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Snx5, as a Mind bomb-binding protein, is expressed in hematopoietic and endothelial precursor cells in zebrafish

Kyeong-Won Yoo[a], Eun-Hye Kim[a], Seung-Hyun Jung[a], Myungchull Rhee[a], Bon-Kyoung Koo[b], Ki-Jun Yoon[b], Young-Yun Kong[b], Cheol-Hee Kim[a,*]

[a] *Department of Biology, Chungnam National University, Daejeon 305-764, South Korea*
[b] *Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang, Kyungbuk 790-784, South Korea*

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Abstract **Notch signaling has an evolutionarily conserved function for cell fate determination and stem cell maintenance. Previously, we identified a novel component of the Notch signaling pathway in zebrafish, *mind bomb*, which encodes an E3 ubiquitin ligase essential for Notch signal activation. Further studies showed that *Mind bomb*$^{-/-}$ mouse embryos exhibited pan-Notch phenotypes in various tissues, suggesting that Mind bomb function is conserved in mammals. Therefore we sought to understand the various molecular partners of Mind bomb using yeast two-hybrid screening. In this search we identified Sorting nexin 5 (Snx5) as a novel interacting partner of Mind bomb. Furthermore we demonstrated that Snx5 colocalizes with Mind bomb in early endosomal compartments, suggesting that Snx5 is important for Mind bomb trafficking. In addition, we identified zebrafish orthologue of Snx5 and showed that *snx5* is predominantly expressed in hematopoietic and endothelial precursor cells in zebrafish. We also found defects in hematopoiesis and blood vessel development in *snx5* morpholino-injected embryos. Taken together, we show that Snx5, a novel interacting partner of Mind bomb, may have an essential role for cell fate determination in early development.**

Keywords: Mind bomb; Sorting nexin 5; Notch signaling; Hematopoietic precursor cells; Endothelial precursor cells; Zebrafish

1. Introduction

Notch signaling is an evolutionarily conserved mechanism that is used in metazoans. This signaling mechanism controls an extraordinarily broad spectrum of cell fates and developmental processes through local cell–cell interactions [1]. Defects in Notch signaling have been implicated in a wide variety of developmental defects and often lead to cancer in mammals [2,3].

Both the Notch receptors and their ligands, Delta/Jagged, are single-pass type I transmembrane glycoproteins, which function on opposing cellular membrane for interactions. Notch signaling is initiated when the extracellular domain of the ligand interacts with the receptor, resulting in the proteolytic cleavage of an intracellular domain of Notch, NICD. Then NICD translocates to the nucleus and regulates transcription of various target genes [4–6].

Endocytosis is an integral process for the efficient activation of Notch receptor signaling and is regulated spatiotemporally by several distinct mechanisms [7–9]. Recently, many studies highlighted an essential role of endocytosis in Notch ligand signaling. Post-translational modification, especially involving ubiquitination, plays a critical role in regulating Notch-Delta activity [10,11]. *Drosophila* Neuralized was identified as an E3 ligase that ubiquitinates and promotes the endocytosis of the Notch ligand Delta [12–15]. In vertebrate systems, however, Neuralized is not essential for Notch signaling, suggesting that other E3 ligases may be involved in the signaling [9,16,17].

We previously identified a novel component of the Notch pathway, Mind bomb, from the positional cloning of zebrafish neurogenic mutants [8] and showed its essential role in Notch signaling [8,9,17]. Mind bomb encodes a highly conserved and previously uncharacterized E3 ligase that functions similarly to Neuralized [8,19]. In the neural plate of *mind bomb* (*mib*) mutants, neurons are produced in the proper spatial regions, but are produced in excessive numbers. Furthermore, the neurons are expressed contiguously, rather than in a septate manner, separated by intervening non-neuronal cells [18]. This pattern is similar to that seen in embryos where Delta-Notch signaling is blocked by the injection of RNA coding for the dominant negative Delta construct [20,21]. *mib* mutations also cause defects in a variety of tissues in which Delta-Notch signaling is thought to play an important role such as somites, pancreas, intestine, definitive hematopoietic stem cells, and vascular tissue [22–27].

In this study we attempted to identify proteins that associate with Mind bomb for Notch signaling, using the yeast two-hybrid screening method. In this search we isolated one intriguing candidate, Sorting nexin 5 (Snx5), that localized in the early endosomal compartment [28,29]. Sorting nexins are a large family of proteins that contain the phosphoinositide-binding Phox homology (PX) domain. Recently, the sorting nexin (Snx) family has emerged as a group of interesting proteins associated with endosomal compartments to regulate the membrane trafficking of plasma membrane proteins ([see reviews for details [30–33]). The binding of Snx5 with Mind bomb was confirmed by in vitro coimmunoprecipitation and subcellular colocalization assays in cultured cells. From the

*Corresponding author. Fax: +82 42 822 9690.
E-mail address: zebrakim@cnu.ac.kr (C.-H. Kim).

Abbreviations: Mib, mind bomb; Snx5, sorting nexin 5; EEA1, early endosome antigen 1; HEK, human embryonic kidney; hpf, hours postfertilization

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whole-mount in situ hybridization analysis, the spatiotemporal expression pattern of *snx5* implicated that Snx5 may be required for the early development of hematopoietic and endothelial precursor cells in zebrafish. Finally, we performed a knockdown experiment using morpholino oligonucleotide to examine the functional role of *snx5* in zebrafish hematopoiesis and blood vessel development.

2. Materials and methods

2.1. Yeast two-hybrid screen

Yeast two-hybrid analysis was performed using the Pretransformed Matchmaker Library (Clontech) human brain cDNA library according to the manufacturer's instructions. We used a human cDNA library because it was commercially available and was better established than that of zebrafish. For bait construction, the middle region (encoding the ankyrin repeat domain from amino acids 410–744) of the human MIB cDNA (GenBank Accession No. AY147849) was amplified by PCR. Used primer sequences were 5′-CAGAATTCGTGGTGGAAGGGGTTGGCGCTCGGGT-3′ as forward primer and 5′-GATGCATTGCTAATGGCTGATCCTG-3′ as reverse primer. The PCR fragment was cloned into the pGEM-T easy vector (Promega) and subcloned into the *Eco*RI site of pGBT9, respectively. The pretransformed cDNA library in yeast strain Y187 was screened for MIB-interaction partners. The effect of baits on cell toxicity and transcriptional activation was tested in the yeast strain AH109. Another bait construct, encoding the N-terminal region from amino acids 11–411, was also tested and exhibited autonomous transcriptional activation on the host strain. This construct was not used further. In the bait mating 1.44×10^6 colonies were screened. The positive candidate colonies grew on minimal synthetic dropout medium for yeast (SD)/-Ade-His-Trp-Leu. All sequences of constructs were confirmed by automatic sequencing.

2.2. Isolation of snx5 cDNA and sequence analysis

To isolate zebrafish *snx5*, we performed RT-PCR with total RNA isolated from several stages of zebrafish embryos by using specific primers based on several ESTs and genomic sequences. Used primer sequences were 5′-TGAGGAACGAGCTTGCTGCTGGAA-3′ as forward primer and 5′-CAACTGAGGACATCAGTCAGTCCTT-3′ as reverse primer for *snx5*. The amplified fragment of the full-length zebrafish *snx5* cDNA was subcloned into the pGEM-T easy vector and sequenced by automatic sequencer.

To study the evolutionary relationships between the Snx5 and other related proteins, deduced amino acid sequences of the genes were aligned with the "CLUSTALW" protein alignment program with a parameter of N–J method unrooted, GenomeNet Computation Service (http://align.genome.jp/). Protein sequence data reported are available in the NCBI database under accession numbers Q92331 (yeast Vps5p), NP609199 (*Drosophila* Snx5/6), NP608777 (*Drosophila* Snx1/2), DQ377334 (zebrafish Snx5), DQ486138 (zebrafish Snx6), XP684658 (zebrafish Snx1), AAI09442 (zebrafish Snx2), NP077187 (mouse Snx5), NP081274 (mouse Snx6), NP062701 (mouse Snx1), NP080662 (mouse Snx2), NP689413 (human SNX5), NP689419 (human SNX6), NP003090 (human SNX1), and NP003091 (human SNX2).

2.3. Transient transfection

Human embryonic kidney (HEK)293 cells and COS7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and antibiotics. The cDNA coding for zebrafish Snx5 was subcloned into pCS2 + MT via *Eco*RI and *Xho*I. pCS2 + Myc-Snx5 (or HA-Snx 5) was cotransfected with pcDNA-mouse HA-Mib1, HA-Mib2, or XDelta1-myc into the cultured cells. Cells were transfected with appropriate amounts of plasmid DNA using Lipofectamine Plus (Invitrogen). Cells were harvested 24–48 h after transfection in 1 ml of lysis buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA) containing protease inhibitors (Roche). The lysates were sonicated several times, and the insoluble debris was removed by centrifugation. Aliquots of the supernatants, containing 15–40 μg of protein, were separated on a polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was blotted with the primary antibody of interest and a secondary antibody. To detect the immunoreactive bands, ECL plus was used (Amersham Biosciences).

2.4. Coimmunoprecipitation and Western blotting

HEK293 cells were transfected with 4 μg of plasmid DNA per 10-cm plate, harvested after 24–48 h, and resuspended in IP buffer (50 mM HEPES/NaOH (pH 7.5), 3 mM EDTA, 3 mM $CaCl_2$, 80 mM NaCl, 1% Triton X-100, 5 mM dithiothreitol). After the cells were lysed, the extracts were centrifuged to remove the debris. The supernatants were cleared using protein A/G Plus-agarose at 4 °C for 1 h. The indicated proteins were immunoprecipitated by the addition of protein A/G beads pre-complexed with an anti-HA antibody (Santa Cruz Biotechnology) or anti-Myc (9E10, Santa Cruz Biotechnology) antibody at

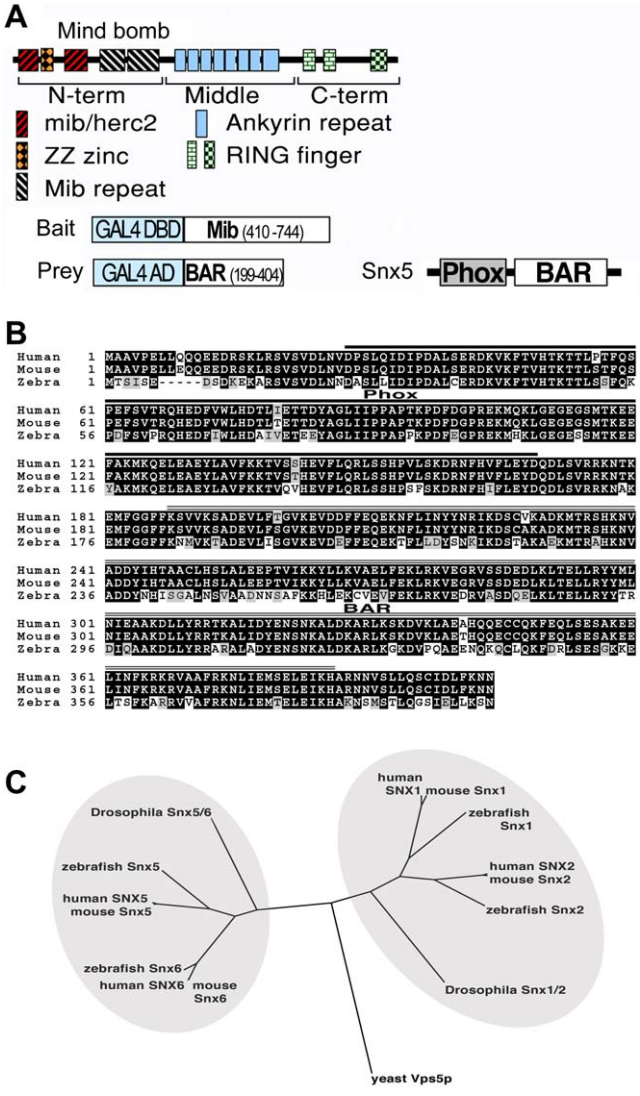

Fig. 1. Isolation of Snx5 as a novel binding partner of Mind bomb. (A) Schematic structures of the Mind bomb bait and Snx5 prey proteins. The bait contains the human Mib ankyrin repeats portion from amino acids 410–744. The isolated prey corresponds to the BAR domain of Snx5 from amino acids 199–404. (B) Sequence alignment of human, mouse and zebrafish Snx5 orthologues. The Phox homology domain and BAR domain are indicated by the single line and double line, respectively. Darkly shaded residues are conserved, light-shaded residues are similar, and hyphen indicates deletions. (C) Unrooted phylogenetic tree of yeast to human Snx proteins was generated using the CLUSTALW program with the N–J method. The Snx5/6 and Snx1/2 subgroups that are present on the separate branches in the tree are shown in two ellipses.

4 °C for 8 h. The immune complexes bound to the protein A/G beads were washed with IP buffer and boiled in 2 × SDS gel loading buffer, and the eluted proteins were electrophoresed on a polyacrylamide gel. Immunoreactive proteins were analyzed by immunoblotting with an anti-Myc antibody followed by either a goat anti-mouse horseradish peroxidase-conjugated antibody (Promega) or an anti-HA horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology).

2.5. Immunocytochemistry

HEK293 cells and COS7 cells were transfected with various plasmids. At 24 h post-transfection, the cells were washed in phosphate-buffered saline and fixed in 4% paraformaldehyde with 3% sucrose for 30 min at 4 °C. The fixed cells were incubated in blocking solution (3% skim milk and 0.1% Triton X-100 in PBS) overnight at 4 °C, and then stained with appropriate primary antibody in 3% skim milk in PBS for 1 h at room temperature. Subsequently, the cells were incubated with an anti-mouse antibody conjugated with Alexa546 (orange red) and/or Alexa350 (blue) for 30 min at room temperature. For DNA staining, cells were stained with Hoechst (10 μg/ml) for 2 min. After rinsing with 0.1% Triton X-100 in PBS, the cells were mounted on glass slides and analyzed with a Zeiss fluorescent microscope.

2.6. Zebrafish maintenance

Wild-type and mutant zebrafish were obtained from "Zebrafish Organogenesis Mutant Bank" in Kyungpook National University (Daegu, Korea). The *flk1:EGFP* transgenic fish [34] (kindly provided by Tao P. Zhong) was used in morpholino experiments. The *cloche* mutant, which produces embryos with profound defects in both hematopoietic and endothelial lineages [35], was used to characterize the origin of *snx5*-expressing cells. Zebrafish were raised and maintained as described by Westerfield [36]. Embryos were obtained from natural spawning and staged as previously described [37].

2.7. Whole-mount in situ hybridization and cryostat section

Whole-mount in situ hybridization [38] and two-color in situ hybridization [39] were performed as previously described with a few modifications. DIG-labeled antisense RNA probe for *snx5* or *mib* was synthesized from linearized plasmids by using SP6 RNA polymerase according to the manufacture's instruction (Ambion). Fluorescein-labeled riboprobes for *gata1* [40] and *flk-1* [41] were synthesized as previously described. Embryos for section were soaked in 30% sucrose overnight at 4 °C to equilibrate and then embedded in 1.5% agarose and 5% sucrose. Sections (8 μm) were cut using a cryostat microtome.

2.8. snx5 knockdown experiment by morpholino oligonucleotide

An antisense morpholino oligonucleotide was designed to target the exon 4 splicing donor site of the *snx5* gene. The synthetic oligonucleotide (MO-*snx5*) sequence was 5′-CAGAGTTAGACTCACGCCTCAAGTT-3′. The control morpholino sequence was 5′-CGTGATCTTCAGGACATATTTGGCT-3′. The oligonucleotide was resuspended in distilled nuclease-free water and 0.5 nl of 0.75 mM oligonucleotides (3–4 ng/embryo) was microinjected into one- to two-cell-stage zebrafish embryos. Efficacy of morpholino on the pre-mRNA splicing was evaluated using RT-PCR with forward primer designed to exon 2 (5′-GACAAAGTCAAATTTACCGTCCACACCA-3′) in conjunction with reverse primer designed to exon 5 (5′- ACACTTCATGAACCTGCACAGTCTTCT-3′).

3. Results and discussion

3.1. Identification of Snx5 as a Mind bomb-binding protein by yeast two-hybrid screen

To identify putative members of Mind bomb-mediated Notch signaling pathways, we performed yeast two-hybrid screens using the middle region of Mind bomb as baits (Fig. 1A). *Mind bomb* is highly conserved during the evolution from *Drosophila* to human [8]. After screening 1.44×10^6 colonies of a cDNA library, several candidate genes were found using the middle region containing ankyrin repeats. One of the prey genes was a partial fragment of cDNA encoding the C-terminal half of SNX5 (amino acids 191–404 containing the BAR domain) (Fig. 1A).

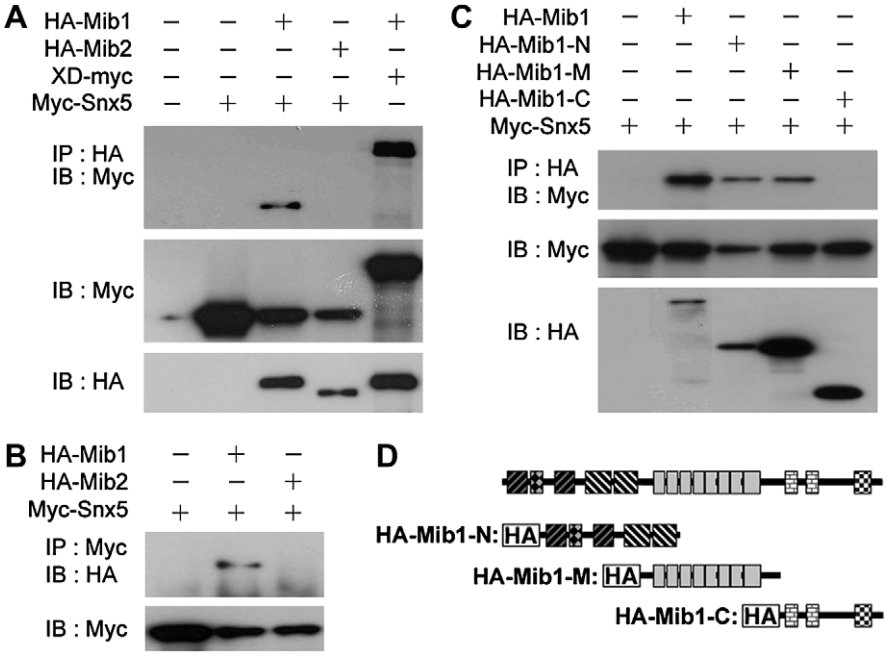

Fig. 2. Snx5 specifically binds to Mib1 in immunoprecipitation analysis. (A) Coimmunoprecipitation (IP) of Myc-Snx5 by HA-Mib constructs. The top panel shows Co-IP of Myc-Snx5 by HA-Mib1, but not by HA-Mib2. The Notch ligand Xdelta1-myc was used as a positive control. The middle and bottom panels are immunoblots showing expression of Myc-Snx5, XDelta1-myc and HA-Mib constructs, respectively. (B) Reversal Co-IP of HA-Mib constructs by Myc-Snx5. (C) Co-IP of Myc-Snx5 by HA-Mib1 deletion constructs. The top panel shows Co-IP of Myc-Snx5 by HA-Mib1 and its deletion constructs. The middle and bottom panels are immunoblots showing expression of Snx5 and Mib1 deletion constructs, respectively. (D) A schematic diagram of Mib deletion constructs used in this experiment. IP, coimmunoprecipitation; IB, immunoblot.

After zebrafish EST and genomic database search, several *snx5* ESTs and genomic fragments were identified. Based on this information, the zebrafish *snx5* was amplified by RT-PCR with specific primers from cDNA library at 24 hours postfertilization. Zebrafish Snx5 is 399 amino acid residues long and is highly conserved in vertebrates (Fig. 1B). Snx5 proteins have Phox-homology and BAR (bin/amphiphysin/rvs) domains (Figs. 1A and B). A phylogenetic tree shows the relationship of vertebrate, *Drosophila*, and yeast Snx-like proteins (Fig. 1C). Snx5 shares a significant amino acid identity with Snx6 (63–66%) and both contain an insert in their PX domains [30].

3.2. Physical interaction of Snx5 with Mib

We tested for the physical interaction of Snx5 and Mind bomb by coimmunoprecipitation of proteins expressed in cultured cells. Myc-tagged Snx5 (Myc-Snx5) was cotransfected with HA-tagged mouse Mib1 (HA-Mib) [9] and Mib2 (HA-Mib2) [17], respectively, into HEK293 cells. Myc-tagged *Xenopus* Delta1 (XD-myc) was used as a positive control for a Mib-binding protein. Previous reports showed that X-Delta1 binds to Mib2 as well as Mib1 [8,17]. As expected, Myc-Snx5 was coimmunoprecipitated with HA-Mib1, as was XD-myc. However, Myc-Snx5 was not coimmunoprecipitated with HA-Mib2 (Figs. 2A and B). This result indicates that Snx5 specifically binds to Mib1.

To address the region of Mib1 that interacts with Snx5, Myc-Snx5 was cotransfected with truncated forms of Mib1, namely N-terminal, Middle, and C-terminal fragments. The N-terminal region is comprised of mib/herc2, ZZ and Mib repeats; the Middle region includes the ankyrin-repeat domain, and the C-terminal form includes three RING-finger domains of the C-terminus, respectively (Fig. 2D). All of the Mib1 derivatives were immunoprecipitated with an anti-HA antibody and immunoblotted with an anti-Myc antibody to detect the coimmunoprecipitation with Myc-Snx5 (Fig. 2C). The result indicates that Mib1 interacts with Snx5 through its N-terminal and middle region.

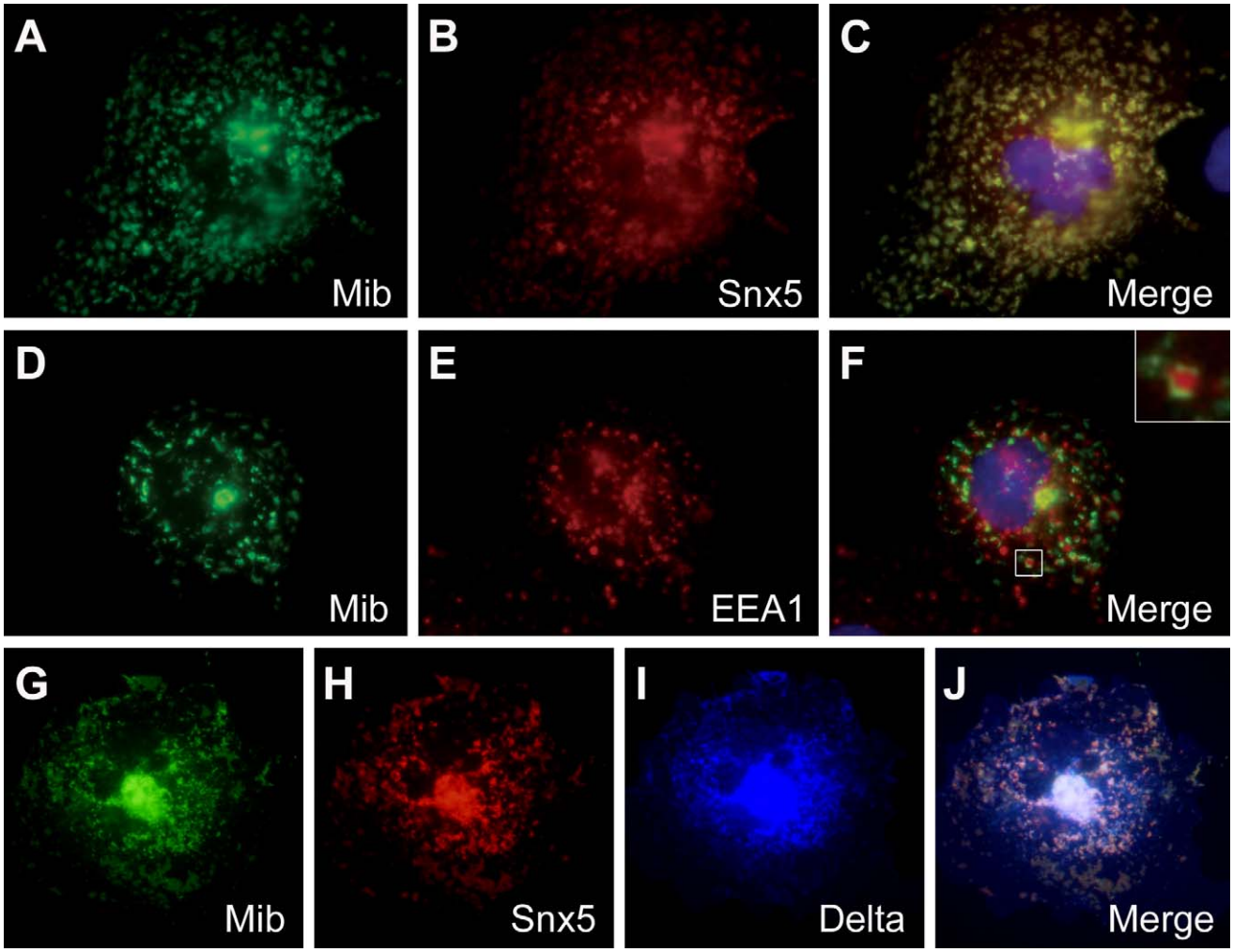

Fig. 3. Intracellular colocalization of Mib1, Snx5 and Notch ligand Delta. HEK293 cells were transiently transfected with expression vectors encoding Mib1-GFP and Myc-Snx5 (A–C), or Mib1-GFP alone (D–F). Transfected cells were fixed and stained with anti-Myc (A–C) or anti-EEA1 (D–F) antibodies, respectively. (A,D) Mib-GFP (green) was detected in a punctate pattern similar to that seen with Snx5 (B) and EEA1 (E). The yellow color in the merged image in (C) indicates the complete colocalization of Mind bomb and Snx5 proteins. Hoechst dye staining (blue) shows the nucleus. (E) A similar punctate distribution of EEA1-positive structures, which are known to represent an early endosome subdomain. (F) Most of Mib1-GFP (green) signals do not overlap with EEA1-positive structures (red). The inset is a high magnification of a spot which shows double-positive but distinct localization of two proteins. (G–J) Colocalization of Mib–GFP (green), HA-Snx5 (red), and Delta1-myc (blue) in transfected cells.

3.3. Subcellular colocalization of Snx5 with Mib1

To determine whether Snx5 colocalizes with Mib1 in cultured cells, HEK293 cells were cotransfected with expression vectors encoding both Myc-Snx5 and Mib1-GFP. After 24 h of transfection, the cells were stained with anti-Myc and then Alexa546-conjugated secondary antibodies. Mib1-GFP was detected in a punctate pattern and overlapped with myc-Snx5 (Figs. 3A–C). This result indicated that Snx5 associates with Mib1 on vesicular structures. In a previous report, Snx5 was localized to an early endosomal subdomain distinct from EEA1-positive early endosomes [29]. To further define the Mib1-GFP-positive structures, transfected HEK293 cells were labelled with antibody against the early endosome antigen 1 (EEA1). As shown in Figs. 3D–F, no colocalization of Mib1-GFP and EEA1 was detected, suggesting that association of Snx5 with Mind bomb might be localized in the other subdomain of early endosomes which is different from the EEA1-positive subdomain. We also observed that Snx5 is colocalized to the Mib and Delta complex (Figs. 3G–J). In previous reports, we showed that Mib directly bind to the intracellular domain of Delta and causes internalization of membrane protein Delta into cytosolic vesicles and perinuclear structures [8,9]. The identity of these cytosolic vesicles and perinuclear structures is currently unclear. In *Drosophila*, however, it is reported that another endosome-associated protein, Hrs/Vps27p, affects sorting and degradation of Notch receptor as well as other signaling receptors [42]. Further studies should be done to verify the possible role of Sorting nexin proteins in Mind bomb-mediated endocytosis of Notch ligand proteins.

3.4. Zebrafish snx5 expression in hematopoietic and endothelial precursor cells

Zebrafish and mouse orthologues of *mind bomb* are expressed in a variety of tissues [8,9,22,26,27]. To determine the spatial and temporal expression pattern of *snx5*, whole-mount in situ hybridization was performed at various developmental stages. *snx5* mRNA was expressed maternally and found throughout the embryo during blastula and gastrula stages. At tailbud and early somite stages, expression was also detected ubiquitously in zebrafish embryos (Figs. 4A and B). Around the 10-somite stage, however, a tissue-specific expression pattern was detected in bilateral rows of cells in the caudal lateral plate mesoderm (Fig. 4C). This group of cells continued to express *snx5* and converged into the midline to form the hematopoietic and blood vessel precursor cells (Figs. 4D–H). The *snx5* gene was also expressed in the central nervous system (Figs. 4B, D and G). To identify the anatomic sites of early *snx5* expression in the zebrafish embryos, we performed two-color in situ hybridization assays with the hematopoietic marker *gata1* [40] and the endothelial marker *flk-1* [41]. *snx5*-expressing cells in the ventral trunk region included both *gata1*-positive hematopoietic cells (Fig. 4E) and *flk-1*-positive

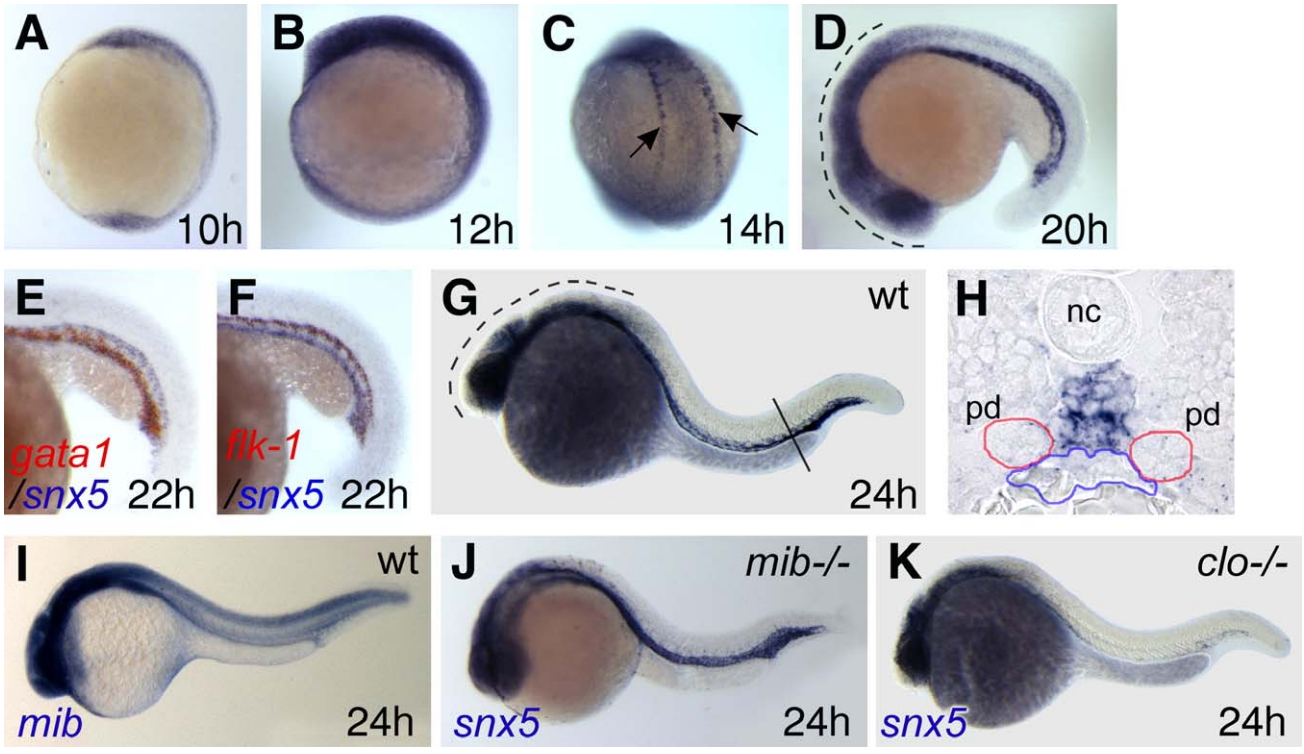

Fig. 4. Spatiotemporal expression pattern of *snx5* in wild-type and mutant zebrafish embryos. All lateral views except for (C). (A) At 10 hpf, *snx5* expression is ubiquitous along the body axis. (B) As development proceeds, transcripts were enriched in the brain primordia. Broken lines in (D,G) indicate the brain region. (C) By the 10-somite stage, *snx5* expression is predominantly expressed in specific group of cells in the lateral plate mesoderm (arrows). Dorsolateral view at the posterior region. At 20 hpf (D) and 22–24 hpf (E–H), *snx5*-positive cells were restricted to ventral region in the trunk and tail. Two-color in situ hybridization with probes, *gata1*/*snx5* (E) and *flk-1*/*snx5* (F), to identify the specific cell populations. (H) Transverse sections of the embryo shown in (G), showing a restricted expression of *snx5* in the ventromedial cells. Cells in the pronephric duct (pd, red circles), endodermal tissue (blue circle), and notochord (nc) were not stained. (I) *mib* was ubiquitously expressed in wild-type embryos, including *snx5*-positive tissues. (J) *snx5* expression was not changed in *mib* mutant embryos. (K) In *cloche* mutant, however, *snx5*-positive cells were almost absent in the ventral trunk region, although the brain region was not affected.

endothelial precursor cells (Fig. 4F). Transverse section of the *snx5*-stained embryo at 24 hpf showed a restricted expression pattern in the ventromedial mesoderm, from which both hematopoietic and blood vessel precursor cells arise. (Fig. 4H). *snx5* expression was not detected in the neighboring pronephric duct or endodermal tissues (Fig. 4H). We also examined whether the expression of *mind bomb* is colocalized with that of *snx5* in time and space. As we previously reported, *mind bomb* was expressed ubiquitously in various tissues, including the ventral mesoderm (Fig. 4I).

We further examined *snx5* expression in the *mind bomb* (*mib*) and *cloche* (*clo*) mutant embryos. In *mib* embryos, we could not detect any significant change in the *snx5* expression, suggesting that at least the transcriptional regulation of *snx5* gene expression is not a direct target of the Notch signaling pathway (Fig. 4J). The zebrafish mutant *cloche* (*clo*) displays hematopoietic and vascular defects [35]. The *clo* gene acts at the level of the putative hemangioblast originated from mesodermal tissue that gives rise to both blood and blood vessel progenitors [41,43,44]. In *cloche* mutants, *snx5* expression and/or *snx5*-expressing cells were completely absent in ventral mesoderm (Fig. 4K), but expression was retained in other tissues, such as brain. This result suggests that *snx5* expression is directly or indirectly involved in the development of hematopoietic and blood vessel precursor cells.

3.5. Snx5 knockdown and defects in blood vessel development

To examine the functional role of *snx5* in zebrafish development, we performed gene knockdown experiments using morpholino antisense oligonucleotides. A morpholino oligonucleotide was designed to target the exon 4 splice donor site to interfere with pre-mRNA splicing, resulting in intron retention and hence premature translation termination (Figs. 5A–C). We verified the MO efficacy by RT-PCR and found a product of 410 bp in morphant embryos, compared to 300 bp in control embryos (Fig. 5B). The effect of the morpholino was stable for more than 73 h in MO-injected embryos. We also confirmed the retention of intron 4 in the morphant embryos by direct sequencing of wild-type and morphant mRNA (Fig. 5C). Conceptual translation of the

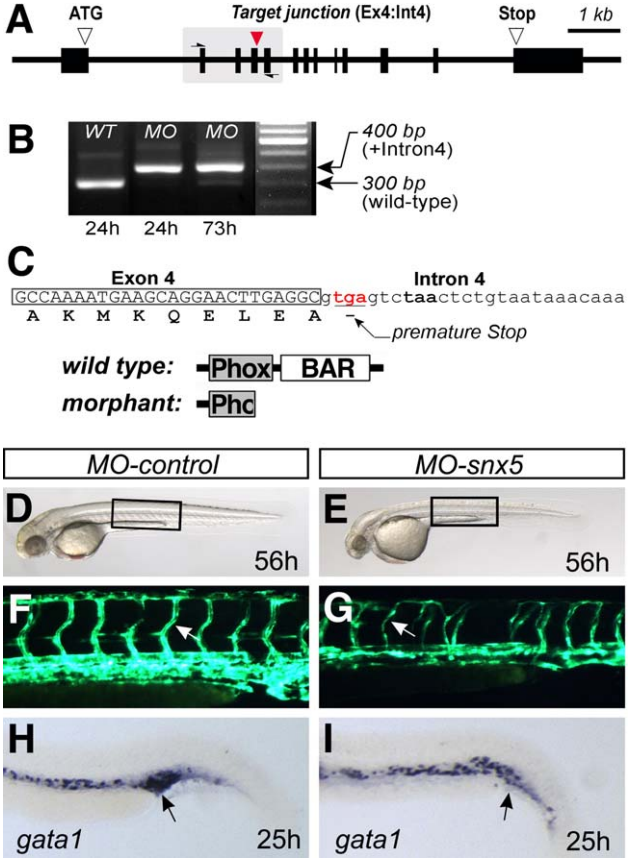

Fig. 5. Mopholino-based knockdown of *snx5* and blood vessel defects. (A) Genomic structure of *snx5*. Arrowhead indicates morpholino target site (Ex4:Int4) for splicing variation. Primers (small arrows) were designed for the test of morpholino efficacy. (B) Test and quantification of morpholino efficacy by RT-PCR. In morphant embryo, the wild-type *snx5* mRNA was undetectable by RT-PCR at 24 h and the effect was maintained at 73 h. (C) Identification of a premature stop codon in the morphant mRNA, which encodes a truncated form of Snx protein. (D–I) Blood vessel defects in *snx5* morpholino-injected embryos. The *flk1:EGFP* transgenic zebrafish embryos were microinjected with MO-control (D,F) or MO-*snx5* (E,G), and their vessel formation was examined at a cellular level in living embryos at 56 h. (F,G) Higher magnification of the boxed region in D and E under the fluorescent microscope. Regular formation of intersegmental vessels (arrow) by the GFP-positive endothelial cells was shown in control MO-injected embryos (F), but severe vascular defects were detected in *snx5* MO-injected embryos (G). (H,I) Whole-mount in situ hybridization of injected embryos with *gata1* at 25 hpf. A significant reduction of *gata1*-positive cells was detected in *snx5* MO-injected embryos, especially in the intermediate cell mass (arrow).

transcripts generates a premature stop codon just after the exon-intron junction, resulting in a truncated form of Snx5 protein (Fig. 5C).

After microinjection of MO-*snx5* into zebrafish embryos, we examined the circulating blood cells and blood vessel formation. We observed a reduction in blood circulation in 78.6% of *snx5* MO-injected embryos ($n = 42$), in comparison with control MO-injected embryos (10%, $n = 20$). These embryos exhibited defects in blood vessel formation, were largely poor in axial vasculature, and showed an abnormal sprouting and meagerness of intersegmental vessels (Figs. 5F and G). Previously, we reported that *mib* mutant embryos have defects in blood circulation, and demonstrated a role of Notch signaling in blood vessel patterning [27]. In addition, most recently, similar vascular defects were also observed in zebrafish *beamter/deltaC* mutant and *DeltaC* morphant embryos [45]. These results suggest that Notch components, including Mib and Notch ligand DeltaC, have important roles in zebrafish blood vessel development. We also observed a significant reduction in the number of *gata1*-expressing hematopoietic cells in morphant embryos (Figs. 5H and I). The exact role of Snx5 in embryonic hematopoietic cell development is currently unclear. However, recently, it was reported that *mib* mutant fails to specify adult hematopoietic stem cells (HSC). They suggested that the Notch-Runx pathway is critical for the specification of HSC fate and the subsequent homeostasis of HSC number [26]. Snx5 was also identified as a gene differentially expressed between hematopoietic stem cells and committed progenitor cells [46]. In human, interestingly, Snx5 has been reported to bind the Fanconi anemia complementation group A protein (FANCA) [47]. Fanconi anemia (FA) is an autosomal-recessive genetic syndrome that is characterized by progressive bone-marrow failure, several developmental abnormalities and a predisposition to malignancy [48]. Together with these reports, our observations imply that Snx5 may have an important role in hematopoiesis and blood vessel developments.

Mind bomb plays a central part in the development and maintenance of a variety of tissues in vertebrates through Notch signaling. However, the mechanism of how the signal is promoted in association with endocytosis is largely unknown. Here, we have identified the early endosomal protein Snx5 as a Mind bomb-binding partner. The mechanisms by which Snx5 associate with Mind bomb in the endocytic pathway and participate in Notch signaling would be an important area of investigation in the future.

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