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Dispensable role of protein 4.1B/DAL-1 in rodent adrenal medulla regarding generation of pheochromocytoma and plasmalemmal localization of TSLC1

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ABSTRACT

Protein 4.1B is a membrane skeletal protein expressed in various organs, and is associated with tumor suppressor in lung cancer-1 (TSLC1) in vitro. Although involvement of 4.1B in the intercellular junctions and tumor-suppression was suggested, some controversial results posed questions to the general tumor-suppressive function of 4.1B and its relation to TSLC1 in vivo. In this study, the expression of 4.1B and its interaction with TSLC1 were examined in rodent adrenal gland, and the involvement of 4.1B in tumorigenesis and the effect of 4.1B deficiency on TSLC1 distribution were also investigated using rodent pheochromocytoma and 4.1B-knockout mice. Although plasmalemmal immunolocalization of 4.1B was shown in chromaffin cells of rodent adrenal medulla, expression of 4.1B was maintained in developed pheochromocytoma, and morphological abnormality or pheochromocytoma generation could not be found in 4.1B-deficient mice. Furthermore, molecular interaction and colocalization of 4.1B and TSLC1 were observed in mouse adrenal gland, but the immunolocalization of TSLC1 along chromaffin cell membranes was not affected in the 4.1B-deficient mice. These results suggest that the function of 4.1B as tumor suppressor might significantly differ among organs and species, and that plasmalemmal retention of TSLC1 would be maintained by molecules other than 4.1B interacting in rodent chromaffin cells.

1. Introduction

Proteins associated with membrane skeletons are essential for structural integrity via cellular morphogenesis and intercellular adhesion, and also clearly related to intracellular signal transduction [1–4]. Protein 4.1B (4.1B), which is also known as differentially expressed in adenocarcinoma of the lung-1 (DAL-1), is one of the proteins associated with the membrane skeleton. It was originally identified as a molecule composing the junctional complex between axons and myelinating cells in the paranodal and juxtaparanodal regions of the peripheral and central nervous systems [5–7]. The distribution of 4.1B along cell membranes in organs outside the nervous system, such as kidney, intestine and pancreatic islets, suggest that 4.1B would also be involved in intercellular junctions via transmembranous molecules in different organs [8–11]. On the other hand, 4.1B was identified as a molecule whose expression decreased in non-small cell lung carcinoma [12]. Several studies in different cancers such as non-small cell lung carcinoma, meningioma, ependymoma, breast cancer, colorectal carcinoma and prostate cancer supported the tumor-suppressive function of 4.1B in general [13–17]. Tumor suppressor in lung cancer-1 (TSLC1) is another tumor suppressor responsible for the development of various cancers, and is also known as IGSF4/Necl-2/SgIGSF/RA175/SynCAM1. Direct binding of 4.1B to TSLC1 was postulated to mediate the interaction between TSLC1 and actin membrane skeletons, and impairment of the 4.1B-TSLC1 system was found in lung and breast cancers [18–23]. Although these results showed significant involvement of 4.1B in multi-organ carcinogenesis and related tumor-suppressive functions of 4.1B and TSLC1, genetic loss of 4.1B in mice did not result in abnormal development or higher frequency of tumor generation [24]. In addition, reduced expression of TSLC1, but not 4.1B, was found in the generation of rat hepatocellular carcinoma [25]. These discordant results pose questions concerning the general tumor-suppressive function of 4.1B, as well as its relationship with TSLC1 in vivo.

In the present study, expression of 4.1B and its interaction with TSLC1 were examined in rodent adrenal gland. The involvement of 4.1B in tumorigenesis and the effect of 4.1B deficiency on TSLC1

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distribution were also investigated using rodent pheochromocytoma specimens and our 4.1B-knockout mouse model.

2. Materials and methods

2.1. Derivation of rodent pheochromocytoma and 4.1B-knockout mice

All animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee, University of Yamanashi. Rat spontaneous pheochromocytomas were obtained from Wistar and Sprague–Dawley rats, as described previously [26]. A mouse model of multiple endocrine neoplasia type 2B (MEN2B) was produced by substituting methionine at the codon 919 of Ret oncogene to threonine, as described previously [27]. The MEN2B mice were inbred on C57BL/6J background, and used for histological analyses. For 4.1B-knockout mice, DNA fragments located upstream and downstream of the 4.1B gene exons 2 and 3 were obtained from genomic DNA of 129/Sv embryonic stem cells by PCR using TaKaRa LA Taq (TaKaRa Bio, Otsu, Shiga, Japan). The fragments were directly cloned and sequenced with a TOPO XL PCR cloning kit (Invitrogen, Carlsbad, CA) to be >99.8% identical to the original 129/Sv mouse genomic sequences. The fragments were then subcloned to the PGKneolox2DTA vector [28], and flanked a neo^R expression cassette (Fig. 1A). This targeting vector replaces a 2.8-kb genomic fragment containing exons 2 and 3. The lineated construct was electroporated into 129/S4-derived AK7 embryonic stem (ES) cells [28], and colonies were selected with G418. Homologous recombination events were screened by the PCR, using a set of primers corresponding to the Neo gene and a genomic sequence outside the targeting construct. ES cells were injected into blastocysts of C57BL/6 mice, and the chimeric mice generated were crossed with C57BL/6 mice for several generations.

2.2. Genotyping of 4.1B-knockout mice by Southern blotting and PCR

First, we genotyped wild type and heterozygote mice obtained from the chimeric mice. For genotyping, Southern blotting was performed with the digoxigenin application system according to the manufacturer's instructions (Roche Diagnostic Systems, Basel, Switzerland). The DNA obtained from liver biopsy was digested with XbaI and blotted on Hybond-N+ (GE Healthcare, Buckinghamshire, England). A probe was produced and labeled with digoxigenin through PCR of a region located in the short arm (Fig. 1A), using a PCR DIG Probe Synthesis Kit (Roche Diagnostic Systems, Basel, Switzerland). The blotted DNA and the probe were hybridized with Easy Hyb, anti-DIG antibody, blocking solution and CDP Star (Roche Diagnostic Systems, Basel, Switzerland). The XbaI digestion generated a 5.8-kb band in the wild type allele and a 2.0-kb band in the mutant allele following homologous recombination (Fig. 1A, B).

Subsequent offspring were genotyped by PCR, using DNA obtained from tail biopsy and primers of 5′-AGGTGACTTCGCAGTGTTCC-3′, 5′-ATTGGAAGCCTTGAGCAGCA-3′ and 5′-TATCGCCTTCTTGACGAGTTC-3′. A PCR reaction for the wild type allele produced a 0.5-kb DNA fragment in wild type and heterozygous mice, and for the mutant allele produced a 0.5-kb DNA fragment in heterozygous and homozygous mice (Fig. 1A, C).

2.3. Antibodies

The following antibodies were commercially obtained: a rabbit polyclonal anti-4.1B (ProteinExpress, Chiba, Japan), which was produced as described previously [7], goat anti-synaptosomal-associated protein of 25 kDa (SNAP-25; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-neural cell adhesion molecule (NCAM; Chemicon, Temecula, CA, USA) and chicken anti-TSLC1 (clone 3E1; Medical and Biological Laboratories, Nagoya, Japan). Secondary antibodies and streptavidin conjugated with Alexa fluorescent dye were obtained from Invitrogen (Carlsbad, CA). Biotinylated goat anti-rabbit and donkey anti-rabbit antibodies were obtained from Vector laboratories (Burlingame, CA) and chicken antibodies were obtained from Jackson immunoresearch laboratories (West Grave, PA, USA).

2.4. Western blotting and immunoprecipitation analyses

The tissue of C57BL/6 mice at 8 weeks of age was homogenized in Tris buffer (40 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA,

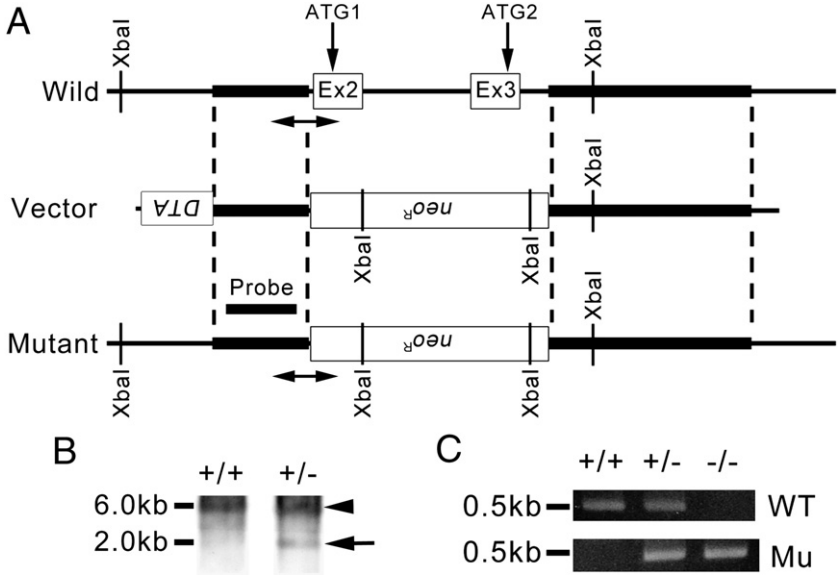

Fig. 1. (A) Construction of the targeting vector (Vector) and organization of the 4.1B gene of wild type (Wild) and mutant mice (Mutant). DTA: diphtheria toxin; Ex: exon; neo^R: neo-resistance gene; ATG1 and ATG2: the translation initiation codons of 4.1B; Probe: a region of a digoxigenin-labeled probe for Southern blotting; bidirectional arrows: the target of PCR reaction for genotyping of offspring. (B) In the Southern blotting, using the 1-kb region upstream of exon 2 as a probe, the genomic DNA of wild type mice (+/+) digested with XbaI provided a 5.8-kb fragment (arrowhead), whereas that of heterozygous mice (+/−) provided 5.8-kb (arrowhead) and 2.0-kb (arrow) fragments. (C) The PCR reaction for wild type allele (WT) produces an approximately 0.5-kb DNA fragment in wild type and heterozygous mice, whereas that for the mutant allele (Mu) produces an approximately 0.5-kb DNA fragment in heterozygous and homozygous mice (−/−).

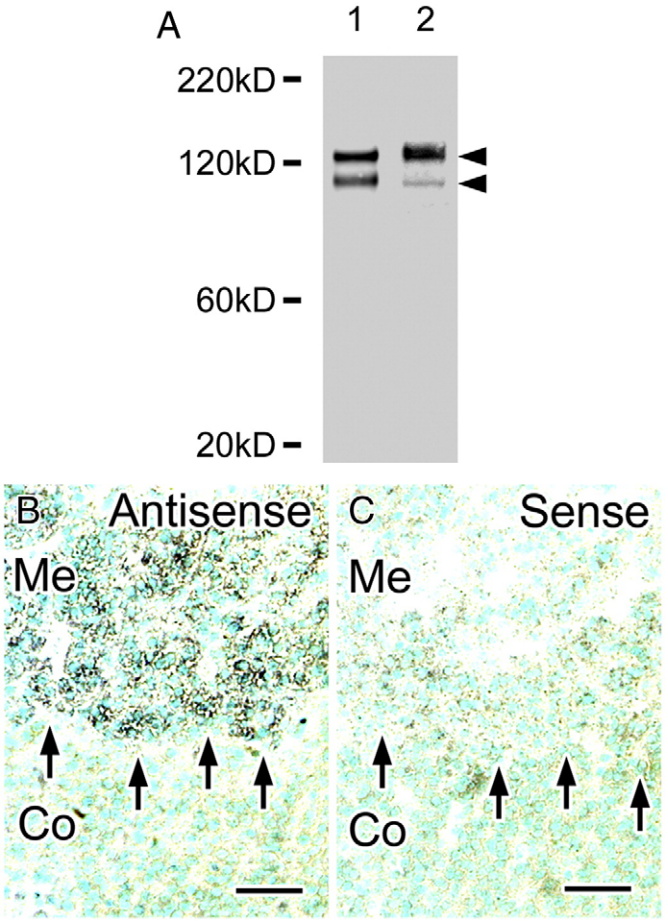

Fig. 2. (A) Western blotting analysis of 4.1B/differentially expressed in adenocarcinoma of the lung-1 (4.1B) in mouse brain (lane 1) and adrenal gland (lane 2). Specific bands with molecular weights of approximately 130 and 110 kDa can be detected in both organs (arrowheads). (B, C) In situ hybridization for 4.1B mRNA in rat adrenal gland. Reaction products can be observed in adrenal medulla (Me) with the antisense probe (B, arrows), but not with the sense probe (C, arrows). Co: adrenal cortex. Bars: 50 μm.

250 mM sucrose) containing 1% Triton X-100 and 10% v/v protease inhibitor cocktail (Sigma, St. Louis, MO, USA), incubated at 4 °C for 1 h, and centrifuged at 10,000 *g* for 20 min. Protein concentration was measured using a BCA Protein Assay Kit (Pierce Chemicals, Rockford, IL). For western blotting, the supernatants were mixed with an equal amount of Laemmli sample treatment buffer, and SDS-PAGE and western blotting were performed with the anti-4.1B or TSLC1 antibodies, as described previously [15]. For the immunoprecipitation assays, the supernatant was diluted with an equal volume of Tris buffer and incubated with protein G-Sepharose (GE Healthcare Bio-Science, Piscataway, NJ) for 1 h, with 15 μg anti-4.1B antibody or purified rabbit control IgG (Zymed Laboratories, South San Francisco, CA) overnight, and with the protein G-Sepharose for 1 h. Thereafter, the beads were washed with Tris buffer containing 0.5% TritonX-100 three times for 10 min each, and boiled in Laemmli sample treatment buffer. SDS-PAGE and western blotting were performed as described above.

2.5. In situ hybridization

Adrenal gland tissue of 8-weeks-old Wistar rats was quickly frozen by plunging into isopentane–propane cryogen, as described previously [29]. Then 14 μm-thick sections were mounted on glass slides, air-dried, briefly incubated in chloroform, and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min. Both anti-sense and sense complementary RNA probes against 4.1B mRNA were prepared, quantified and standardized, as described previously [6]. They were then treated with 0.2 M hydrogen chloride for 20 min and 1 mg/l protease K in PBS at 37 °C for 15 min. After post-fixation in 4% paraformaldehyde in PBS for 10 min, they were incubated in 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 20 min, in 2× saline-sodium citrate (SSC) with 50% formamide at 55 °C for 30 min, and in Hybridization buffer (Nippon Gene, Tokyo, Japan) containing approximately 200 ng/ml sense or anti-sense probe at 55 °C for 16 h. The sections were washed in 4× SSC at 55 °C for 5 min and in 2× SSC containing 50% formamide for 30 min, and incubated in NTE buffer (500 mM NaCl, 10 mM Tris–HCl, 1 mM

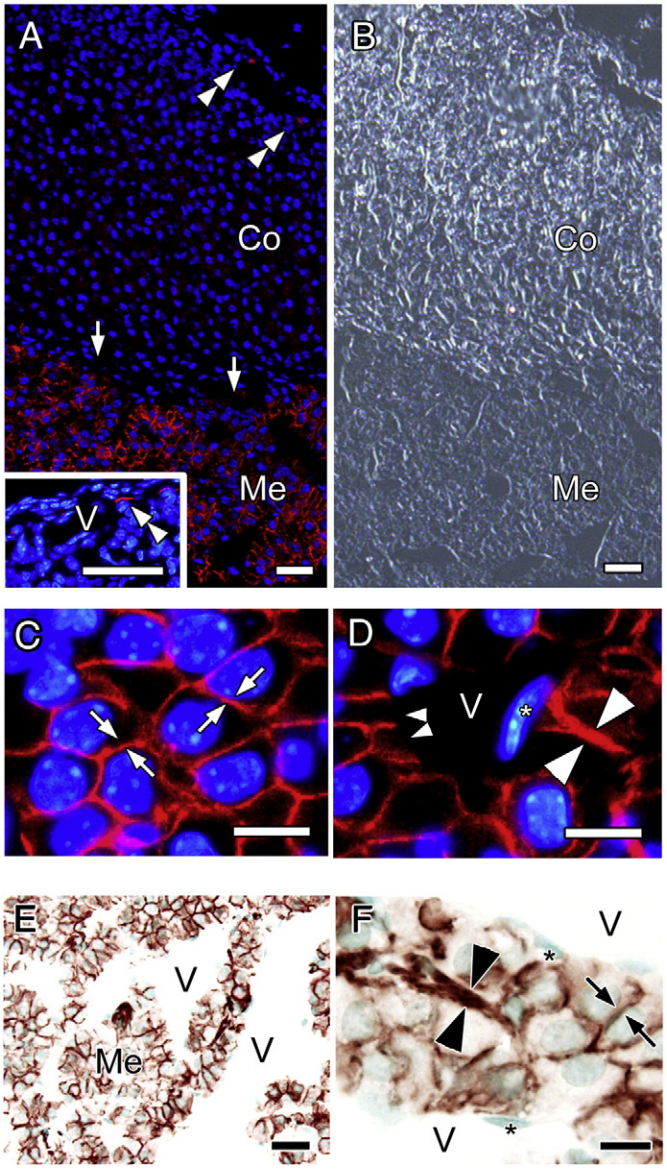

Fig. 3. Immunostaining for 4.1B in mouse (A–D) or rat (E, F) adrenal gland. A differential interference contrast image of the area in (A) is shown in (B). Immunoreactivity of 4.1B is detected in adrenal medulla (Me; A, arrows), and fascicular immunostaining (A, double arrowheads) is also detected in the cortex (Co). At higher magnification, the immunoreactivity in the cortex (A inset, double arrowhead) is frequently detected around blood vessels (V). In the medulla, the immunoreactivity appears to localize along cell membranes of chromaffin cells (C, arrows), and the fascicular staining is also observed (D, large arrowheads). The immunoreactivity cannot be detected on the side facing blood vessels (D, small arrowheads). In rat adrenal gland (E), similar immunostaining for 4.1B along cell membranes (F, arrows) and fascicular immunostaining (F, large arrowheads) are observed. Asterisks: endothelial cells. Bars: 30 μm in A, B, and E; 10 μm in C, D and F.

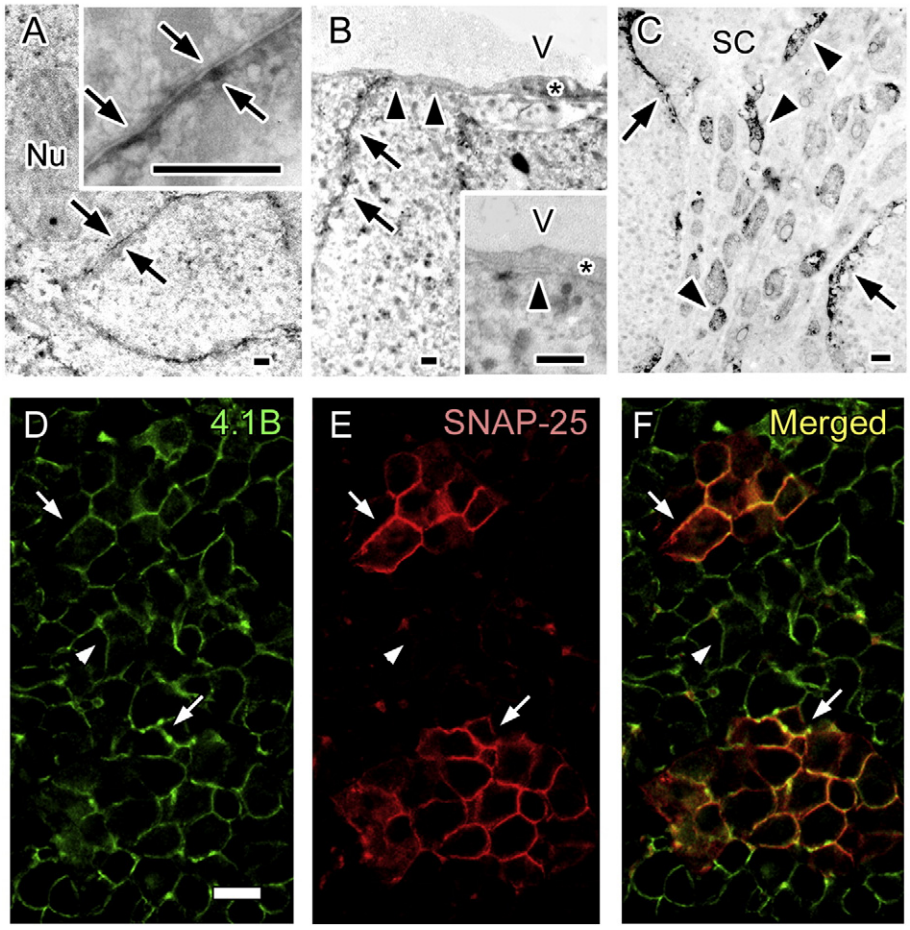

Fig. 4. (A–C) Immunoelectron micrographs for 4.1B in mouse adrenal medulla. The immunoreaction products are detected along cell membranes of chromaffin cells facing adjacent chromaffin cells (A, B, arrows) and unmyelinated nerve fibers (C, arrows) but not along those facing endothelial cells (B, arrowheads). The immunoreactivity is also detected in unmyelinated neuronal fibers (C, arrowheads). Asterisk: an endothelial cell. Nu: a nucleus of a chromaffin cell; V: a blood vessel; SC: a Schwann cell. Bars: 50 μm. (D–F) Double immunofluorescence staining for 4.1B (green) and SNAP-25 (red) in mouse adrenal medulla. Immunoreactivity of 4.1B can be detected in chromaffin cells strongly (D–F, arrows) or weakly (D–F, arrowheads) immunopositive for SNAP-25. Bar: 20 μm.

EDTA, pH 8.0) containing 10 mg/l RNase A (Nippon Gene, Tokyo, Japan) at 37 °C for 30 min. They were then washed in 2× SSC for 15 min, and in 0.2× SSC for 15 min. After a brief wash in Tris–saline buffer (100 mM Tris–HCl, 150 mM NaCl, pH 7.5), they were incubated with blocking solution (2% bovine serum albumin in Tris–saline buffer) for 1 h, with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche, Penzberg, Germany) in blocking solution for 1 h, and with visualization buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) containing 0.05 mg/ml 5-bromo-4-chloro-3-indolylphosphate and 0.1 mg/ml nitroblue tetrazolium (Kirkegaard and Perry laboratories, Guildford, UK) for approximately 12 h. They were briefly counterstained with 0.1% methylgreen and embedded in glycerol.

2.6. Immunohistochemistry

Rat pheochromocytoma was resected, fixed in 10% buffered formalin, and embedded in paraffin, as described previously [26]. Tissue preparation of other specimens and immunohistochemistry were performed as described previously [30]. Briefly, following perfusion with buffered 2% paraformaldehyde, adrenal glands of adult C57BL/6, MEN2B model mice, 4.1B-knockout mice, and Wistar rats were removed and embedded in paraffin or OCT compound. The adrenal sections on glass slides were irradiated in a microwave oven. The sections for diaminobenzidine and immunofluorescence staining were incubated in 1% hydrogen peroxide and 0.1% Triton X-100 in PBS, respectively. The sections were treated with a blocking solution containing 2% bovine serum albumin (Sigma, St. Louis, MO) in PBS, primary antibodies in the blocking solution, and secondary antibodies conjugated with biotin or Alexa in the blocking solution with or without TO-PRO-3 (Invitrogen, Carlsbad, CA, USA). Immunocontrol sections were prepared by omitting the primary antibody. The sections for immunofluorescence microscopy were embedded in Vectashield containing DAPI (Vector Laboratories. Burlingame, CA, USA) and observed with a light microscope (BX-61; Olympus, Tokyo, Japan) or with a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan). The other sections were visualized with the avidin–biotin complex and diaminobenzidine, and observed under the light microscope. Immunostaining for tyrosine hydroxylase and S-100 protein was performed as described previously [26].

2.7. Electron microscopy

Immunoelectron microscopy was performed as described previously [31]. Briefly, sections of mouse adrenal gland were immunostained for 4.1B with diaminobenzidine, and sequentially post-fixed with buffered 2.5% glutaraldehyde and 1% osmium tetroxide. The sections were dehydrated in ethanol, embedded into epoxy resin by the conventional inverted capsule method, sectioned at a thickness of 70 nm. They were then observed with a transmission electron microscope (H-7500; Hitachi, Ibaragi, Japan). For the ultrastructural analyses of 4.1B-knockout mice, mice were transcardially perfused

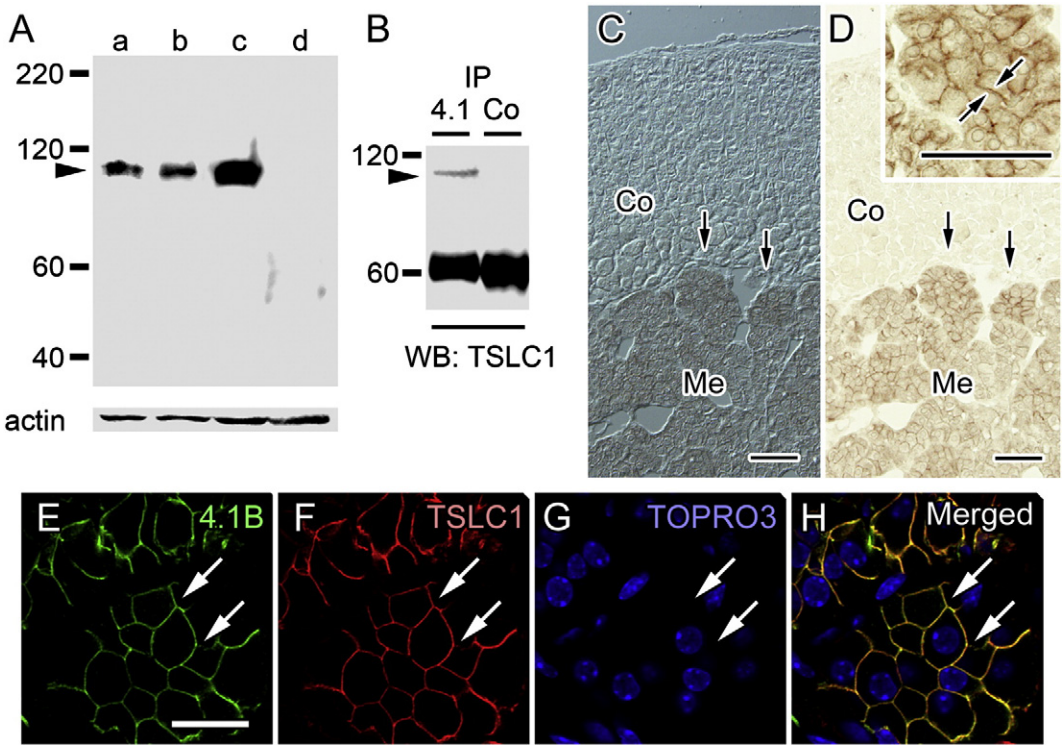

Fig. 5. (A,B) Western blotting (A, lanes a–d) and immunoprecipitation analyses (B, lanes 4.1, Co) for tumor suppressor in lung cancer-1 (TSLC1) in mouse adrenal glands. The western blotting for TSLC1 shows single bands (A, arrowhead) in adrenal gland (A, lane a), kidney (A, lane b), and lung (A, lane c), but not in heart (A, lane d), although actin is detected in all organs. A band (B, arrowhead) can be detected following immunoprecipitation with the anti-4.1B/differentially expressed in adenocarcinoma of the lung-1 (4.1B) antibody (B, lane 4.1) but not with the control IgG (B, lane Co). (C, D) Immunostaining for TSLC1 in mouse adrenal gland. The immunostained section (D) is also shown as a differential interference contrast image (C). The immunoreactivity of TSLC1 is observed in adrenal medulla (Me; C, D, arrows), and appears to localize along cell membranes of chromaffin cells (D, inset, arrows). Co: adrenal cortex. Bars: 50 μm. (E–H) Double immunofluorescence staining for 4.1B (green) and TSLC1 (red) in mouse adrenal medulla. Cellular nuclei are labeled with TOPRO3 (blue). Immunoreactivity of TSLC colocalizes with that of 4.1B on cell membranes of chromaffin cells (E–H, arrows). Bar: 20 μm.

with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer, and the collected adrenal gland specimens were embedded, sectioned, stained and analyzed as described previously [32]. All statistical comparisons were conducted utilizing a two-sided Student's *t*-test, and difference with $p<0.05$ was considered significant.

3. Results

Two isoforms of 4.1B are reported to be expressed in rat brain tissue [7]. In the western blot analyses of 4.1B using rat adrenal gland and brain tissue, two bands with similar molecular weights were detected in both organs (Fig. 2A). The lower band corresponds to a smaller isoform lacking the 5′ terminal region [33]. In situ hybridization for 4.1B mRNA was also conducted in the rat adrenal gland, and hybridization signals for 4.1B could be detected in the adrenal medulla with an antisense probe (Fig. 2B), but not with a sense probe (Fig. 2C). In the immunohistochemical analysis, immunoreactivity of 4.1B was mainly localized in the adrenal medulla (Fig. 3A, B), and fascicular immunostaining was also observed in the adrenal cortex (Fig. 3A). The fascicular immunostaining was frequently observed around blood vessels (Fig. 3A), and considered to represent unmyelinated nerve fibers [9]. There was no immunostaining for 4.1B in the immunocontrol sections (data not shown). At higher magnification, the immunoreactivity appeared to distribute along the cell membranes of chromaffin cells in the adrenal medulla (Fig. 3C). The fascicular immunostaining similar to that detected in cortex was also observed among the chromaffin cells (Fig. 3D). In the chromaffin cells, the immunoreactivity of 4.1B was not detected on the side facing the blood vessels, which was characterized by spaces delimited by endothelial cells (Fig. 3D). Similar findings were also observed in rat adrenal glands as well (Fig. 3E, F). Immunoelectron microscopy showed that the immunoreaction products for 4.1B were localized along cell membranes of chromaffin cells not in contact with the endothelial cells (Fig. 4A, B). The immunoreaction products were also observed on the unmyelinated axolemma (Fig. 4C). In the double immunofluorescence staining with SNAP-25, which predominantly immunolocalizes in noradrenergic cells of the adrenal medulla [34], the immunoreactivity of 4.1B was observed in both adrenergic and noradrenergic cells of the mouse adrenal medulla (Fig. 4D–F). These results suggest that 4.1B in rodent adrenal medulla is localized along cell membranes of all chromaffin cells.

TSLC1 expression was also examined and compared with 4.1B in the mouse adrenal glands. TSLC1 expression has been reported previously in kidney and lung tissue [35]. In western blot analyses of mouse adrenal glands using an anti-TSLC1 antibody, bands with the same molecular weight as those in mouse kidney and lung tissue were observed (Fig. 5A). Additionally, a band could be detected by western blotting against TSLC1 following the immunoprecipitation assay with the anti-4.1B antibody (Fig. 5B). The immunostaining for TSLC1 was predominantly observed in the adrenal medulla, and it appeared to immunolocalize along cell membranes of chromaffin cells (Fig. 5C, D). There was no immunostaining for TSLC1 in the immunocontrol sections (data not shown). Double immunofluorescence labeling for 4.1B and TSLC1 confirmed that 4.1B and TSLC1 co-localized on cell membranes of chromaffin cells (Fig. 5E–H). These results suggest that in the mouse adrenal medulla, TSLC1 interacts with 4.1B, and is distributed along the cell membranes of chromaffin cells.

Given the expression of 4.1B and its interaction with TSLC1, the relationship between the loss of 4.1B and tumorigenesis was examined in the adrenal medulla. First, loss of 4.1B expression in tumors was investigated immunohistochemically using the rat spontaneous pheochromocytoma [26]. In rat pheochromocytomas

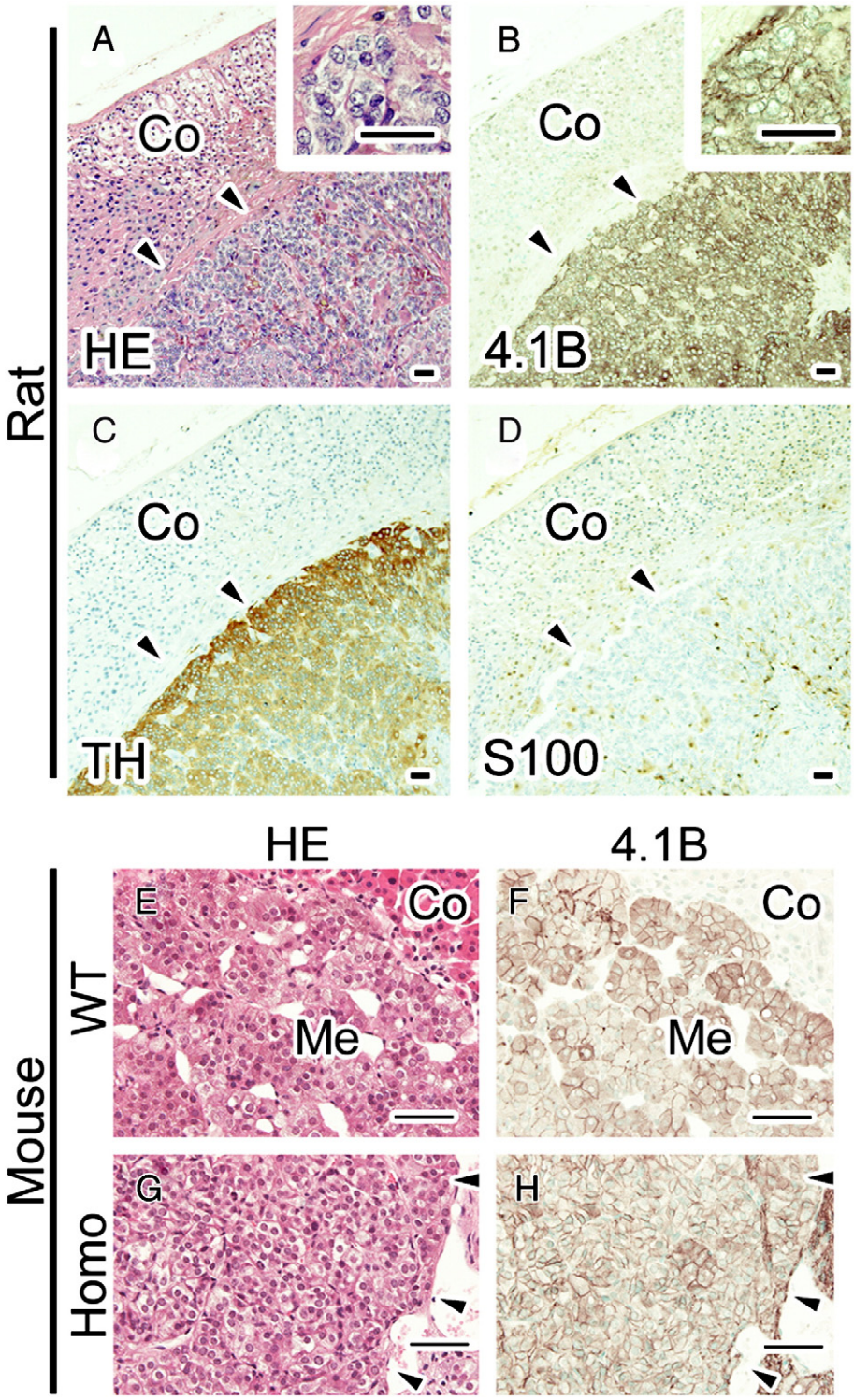

Fig. 6. (A–D) Immunostaining for 4.1B/differentially expressed in adenocarcinoma of the lung-1 (4.1B) in the rat spontaneous pheochromocytoma. Strong immunoreactivity of 4.1B can be detected along cell membranes (A, B, arrowheads). The tumor is immunopositive for tyrosine hydroxylase (TH; C, arrowheads) but not for S100 (D, arrowheads). Co: adrenal cortex. HE: hematoxylin–eosin. Bars: 30 μm. (E–H) Immunostaining for 4.1B in the pheochromocytoma developed in a MEN2B homozygous mouse. As in normal adrenal medulla (Me) of wild type mice (WT; E, F), strong immunoreactivity of 4.1B can be detected in pheochromocytoma developed in adrenal gland of the homozygous mouse (Homo; G, H, arrowheads). Bars: 50 μm.

which were immunopositive for tyrosine hydroxylase but immunonegative for S-100, immunostaining for 4.1B could be clearly observed on cell membranes as seen in normal chromaffin cells (Fig. 6A–D). Spontaneous pheochromocytomas obtained from 20 rats were examined, and immunoreactivity for 4.1B was found to be detected in all tumors, although it was decreased in some limited areas of 5 tumor specimens. However, considering the possibility that the genetic background of pheochromocytomas in rats is different from that in human [36], pheochromocytomas of MEN2B model mice generated by mutation in Ret oncogene were also investigated. In this model, a small fraction of heterozygous mice (Met919Thr/Met919) developed diffuse hyperplasia and pheochromocytoma, whereas most

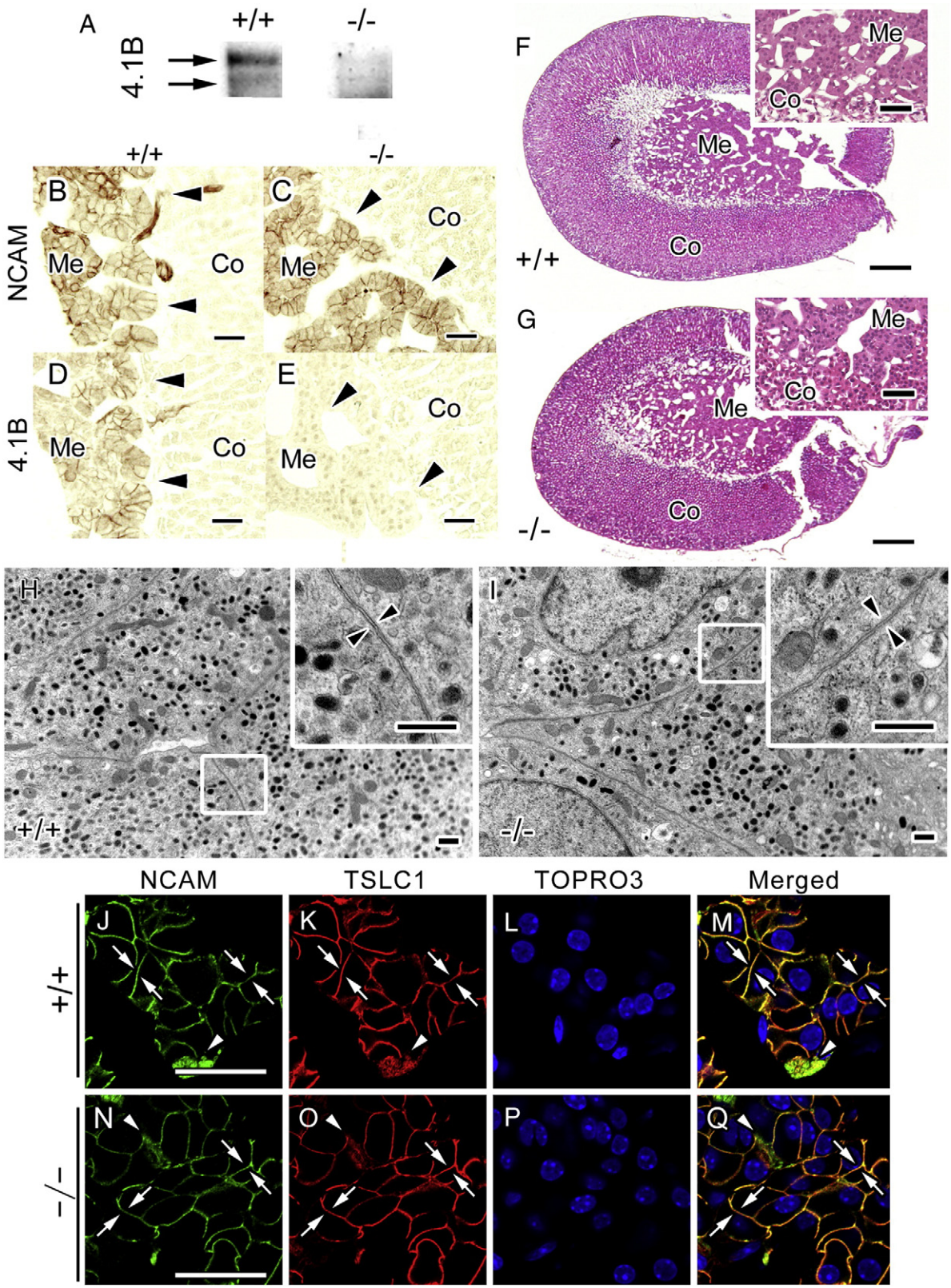

Fig. 7. (A) Western blot analysis of 4.1B/differentially expressed in adenocarcinoma of the lung-1 (4.1B) in brain of wild type (+/+) or homozygote (−/−) mice. The specific bands with molecular weights of approximately 130 and 110 kDa (arrows) can be detected in +/+ mice, but not in −/− mice. The amount of protein loaded in each well was 5 μg. (B–E) Immunostaining for neural cell adhesion molecule (NCAM; B, C) and 4.1B (D, E) in serial sections of adrenal glands obtained from +/+ (B, D) or −/− (C, E) littermates. Adrenal medulla (Me) can be distinguished from adrenal cortex (Co) by its positive immunostaining for NCAM (B, C, arrowheads). Immunoreactivity of 4.1B is observed in adrenal medulla of +/+ mice (D, arrowheads), but absent in −/− mice (E, arrowheads). Bars: 30 μm. (F, G) Light micrographs of adrenal glands obtained from +/+ (F) or −/− (G) mice and stained with hematoxylin–eosin. Me: adrenal medulla; Co: adrenal cortex. Bars: 200 μm; 50 μm in insets. (H, I) Electron micrographs of chromaffin cells in adrenal gland of +/+ (H) or −/− (I) mice. Areas marked with rectangles are shown at higher magnification in insets. Opposed arrowheads: intercellular spaces between chromaffin cells. Bars: 500 nm. (J–Q) Double immunofluorescence labeling of NCAM (green) and tumor suppressor in lung cancer-1 (TSLC1; red) in adrenal glands of +/+ (J–M) or −/− (N–Q) mice. Immunolocalization of NCAM and TSLC1 along cell membranes of chromaffin cells (arrows) and in nerve fibers (arrowheads), observed in +/+ mice, is not changed in −/− mice. Bars: 30 μm.

homozygous mice (Met919Thr/Met919Thr) developed pheochromocytomas at the ages of 6–10 months [27]. Therefore, immunohistochemistry for 4.1B was performed in the adrenal medulla of 5 homozygote and 3 wild type (Met919/Met919) MEN2B model mice at 6 months of age. There was no histological abnormality in the adrenal gland of any of the wild type mice (Fig. 6E). By contrast, in the adrenal medulla of all homozygous mice, normal polyhedral secretory cells were replaced by abnormal cells with higher density of nuclei and less volume of basophilic cytoplasm, and their mass mostly compressed the surrounding adrenal cortex (Fig. 6G). This morphological appearance was suggestive of pheochromocytoma development in most of the homozygous mice, as reported previously [27]. In the pheochromocytomas of the homozygotes, the immunostaining of 4.1B was also strongly observed in all examined tumors, as obtained in normal adrenal medulla of wild type (Fig. 6F, H).

Adrenal glands of 4.1B-knockout mice were further examined for the development of tumors in the adrenal medulla. The 4.1B-deficient mice were born and developed normally without any apparent difference from wild type littermates, as reported previously in another 4.1B-knockout model [24]. First, lack of 4.1B expression was confirmed with western blotting in brain tissue, and bands reflecting the expression of 4.1B in brain tissue of wild type mice were not observed in those of homozygous mice (Fig. 7A). The expression of 4.1B protein was also immunohistochemically examined on cryosections of adrenal glands. In the adrenal medulla, immunoreactivity of 4.1B, which was clearly observed in wild type mice, was not present in the homozygous mice, as determined by immunostaining for NCAM [37] (Fig. 7B–E). However, the histological and ultrastructural analyses of adrenal glands in the 4.1B-knockout mice revealed no significant difference between the homozygous mice and wild type littermates; the sizes of the cortex and medulla were not significantly different, and no apparent changes indicating abnormal proliferation of chromaffin cells could be observed in the adrenal medulla up to the age of 18 months (Fig. 7F, G). In addition, although 4.1B is mainly immunolocalized along cell membranes of chromaffin cells, their ultrastructures were not changed in the knockout mice (Fig. 7H, I), and the intercellular spaces were not significantly different in the quantitative comparison (Mean±SD; 15.6±1.3 nm for wild type mice; 15.7±1.6 nm for knockout mice; $N=40$, $p>0.05$). Considering the potential involvement of 4.1B in the linkage of TSLC1 to cell membranes via the actin cytoskeleton, immunostaining for TSLC1 was also investigated in the adrenal medulla. Immunoreactivity of TSLC1 was colocalized with that of NCAM along cell membranes of chromaffin cells without any translocation or decrease, and thus immunostaining of TSLC1 appeared to be unchanged in mutant and wild type littermates of 4.1B-knockout mice (Fig. 7J–Q).

4. Discussion

In the present study, localization of 4.1B along cell membranes, and its co-localization and association with TSLC1, were found for the first time in rodent adrenal medulla in vivo. These results support the previously reported presence of 4.1B mRNA in the adrenal gland of mouse fetus [38]. The adrenal medulla is composed of chromaffin cells, and stimulated to secrete catecholamines through elaborate mechanisms upon a “fight or flight” response [39]. The intercellular spaces among adrenal chromaffin cells are believed to be crucial for the transport of the secreted catecholamines and also for intercellular communication [40]. Although expression of some cell adhesion molecules such as NCAM and L1 are reported in the adrenal medulla, the precise structural and molecular basis for maintaining those structures are not well understood. TSLC1 expression was already reported in several organs, including testes, kidneys, and lungs [35,41,42], and TSLC1 is known to be involved in Ca^{2+}-independent intercellular junctions, which is critical in spermatogenesis of mouse testes [35,43,44]. Given that 4.1B is involved in the intercellular junctional complex through interaction with transmembranous proteins such as Caspr in the nervous system [5], our results suggest a physiological association between 4.1B and TSLC1 in the adrenal medulla as well as in cancer cells [23], and also the potential involvement of 4.1B in the intercommunication among adjacent chromaffin cells. At the same time, other new functions of the TSLC1–4.1B interaction should be also evaluated in further studies. For example, NCAM-deficiency resulted in impaired granule trafficking rather than morphological abnormalities [37], although a significant difference in the density of secretary granules could not be found in our quantitative comparison (data not shown).

In the present study, immunoreactivity of 4.1B in chromaffin cells was not detected on the side of endothelial cells. Expression of 4.1B has been found in different epithelial cells, such as mammary, intestinal and renal epithelial cells [8,11,15,45,46]. In all of these cells, immunostaining for 4.1B was observed along lateral and basal plasma membranes directly facing adjacent cells or extracellular matrices. From these findings, it was considered that 4.1B would stabilize molecules for attachment to adjacent structures, including those against extracellular matrices [47]. This hypothesis is also supported by the finding that deletion of the intracellular domain of Caspr resulted in its impaired binding to 4.1B and abnormal distribution in axonal cytoplasm [48]. On the other hand, adrenal medulla is one of the organs with sinusoidal capillaries, and the basement membranes are located between the fenestrated sinusoidal endothelial cells and chromaffin cells [49]. In the pancreatic islets where endocrine cells also have sinusoidal capillaries and basement membranes on the side of endothelial cells [50], similar findings have been observed [51]. Plasma membranes of β-cell on the side of endothelial cells had less 4.1B immunoreactivity compared to those facing adjacent endocrine cells. These findings suggest that the distribution of 4.1B is significantly influenced by the molecular mechanisms of adhesion, which would be distinct on the side of basement membrane facing endothelial cells in chromaffin cells. The molecular complex of intercellular and/or cell-extracellular junctions in adrenal medulla should be examined in future studies.

Although 4.1B has been implicated in carcinogenesis in several organs, its expression was well maintained in rodent pheochromocytoma, and also 4.1B-knockout mice did not developed pheochromocytoma by our examination. The possibility that some unknown isoforms are initiated from unidentified initiation sites but undetectable with our antibody could not be excluded. However, known 4.1B gene products from the two initiation sites, which are expressed in brain and adrenal glands, are eliminated by the genetic mutation in our knockout mice [33,52]. Our results were consistent with the previous observation that a murine 4.1B-knockout model does not produce increased incidence of cancer [24]. FERM and U2 domains of 4.1B are thought to be essential for growth suppression of meningioma-derived cells [53], and post-translational methylation of arginine residues is a proposed pathway of 4.1B-induced apoptosis [54]. However, the mechanism by which 4.1B influences the carcinogenesis in various organs is not well known. Our results suggest that loss of 4.1B is not essential for the generation of pheochromocytoma in the rodent adrenal gland, and one possible explanation for this might be that rodent adrenal glands lack such molecules related to the tumor suppressive functions of 4.1B in other organs. Another possibility is that the tumor-suppressive function of 4.1B varies in different species (including humans), as we found that immunoreactivity of 4.1B was significantly decreased in human pheochromocytomas compared with the residual part of the normal adrenal medulla (Ohno et al. unpublished data). This possibility would be supported by the distinctive immunolocalization of human 4.1B in blood vessel walls [13], where immunoreactivity of rodent 4.1B could not be observed. Recently, some reports suggested that 4.1B was mainly involved in the progression or metastatic process of cancers, rather than cancer generation [17,55]. Compared to several key

molecules associated with hereditary pheochromocytoma, such as Ret, Menin and NF1 [56,57], other genes involved in the generation and behavior of sporadic pheochromocytomas are still poorly understood. Further studies on the role of 4.1B in the progression or metastasis of pheochromocytoma would be essential to elucidate the functional significance of 4.1B in the adrenal medulla.

In our present investigation, TSLC1 distribution was not affected by genetic ablation of 4.1B in mice. TSLC1 contains a domain essential for its efficient binding to 4.1B, and deletion of the domain causes diminished binding to 4.1B [23]. However, although inhibition of actin polymerization resulted in reduced immunoreactivity of 4.1B and TSLC1 along cell membranes [23], dispensability of 4.1B on the plasmalemmal localization of TSLC1 in vivo has remained unclear. TSLC1 also contains the PSD-95, Discs Large, and Zona Occludens 1 (PDZ) binding motif in its C-terminal region which mediates the binding to membrane-associated guanylate kinase homologs (MAGuK) [58]. Our results in 4.1B-knockout mice showing that 4.1B is not essential to the localization of TSLC1 along cell membranes suggest that the PDZ domain in TSLC1 would compensate for the linkage to the actin membrane skeleton via other cytoskeletal molecules associated with MAGuKs. Further studies on TSLC1-binding proteins including MAGuKs in adrenal medulla are needed to reveal the interaction network responsible for the plasmalemmal distribution of TSLC1, which is considered to be crucial for its tumor-suppressive function [22].

Another possible explanation for the lack of obvious phenotype and unaffected distribution of TSLC1 is the compensatory effect of other molecules in the protein 4.1 family. The protein 4.1 family is composed of four homologous proteins, 4.1R, 4.1N, 4.1G and 4.1B. They are characterized by three highly conservative domains; the FERM domain, the spectrin-actin binding domain and the C-terminal domain [52,59]. Multiple members of protein 4.1 family can be expressed in single cells and localize in same areas of different organs, which lead to the hypothesis of compensation among family members [6,10,31,60–62]. In our studies, we found that immunoreactivity of 4.1N is detected along intercellular membranes of chromaffin cells in mouse adrenal gland (Ohno et al., unpublished data). By our observation, 4.1N did not co-immunoprecipitated with TSLC-1, and the immunoreactivity of 4.1N did not significantly change in the 4.1B-knockout mice. However, single binding proteins can interact with different members of protein 4.1 family via highly conserved domains [5,47], and 4.1B is considered to associate with TSLC1 via the FERM domain [5,23,52,59]. Further studies, such as genetic ablation of 4.1N in 4.1B knockout mice, would be necessary to examine the compensatory role among protein 4.1 family more precisely. In addition, potential expression of protein 4.1 family isoforms which could not be detected in our paradigm should be explored in future studies.

In conclusion, although expression and plasmalemmal localization of 4.1B were shown in chromaffin cells of rodent adrenal medulla, the expression of 4.1B was maintained in developed pheochromocytomas, and generation of pheochromocytomas in 4.1B-deficient mice could not be detected in our studies. Furthermore, interaction and colocalization of 4.1B and TSLC1 were observed in the adrenal medulla, but 4.1B was not essential for the immunolocalization of TSLC1 along chromaffin cell membranes. These results suggest that the function of 4.1B as a tumor suppressor might differ significantly among organs and species, and that retention of TSLC1 on the cell membranes would be maintained by interacting molecules other than 4.1B in rodent chromaffin cells.

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