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Author(s)	Nakazawa, Shiori; Gotoh, Naoya; Matsumoto, Hirotaka et al.
Citation	Journal of Histochemistry & Cytochemistry, 59(2), 202-213 https://doi.org/10.1369/0022155410392231
Issue Date	2011-02
Doc URL	https://hdl.handle.net/2115/48172
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Type	journal article
File Information	JHC59-2_202-213.pdf



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Title: Expression of sorting nexin 18 (SNX18) is dynamically regulated in developing spinal motor neurons.

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Abstract

The sorting nexin (SNX) family proteins, which contain a Phox homology (PX) domain, play crucial roles in regulating the intracellular membrane trafficking of the endocytic pathway. The proper coordination of this pathway is important for axonal elongation; however, little is known about the expression and intracellular dynamics of the SNX members during the formation of the nervous system. Here we found that *SNX18*, which belongs to the Src-homology-3-PX-Bin/Amphiphysin/Rvs domain-containing SNX subfamily, was specifically expressed in differentiating motor neurons in the chick and mouse embryonic spinal cord. The expression of *SNX18* in embryonic spinal motor neurons was transient, and was down-regulated as the neurons matured. We further demonstrated that the localization of EGFP-SNX18 in growth cones was dynamically regulated, and accumulated especially at areas in contact with permissive substrates. Our findings collectively suggest that SNX18 may play an active role in axonal elongation.

Keywords: sorting nexin, SNX18, SNX9-family, motor neuron, DRG neuron, growth cone

Introduction

Axonal growth is precisely regulated by the concerted actions of multiple factors, ranging from extracellular guidance cues and the factors that recognize them to the intracellular molecules that process such information and alter growth-cone behavior (Chilton, 2006; Geraldo and Gordon-Weeks, 2009). To facilitate the progression of this complex process, the participating molecules need to be actively distributed and sorted. Given that the expression of axon guidance factors is tightly controlled throughout the process of neural circuit formation, it is plausible that the expression of some of their regulatory molecules is also developmentally regulated. However, little has been reported about the differential expression of molecules involved in intracellular protein sorting during nervous system development.

The sorting nexin (SNX) family proteins are cytoplasmic/membrane-associated proteins containing a phosphoinositide-binding module called the Phox homology (PX) domain, which targets these proteins to phosphoinositide-enriched membranes (Ellson et al., 2002). SNXs play crucial roles in the endosomal sorting system that regulates the intracellular trafficking of plasma membrane receptors (Worby and Dixon, 2002; Carlton et al., 2005; Seet and Hong, 2006; Cullen, 2008). The SNX family has more than 30 members, which are categorized into subgroups according to their structural organization and function. This diversity of SNX family members may reflect the complexity and variety of the endocytic system, of which parts must be regulated differently in different neurons during neural circuit formation.

The SNX9 family is a subgroup of SNXs consisting of SNX9, SNX18, and SNX33 (Lundmark and Carlsson, 2009). These three proteins share the same structural architecture: Src-homology-3 (SH3) at the N-terminus followed by PX and Bin/Amphiphysin/Rvs (BAR) domains; these features are not shared by other SNX molecules (Cullen, 2008). SNX9 is a major binding partner of dynamin, and it is required for efficient clathrin-mediated endocytosis (Lundmark and Carlsson, 2004; Soulet et al., 2005). SNX9 also interacts with N-WASP through its SH3 domain, to couple actin dynamics with membrane remodeling during endocytosis (Yarar et al., 2007; Shin et al., 2007), further indicating its important role in regulating the endocytic pathway. All the SNX9 family members interact and co-localize with dynamin 2 (Håberg, et al., 2008), suggesting that they may function in part through a common

molecular mechanism. Interestingly, the SNX9 family proteins are differentially localized in HeLa cells; SNX18 interacts and partly co-localizes with AP-1, but not AP-2 in these cells (Håberg, et al., 2008), while SNX9 interacts with AP-2 (Lundmark and Carlsson, 2003). These observations collectively suggest that the SNX9 family members may play different roles in the complex endosomal sorting machinery, with some members being selectively utilized during nervous system development.

Here we report that the expression of *SNX18* is regulated in embryonic spinal neurons. *SNX18* was specifically expressed by newly generated motor neurons in the chick and mouse embryonic spinal cord, and its expression was later down-regulated as the motor neurons matured. We also observed that the localization of EGFP-SNX18 was dynamically regulated in growth cones. Collectively, our findings suggest that this member of the SNX9 family could play a specific role in neural circuit formation.

Materials and Methods

Isolation of chick and mouse TY35/SNX18 cDNAs.

The cDNA fragment of the 3'-UTR, obtained by screening genes selectively expressed by chick embryonic motor neurons (Tanabe et al., 1998; Fujimura et al., 2006), was used to isolate longer cDNAs to identify their coding sequences. Of these, one named *TY35* was later identified as the chick ortholog of *SNX18*. Mouse *TY35/SNX18* cDNAs were isolated from a cDNA library made from the brain of a neonatal mouse under low-stringency hybridization and washing conditions, using the coding regions of chick *TY35/SNX18* as a probe. The cDNAs for mouse and chick *SNX9* and a fragment of *SNX33* were purchased from Invitrogen (Carlsbad, CA) or Delaware Biotechnology Institute (Newark, DE). The cRNA probes were prepared from these plasmids (3.6 kb, 2.2kb, 3 kb, and 3.1kb for chick SNX9, SNX18, SNX33, and mouse SNX18 respectively).

In situ hybridization.

In situ hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993; Tsuchida et al., 1994). Briefly, embryos were fixed in 4% PFA/PBS overnight at 4°C. The fixed embryos were successively placed in 10%, 20%, and 30% sucrose solutions, overnight. The fixed and cryo-protected embryos were embedded in OCT compound (Sakura Finetech, Tokyo, Japan) and sectioned into serial 12-μm sections on a CM3000 cryostat (Leica Microsystems, Wetzler, Germany). The resultant sections were post-fixed in 4% PFA/PBS at room temperature (RT) for 5 min, washed three times with PBS, and incubated in 1 μg/ml Proteinase K (Roche Applied Science) in 6.25 mM EDTA pH 8.0 (Dojindo Laboratories, Kumamoto, Japan) and 50 mM Tris pH 7.5 (Wako Pure Chemical Industries, Osaka, Japan) at RT for 5 min. The sections were re-fixed in 4% PFA/PBS at RT for 5 min, washed three times with PBS, and acetylated in 1.33% triethanol amine (Sigma-Aldrich; St. Louis, MO), 0.75% acetic anhydride solution (Wako Pure Chemical Industries) at RT for 10 min. The acetylated sections were washed three times with PBS and incubated in hybridization buffer (50% formamide (Sigma-Aldrich), 0.25 mg/ml Yeast RNA (Sigma-Aldrich), 0.5 mg/ml herring sperm DNA (Roche Applied Science), 5x Denhard's (Sigma-Aldrich), 5x SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0)) at RT for 2 hr, then with

digoxigenin-labeled cRNA probes in hybridization buffer (~1 ng/μl) at 72°C for 16 hr. The hybridized sections were washed in 5x SSC at 72°C for 10 min and then in 0.2x SSC for 1 hr. The washed sections were incubated with 10% heat-inactivated goat serum (Roche Applied Science) in 100 mM Tris pH 7.5, 0.15 M NaCl solution at RT for 1 hr, followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science; 1:5000) in the same solution at 4°C overnight. The sections were washed three times with 100 mM Tris pH 7.5, 0.15 M NaCl solution, and twice with 100 mM Tris pH 9.5, 0.1 M NaCl, 50 mM MgCl2 solution, followed by incubation with NBT/BCIP (Roche Applied Science) in the same solution containing 0.24 mg/ml levamisole (Sigma-Aldrich) at RT in the dark. The reaction was stopped by immersing the sections in PBS-5 mM EDTA. Whole-mount *in situ* hybridization was performed as described (Streit and Stern, 2001) using the same probe. Briefly, the fixed embryos were washed with PBS for three times, and immersed in absolute methanol. The embryos were rehydrated in PBT (PBS-0.1% Tween 20 (Sigma-Aldrich)), bleached for 1 hour in 6% H2O2, and digested for 10 min in 10 μg/ml Proteinase K/PBT at RT. The embryos were washed with PBT for three times and post-fixed for 20 min at RT in 0.2% glutalaldehyde (Sigma-Aldrich)-4% PFA/PBT. After washing with PBT, the treated embryos were pre-hybridized in hybridization solution (50% formamide, 5X SSC, 50 μg/ml Yeast RNA, 50 μg/ml heparin (Sigma-Aldrich)) for 2 hrs at 65°C followed by hybridization with the indicated cRNA probes (~0.2 ng/μl) overnight at 65°C. The embryos were washed twice with 50% formamide, 5X SSC, 1% SDS solution at 65°C for 30 min, three times with TBS-T (20 mM Tris pH 7.5, 0.13 M NaCl, 0.1% Tween20). The washed embryos were incubated with 20% heat-inactivated goat serum in TBS-T for 2 hr at RT, followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:5000) in the same solution at 4°C overnight. The embryos were washed with TBS-T three times for 5min, four times for 1 hr at RT and overnight at 4°C. The washed embryos were incubated with 100 mM Tris pH 9.5, 0.1 M NaCl, 50 mM MgCl2, 0.1% Tween20 solution followed by incubation with NBT/BCIP in the same solution containing 0.24 mg/ml levamisole at RT in the dark. The reaction was stopped by immersing the sections in PBS-5 mM EDTA. The developmental stages of chick embryos were determined according to the table of Hamburger and Hamilton (1951).

Plasmids.

Mouse N-WASP cDNA was purchased from Invitrogen. For the construction of pACT-N-WASP, a cDNA fragment of N-WASP containing the whole open reading frame was inserted into the *Eco*RI site of pACT2 (Clontech; Mountain View, CA). pAS-SNX18, pAS-SNX18N, pAS-SNX18C, and pAS-SNX18SH3 were generated by inserting the *Nco*I, *Nco*I-*Stu*I, *Nae*I-*Sca*I, and *Nco*I-*Sma*I fragments of mouse SNX18 cDNA, respectively, into the *Nco*I or *NcoI-SmaI* site of pAS2 (Clontech). pEGFP-SNX18 was prepared by inserting the *Bam*HI-*Eco*RI fragment of mouse SNX18 cDNA into the *Bgl*II-*Eco*RI site of pEGFP-C1 (Clontech). pDsRed-NWASP was generated by inserting the corresponding coding sequences into the *Hin*dIII-EcoRI (N-WASP) site of pDsRed-Monomer (Clontech).

Generation of an anti-SNX18 antibody.

An expression construct for producing a GST-fusion protein of mouse SNX18 (1-232) in *E. coli* was generated by inserting the *Bam*HI-*Stu*I fragment of the mouse SNX18 cDNA fragment into the *Bam*HI-*Sma*I site of pGEX-5X-3 (Pharmacia, Sweden). The purified recombinant proteins were used to immunize guinea pigs. Immunohistochemistry was performed as described (Fujimura et al, 2006). The mouse anti-Islet1/2 antibody was purchased from Developmental Studies Hybridoma Bank (Iowa City, IA). Donkey secondary antibodies (which show minimal cross-reaction to the serum proteins of other species) were purchased from Jackson Immuno Research Laboratories (West Grove, PA).

Ectopic generation of motor neurons.

Ectopic motor neurons were generated as described (Briscoe et al, 2000) by introducing RCAS-Nkx6.1 (gift from Dr. Jessell, Columbia University) into HH12 chick embryonic spinal cord using CUY21 electroporator (NEPA GENE, Chiba, Japan). After electroporation, the eggs were incubated for 2 days at 39°C, and the embryos were fixed in 4%PFA-PBS for 2 hrs at 4°C. The fixed embryos were cryo-protected in 30% sucrose, embedded in OCT compound, and sectioned into 12-μm slices on a CM3000 cryostat for immunohistochemistry as described above.

Yeast two-hybrid assay.

Mouse N-WASP cDNA was purchased from Invitrogen. For the construction of pACT-N-WASP, the cDNA fragment of N-WASP containing the whole open reading frame was inserted into the *Eco*RI site of pACT2. pAS-SNX18, pAS-SNX18 (1-232), pAS-SNX18 (182-615), and pAS-SNX18SH3 were generated by inserting the *Nco*I, *Nco*I-*Stu*I, *Nae*I-*Sca*I, and *Nco*I-*Sma*I fragments of mouse SNX18 cDNA, respectively, into the *Nco*I, *Nco*I-*Sma*I, or *Sma*I site of pAS2 (Clontech). The yeast two-hybrid assay was performed by following the manufacturer's instructions (Clontech). Briefly, the indicated pAS2-derived plasmids and pACT2 or pACT-N-WASP were co-transfected into Y190 cells by the lithium acetate-mediated method. The resultant transformants were grown on SD-Leu-Trp-His plates containing 50 mM 3-amino-1,2,4-triazol (Sigma-Aldrich; St. Louis, MO).

GST-pull-down assay.

The N-WASP cDNA was inserted into the *Eco*RI site of pcDNA3.1 (Invitrogen) to produce ^{35}S-labeled N-WASP protein, using a TNT coupled reticulocyte lysate system, following the manufacturer's instructions (Promega; Madison, WI). GST- or GST-SNX18 (1-232)-bound beads were prepared by incubating 50 μg of the purified proteins with 10 μl of Glutathione-Sepharose 4B beads (GE Healthcare; Piscataway, NJ) for 10 min at room temperature, followed by washing twice with PBS-0.1% Triton-X100 (Sigma). The resultant beads were preincubated at 4°C for 30 min with the reticulocyte lysate to minimize non-specific interactions. Twenty-five microliters of the resultant lysate containing the ^{35}S-labeled N-WASP was incubated with 5 μl of GST- or GST-SNX18 (1-232)-bound beads at 4°C for 30 min with occasional mixing. The beads were extensively washed with PBS-0.1% Triton X100 and subjected to SDS-PAGE. The resultant gel was dried and autoradiographed.

Phospholipid filter binding assay.

The phospholipid filter binding assay was performed using PIP MicroStrips (Invitrogen), following the manufacturer's instructions. Briefly, each PIP MicroStrip filter was incubated with 1 ng/μl purified GST-SNX18 (1-232) or GST-SNX18 (182-411) in TBS (10 mM Tris-HCl pH 8.0, 150 mM NaCl)-0.1% Tween20 (Sigma-Aldrich) containing 30 mg/ml fatty acid-free BSA (Sigma-Aldrich), for 30 min at room temperature. After extensively washing the filters with TBS-0.1% Tween20, the bound GST fusion proteins

were detected using an anti-GST antibody (Medical and Biological Laboratories; Nagoya, Japan) and visualized by DAB staining (Vector; Burlingame, CA). For the preparation of GST-SNX18 (182-411), the fragment of mouse SNX18 PCR-amplified using the following oligonucleotides as a primer (CGCACTAGTCGCTACCGCCTGTCCACCCGCT and CCGCTCGAGTCAGTCCAGGGCGGCGGCT) was digested with *Spe*I and *Xho*I, and inserted into the *Spe*I-*Xho*I site of pET42b (Merck; Darmstadt, Germany). The resultant expression plasmid was transfected into BL21(DE3);pLysS cells (Merck). The cells were cultured and treated with 0.5 mM IPTG for 5 min at 30°C to minimize aggregation of the protein product. The produced GST-fusion protein was immediately purified from the cell lysate, and the purified protein (~20 ng/μl) was quickly frozen in small aliquots.

Live cell imaging.

CAD mouse neuroblastoma cells were plated on a chambered coverglass (Nalge Nunc; Rochester, NY) and cultured in DMEM/F12 (1:1) containing 8% Calf Serum. pEGFP-SNX18 and pDcRed-N-WASP were co-transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Twenty-four hours after transfection, the culture medium was changed to DMEM/F12 (1:1; without phenol red) containing 8% Calf Serum and 25 mM HEPES (pH 7.4), and the cells were observed using an LSM510 confocal microscope (Carl Zeiss, Germany).

Observation of EGFP-SNX18 in the growth cones of elongating DRG neurons.

pEGFP-SNX18 was electroporated into the neural tube of HH 12 chick embryos as described previously (Arakawa, et al., 2008). At this stage, the expression plasmids could be introduced into the neural crest cells that become DRG neurons. After electroporation, the eggs were incubated for 5 days at 39°C, and the embryos were dissected in L-15 medium (Sigma-Aldrich) under a fluorescence microscope MZ10F (Leica Microsystems; Wetzlar, Germany). The DRGs showing faint green fluorescence were collected and plated on a polyethyleneimine-coated glass-bottom dish (Matsunami Glass; Kishiwada, Japan). The dissected DRG explants were cultured in DMEM/F12 containing 1xITS3, 10 ng/ml NGF, and 10 ng/ml NT3 (Sigma-Aldrich) at 37°C for 16 hrs. The fluorescent signals in the growth cones of elongated axons were observed, and time-lapse images were collected at 37°C with a BZ-9000 microscope (Keyence; Osaka,

Japan).

Results

SNX18 is specifically and transiently expressed by motor neurons in the embryonic chick and mouse spinal cord.

In the course of searching for genes selectively expressed in chick embryonic motor neurons, using a single-cell PCR technique (Fujimura et al., 2006), we isolated a cDNA fragment of chick *SNX18*. *SNX18* encodes a protein containing one PX domain in its middle region (Figure 1A), and is categorized as a member of the SNX family proteins. To verify that *SNX18* was expressed in motor neurons of the developing spinal cord, we performed *in situ* hybridization. In the developing chick brachial spinal cord, *SNX18* expression was first observed in some ventral neurons around Hamburger and Hamilton stage (HH) 15, when the motor neurons begin to be generated (Hollyday and Hamburger, 1977), as confirmed by the expression of *Isl1*, a marker for motor neurons at this stage, in the same regions (Figure 1B-a and b). At HH 20, *SNX18* was robustly expressed by ventral spinal neurons, whose position was confirmed by the expression of *Isl1* (Figure 1B). The whole-mount *in situ* hybridization of HH 20 embryos showed that *SNX18* was expressed in the ventral spinal cord along the entire A-P axis where motor neurons reside (Figure 2B). These observations collectively suggested that *SNX18* was selectively expressed by embryonic motor neurons in the spinal cord.

Interestingly, the signal strength of the *SNX18* expression in motor neurons decreased as the neurons moved laterally, which was clearly seen at HH 20-23 (Figure 1B), when the spinal motor neurons are most rapidly generated (Ericson et al., 1992). At HH20, all embryonic motor neurons generated by this stage express *Isl1*. Of the *Isl1*-expressing motor neurons, *SNX18* mRNA was barely detected in the laterally-located motor neurons that were generated earlier. At HH23, Isl1 was expressed by newly born motor neurons residing near the ventricular zone (Figure 1B-f, pointed by the arrowhead) and LMCm motor neurons located in the ventral horn (Figure 1B-f). *SNX18* mRNA was detected around the region where newly generated *Isl1*-positive neurons reside (Figure 1B-e, pointed by the arrowhead), but rarely detected in mature LMCm motor neurons. At HH28, *SNX18* mRNA was detected near the ventricular zone at the area where *Isl1*-expressing neurons were still observed (Figure 1B-g and h, pointed by arrowheads). These observations collectively indicated that expression of *SNX18* was observed

around the area where newly generated motor neurons reside, further suggesting that the expression of *SNX18* is transient in motor neurons, peaking soon after their birth and then becoming down-regulated as these neurons mature. *SNX18* expression was almost undetectable in the spinal cord at HH28 (Figure 1B-g), confirming that little *SNX18* was expressed by mature motor neurons. The expression profiles of two closely related family members, SNX9 and SNX33, were dissimilar to that of *SNX18* (Figure 2).

To examine whether this characteristic expression takes place in other animals, we isolated the mouse ortholog of chick *SNX18*. The deduced amino acid sequence of mouse SNX18 shared high identity with the chick ortholog (82% identity; Figure 1A), suggesting that SNX18 plays an evolutionally conserved role. As anticipated, the mouse *SNX18* was also specifically and transiently expressed in the ventral spinal cord, where motor neurons reside at E10.5 (Figure 1B). Its expression became faint at E11.5 (Figure 1B), indicating that *SNX18* functions at relatively early stages of motor neuron differentiation and maturation. Later in development, the dorsal root ganglia neurons began to express SNX18 (Figure 1B).

To confirm whether SNX18 protein was selectively expressed by motor neurons, we generated an anti-SNX18 antibody. The antibody specifically recognized exogenous chick and mouse SNX18 expressed in Neuro2a cells, but did not react with mouse SNX9 (Supplemental figure 2). Immunohistochemistry using the prepared anti-SNX18 antibody clearly showed the SNX18 protein around all and only the ventral Isl1/2-positive nuclei. Since *Isl2* is also expressed by LMCl motor neurons that cease expressing *Isl1* soon after their births (Tsuchida, et al., 1994), this anti-Isl1/2 antibody can label all motor neurons in the ventral spinal cord. This observation confirmed that the SNX18 protein was specifically expressed by motor neurons in both the mouse and chick developing spinal cord (Figure 3A). Next, to test whether SNX18 was expressed by any types of motor neurons, we verified its expression in artificially and ectopically induced motor neurons. The mis-expression of a homeobox-containing transcription factor Nkx6.1 induces motor neurons ectopically (Briscoe et al., 2000; Figure 3C). SNX18 was also expressed in these ectopically induced motor neurons (Figure 3C, in the area surrounded by the dotted line). This observation together with the fact that we failed to find any spinal motor neurons that did not express SNX18 (data not shown) strongly suggest that SNX18 is expressed by all types of spinal motor neurons.

SNX18 proteins were detected in virtually all motor neurons at HH24 (Figure 3A),

supporting the conclusion that *SNX18* is once expressed by all motor neurons. Considering that SNX18 mRNA was barely detected in earlier generated LMCm neurons by HH23 (Figure 1B), this observation may also suggest that SNX18 protein is relatively stable in the cell. The relative amounts of SNX18 protein in the motor neuron cell bodies were estimated by immunohistochemistry using simultaneously prepared specimen. SNX18 proteins in the motor neuron cell bodies decreased over time but were still slightly detectable at HH28 (Figure 3B), confirming that *SNX18* is transiently expressed by embryonic motor neurons.

SNX18 interacts with N-WASP through its SH3 domain, and its PX domain binds to a broad range of phosphoinositides.

It has been shown that SNX9 family members share the ability to bind dynamin through their SH3 domain (Håberg, et al., 2008; Schöbel et al., 2008). N-WASP is another molecule that interacts with SNX9 and SNX33 through their SH3 domain (Yarar, et al., 2007; Zhang, et al., 2009); however, it is not reported whether SNX18 also binds to N-WASP. To test this, we performed a yeast two-hybrid assay. In this assay, SNX18 showed an interaction with N-WASP, and its SH3 domain was essential and sufficient for the binding (Figure 4A). This interaction was further confirmed by a pull-down assay using *in vitro*-translated N-WASP, in which ^{35}S-methione-labeled N-WASP bound GST-SNX18 (1-232) (Figure 3B). These findings together indicated that SNX18 interacts with N-WASP. Moreover, the co-expression of SNX18 and N-WASP in CAD mouse neuroblastoma cells showed they were co-localized in live cells as previously reported for SNX9 (Yarar et al., 2007), further supporting the idea that SNX18 interacts with N-WASP.

The PX domain is a phosphoinositide-binding domain (Ellson et al., 2002), and each PX domain of the different SNX family members shows a different PIP-binding activity (Seet and Hong, 2006; Cullen, 2008). Full-length SNX9 binds liposomes containing a relatively broad range of PIPs (Lundmark and Carlsson, 2003; Yarar et al., 2008), while the SNX9 PX domain alone preferentially binds to PIP3 spotted on a filter (Badour et al., 2007). The PX and BAR lipid-binding domains of SNX9 also act cooperatively to promote PIP-containing lipid targeting and tubulation (Pylypenko et al., 2007), and the broad specificity of SNX9 seems to be conferred by structures outside of the PX domain.

Full-length SNX18 was previously shown to preferentially bind liposomes containing PIP2s (Håberg, et al., 2008). We therefore performed a filter-binding assay to examine whether the lipid-binding preference of the SNX18 PX domain was narrow or broad. We found that GST-SNX18 (182-411), which consisted of SNX18's PX domain, bound all of the PIPs spotted on a filter (Figure 4D), suggesting that the broad lipid-binding potential of the PX domain of SNX18 may be modulated by the BAR domain to ensure a stricter PIP-binding preference.

The localization of SNX18 is dynamically regulated in the growth cones of DRG neurons.

Transient expression of SNX18 in motor neurons at their births, when motor neurons start extending their axons out from the spinal cord, suggested that SNX18 might play a role in axonal growth regulation. Since axonal growth is closely correlated with the behavior of growth cones, we used chick embryonic DRG neurons, which express SNX18 after HH 23, because their growth cones are readily visible.

Because SNX9's expression is known to change dynamically in live cells (Yarar et al., 2007), we next investigated the distribution of EGFP-SNX18 proteins in the growth cones of living DRG neurons during axon elongation. Plasmids expressing EGFP-SNX18 were electroporated into the neural tubes of HH 10-12 chick embryos to introduce the expression plasmids into neural crest cells. To minimize the possible side effects caused by the over-expression of EGFP-SNX18, we selected and cultured DRGs that only faintly expressed the EGFP-SNX18 protein. As shown in Figure 5A, the distribution of the EGFP-SNX18 protein in the growth cones was very dynamic. It appeared transiently as a radial line, and the fluorescence then disappeared within a minute (Figure 5A, arrowheads). In the course of axonal growth, the EGFP-SNX18 protein also appeared to be actively redistributed where growth cones formed contacts with permissive substrates (Figure 5B, arrowheads). These observations collectively suggest that SNX18 may function in the growth cones to affect the elongation of peripheral nerves.

Discussion

Here we provide evidence that the expression of *SNX18* is differentially regulated during neural development. In the embryonic spinal cord, *SNX18* expression was obvious in newly generated motor neurons. Our observations suggest that the endosomal system could be dynamically controlled by the expression of the appropriate components in accordance with a cell's particular needs. The relative amounts of SNX9 family proteins are known to vary among tissues in mice (Håberg, et al., 2008), collectively suggesting that they function in cell- and tissue-specific manners. It is still unknown which, if any, aspect of motor neuron differentiation and/or axonal elongation requires SNX18; however, the specificity and transience of its expression suggest that it might play a role in responding to situations that are specifically experienced just after these neurons are born. In the central nervous system, motor neurons are the only ones that project their axons into the periphery. Soon after motor neurons are generated, they begin to elaborate axons, and their growth cones must exit the ordered neuroepithelial tissue of their birth to enter the relatively unstructured region of the outer mesenchymal cells, which are surrounded by a different extracellular matrix. That is, the growth cones immediately encounter a completely novel environment. *SNX18* might help motor neurons prepare for and/or deal with these drastic changes by facilitating the intracellular trafficking of plasma membrane receptors. Further investigations are obviously required to examine these possibilities.

A characteristic transience of the *SNX18* expression in embryonic spinal motor neurons was observed in both the chick and mouse, suggesting it might be functionally relevant. It is possible that SNX18 later disrupts the proper regulation of the endosomal pathway in motor neurons if its expression is maintained. In addition, although the *SNX18* mRNA of laterally located earlier-born motor neurons at HH 20 was below the detection level of our ISH, the SNX18 protein was still observed in the motor neuron cell bodies even at HH 24, suggesting that once produced, the SNX18 protein is relatively stable. Our observations collectively suggest that the expression of *SNX18* in motor neurons may have to be transient because the level of SNX18 protein would soon become too high to support normal axonal elongation. The prolonged expression of exogenous *SNX18* by *in ovo* electroporation slightly but significantly attenuated the growth of motor axons (SN, unpublished observation) may support our speculation. Future studies

aimed at determining the specific functional role of SNX18 in the endosomal pathway should help explain the functional significance of this characteristic transience of the *SNX18* expression.

We have also showed evidence suggesting that SNX18 may function with N-WASP in the cells. During preparation of the manuscript, Park, et al reported that SNX18 interacts with N-WASP (Park, et al, 2010). Our observation confirms their conclusion, and SNX18 appears to share the same binding partners. However, the PX domain of SNX18 bound a broad range of phopholipids spotted on a filter, while the PX domain of SNX9 preferentially binds to PIP3 on a filter (Badour et al., 2007). As a whole molecule, it has been shown that SNX18 preferentially binds to liposome containing PIP2s, while SNX9 binds a broad range of lipids (Håberg, et al., 2008; Lundmark and Carlsson, 2003; Yarar et al., 2008). These observations collectively suggest that the different lipid binding preference of SNX9 family members might be partly attained by a combination of different nature of each lipid binding domains, which could be differently utilized in the cells.

Endosomal sorting is a highly dynamic process, and the localization of EGFP-SNX18 proteins in growth cones was also actively regulated, as we anticipated. Interestingly, EGFP-SNX18 tended to accumulate at sites of contact between growth cones and an attractive substrate. In *Drosophila*, SNX9 is found in a complex containing DSCAM, an axon-guidance molecule, and AP50, a clathrin adaptor protein (Worby et al., 2001). SNX18 may play a similar role in sorting molecules that have interacted with the outer surrounding environment in growth cones. We cannot exclude the possibility that its observed accumulation might represent stacked overexpressed SNX18 proteins. However, we sought to minimize this possibility by examining growth cones that showed a dim fluorescence that was just sufficient to acquire images. EGFP-SNX18 was electroporated into neural crest cells just before they moved out from spinal cord, and the resultant observed DRG neurons were descendants of the neural crest cells that migrated, differentiated and elongated axons apparently normally, suggesting that the amount of introduced EGFP-SNX18 should be below the level affecting such complex biological events. Even if too much SNX18 protein was still present, our observations at least indicate that SNX18 is actively recruited to such contact sites. Future studies on the function of SNX18 together with its family members in growth cones could help elucidate the significance of the endosomal pathway in regulating axonal behavior.

Acknowledgements

We thank Dr. Thomas M. Jessell (Columbia University) and Fumio Matsuzaki (RIKEN) for encouraging us to pursue the project. This work was partly supported by Grant-in-Aid for Scientific Research 22590055 (TY) and Grant-in-Aid for Scientific Research on Innovative Areas 20200011 (TY) from the Ministry of Education, Science, Culture, Sports, and Technology, Japan.

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Figures

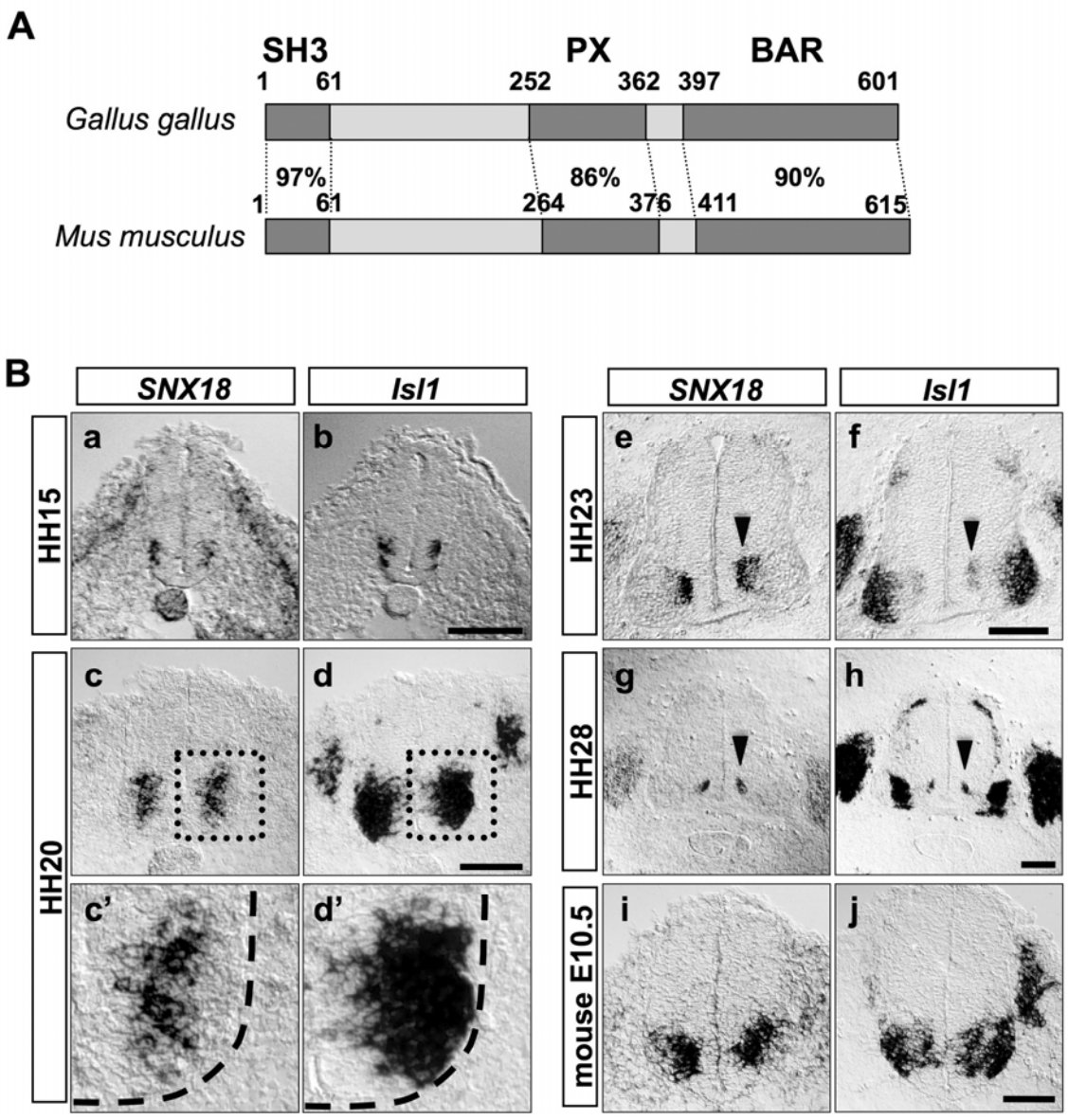


Figure 1. *SNX18* is specifically and transiently expressed by motor neurons in the embryonic spinal cord.

(A) Primary structures of chick and mouse SNX18. The percentage of identical amino acid residues between the structural domains of the chick and mouse orthologs is indicated.

(B) *SNX18* expression in the embryonic spinal cord. The expression of *SNX18* in the brachial level embryonic spinal cord was observed by *in situ* hybridization in chick embryos at HH15 (a), HH20 (c), HH23 (e), HH28 (g) and in mouse at E10.5 (i),

E11.5 (k). The expression of *Isl1* in the adjacent sections (b, d, f, h, j, l) is shown to indicate the areas where motor neurons reside. The magnified view of the dotted boxes in c and d is indicated by a dashed line in c' and d'. The outline of the spinal cord is shown in c' and d'. The expression of *SNX18* was down regulated in the chick and mouse motor neurons that moved laterally and became mature. Highest expression of *SNX18* at HH23 and HH28 is observed in the region where newly generated motor neurons reside shown by the expression of *Isl1* (e-h, arrowheads). DRG neurons started expressing SNX18 around HH23. Scale bar: 100 μm.

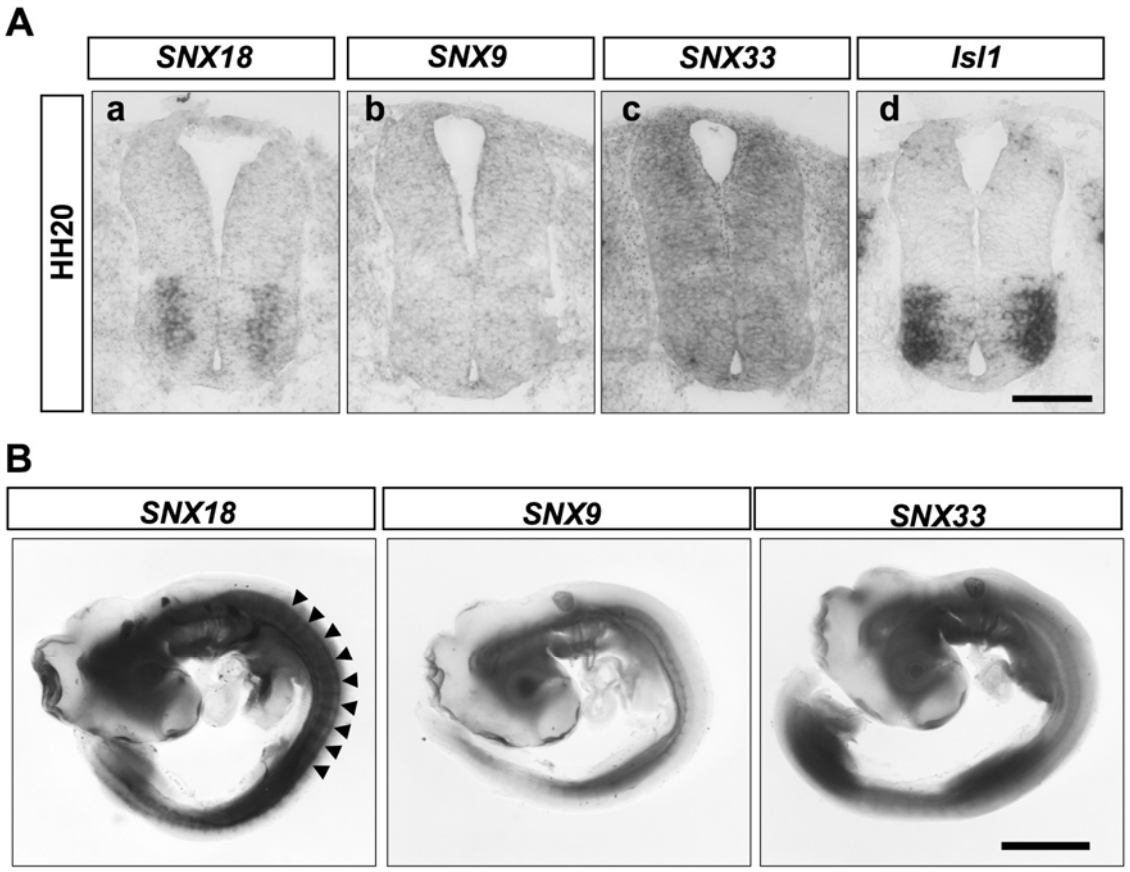


Figure 2. SNX9 family members are differently expressed in chick HH20 embryo. Expression of the indicated SNX9 family members in HH20 chick spinal cord (A) and whole embryo (B). The column of spinal motor neurons expressing *SNX18* is partly highlighted by the line of arrowheads. Scale bar: 100μm (A) and 2mm (B).

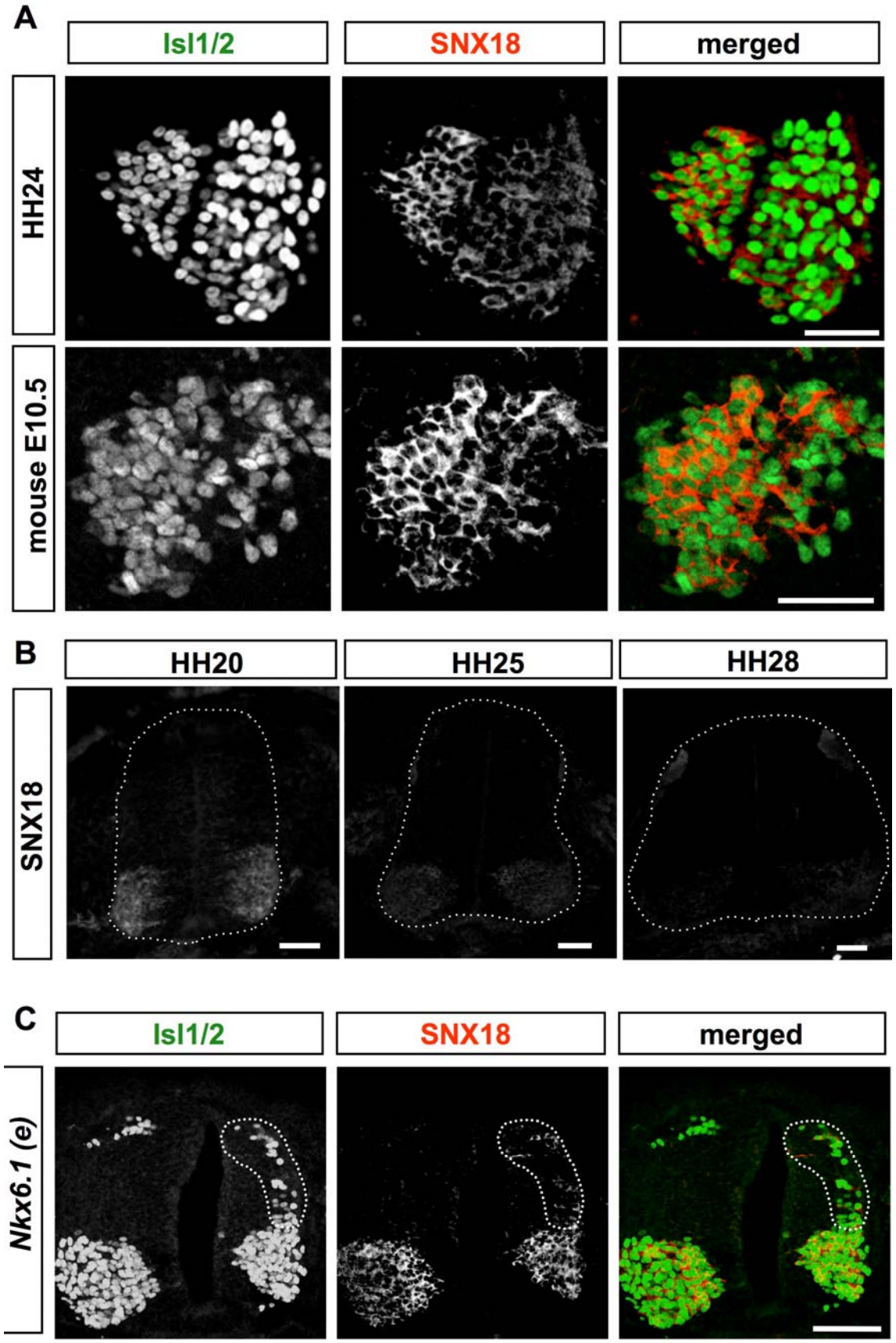

A
Isl1/2
SNX18
merged
HH24
mouse E10.5
B
HH20
HH25
HH28
SNX18
C
Isl1/2
SNX18
merged
Nkx6.1 (e)

Figure 3. SNX18 protein is expressed in embryonic motor neurons and in ectopically induced chick embryonic spinal motor neurons.

(A) Localization of SNX18 protein in the ventral spinal cord of chick HH24 and mouse E10.5 embryos. The indicated proteins were visualized by FITC-anti mouse (Isl1/2) and Cy3-anti guinea pig (SNX18) antibodies. Scale bar: 50 μm.

(B) Distribution of SNX18 at HH20, 25, and 28 chick spinal cord. The chick embryos were simultaneously fixed, embedded, cryosectioned and immunostained with anti-SNX18 antibody. Images were taken from the same slide in the same conditions. Scale Bar: 50μm.

(C) SNX18 is expressed by ectopically generated motor neurons. An expression plasmid for rat Nkx6.1 was introduced into the right side of spinal neuron progenitors by *in ovo* electroporation at HH12. The ectopically generated motor neurons expressing Isl1/2 at the dorsal region of the elctroporated side (surrounded by dotted line) expressed SNX18. Scale bar: 100 μm.

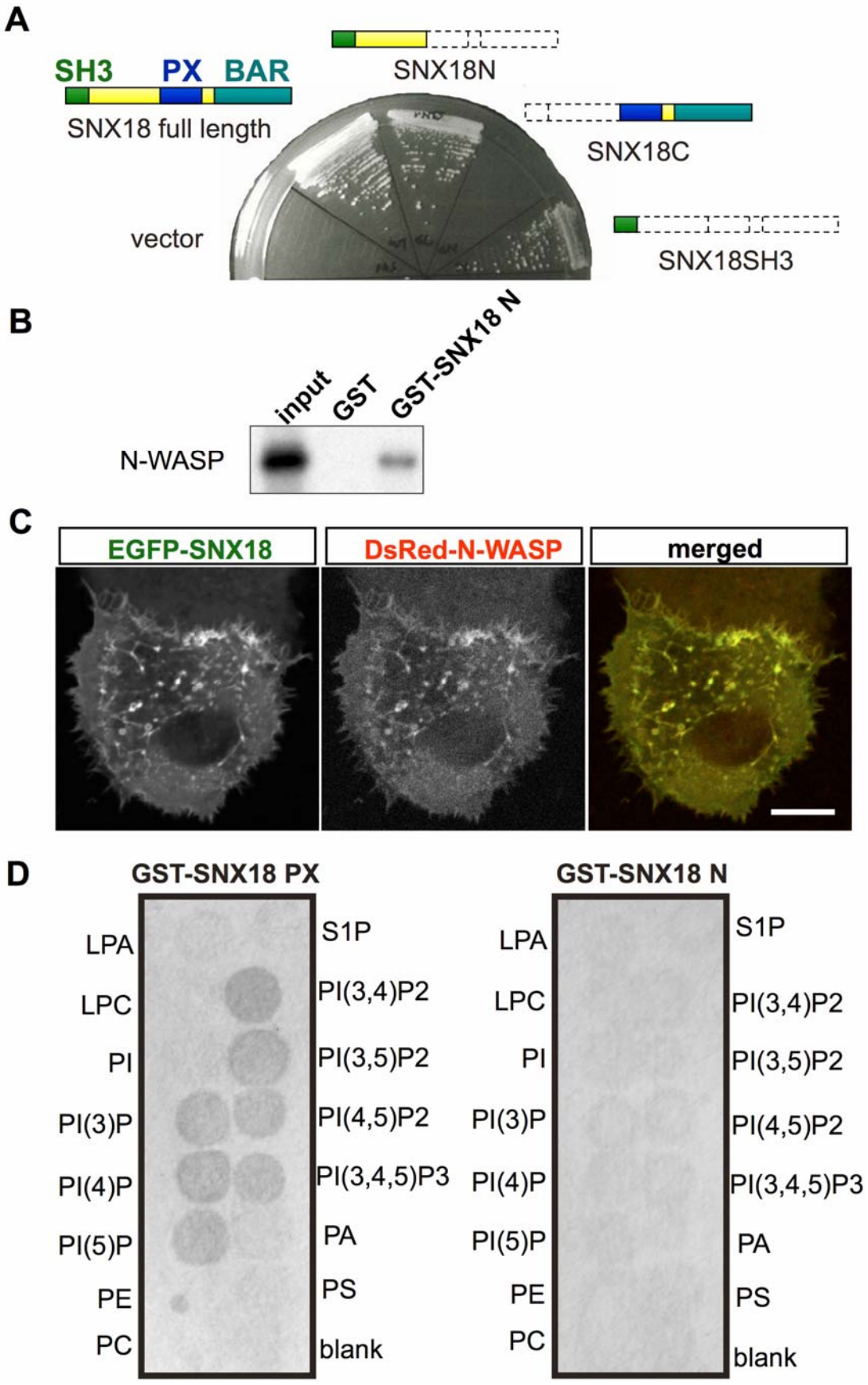

A
SH3
PX
BAR
SNX18 full length
SNX18N
SNX18C
SNX18SH3
vector
B
input
GST
GST-SNX18 N
N-WASP
C
EGFP-SNX18
DsRed-N-WASP
merged
D
GST-SNX18 PX
GST-SNX18 N
LPA
LPC
PI
PI(3)P
PI(4)P
PI(5)P
PE
PC
S1P
PI(3,4)P2
PI(3,5)P2
PI(4,5)P2
PI(3,4,5)P3
PA
PS
blank

Figure 4. SNX18 interacts with N-WASP and phosphatidylinositol phosphates.

(A) Yeast two-hybrid analysis. The constructs expressing the indicated SNX18 deletion mutants were co-introduced with N-WASP-expressing constructs.

(B) GST-pull-down assay. ^{35}S-labelled N-WASP was incubated with GST- or GST-SNX18 (1-232)-bound beads. The beads were extensively washed and subjected to SDS-PAGE. The resultant gel was dried and autoradiographed.

(C) Confocal image of live CAD cells expressing EGFP-SNX18 and DsRed-N-WASP. Scale bar: 10 μm.

(D) PIP filter-binding assay. The GST-fusion proteins of the SNX18 PX domain (GST-SNX18PX) or the N-terminal region before the PX domain (GST-SNX18N) were incubated with the filter, and the bound proteins were detected by an anti-GST antibody. LPA: lysophosphatidyl acid, LPC: lysophosphatidylcholine, PI: phosphatidylinositol, PI(3): phosphatidylinositol 3-phosphate, PI(4): phosphatidylinositol 4-phosphate, PI(5): phosphatidylinositol 5-phosphate, PE: phosphatidylethanolamine, PC: phosphatidylcholine, S1P: sphingosine 1-phosphate, PI(3,4)P2: phosphatidylinositol 3,4-diphosphate, PI(3,5): phosphatidylinositol 3,5-diphosphate, PI(4,5)P2: phosphatidylinositol 4,5-diphosphate, PI(3,4,5)P3: phosphatidylinositol 3,4,5-triphosphate, PA: phosphatidic acid, PS: phosphatidylserine.

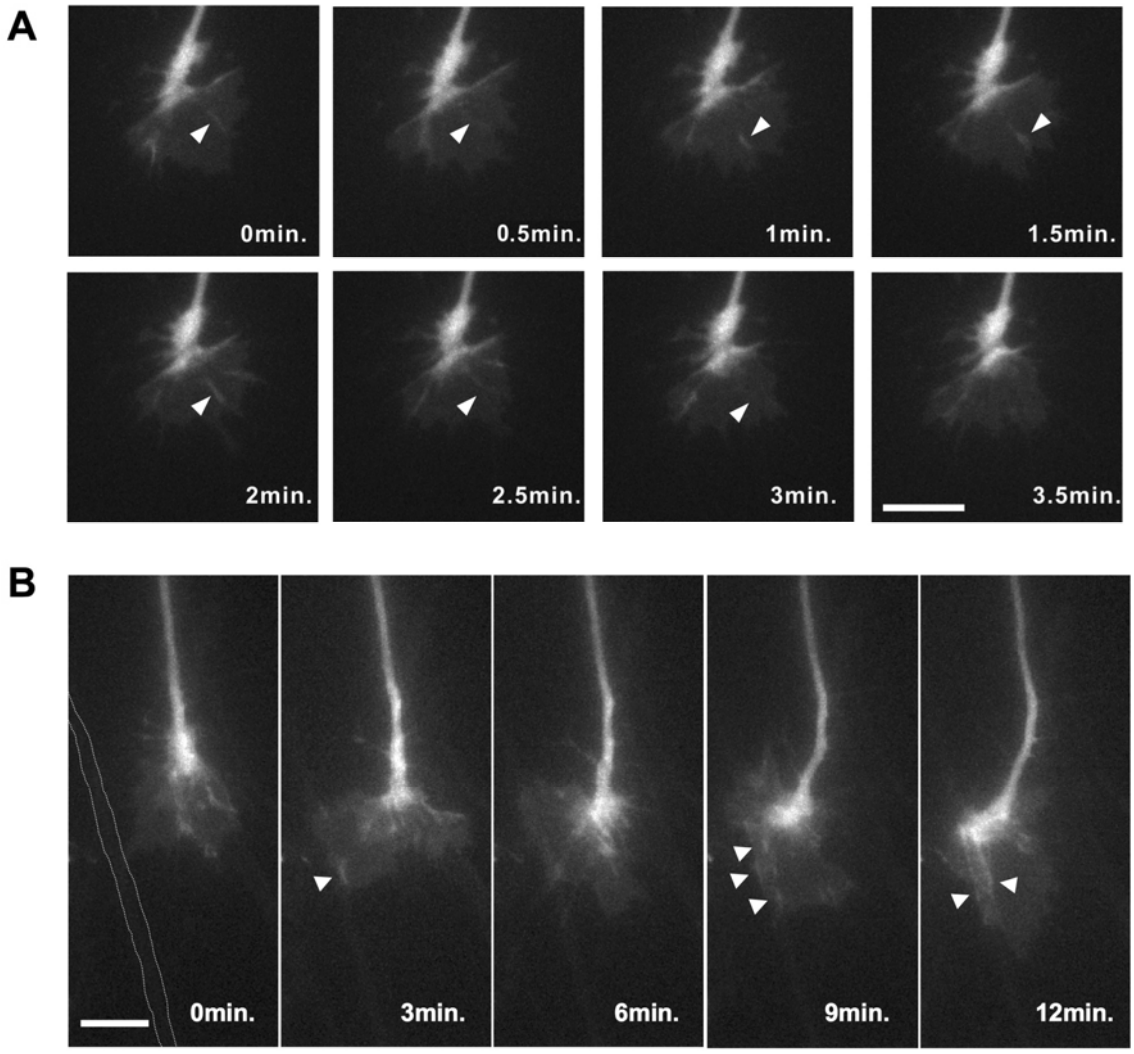


Figure 5. SNX18 is dynamically distributed in the growth cones of elongating DRG neurons.

(A) Time-lapse images of EGFP-SNX18 in the growth cone of an elongating chick DRG axon. Arrowheads highlight the transient radial accumulation of EGFP-SNX18. Scale bar: 20 μm.

(B) Time-lapse images of an elongating EGFP-SNX18-expressing DRG axon. The axon shaft that attracted the growth cone is highlighted by white dotted lines at 0 min. EGFP-SNX18 proteins were actively and dynamically distributed along the contact area (arrowheads). Scale bar: 20 μm.