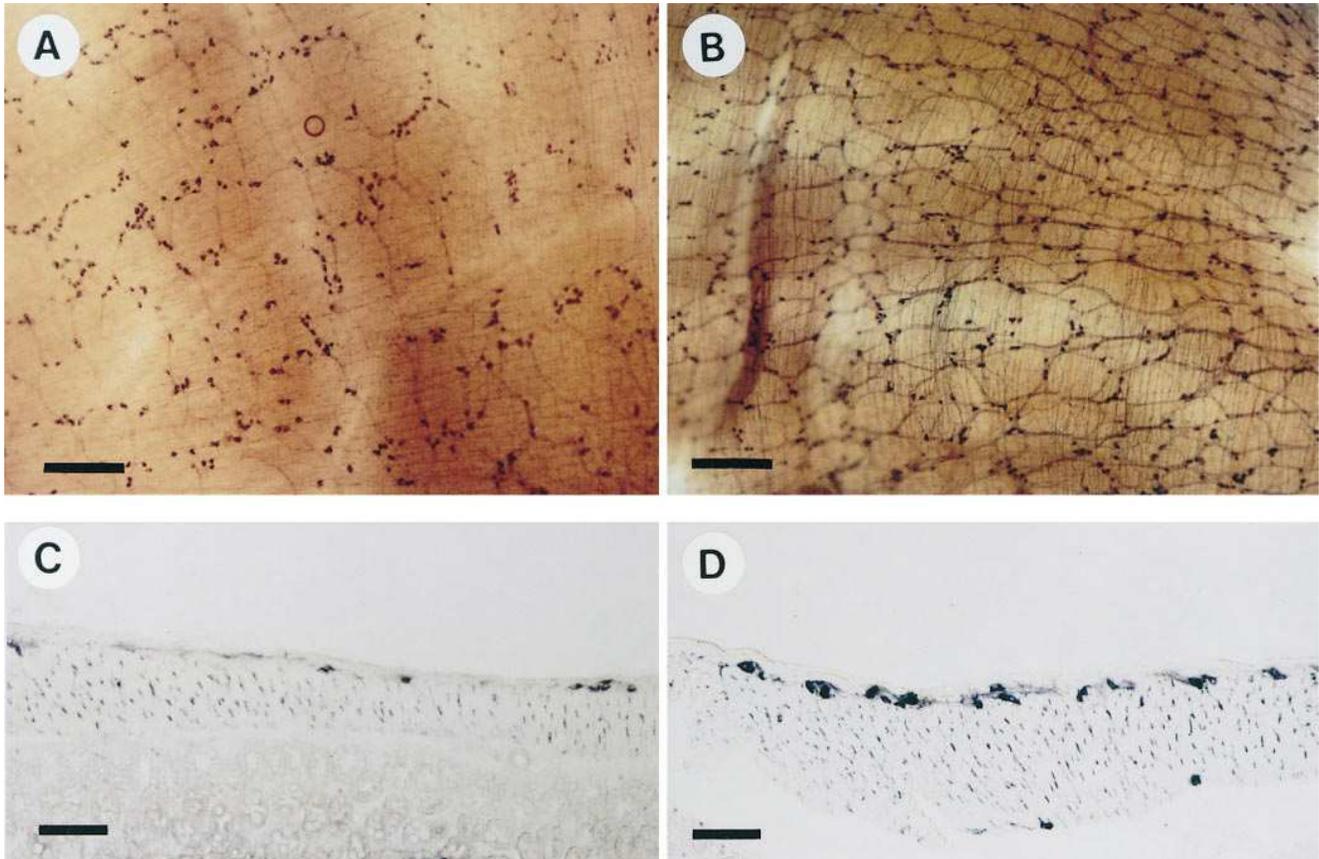
**A****B**

**Figure 2.** Megacolon in *Ncx/Hox11L.1* deficient mice. (A) Autopsy of wild-type littermate (WT) and *Ncx*<sup>-/-</sup> (KO) mice. (B) The dissected entire gastrointestinal tracts from wild-type (WT) and *Ncx*<sup>-/-</sup> (KO) mice. An arrow indicates the transitional zone between the proximal distended colon and the distal narrow segment.

pendix, cecum, and small intestine were dilated in all cases examined (Fig. 2B). A narrow segment spanned from the proximal colon to the distal rectum in all megacolon cases (34/34).

*Hyperinnervation of enteric neurons in the proximal colon from Ncx/Hox11L.1 deficient mice.* To examine the pathogenesis of megacolon in *Ncx*<sup>-/-</sup> mice, histological analyses of enteric ganglia were performed in the narrow segment of the megacolon. Unexpectedly, enteric ganglia were detected in the segment, although the numbers of ganglia were variable among the mice. Thus, functions of the ganglia were analyzed by examining the production of NADPH diaphorase and substance P in the neuronal cells. NADPH diaphorase is identical to nitric oxide synthase (26–29) and nitric oxide functions as an inhibitory neurotransmitter to relax smooth muscle (30), while substance P is an excitatory neurotransmitter to contract circu-

lar smooth muscles (31, 32). NADPH diaphorase positive neuronal cells were dense in the proximal colon of *Ncx*<sup>-/-</sup> mice with (data not shown) or without megacolon (Fig. 3, B and D). Numbers of the neuronal cells were counted in ileum and proximal colon from *Ncx*<sup>-/-</sup> mice at 3 wk of age without megacolon (Fig. 4). The number ( $185 \pm 41$  cells/mm<sup>2</sup>) in the proximal colon from *Ncx*<sup>-/-</sup> mice exceeded that ( $78 \pm 15$  cells/mm<sup>2</sup>) from wild-type littermates, although the numbers in the cecum were similar between them. The number of substance P-positive neuronal cells and fibers also increased in the proximal colon of *Ncx*<sup>-/-</sup> mice (Fig. 5, A and B). This increase was confirmed using antibodies specific for another neurotransmitter, neuropeptide Y (33) (Fig. 5, C and D). These results indicate that these enteric neurons hyperinnervate in the colon.



**Figure 3.** Histological analysis of proximal colon from *Ncx/Hox11L.1* deficient mice. (*A* and *B*) A whole mount NADPH diaphorase staining of the proximal colon. (*C* and *D*) NADPH diaphorase histochemistry in longitudinal sections of the proximal colon. The proximal colons were isolated from a 3-wk-old wild-type littermate (*A* and *C*) and an asymptomatic *Ncx*<sup>-/-</sup> mouse (*B* and *D*). Bars: *A* and *B*, 300  $\mu\text{m}$ ; *C* and *D*, 100  $\mu\text{m}$ .

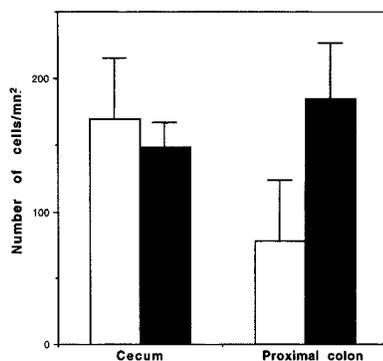
*Degeneration of the enteric ganglia in the narrow segment of colon from Ncx/Hox11L.1 deficient mice.* In some cases of the megacolon (7/12) from *Ncx*<sup>-/-</sup> mice after 6 wk of age, each ganglion was flat and degenerated in the narrow segment (Fig. 6 *B*). A thickness of smooth muscle layer also decreased in the segment. The TdT-mediated dUTP-biotin nick end labeling method (34) identified much cell death in areas of the ganglia (Fig. 6, *C* and *D*). Ultrastructurally, a ganglion of the enteric plexus exhibited degenerative changes with an irregu-

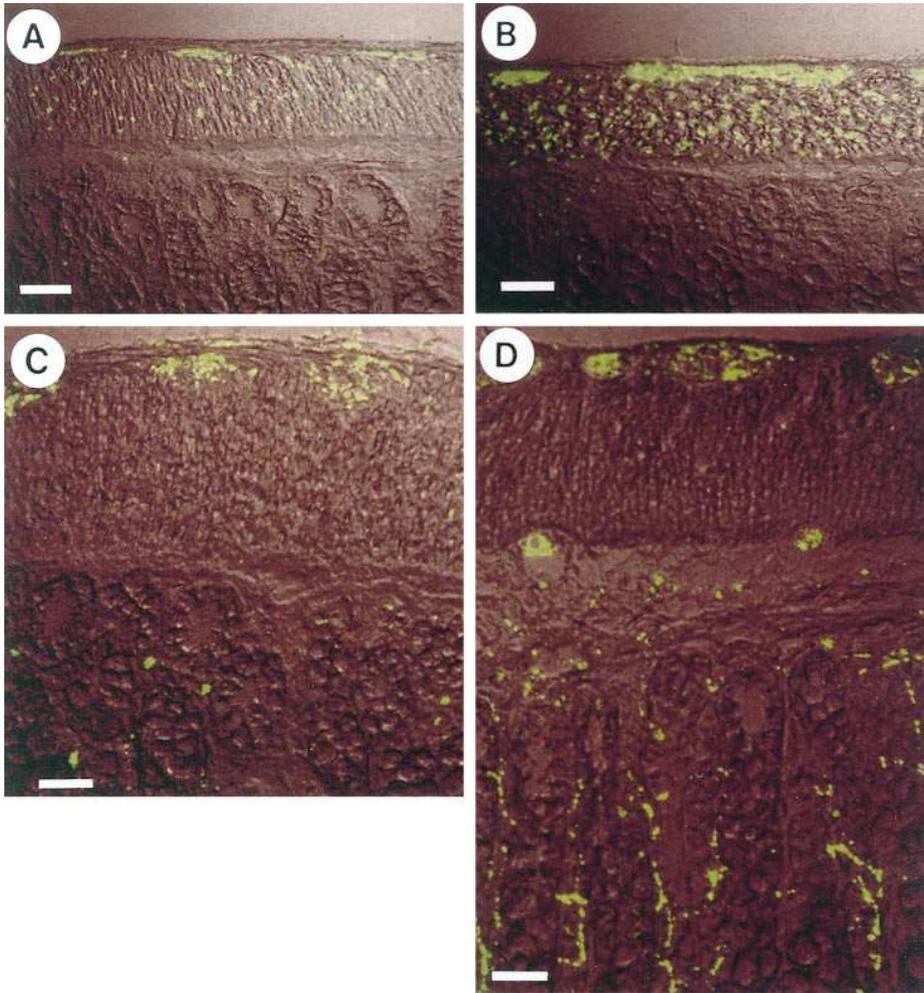
larly outlined nucleus containing a condensed chromatin (Fig. 6 *E*). On the other hand, degenerative changes were not detected in ganglia of the dilated segment of colon from the same *Ncx*<sup>-/-</sup> mouse and in ganglia of the proximal colon from wild-type littermates (data not shown).

## Discussion

Megacolon developed in *Ncx*<sup>-/-</sup> mice has several distinct characteristics from that in mutant mice lacking the c-Ret (18, 19) and its ligand, the glial cell line-derived neurotrophic factor (35, 36), or the endothelin-B receptor (21) and its ligand, the endothelin-3 (20), or the dominant megacolon (*dom*) (37–39). First, *Ncx*<sup>-/-</sup> mice develop megacolon with hyperinnervated enteric neurons although megacolon in the latter mutant mice is caused by the absence of enteric neurons in colon. In addition, the hyperinnervation was observed specifically in the proximal part of colon from *Ncx*<sup>-/-</sup> mice. Second, the transitional zone between narrow and dilated segments of megacolon is almost constant in the proximal colon from *Ncx*<sup>-/-</sup> mice although the zone is variable in the hindgut from the latter mutant mice. As signals through c-Ret and endothelin-B receptor in neural crest cells are essential for their migration into colon (40), the neural crest cells cannot colonize into their proper position in colon from the latter mutant mice. However, neural crest cells migrated and colonized well in *Ncx*<sup>-/-</sup>

**Figure 4.** Numbers of NADPH positive neurons in the colon from *Ncx/Hox11L.1* deficient mice. The colons were isolated from 3-wk-old wild-type littermates and asymptomatic *Ncx*<sup>-/-</sup> mice. Numbers of NADPH-positive neuronal cells per  $\text{mm}^2$  in the cecum and the proximal colon were calculated in the wild-type (*open bar*) and the *Ncx*<sup>-/-</sup> mice (*closed bar*).





**Figure 5.** Immunohistochemistry of the proximal colon from *Ncx/Hox11L.1* deficient mice. (A and B) Fluorescent immunohistochemistry with an antibody to substance P. (C and D) Fluorescent immunohistochemistry with an antibody to neuropeptide Y. The proximal colons were isolated from a 3-wk-old wild-type littermate (A and C) and an asymptomatic *Ncx*<sup>-/-</sup> mouse (B and D). Bars: A and B, 50  $\mu$ m; C and D, 25  $\mu$ m.

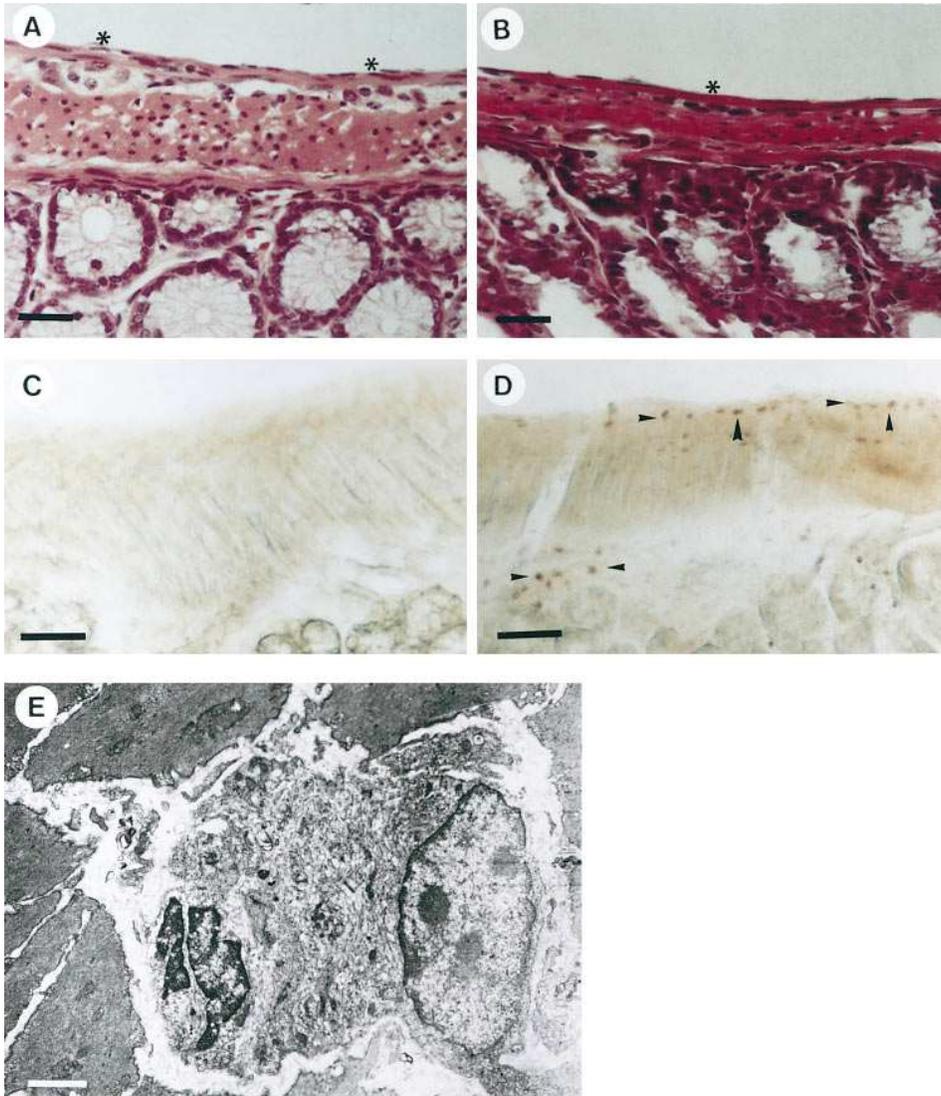
mice, indicating that the pathogenesis of megacolon in *Ncx*<sup>-/-</sup> mice is novel.

The pathogenesis of megacolon with the absence of enteric neurons can be explained by the abnormal movement of colon. Loss of neurons below normal range is also responsible for the pathological changes in some neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinsonism, and Alzheimer's disease (41–45), and in normal aging (42). In contrast, pathological significance of an increased number of neurons is not well established. The similar results were observed in *fyn* deficient mice in which an increased number of neurons in hippocampus causes learning abnormality (46). The motor activity of alimentary tract is controlled by the balance of activities between inhibitory and excitatory neurons in a complex manner. Immunohistochemical analysis of the enteric ganglia in *Ncx*<sup>-/-</sup> mice showed that both inhibitory and excitatory neurons hyperinnervated (Figs. 4 and 5). The hyperinnervation may cause inappropriate activation of enteric neurons and may result in a functional abnormality of the colon.

The hyperinnervation of enteric neurons in *Ncx*<sup>-/-</sup> mice suggests the function of *Ncx/Hox11L.1* in the development of enteric neurons. Neural crest cells migrate into a gastrointestinal tract at E9.5 and differentiate into enteric ganglia (47). Since enteric neurons hyperinnervated in the proximal part of colon from *Ncx*<sup>-/-</sup> mice, *Ncx/Hox11L.1* might negatively reg-

ulate the migration of neural crest cells. However, the hyperinnervation was not distinct in *Ncx*<sup>-/-</sup> mice by 2 wk of age (data not shown). These results suggested the following possibilities to explain the hyperinnervation. First, cell death in the enteric neurons might be prevented in *Ncx*<sup>-/-</sup> mice since neuronal cell death takes place during the normal development of enteric neurons (48). Second, the enteric neuroblasts in *Ncx*<sup>-/-</sup> mice might proliferate more than these in wild-type mice. However, it is not likely because the number of NADPH diaphorase positive neuronal cells did not increase in *Ncx*<sup>-/-</sup> mice after birth (our unpublished observation). Third, the hyperinnervation might be a secondary reaction to compensate for a functional abnormality in the enteric nervous system of *Ncx*<sup>-/-</sup> mice. However, it is less likely because both inhibitory and excitatory neurons hyperinnervated (Figs. 4 and 5). Further study is required to elucidate the function of *Ncx/Hox11L.1* in the development of enteric neurons.

The human homologue of the *Ncx/Hox11L.1* gene may be involved in the human congenital megacolon. Hirschsprung's disease is characterized by the congenital absence of the enteric ganglia from the hindgut. Mutations in the *c-RET* gene (22, 24), the glial cell line-derived neurotrophic factor gene (49, 50), the endothelin-3 gene (51), and the endothelin-B receptor gene (23) were identified in Hirschsprung's disease. Neuronal intestinal dysplasia is a human congenital disorder



**Figure 6.** Histology of a megacolon from *Ncx/Hox11L.1* deficient mice. (A and B) Hematoxylin and eosin staining of longitudinal sections of the proximal colon from 4-wk-old wild-type mouse (A) and *Ncx*<sup>-/-</sup> mouse (B). Asterisks indicate each enteric ganglion. (C and D) In situ TdT-mediated dUTP-biotin nick end labeling method analysis of the proximal colon from a wild-type littermate (C) and a *Ncx*<sup>-/-</sup> mouse (D). Arrowheads indicate apoptotic myenteric neurons or submucosal neurons. (E) Transmission electron micrograph of enteric ganglion in the proximal colon from *Ncx*<sup>-/-</sup> mouse with megacolon. Bars: A and B, 30  $\mu$ m; C and D, 50  $\mu$ m; E, 2  $\mu$ m.

which is characterized by a megacolon with a normal number of ganglia or hyperplasia of enteric neurons (52, 53). Therefore, the human homologue of the *Ncx/Hox11L.1* gene can be a candidate gene for neuronal intestinal dysplasia. Overall, our animal model provides a unique opportunity to investigate a novel pathogenesis of megacolon and neuronal cell death.

## Acknowledgments

We thank Drs. S. Yuasa, S. Okada, and G. Ishii for discussion, Y. Iwata for skillful technical assistance, and E. Furusawa for secretarial assistance.

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science, Sports and Culture of Japan and Mochida Memorial Foundation for Medical and Pharmaceutical Research.

## References

1. Krumlauf, R. 1994. Hox genes in vertebrate development. *Cell* 78:191-201.
2. Lin, C., S.-C. Lin, C.-P. Chang, and M.G. Rosenfeld. 1992. Pit-1-dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. *Nature (Lond.)* 360:765-768.
3. Jonsson, J., L. Carlsson, T. Edlund, and H. Edlund. 1994. Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature (Lond.)* 371:606-609.
4. Lyons, I., L.M. Parsons, L. Hartley, R. Li, J. Andrews, L. Robb, and R.P. Harvey. 1995. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene *Nkx2-5*. *Genes Dev.* 9:1654-1666.
5. Hentsch, B., I. Lyons, R. Li, L. Hartley, T.J. Lints, J.M. Adams, and R.P. Harvey. 1996. *Hlx* homeo box gene is essential for an inductive tissue interaction that drives expansion of embryonic liver and gut. *Genes Dev.* 10:70-79.
6. Dubé, I.D., S. Kamel-Reid, C.C. Yuan, M. Lu, X. Wu, G. Corpus, S.C. Raimondi, W.M. Crist, A.J. Carroll, J. Minowada, and J.B. Baker. 1991. A novel human homeobox gene lies at the chromosome 10 breakpoint in lymphoid neoplasias with chromosomal translocation t(10;14). *Blood* 78:2996-3003.
7. Hatano, M., C.W. Roberts, M. Minden, W.M. Crist, and S.J. Korsmeyer. 1991. Deregulation of a homeobox gene, HOX11, by the t(10;14) in T cell leukemia. *Science (Wash. DC)* 253:79-82.
8. Kennedy, M.A., R. Gonzalez-Sarmiento, U.R. Kees, F. Lampert, N. Dear, T. Boehm, and T.H. Rabbitts. 1991. HOX11, a homeobox-containing T-cell oncogene on human chromosome 10q24. *Proc. Natl. Acad. Sci. USA* 88:8900-8904.
9. Lu, M., Z. Gong, W. Shen, and A.D. Ho. 1991. The *tel-3* proto-oncogene altered by chromosomal translocation in T-cell leukemia codes for a homeobox protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2905-2910.
10. Roberts, C.M.W., J.R. Shutter, and S.J. Korsmeyer. 1994. Hox11 controls the genesis of the spleen. *Nature (Lond.)* 368:747-749.
11. Dear, T.N., W.H. Colledge, M.B. Carlton, I. Lavenir, T. Larson, A.J. Smith, A.J. Warren, M.J. Evans, M.V. Sofroniew, and T.H. Rabbitts. 1995. The *Hox11* gene is essential for cell survival during spleen development. *Development (Camb.)* 121:2909-2915.

12. Dear, T.N., I. Sanchez-Garcia, and T.H. Rabbitts. 1993. The HOX11 gene encodes a DNA-binding nuclear transcription factor belonging to a distinct family of homeobox genes. *Proc. Natl. Acad. Sci. USA.* 90:4431–4435.
13. Raju, K., S. Tang, I.D. Dubé, S. Kamel-Reid, D.M. Bryce, and M.L. Breitman. 1993. Characterization and developmental expression of Tlx-1, the murine homolog of HOX11. *Mech. Dev.* 44:51–64.
14. Wen, X.-Y., S. Tang, and M.L. Breitman. 1994. Genetic mapping of two mouse homeobox genes, Tlx-1 and Tlx-2 to murine chromosome 19 and 6. *Genomics.* 24:388–390.
15. Hatano, M., Y. Iitsuka, H. Yamamoto, M. Dezawa, S. Yusa, Y. Kohno, and T. Tokuhisa. 1997. Ncx, a Hox11 related gene, is expressed in a variety of tissues derived from neural crest cells. *Anat. Embryol.* 195:419–425.
16. Rugh, R. 1990. Organogeny. In *The Mouse*. R. Rugh, editor. Oxford University Press, New York. 237–262.
17. Selleck, M.A.J., T.Y. Scherson, and M. Bronner-Fraser. 1993. Origins of neural crest cell diversity. *Dev. Biol.* 159:1–11.
18. Schuchardt, A., V. D'Agati, L. Larsson-Blomberg, F. Costantini, and V. Pachnis. 1994. Defect in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature (Lond.)*. 367:380–383.
19. Durbec, P.L., L.B. Larsson-Blomberg, A. Schuchardt, F. Costantini, and V. Pachnis. 1996. Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. *Development (Camb.)*. 122:349–358.
20. Baynash, A.G., K. Hosoda, A. Giaid, J.A. Richardson, N. Emoto, R.E. Hammer, and M. Yanagisawa. 1994. Interaction of endothelin-3 with endothelin-B receptors is essential for development of epidermal melanocytes and enteric neurons. *Cell*. 79:1277–1285.
21. Hosoda, K., R.H. Hammer, J.A. Richardson, A.G. Baynash, J.C. Cheung, A. Giaid, and M. Yanagisawa. 1994. Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell*. 79:1267–1276.
22. Edery, P., S. Lyonnet, L.M. Mulligan, A. Pelet, E. Dow, L. Abel, S. Holder, C. Nihoul-Fekete, B.A.J. Ponder, and A. Munnich. 1994. Mutation of the RET proto-oncogene in Hirschsprung's disease. *Nature (Lond.)*. 367:378–380.
23. Puffenberger, E.G., K. Hosoda, S.S. Washington, K. Nakao, D. de Wit, M. Yanagisawa, and A. Chakravarti. 1994. A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. *Cell*. 79:1257–1266.
24. Romeo, G., P. Ronchetto, Y. Luo, V. Barone, M. Seri, I. Ceccherini, B. Pasini, R. Bocciarelli, M. Lerone, H. Kaariainen, and G. Martucciello. 1994. Point mutation affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. *Nature (Lond.)*. 367:377–378.
25. Wood, S.A., N.D. Allen, J. Rossant, A. Auerbach, and A. Nagy. 1993. Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature (Lond.)*. 365:87–89.
26. Hope, B.T., G.J. Michael, K.M. Knigge, and S.R. Vincent. 1991. Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc. Natl. Acad. Sci. USA.* 88:2811–2814.
27. Belai, A., H.H.H.W. Schmidt, C.H.V. Hoyle, C.J.S. Hassall, M.J. Safrey, J. Moss, U. Forstermann, F. Murad, and G. Burnstock. 1992. Colocalization of nitric oxide synthase and NADPH-diaphorase in the myenteric plexus of the rat gut. *Neurosci. Lett.* 143:60–64.
28. Young, H.M., J.B. Furness, C.W.R. Shuttleworth, D.S. Bredt, and S.H. Snyder. 1992. Co-localization of nitric oxide synthase immunoreactivity and NADPH diaphorase staining in neurons of the guinea-pig intestine. *Histochemistry*. 97:375–378.
29. Neuhuber, W.L., J. Worl, H. Berthoud, and B. Conte. 1994. NADPH-diaphorase-positive nerve fibers associated with motor endplates in the rat esophagus: new evidence for co-innervation of striated muscle by enteric neurons. *Cell Tissue Res.* 276:23–30.
30. Bult, H., G.E. Boeckstaens, P.A. Pelckmans, F.H. Jordaens, Y.M. Van Maercke, and A.G. Herman. 1990. Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature (Lond.)*. 345:346–347.
31. Stanfield, P.R., Y. Nakajima, and K. Yamaguchi. 1985. Substance P raises neuronal membrane excitability by reducing inward rectification. *Nature (Lond.)*. 315:498–501.
32. Peters, S., and D.L. Kreulen. 1986. Fast and slow synaptic potentials produced in a mammalian sympathetic ganglion by colon distension. *Proc. Natl. Acad. Sci. USA.* 83:1941–1944.
33. Su, H.C., A.E. Bishop, R.F. Power, Y. Hamada, and J.M. Polak. 1987. Dual intrinsic and extrinsic origins of CGRP- and NPY-immunoreactive nerves of rat gut and pancreas. *J. Neurosci.* 7:2674–2687.
34. Gavrieli, Y., Y. Sherman, and S.A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119:493–501.
35. Pichel, J.G., L. Shen, H.Z. Sheng, A.C. Granholm, J. Drago, A. Grinberg, E.J. Lee, S.P. Huang, M. Saarma, B.J. Hoffer, H. Sariola, and H. Westphal. 1996. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature (Lond.)*. 382:73–75.
36. Sanchez, M.P., I. Silos-Santiago, J. Frisen, B. He, S.A. Lira, and M. Barbacid. 1996. Renal agenesis and absence of enteric neurons in mice lacking GDNF. *Nature (Lond.)*. 382:70–73.
37. Bolande, R.P. 1975. Hirschsprung's disease, aganglionic or hypoganglionic megacolon. Animal model: aganglionic megacolon in piebald and spotted mutant mouse strains. *Am. J. Pathol.* 79:189–192.
38. Lane, P.W., and H.M. Liu. 1984. Association of megacolon with a new dominant spotting gene (Dom) in the mouse. *J. Hered.* 57:29–31.
39. Kapur, R.P., R. Livingston, B. Doggett, D.A. Sweetser, J.R. Siebert, and R.D. Palmiter. 1996. Abnormal microenvironmental signals underlie intestinal aganglionosis in dominant megacolon mutant mice. *Dev. Biol.* 174:360–369.
40. Kapur, R.P., D.A. Sweetser, B. Doggett, J.R. Siebert, and R.D. Palmiter. 1995. Intercellular signals downstream of endothelin receptor-B mediate colonization of the large intestine by enteric neuroblast. *Development (Camb.)*. 121:3787–3795.
41. Appel, S.H. 1981. A unifying hypothesis for the cause of amyotrophic lateral sclerosis, Parkinsonism and Alzheimer disease. *Ann. Neurol.* 103:443–445.
42. Coleman, P.D., and D.G. Flood. 1987. Neuron numbers and dendritic extent in normal aging and Alzheimer's disease. *Neurobiol. Aging*. 8:521–545.
43. Saper, C.B., B.H. Wainer, and D.C. German. 1987. Axonal and transneuronal transport in the transmission of neurological disease: potential role in systems degenerations, including Alzheimer's disease. *Neuroscience*. 23:389–398.
44. Snider, W.D., and E.M. Johnson. 1989. Neurotrophic molecules. *Ann. Neurol.* 26:489–506.
45. Griffin, J.W., and D.F. Watson. 1988. Axonal transport in neurological disease. *Ann. Neurol.* 23:3–13.
46. Grant, S.G.N., T.J. O'Dell, K.A. Karl, P.L. Stein, P. Soriano, and E.R. Kandel. 1992. Impaired long-term potentiation, spatial learning, and hippocampal development in *fyn* mutant mice. *Science (Wash. DC)*. 258:1903–1910.
47. Serbedzija, G.N., S. Burgan, S.E. Fraser, and M. Bronner-Fraser. 1991. Vital dye labeling demonstrates a sacral neural crest contribution to the enteric nervous system of chick and mouse embryos. *Development (Camb.)*. 111:857–866.
48. Oppenheim, R.W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14:453–501.
49. Angrist, M., S. Bolk, M. Halushka, P.A. Lapchak, and A. Chakravarti. 1996. Germline mutations in *glis1* cell line-derived neurotrophic factor (GDNF) and RET in a Hirschsprung disease patient. *Nat. Genet.* 14:341–344.
50. Salomon, R., T. Attie, A. Pelet, C. Bidaud, C. Eng, J. Amiel, S. Sarnacki, O. Goulet, C. Ricour, C. Nihoul-Fekete, A. Munnich, and S. Lyonnet. 1996. Germline mutations for the RET ligand GDNF are not sufficient to cause Hirschsprung disease. *Nat. Genet.* 14:345–347.
51. Edery, P., T. Attie, J. Amiel, A. Pelet, C. Eng, R.M. Hofstra, H. Martelli, C. Bidaud, A. Munnich, and S. Lyonnet. 1996. Mutation of the endothelin-3 gene in the Waardenburg-Hirschsprung disease (Shah-Waardenburg syndrome). *Nat. Genet.* 12:442–444.
52. McMahon, R.A., C.C.M. Moore, and L.J. Cussen. 1981. Hirschsprung-like syndromes in patients with normal ganglion cells on suction rectal biopsy. *J. Pediatr. Surg.* 16:835–839.
53. Munakata, K., K. Morita, I. Okabe, and H. Sueoka. 1985. Clinical and histologic studies of neuronal intestinal dysplasia. *J. Pediatr. Surg.* 20:231–235.