CELL REGENERATION

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SNX17 regulates Notch pathway and pancreas development through the retromer-dependent recycling of Jag1

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Abstract

Background: Notch is one of the most important signaling pathways involved in cell fate determination. Activation of the Notch pathway requires the binding of a membrane-bound ligand to the Notch receptor in the adjacent cell which induces proteolytic cleavages and the activation of the receptor. A unique feature of the Notch signaling is that processes such as modification, endocytosis or recycling of the ligand have been reported to play critical roles during Notch signaling, however, the underlying molecular mechanism appears context-dependent and often controversial.

Results: Here we identified SNX17 as a novel regulator of the Notch pathway. SNX17 is a sorting nexin family protein implicated in vesicular trafficking and we find it is specifically required in the ligand-expressing cells for Notch signaling. Mechanistically, SNX17 regulates the protein level of Jag1a on plasma membrane by binding to Jag1a and facilitating the retromer-dependent recycling of the ligand. In zebrafish, inhibition of this SNX17-mediated Notch signaling pathway results in defects in neurogenesis as well as pancreas development.

Conclusions: Our results reveal that SNX17, by acting as a cargo-specific adaptor, promotes the retromer dependent recycling of Jag1a and Notch signaling and this pathway is involved in cell fate determination during zebrafish neurogenesis and pancreas development.

Background

In the canonical Notch pathway, membrane-bound ligand binds to the Notch (receptor) in the target cells and induces a series of proteolytic cleavages to release the Notch intracellular domain (NICD) from the plasma membrane. The NICD then translocates into the nucleus and activates the expression of Notch target genes [1,2]. Studies during the past decade have revealed that ubiquitylation by the Neur or Mib family E3 ubiquitin ligases and subsequent endocytosis of Notch ligands are essential for the activation of this pathway [3,4]. It has been proposed that endocytosis generates a pulling force on the Notch receptor and promotes the cleavage and the activation of the receptor. Otherwise, the internalized ligand can be recycled back to specific micro-

domains on the plasma membrane conducive for Notch signaling. It has also been suggested that ligands can be activated by certain modifications during the recycling process [3-6]. However, the molecular nature of the modification remains elusive.

Sorting nexin (SNX) family proteins play diverse roles in processes such as endocytosis, intracellular protein sorting and endosomal signaling [7]. The PX-BAR subfamily members of SNXs are able to induce membrane tubulation which is essential for the retromer-dependent cargo trafficking [8]. The classic retromer is a multiprotein complex consists of a cargo-selective adaptor (Vps26-29-35) and a membrane-bound heterodimer of Vps5 (SNX1/2 in vertebrate) and Vps17 (SNX5/6 in vertebrate) [9]. It regulates the retrograde trafficking of cargos such as the cation-independent mannose-6-phosphate receptor (CI-MPR) from endosome to the Golgi apparatus. Recently, retromer is found to play a critical role in Wnt signaling by promoting the recycling of Wntless [10-16]. Interestingly, it is SNX3 but not

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SNX1/2 or 5/6 that regulates the retromer-dependent recycling of Wntless [17,18]. Another SNX family member, SNX27, can bind to the β2 adrenergic receptor and regulates the retromer-dependent endosome-to-plasma membrane trafficking of it [19]. It remains to be investigated whether other SNXs play similar cargo specific regulatory roles in retromer-dependent trafficking.

We report here that SNX17 regulates Notch pathway and cell-fate determination during zebrafish neurogenesis and pancreas development. SNX17 binds to Jag1a and facilitates the retromer-dependent recycling of the ligand. In fact, direct inhibition of the retromer pathway also reduces Notch activation. Thus, our study revealed a novel SNX17/retromer pathway in the regulation of Notch signaling.
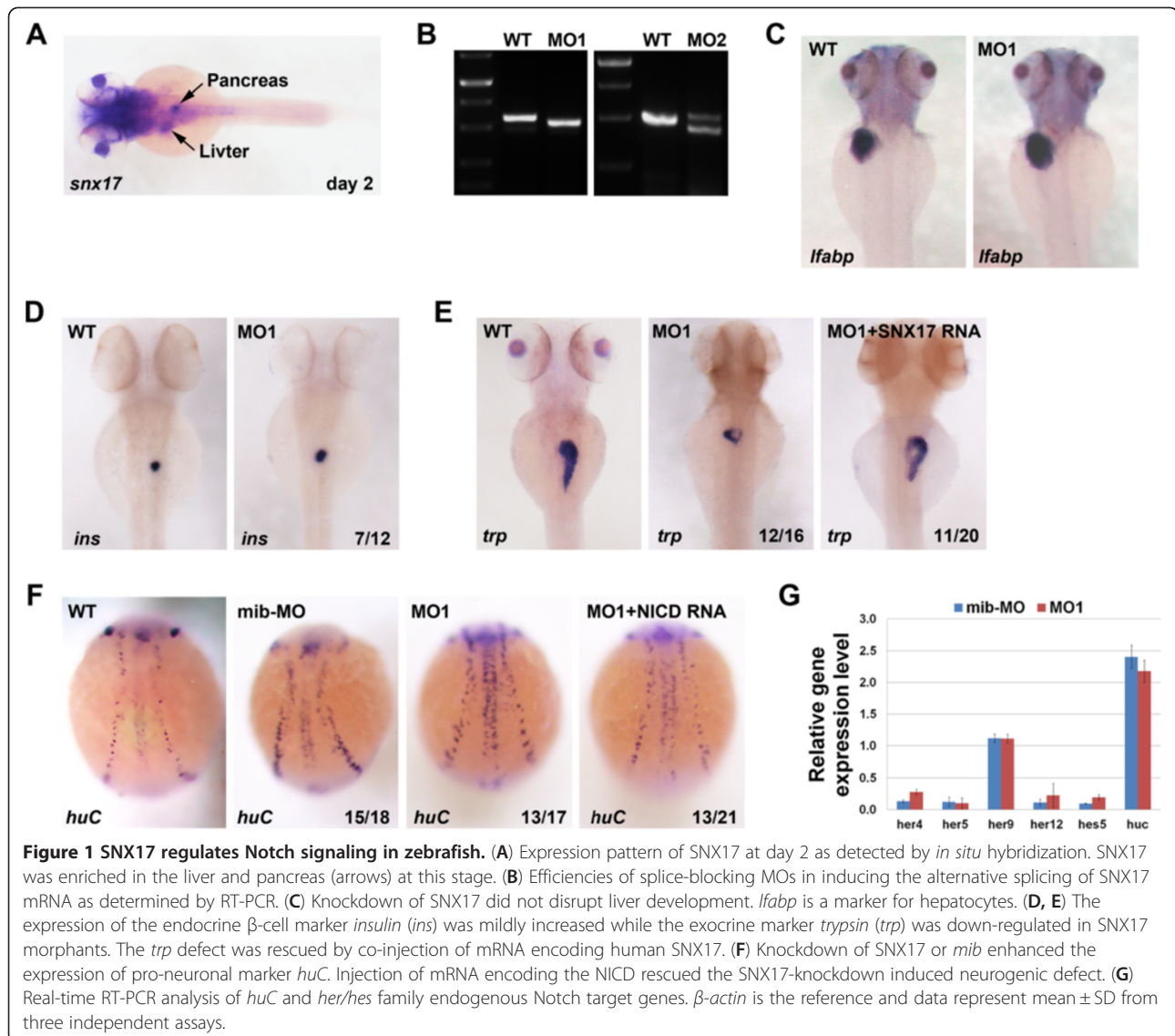
Results

SNX17 regulates Notch signaling during zebrafish embryogenesis

Previous studies in various cultured cells have reported that SNX17 is involved in the endocytosis of LDLR [20] and P-Selectin [21], the recycling of LRP [22], the scavenger receptor FEEL-1 [23] and integrins [24,25] to plasma membrane, as well as the escape of the lysosomal degradation of human papillomavirus L2 capsid protein during virus infection [26], however, the physiological function of SNX17 remains unknown. We used the zebrafish model to investigate the *in vivo* function of SNX17. We first examined the embryonic expression pattern of SNX17 by *in situ* hybridization and found it was not spatially restricted during gastrulation and early somitogenesis stages, but became enriched in the pancreas, liver, eye and neuronal tissues starting from day 2 (Figure 1A). We performed morpholino (MO) mediated knockdown of SNX17 and determined the downstream effects of this treatment. MOs targeting the exon 3/intron 3 junction (MO1) or the exon 2/intron 2 junction (MO2) of SNX17 gene were synthesized and both of them induced alternative splicing of SNX17 mRNA effectively (Figure 1B). The reading frames in these abnormally spliced RNAs are shifted and their protein products are non-functional. We treated embryos with these MOs and found that the expression of hepatocyte marker *lfabp* (liver fatty acid binding protein 1a) was normal in the morphants (Figure 1C). Interestingly, the expression of *insulin* (*ins*, a marker for endocrine β-cells) was mildly increased while that of *trypsin* (*trp*, a marker for exocrine pancreas) was severely reduced in SNX17 morphants (Figure 1D, E). Furthermore, the *trp* defect can be rescued by the over-expression of human SNX17, indicating this defect is SNX17 specific and not induced by the off-target effect of MOs. The pancreatic defect in SNX17 morphants is similar to that induced by inhibition of Notch, either in the *mib* mutant or after

DAPT treatment, which promotes the endocrine pancreas at the expense of exocrine pancreas [27]. In addition, Notch is well known to regulate neurogenesis during zebrafish embryogenesis [28], so we tested whether or not SNX17 was required for neurogenesis. As shown in Figure 1F, similar to that in *mib* morphants, the expression of pro-neuronal marker *huC* was enhanced when SNX17 was inhibited. This defect can be reversed by the injection of mRNA encoding the NICD. These data suggest that SNX17 is essential for Notch activation *in vivo*. We then determined the expression levels of several Notch target genes by real-time RT-PCR to further characterize the Notch signaling defect in SNX17 morphants. We found the expression levels of *her4, 5, 12* and *hes5* were significantly reduced in MO1 as well as *mib*-MO injected embryos. On the other hand, the expression of *huC* was increased in both morphants, which was consistent with the previous *in situ* hybridization result (Figure 1G). Taken together, these data clearly demonstrate that SNX17 is required for Notch signaling *in vivo*.

SNX17 is required in the ligand-expressing cell for Notch signaling

We used the Notch luciferase reporter system [29] in 293 T cells to further characterize the molecular mechanisms of SNX17 in Notch signaling. As shown in Figure 2A, over-expression of SNX17 stimulated the Notch reporter activity; on the other hand, siRNA-mediated knockdown of SNX17 reduced the reporter activity (the efficiencies of siRNAs were shown in Additional file 1: Figure S1). We investigated whether the SNX17 regulation of Notch was ligand specific. Jagged (Jag) and Delta family ligands were transfected into the 293 T cells and we found that Jag1 was the most potent one in stimulating the reporter. When SNX17 was knocked-down, the reporter activity was clearly inhibited in all of the ligand-transfected cells (Figure 2B). We further examined the expression levels of several Notch target genes (*hes1/7* and *hey1/2*) by real-time RT-PCR. As shown in Figure 2C, inhibition of SNX17 decreased the expression of *hes7, hes1* and *hey1*. Thus, SNX17 regulated the expression of both the transiently transfected Notch reporter and the endogenous Notch target genes. As Notch signaling requires the direct contact of a ligand-expressing cell (signal-sending cell) with a Notch-expressing cell (signal-receiving cell), we used a cell coculture system [30] to determine in which cell SNX17 functioned. As shown in Figure 1D, when SNX17 was knocked-down in the Jag1-expressing 293 T cells, these cells became less efficient to stimulate the luciferase reporter expressed in the Notch-expressing 3T3 cells. On the other hand, when SNX17 was down-regulated in the Notch-expressing 3T3 cells, luciferase reporter activity

Figure 1 SNX17 regulates Notch signaling in zebrafish. (A) Expression pattern of SNX17 at day 2 as detected by *in situ* hybridization. SNX17 was enriched in the liver and pancreas (arrows) at this stage. **(B)** Efficiencies of splice-blocking MOs in inducing the alternative splicing of SNX17 mRNA as determined by RT-PCR. **(C)** Knockdown of SNX17 did not disrupt liver development. *lfabp* is a marker for hepatocytes. **(D, E)** The expression of the endocrine β-cell marker *insulin (ins)* was mildly increased while the exocrine marker *trypsin (trp)* was down-regulated in SNX17 morphants. The *trp* defect was rescued by co-injection of mRNA encoding human SNX17. **(F)** Knockdown of SNX17 or *mib* enhanced the expression of pro-neuronal marker *huC*. Injection of mRNA encoding the NICD rescued the SNX17-knockdown induced neurogenic defect. **(G)** Real-time RT-PCR analysis of *huC* and *her/hes* family endogenous Notch target genes. *β-actin* is the reference and data represent mean ± SD from three independent assays.

in these cells was still stimulated by the addition of Jag1-expressing 293 T cells. Taken together, these results demonstrate that SNX17 is specifically required in the ligand-expressing cells for Notch signaling.

SNX17 interacts with Jag1a through the FERM-like domain

SNX family proteins can function as cargo-specific adaptors for vesicular trafficking. Our finding that SNX17 regulated Notch signaling in the ligand-expressing cells raised the possibility that it functioned as an adaptor for the trafficking of the ligand. We determined whether SNX17can interact with Jag1, a ligand most effective in stimulating Notch activity in 293 T cells (Figure 2B). We found that Flag-tagged SNX17 pulled down HA-tagged Jag1a and vice versa (Figure 3A, B). As a control, SNX11,

which is a PX-only SNX family member, did not pull-down Jag1a under the same condition. This observation suggested that the interaction between SNX17 and Jag1a is specific. Structurally, SNX17 contains a FERM-like domain which is not present in SNX11 or most of the other SNX family proteins [31]. We investigated whether this FERM-like domain was essential for interaction with Jag1a. As shown in Figure 3B, Jag1a co-immunoprecipitated with the FERM-like, but not the PX domain of SNX17. Thus, the FERM-like domain of NX17 mediated the interaction between SNX17 and Jag1a. We then investigated the subcellular distribution of SNX17 and Jag1a in the same cell line and found that SNX17 accumulated at Jag1a-positive vesicles (Figure 3C). These data further suggest that SNX17 might regulate the intracellular trafficking of Jag1a.
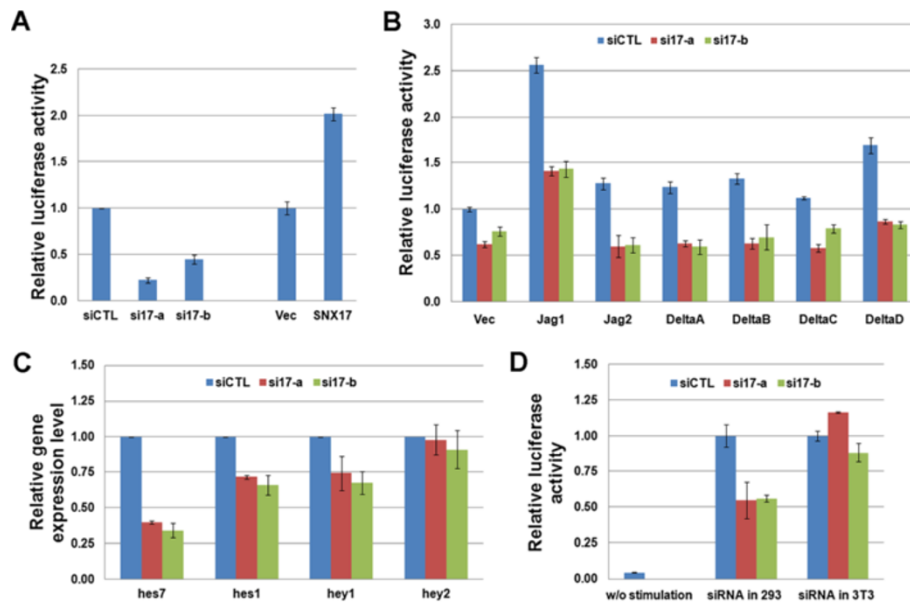
Figure 2 SNX17 is required specifically in the ligand-expressing cells for Notch signaling. (A) Knockdown of SNX17 by siRNAs in 293 T cells down-regulated the Notch luciferase reporter activity while over-expression of SNX17 stimulated the reporter activity. **(B)** Over-expression of Jag and Delta family Notch ligands stimulated the reporter activity which was blocked by the knockdown of SNX17. **(C)** Down-regulation of SNX17 reduced the expression of endogenous Notch target genes *hes1/7* and *hey1* in 293 T cells as determined by real-time RT-PCR (p < 0.05 in all cases). *hey2* was not affected in the same assay (P > 0.1). *β-actin* was the reference. **(D)** Co-culture of the Jag1-expressing 293 T cells with the Notch1-expressing NIH3T3 cells. Knockdown of SNX17 in the ligand-expressing cells reduced the Notch reporter activity while down-regulation of SNX17 in the receptor-expressing cells did not have inhibitory effect. All assays were repeated at least three times and data represent mean ± SD from three independent experiments.

SNX17 regulates the protein level of plasma membrane-associated Jag1a
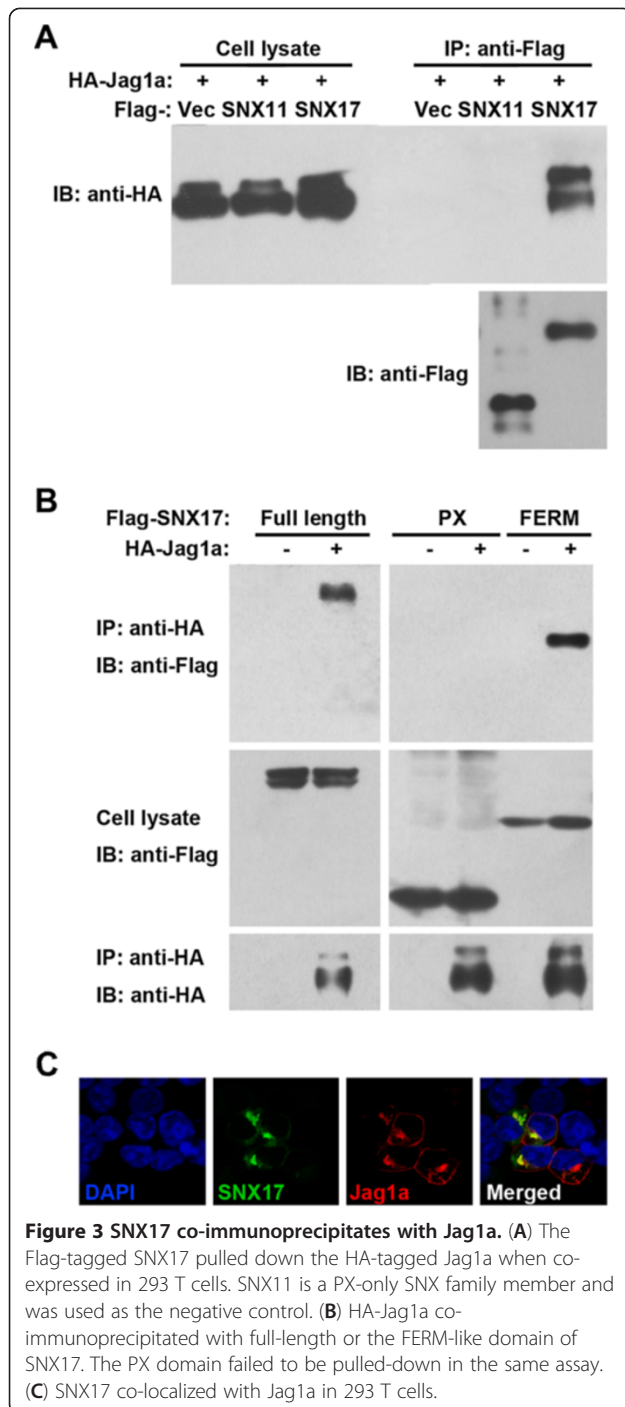
Previous studies indicate that SNX17 is able to regulate the levels of plasma membrane proteins either through the endocytosis or the recycling process. We investigated whether SNX17 regulated the homeostasis of Jag1a by comparing the protein levels of total as well as the plasma membrane associated Jag1a between the control and siSNX17 treated samples. The plasma membrane proteins were first labeled by biotin then isolated with the Pierce Cell Surface Protein Isolation Kit and Jag1a level was determined by western blot. As shown in Figure 4A, Jag1a level in either whole cell lysate or membrane fraction was clearly reduced when SNX17 was knocked-down by siRNAs. On the other hand, over-expression of SNX17 increased Jag1a protein level. As a control, the protein level of the plasma membrane associated transferrin receptor (TFR) was not affected by either knockdown or over-expression of SNX17.

The SNX17-stimulated accumulation of Jag1a on the plasma membrane could be a result of reduced internalization of the ligand, so we investigated whether SNX17 was required for the endocytosis of Jag1a. The endocytosis assay was performed as described [32]. We found that knockdown or over-expression of SNX17 did not affect the endocytosis of Jag1a much (Figure 4B).

The mild enhancement of endocytosis when SNX17 was over-expressed was most likely due to the elevated plasma membrane Jag1a protein level. We concluded that the SNX17 induced membrane accumulation of Jag1a cannot be a result of reduced endocytosis of Jag1a. We determined the subcellular distribution of Jag1a by immunofluorescence staining. As shown in Figure 4C, Jag1a was found at early endosomes, late endosomes and multivesicular bodies (MVB) but not Rab4-positive vesicles which are involved in the endosome-to-plasma membrane trafficking [19]. When SNX17 was knocked-down, we found that Jag1a was internalized and transported to the Hrs-positive vesicle normally (bottom panel). These results further suggest that SNX17 regulates Jag1a through a mechanism independent of endocytosis.

SNX17 regulates the retromer-dependent recycling of Jag1a

The endocytosed membrane proteins can be transported back to the plasma membrane through the recycling endosome pathway (Rab11-dependent) or the retromer pathway (Vps35-dependent). We first determined the subcellular distribution of SNX17 and found that it localized to the Rab5, Rab7 or Vps35 but not Rab11-positive vesicles (Figure 5A). This observation suggested that the
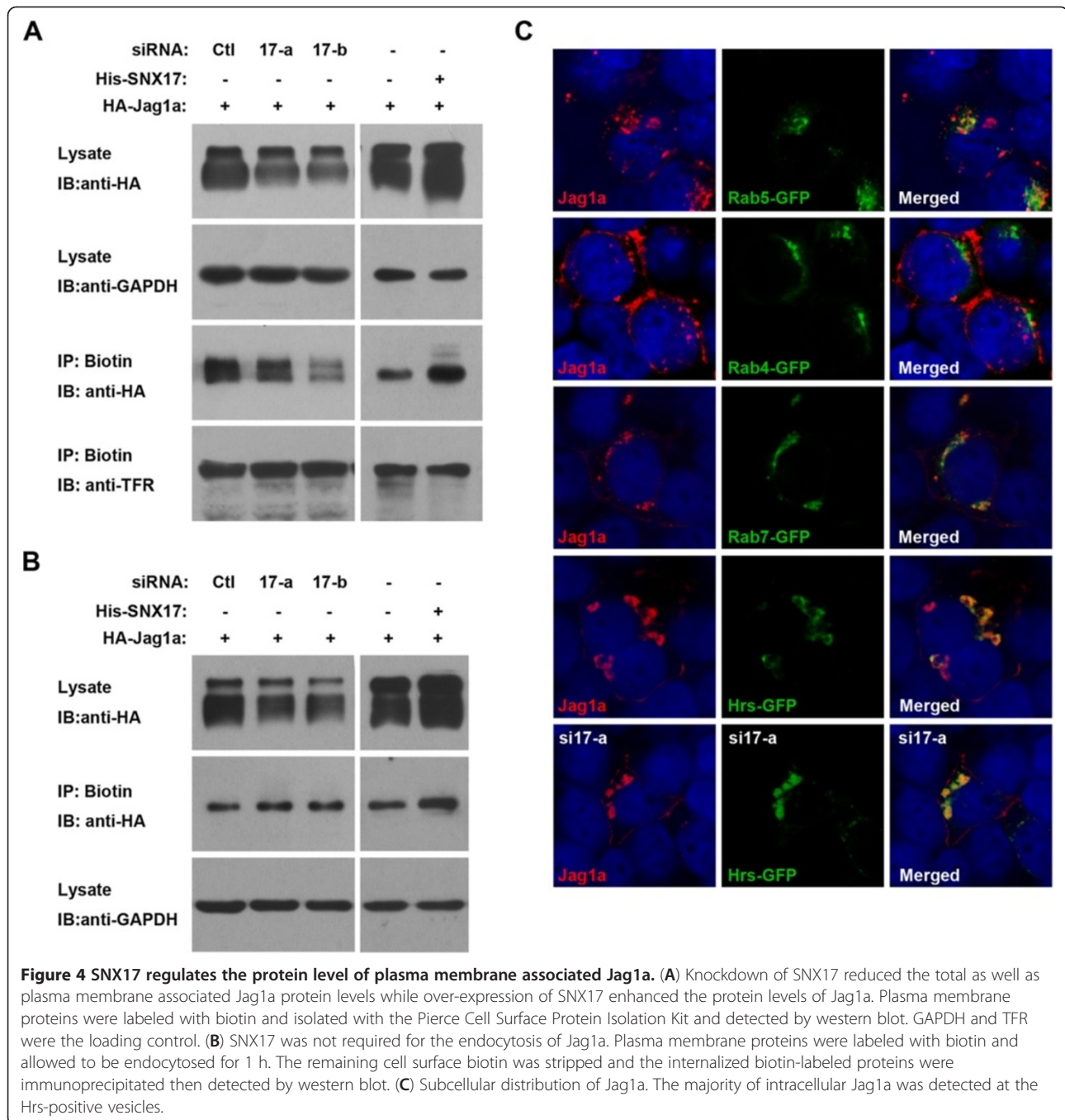
Figure 3 SNX17 co-immunoprecipitates with Jag1a. (**A**) The Flag-tagged SNX17 pulled down the HA-tagged Jag1a when co-expressed in 293 T cells. SNX11 is a PX-only SNX family member and was used as the negative control. (**B**) HA-Jag1a co-immunoprecipitated with full-length or the FERM-like domain of SNX17. The PX domain failed to be pulled-down in the same assay. (**C**) SNX17 co-localized with Jag1a in 293 T cells.

concluded that Jag1a and TFR used different pathways for their recycling and SNX17 regulated the recycling of Jag1a through a Vps35 but not Rab11-dependent pathway. If the retromer pathway is essential for the maintenance of the homeostasis of Jag1a on the plasma membrane, it might be required for Notch signaling. We investigated this possibility by performing the luciferase reporter assay. As shown in Figure 5C, inhibition of Vps35 but not Rab11 reduced the reporter activity. We further found that, similar to SNX17, Vps35 was only required in the ligand-expressing cells for Notch signaling (Figure 5D). Taken together, these results demonstrate that the SNX17-retromer pathway is required for the homeostasis of Jag1a and subsequent Notch signaling.
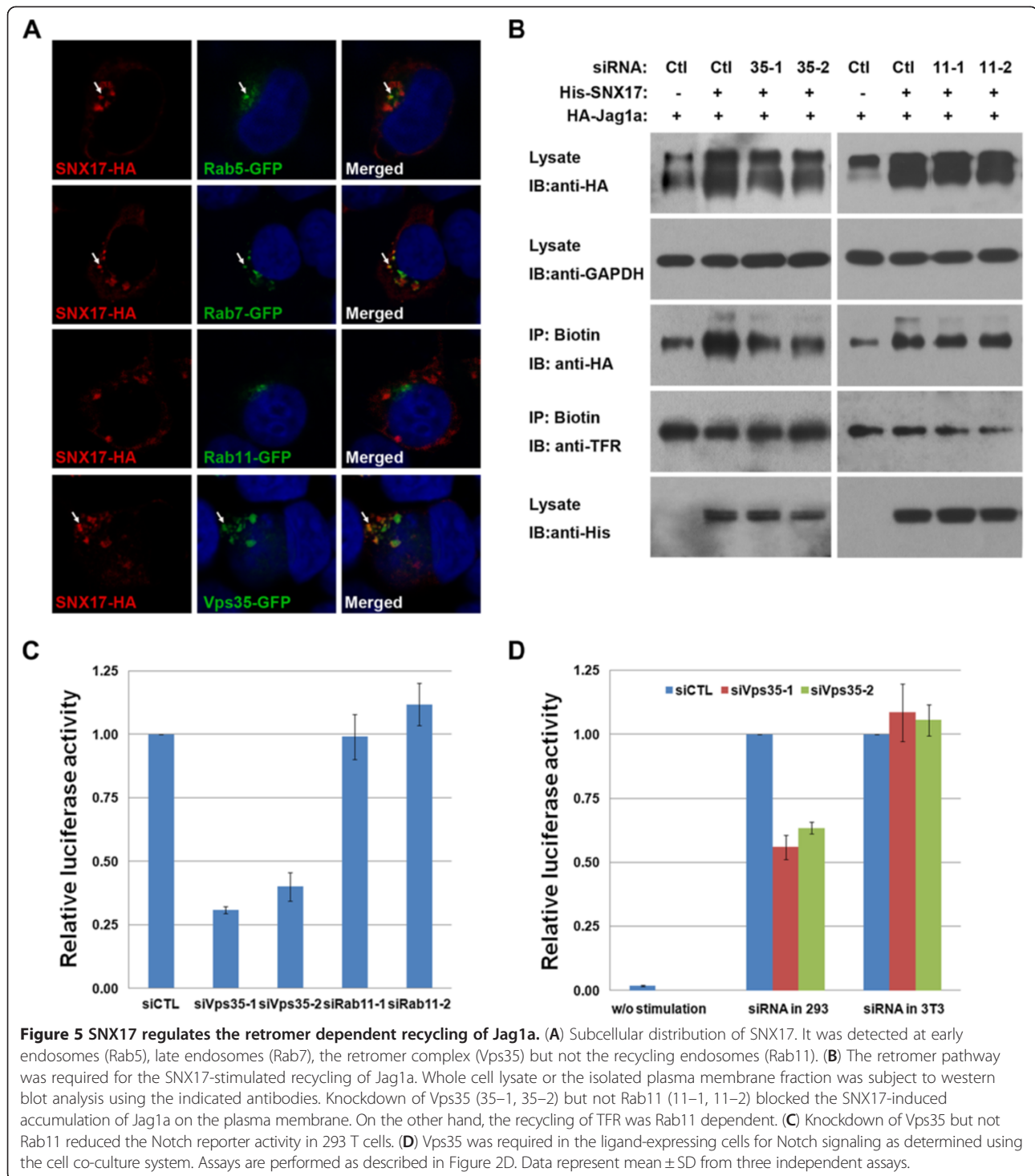
Discussion

It is well established that the endocytosis of ligand is pivotal for the activation of Notch pathway. One generally accepted model is that endocytosis generates a pulling force on the Notch receptor, which promotes the cleavage and the activation of the receptor. In polarized cells, the endocytosed ligand can be relocated to a position on the plasma membrane conducive to signaling. In this model, the transcytosed ligand is clustered or localized to micro-domains adjacent to Notch receptor where it can induce Notch activation efficiently. In this study, we identified vesicular trafficking protein SNX17 as a novel regulator of the Notch signaling both *in vitro* and *in vivo*. We show that SNX17 is not essential for the endocytosis of Jag1a but regulates the homeostasis of plasma membrane associated Jag1a. We propose that, by binding to SNX17, Jag1a escapes from the degradation and enters the recycling pathway which results in the accumulation of Jag1a on the plasma membrane.

Rab11-mediated recycling of ligand has been implicated in Notch activation in the drosophila. For example, it regulates the recycling of Delta in the sensory organ precursors [33,34]. However, the requirement for recycling pathway in Delta signaling appears to be limited to specific cell types, since Rab11 is not essential for Delta signaling in other cells such as the germline cells or eye disc [35,36]. We found that the Rab11 pathway is not involved in the recycling of Jag1a and down-regulation of Rab11 does not reduce the activity of Notch reporter. Unexpectedly, we found that the retromer pathway is required for Notch activation. A few previous studies reported that the retromer pathway is not involved in the regulation of Notch pathway in wing disc or the follicle epithelium in the drosophila [12,13,37]. This is contradictory to what we found here. However, context-dependent regulatory mechanisms are well known for Notch pathway, so it is possible that the retromer-mediated recycling of the ligand is required for Notch signaling in some cell types but not the others. Another

retromer pathway could be involved in the SNX17-induced recycling of Jag1a. We tested this hypothesis by determining whether the recycling of Jag1a is affected when this pathway is blocked. As shown in Figure 5B, pretreatment of cells with siRNAs to Vps35 but not Rab11 blocked the SNX17-stimulated accumulation of Jag1a. On the other hand, the recycling of TFR which is recycling endosome dependent was clearly down-regulated by siRNAs to Rab11 but not Vps35. We

Figure 4 SNX17 regulates the protein level of plasma membrane associated Jag1a. (A) Knockdown of SNX17 reduced the total as well as plasma membrane associated Jag1a protein levels while over-expression of SNX17 enhanced the protein levels of Jag1a. Plasma membrane proteins were labeled with biotin and isolated with the Pierce Cell Surface Protein Isolation Kit and detected by western blot. GAPDH and TFR were the loading control. **(B)** SNX17 was not required for the endocytosis of Jag1a. Plasma membrane proteins were labeled with biotin and allowed to be endocytosed for 1 h. The remaining cell surface biotin was stripped and the internalized biotin-labeled proteins were immunoprecipitated then detected by western blot. **(C)** Subcellular distribution of Jag1a. The majority of intracellular Jag1a was detected at the Hrs-positive vesicles.

possibility is that the role of endocytosis or recycling in Notch activation is ligand specific. For example, Mib-induced ubiquitylation is required for the endocytosis of Delta and subsequent Notch activation [28,38]. Mib also ubiquitylates Jag1a and is required for the Jag1a-induced Notch activation, however, the endocytosis of Jag1a is largely Mib-independent. The exact function of Mib in Jag1a signaling remains to be characterized [39]. It has been proposed that there are two types of ligand

endocytosis: one is the constant endocytosis which regulates the homeostasis of ligand on the plasma membrane; the other is the signaling-specific endocytosis of the ligand. We found a large fraction of intracellular Jag1a at the Hrs vesicles, which usually enter the degradation pathway after fusion to lysosomes. This observation suggests that the rate of internalization and turnover of Jag1a is high in this cell. Under such circumstance, SNX17 could effectively regulates the protein

Figure 5 SNX17 regulates the retromer dependent recycling of Jag1a. (A) Subcellular distribution of SNX17. It was detected at early endosomes (Rab5), late endosomes (Rab7), the retromer complex (Vps35) but not the recycling endosomes (Rab11). **(B)** The retromer pathway was required for the SNX17-stimulated recycling of Jag1a. Whole cell lysate or the isolated plasma membrane fraction was subject to western blot analysis using the indicated antibodies. Knockdown of Vps35 (35–1, 35–2) but not Rab11 (11–1, 11–2) blocked the SNX17-induced accumulation of Jag1a on the plasma membrane. On the other hand, the recycling of TFR was Rab11 dependent. **(C)** Knockdown of Vps35 but not Rab11 reduced the Notch reporter activity in 293 T cells. **(D)** Vps35 was required in the ligand-expressing cells for Notch signaling as determined using the cell co-culture system. Assays are performed as described in Figure 2D. Data represent mean ± SD from three independent assays.

level of plasma membrane associated Jag1a by binding to Jag1a and redirecting it from the degradation pathway to the retromer-dependent recycling pathway,

Vertebrate SNX1/2 and 5/6 are components of the retromer complex. Recent studies revealed that other SNX family proteins can play additional roles in retromer-dependent trafficking. For example, SNX3 functions as a cargo-specific adaptor for the retromer-dependent trafficking of Wntless [17,18] while SNX27 can serve as an adaptor protein for the retromer -dependent endosome-to-plasma membrane trafficking of the β2 adrenergic receptor [19]. We showed here that SNX17 is able to interact with Jag1a and regulate the retromer-dependent recycling of the ligand. It could be a general theme that

SNX family proteins function as cargo-specific regulators in retromer-dependent vesicular trafficking. It will be interesting to test whether other SNX family members can function in similar manners and play regulatory roles in other cell signaling events.

Conclusions

We identified SNX17 as a tissue specific regulator of Notch pathway, and this SNX17-regulated Notch pathway is essential for inhibition of neurogenesis as well as cell fate determination in pancreas development. We revealed the molecular mechanism of SNX17: it does not affect the endocytosis of Jag1a; instead, it promotes the retromer-dependent recycling of the ligand which results in the accumulation of Jag1a protein on plasma membrane and enhanced Notch signaling.

Methods

Constructs

Molecular cloning was performed according to standard protocols. The following constructs were used in this study: N-3Xflag-hSNX17 in pReceiver-M12, N-His-hSNX17 in pReceiver-M01, hSNX17-HA in PCR3.1,N-eGFP-hVps35 in pReceiver-M29, N-3Xflag-SNX17-PX, N-3Xflag-SNX17-FERM in pReceiver-M12, N-eGFP-Hrs in pReceiver-M29, GFP-Rab4 in pReceiver-M29,GFP-Rab5, GFP-Rab7 and GFP-Rab11in PCR3.1, hSNX17-GFP in PCR3.1, 8X CBF1 reporter (Notch luciferase reporter plasmid, a gift from Dr .M. M. Chiu). hJag1 and rNotch1 were cloned into the pBABE-Puro retroviral vector for making the stable cell lines. Jag1, Jag2, DeltaA, DeltaB, DeltaC and DeltaD were cloned in PCR3.1. dJagged1a-HA in pCS2+ was provided by Dr. M. Itoh. Full-length hSNX17 and the rNICD (a.a. 1751–2531) were cloned in the pCS2+ for making mRNAs *in vitro* for rescue experiments. All constructs were confirmed by DNA sequencing. Detailed information about the constructs is available upon request.

Zebrafish

Zebrafish (the Longfin line) and embryos are maintained and staged as previously described [40]. MOs were purchased from Gene-Tools (Corvallis, OR): MO1 (AGACCAACACTTTCTCACCAGCTTG, 3 ng), MO2 (GATGAAAGTGTGTGCTCACCTGTTC, 4 ng) and the standard control MO (CCTCTTACCTCAGTT ACAATTTATA, 4 ng). MOs were injected at 1-cell stage. For rescue experiments, embryos were first injected with MO, 5–10 min later, embryos were injected second time with mRNA (100 pg of hSNX17 mRNA or 20 pg of rNICD mRNA). Injected embryos were fixed at the indicated stages and whole-mount *in situ* hybridization was performed as described [40]. *lfabp, insulin* and *trypsin* were detected at day 3

embryos and *huC* assayed in 2–3 somite stage embryos. Research on animal was performed with the approval of the Guangzhou Institutes of Health ethical committee.

Notch luciferase reporter assay

293T and NIH3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Gibco, CA). Cells were seeded in 24-well plate ($5x10^4$ cells/well) for 16 h then siRNAs (final concentration of 40 nM) were transfected with the DharmaFECT Transfection Reagent according to the manufacturer's protocol (Thermo Fisher, MA). 24 h after transfection, cells were transfected again with the 8X CBF1 reporter plasmid (0.1 μg) and pRL-EF (10 ng). The firefly and renilla luciferase activities were determined with the Promega Dual luciferase assay system (Madison, WI) 48 h post transfection. The pRL-EF plasmid, which expresses renilla luciferase under the control of the EF-1 promoter, was used to normalize the transfection efficiency of the luciferase reporters. For the ligand stimulation assay, 24 h after siRNA treatment, cells were transfected with the ligand plasmid (1 μg), the 8X CBF1 reporter (0.1 μg) and the pRL-EF (10 ng) and luciferase activities were determined as describe above. For co-culture experiment, a 293 T cell line stably expressing human Jag1 and a NIH3T3 cell line stably expressing rNotch were established. In order to test the function of SNX17 in signal sending cells, 293 T-Jag1 cells were transfected with siRNAs as described before. NIH3T3-rNotch cells were seeded in 12-well plate ($1x10^5$cells/ well) for 16 h then the 8X CBF1 reporter (0.4 μg) and pRL-EF (40 ng) were transfected into cells with the Lipofectamine 2000 reagent. 24 h after transfection, these transfected cells ($5x10^4$ each) were mixed and cultured for another 48 h and luciferase activities were assayed. For SNX17 knockdown in signal receiving cells, NIH3T3-rNotch cells were transfected with siRNAs to mouse SNX17 (40 nM) for 16 h then the 8X CBF1 reporter (0.4 μg) and pRL-EF (40 ng) were transfected with the Lipofectamine 2000 reagent. 24 h after transfection, these cells were co-cultured with 293 T-Jag1 cells and luciferase activity measured as describe above.

Real-time RT-PCR

Total RNAs were extracted from 293 T cells or zebrafish embryos using the RNAqueous®-4PCR Kit (Ambion, CA). Reverse-transcription was performed using the ReverTra Ace (TOYOBO, Japan) and PCR reactions were performed with the SYBR®Premix Ex Taq™ Kit (TAKARA, Japan) on the ABI7300 Real-Time PCR System. The relative gene expression level was determined by the delta delta Ct method using the *β-actin* gene as the reference. The sequences of primers used were listed in Additional file 2: Table S1.

Co-IP and western blot

293 T cells in 100-mm dish were transfected with N3FSNX17 (6 μg) and dJag1a HA (3 μg). 36 h after transfection, cells were washed with PBS and lysed in 700 μl TNE buffer (50 mmol/L Tris–HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 1 mmol/L EDTA, 10 mM NaF and 1 mM Na_3VO_4) with the protease inhibitor cocktail (Roche) and PMSF for 30 min. Cell lysates were clarified by centrifugation and the Flag or HA resin (30 μl) was added into the supernatant and incubated for 4 h at 4 °C. The resin was washed with TNE buffer for 3 times and eluted by adding 80 μl of the Flag or HA peptide. The eluant was clarified, boiled in SDS-sample buffer and ready for western blot analysis. Western blot was performed according to the standard protocol with the following antibodies: HRP-conjugated mouse anti-GADPH mouse (1:3000, Abcam), mouse anti-TFR (1:1000, Invitrogen), anti-His mAb (27E8) (1:5000, CST), anti-Myc mAb (9E10) (1: 5000, Sigma), mouse anti-HA (1:1000, Beyotime Institute of Biotechnology), anti-Flag mAb (1:5000, Sigma), goat anti-mouse HRP (1:3000, Amersham Biosciences).

Immunostaining

293 cells on cover slips were transfected with the indicated plasmids for 24 h then washed with PBS and fixed in MeOH at –20 °C for 5 min. Samples were permeablized in PBS containing 0.1% Triton X-100 for 10 min at R.T.. Fixed cells were then blocked in PBS/10% normal goat serum for 1 h, incubated in primary antibody for 1 h at R.T. and washed three times with PBS. Samples were incubated in goat anti- mouse antibody conjugated with TRITC secondary antibody for 1 h at R.T. and washed with PBS, counter-stained with DAPI, mounted on glass slides and ready for imaging. Fluorescence images were taken using the Leica TCS SP2 Spectral Confocal System and manipulated with Adobe Photoshop.

Surface biotinylation and endocytosis assay

293 T cells were first transfected with siRNAs for 24 h, then transfected again with dJag1a (3 μg) and His-SNX17 (6 μg) as indicated for 48 h. For surface biotinylation assay, plasma membrane proteins were labeled with biotin and isolated with the Pierce Cell Surface Protein Isolation Kit. For endocytosis assay, the biotinylation buffer was removed and cells were incubated at 37 °C for 60 min. The remaining cell-surface biotin was stripped by three 25-min incubations with stripping buffer (50 mM MesNa, 50 mM Tris pH 8.3, 100 mM NaCl, 1 mM EDTA, 0.2% BSA) on ice. Cells were then lysed and the biotinylated proteins in the lysate were pulled-down with streptavidin-agarose. The protein level of Jag1a and was then determined by western blot.

Additional files

Additional file 1: Figure S1 Efficiencies of siRNAs used in this study. siRNAs to human SNX17 (**A**), mouse SNX17 (**B**), human vps35 (**C**), mouse vps35 (**D**) and human rab11 (**E**) effectively down-regulated their target genes at the mRNA level as determined by real-time RT-PCR. β-actin was the reference. The sequence of siRNAs and primers used in the PCR reactions were listed in Additional file 2: Table S1 and Additional file 3: Table S2.

Additional file 2: Table S1. List of primers used in the study.

Additional file 3: Table S2. List of siRNAs used in the study.

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Authors' contributions

WY carried out most of the experiments. DL and NL helped biochemical studies and the reporter assay. LX helped the zebrafish analysis. SL provided reagents and zebrafish facility. XS and DP designed and coordinated the study and wrote the manuscript. All authors read and approved the manuscript.

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