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Delta1 family members are involved in filopodial actin formation and neuronal cell migration independent of Notch signaling

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ABSTRACT

Delta family proteins are transmembrane molecules that bind Notch receptors and activate downstream signaling events in neighboring cells. In addition to serving as Notch ligands, Notch-independent roles for Delta have been suggested but are not fully understood. Here, we demonstrate a previously unrecognized role for Delta in filopodial actin formation. Delta1 and Delta4, but not Delta3, exhibit filopodial protrusive activity, and this activity is independent of Notch signaling. The filopodial activity of Delta1 does not depend on the PDZ-binding domain at the C-terminus; however, the intracellular membrane-proximal region that is anchored to the plasma membrane plays an important role in filopodial activity. We further identified a Notch-independent role of DeltaD in neuronal cell migration in zebrafish. These findings suggest a possible functional link between Notch-independent filopodial activity of Delta and the control of cell motility.

1. Introduction

Evolutionarily conserved Notch signaling plays critical roles in cell growth and differentiation in many cell types during development [1,2]. Notch is a transmembrane receptor that is activated by several ligands. Two groups of Notch ligands that are also transmembrane proteins, Delta and Jagged/Serrate, have been identified in numerous species. In the canonical Notch signaling pathway, ligand-stimulated Notch receptors are cleaved, and their intracellular fragments enter the nucleus to activate downstream effector genes.

In addition to acting as Notch ligands, accumulating evidence suggests that Delta and Jagged function independently of Notch in different processes such as cell motility, oncogenic transformation and neurogenesis [3–8]. For example, the potential involvement of the PDZ-binding domain (PDZ-BD) of Delta1 in cell adhesion and motility has been well-studied [3,5,6,9]. These studies suggest that PDZ proteins such as MAGI and Dlg stabilize surface expression of Delta1 and increase the efficiency of binding of Delta or Notch on neighboring cells, which promotes the cohesiveness between cells. However, Delta3 and Jagged2 do not have PDZ-BDs, which are required for binding to PDZ proteins [10], and the function of intracellular regions other than the PDZ-BD of Delta proteins is not fully understood.

Individual cell motility requires rearrangement of the cytoskeleton. The actin cytoskeleton is a dynamic structure that is involved in cell motility in response to various signals. Cells extend two types of protrusions containing actin, sheet-like protrusions called lamellipodia and spine-like protrusions called filopodia [11–14]. A previous study showed that Delta1 co-localized with actin filaments in keratinocytes [3]. In addition, *Drosophila* Delta, which does not have a PDZ-BD, promotes filopodia formation [15]. However, the function of Delta in relation to actin-driven filopodia formation is not known.

In this study, we uncovered a new role for Delta family proteins in filopodial actin extension. Delta1 and Delta4, but not Delta3, promote filopodial protrusions in neuro2a cells, and their functions are independent of Notch signaling activity. This filopodial activity does not require the PDZ-BD of Delta1; however, it does require the membrane-anchored intracellular domain of Delta1. We further identified Notch-independent activity of DeltaD, a Delta1 family protein in zebrafish, in neuronal cell migration *in vivo*. Collectively, these data suggest an unidentified mechanism mediates the actin regulatory function of Delta1 through a Notch-independent pathway that contributes to neuronal cell migration *in vivo*.

Abbreviations: EGF, epidermal growth factor; DSL, Delta/Serrate/Lag-2; PDZ-BD, PSD-95/Discs-large/ZO-1-binding domain; MAGI, MAGUKS with inverted domain structure; Dlg, Discs large; mib, mind bomb; hpf, hours post-fertilization.

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2. Materials and methods

2.1. Plasmids and expression constructs

In full-length mDelta1-Myc (M1 to V722), the stop codon of mouse Delta1 was replaced by histidine to allow fusion with a 6× Myc tag, and the fusion was inserted into pcDNA3 (Invitrogen). Delta1 Myc ΔICD is a deletion form lacking a cytoplasmic region (R570 to D714). Delta1 Myc TMICD is a deletion form lacking the extracellular region (V12 to H535). Delta1 Myc TMICDΔMPR-A is a deletion construct of TMICD lacking the membrane-proximal region A (R570 to C583). Delta1 Myc TMICDΔMPR-B is a deletion form of TMICD lacking the membrane-proximal region B (G584 to A594). Delta1 Myc ECD is a deletion construct lacking the transmembrane domain and intracellular region (M536 to V722). Delta1 Myc ICD is a form containing the intracellular region (V567 to V722). Delta1 HA wt is a full-length form in which an HA tag is inserted between A594 and N595 in pcDNA3.1. Delta1 HA del1 is an HA-tagged deletion form lacking an intracellular region (N595 to V722). mDelta3-Myc was made in a manner similar to mDelta1-Myc. The hDelta4-Flag plasmid was obtained from S. Sakano (Asahi Kasei, Japan). Zebrafish DeltaA-HA and DeltaD-HA expression plasmids were created by replacing the stop codons with HA tags, and the entire coding fragments were inserted into the pCS2 + vector. HA-DeltaD ICD is an HA-tagged form containing the intracellular region (I570 to V717). DeltaD TMICD is a deletion form lacking the extracellular region (G21 to C514). Xsu(H)DN and Notch1ICD (pEFBOSneo-RAMIC) have been previously described [16,17].

2.2. Cell culture, transfection, immunofluorescence, phalloidin staining and chemical treatment

Neuro2a cells were transfected using Fugene6 (Roche, Switzerland). Cells were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Myc-tagged or HA-tagged Delta- or Xsu(H)DN-expressing cells were visualized with primary antibodies, anti-Myc (9E10, Santa Cruz, CA, USA) or anti-HA (3F10, Roche, Switzerland) and then with secondary antibodies, containing Alexa-488 mouse or rat IgG, respectively (Invitrogen, CA, USA). Actin filaments were visualized with rhodamine–phalloidin (Invitrogen, CA, USA) or DY590-phalloidin (Dyomics GmbH, Germany). Twenty-four hours after transfection, cells were treated with 2 μM cytochalasin D (Sigma, MO, USA) or 5 μM nocodazole (Sigma, MO, USA) for 24 h and were then fixed for staining.

2.3. Reporter gene assays

Neuro2a cells (4.5×10^4) were transfected with Xsu(H)DN, Notch1ICD (pEFBOS-neo RAMIC), a wild-type or mutant 8× su(H) Notch reporter (0.1 μg of JH26 or JH28 plasmid, respectively), and pRL-EF (5 ng). Forty-eight hours after transfection, firefly and *Renilla* luciferase activities were determined with the Promega Dual luciferase assay system (Madison, WI, USA). The Notch activity of each transfection was calculated as the ratio of values determined from the wild-type reporter compared to the values determined by the mutant reporter.

2.4. Fish maintenance and mutants

Zebrafish were raised and maintained under standard conditions. The *mib*^{ta52b} mutant line has been previously described [18].

2.5. mRNA injection

mRNAs were synthesized by transcription with SP6 RNA polymerase using the SP6 mESSAGE mACHINE kit (Applied Biosystems, CA, USA), and 600 pg of mRNA were injected into one-cell-stage embryos.

2.6. Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was performed as previously described, except a different blocking buffer was used (5% sheep serum, 1% BMB, 0.1% Tween 20, 1% DMSO, 150 mM NaCl and 100 mM maleic acid, pH 7.5) [19]. *elavl3* utilized in the experiment were previously published [20].

3. Results

3.1. Delta1 induces filopodial protrusions in neuro2a cells

An earlier study demonstrated that overexpression of dominant negative or full-length forms of Delta1 in neuro2a neuroblastoma cells induces neurite extension in reduced serum at low plating density [21]. We performed a similar experiment in regular media containing 10% fetal calf serum and found that overexpression of full-length mouse Delta1 increased the number of cells with short cellular protrusions, as shown by staining with fluorescently labeled phalloidin (85%, $n = 708$) (Fig. 1A). To further define the cellular protrusions induced by Delta1, we treated Delta1 overexpressing cells with cytochalasin D, a potent inhibitor of actin polymerization, and nocodazole, which interferes with the polymerization of microtubules. Treatment with cytochalasin D but not nocodazole inhibited Delta1-induced protrusions (Fig. 1B). These data suggest that the filopodial protrusions induced by Delta1 are composed of actin filaments.

3.2. Delta1 and Delta4 family proteins, but not Delta3, induce cellular protrusions

More than three Delta family members (e.g., Delta-like 1, 3 and 4 in mammals; DeltaA, B, C, D and 4 in zebrafish) have been identified in vertebrates. These proteins show considerable structural homology within their extracellular regions and share a common domain architecture, including multiple epidermal growth factor (EGF)-like repeats and a DSL domain [10]. Therefore, we hypothesized that other Delta family proteins may possess similar protrusive activities. Transfection of Delta1, Delta3 and Delta4 into neuro2a cells revealed differing activities among the Delta protein family, namely Delta1 and Delta4 have protrusive activity but Delta3 does not (Fig. 2A). We next examined if zebrafish homologs of Deltal possess protrusive activity. Although zebrafish DeltaA and DeltaD do not have strong protrusion activity like Delta1, they showed a substantial effect on the formation of filopodial protrusions (Fig. 2B). These data suggest that Delta1 family proteins have conserved functions in inducing actin-based cell protrusions in a cell-autonomous manner.

3.3. A membrane-proximal domain anchored to the plasma membrane is required for the protrusive activity of Delta1

A previous study suggested the potential involvement of Delta in actin cytoskeletal organization [3]. The mechanism of Delta1-mediated regulation of actin dynamics remains unclear; therefore, we sought to identify regions important for the cellular protrusive activity of Delta1. The PDZ-binding domain at the Delta1 C-terminus is important for the cohesiveness of keratinocytes [3]; however, deletion of 128 C-terminal amino acids of Delta1, including the PDZ-binding domain, did not affect its protrusive activity (Delta1 HA del1 in Fig. 2C). In contrast, deletion of almost the entire cytoplasmic region, except the PDZ-binding domain, resulted in a

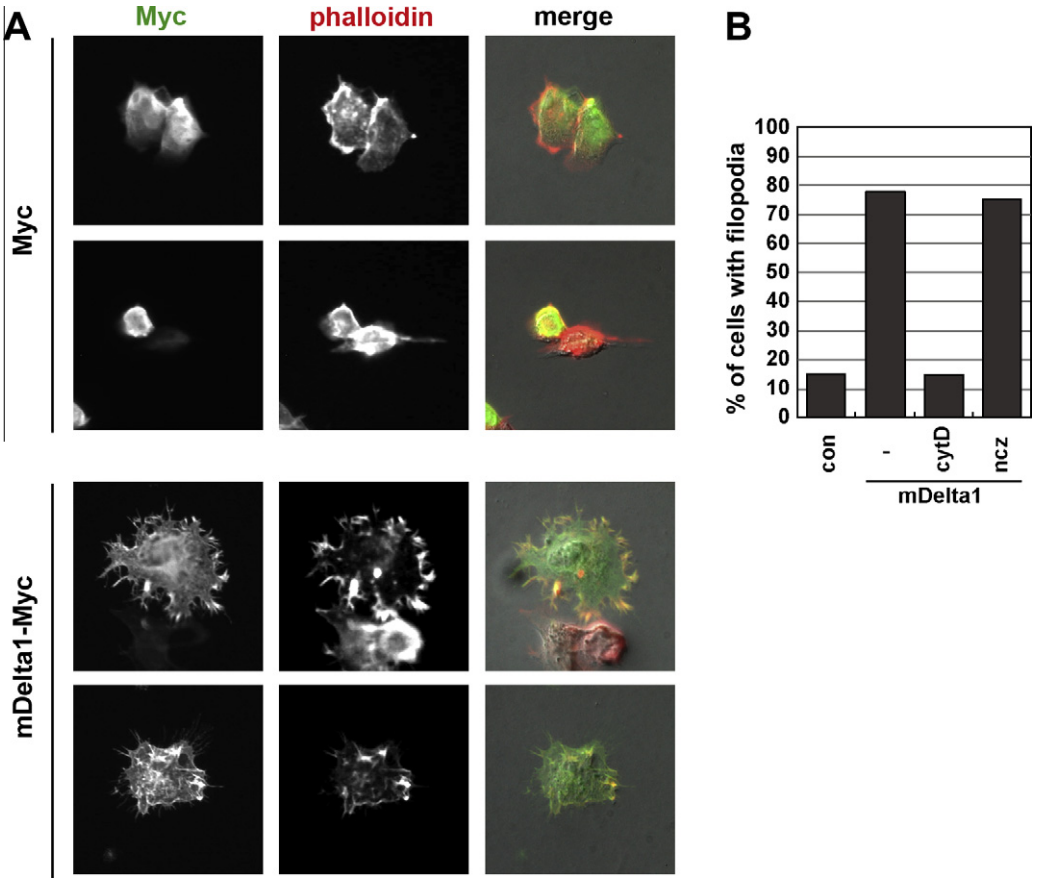

Fig. 1. Delta1 promotes actin-containing filopodia formation. (A) Delta1 induces phalloidin-labeled filopodia. Neuro2a cells were transfected with either control plasmid (Myc) or a plasmid carrying Myc-tagged mouse Delta1 (mDelta1-Myc). Cells were fixed and stained with phalloidin (red) and an anti-Myc antibody (green). Two sets of stained cells transfected with each plasmid are shown. (B) Delta1-induced filopodia formation is repressed by cytochalasin D (cytD) but not nocodazole (ncz). The percentage of cells showing more than three protrusions was scored. More than 100 cells were counted per sample.

marked reduction in protrusive activity (Delta1 Myc ΔICD in Fig. 2C). Furthermore, a membrane-tethered intracellular domain exhibited protrusive activity; however, the extracellular or cytoplasmic domains alone did not demonstrate protrusive activity (Delta1Myc TMICD, ECD, ICD, respectively, in Fig. 2C). These data suggest that the membrane-proximal region of Delta1 that is anchored to the cell membrane plays an important role in its actin reorganizing activity. Comparisons of multiple protein sequence alignments of Delta family membrane-proximal intracellular domains revealed that Delta1 proteins share more than 50% homology, while Delta4 proteins share 30% homology, and Delta3 and *Drosophila* Delta proteins share 12% homology (Fig. 2D). To further explore the importance of this region, we examined the protrusive activity of deletion mutants (Fig. 2E). The deletion analysis revealed that membrane-proximal region A is more important than region B (Fig. 2E). Based on amino acid sequence, the A region is less conserved than the B region. Therefore, we speculate that only a few conserved residues among the Delta1 and Delta4 family proteins and/or their analogous conformational state without amino acid sequence similarity are crucial for protrusive activity.

3.4. The cellular protrusive activity of Delta is independent of its Notch signaling functions

Previous studies have shown that Delta inhibits Notch activity cell-autonomously [21–24]. Delta1 induces cellular protrusions in a cell-autonomous manner; therefore, we examined if filopodia formation is correlated with changes in Notch activity. In neuro2a cells, Notch1 activation mediated by its intracellular domain (RAMIC) was suppressed by the expression of the dominant-negative su(H) mutant Xsu(H)DN, which cannot bind DNA (Fig. 3A) [17]. However, Xsu(H)DN overexpression in neuro2a cells did not cause any obvious change in filopodial formation (Fig. 3B). Therefore, these data indicate that the protrusive function of Delta does not necessarily depend on its inhibitory effects on Notch signaling.

3.5. A membrane-tethered form of DeltaD affects neuronal cell migration independently of Notch signaling during zebrafish development

To explore the Notch-independent *in vivo* functions of Delta, we analyzed motile activity of the zebrafish Delta1 homolog DeltaD, which is expressed in developing neurons. After injection of full-length deltaD mRNA into zebrafish embryos, neuronal differentiation was blocked by the promotion of Notch signaling as previously published ([25], data not shown); therefore, it was difficult to analyze neuronal cell migration. Thus, we next examined the activity of a membrane-tethered intracellular domain (TMICD) and the cytoplasmic domain (ICD) of deltaD in neuronal cell migration. Both of these mRNA injections did not inhibit neuronal differentiation as assessed by *elavl3* expression (Fig. 4); however, some *elavl3*-positive neuronal cells were ectopically positioned at 20 hpf in deltaD TMICD-injected embryos but less in deltaD ICD-injected embryos (TMICD 600 pg, 83%, $n = 24$; ICD 600 pg, 32%, $n = 22$; arrowheads, Fig. 4). In *mib* mutants that have diminished Notch activity compared to wild-type fish, a greater number of

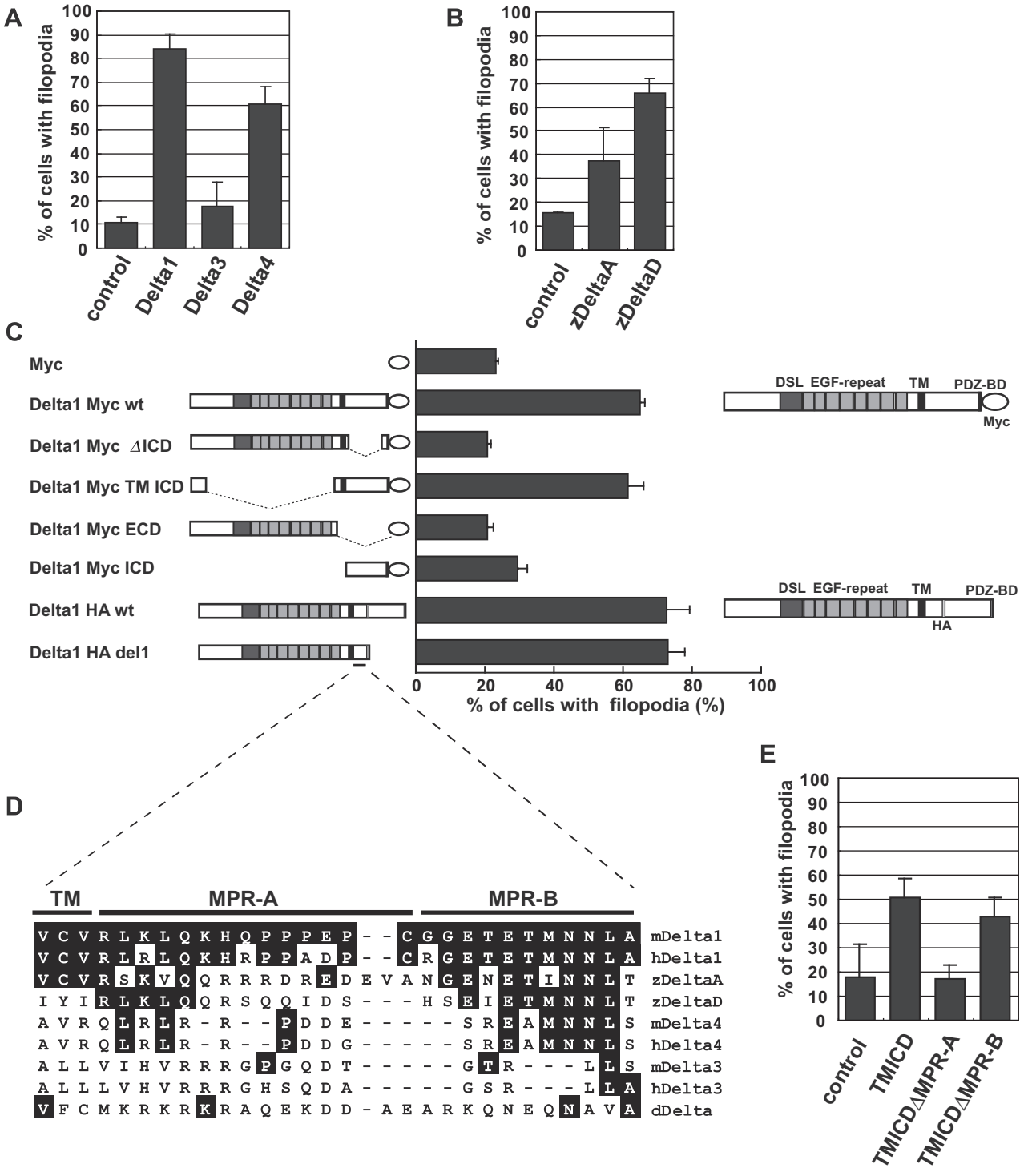

Fig. 2. Filopodia formation by Delta family proteins and domain analysis of Delta1. (A and B) The Delta1 family (Delta1, zDeltaA and zDeltaD) and Delta4, but not Delta3, induce filopodia formation. Neuro2a cells were transfected with plasmids carrying mouse Delta1 (Delta1), mouse Delta3 (Delta3), human Delta4 (Delta4), zebrafish DeltaA (zDeltaA) or zebrafish DeltaD (zDeltaD). (C) The membrane-proximal domain of Delta1 anchored to the plasma membrane is important for filopodia formation. Efficiency of filopodia formation was scored using a series of deletion constructs for Delta1. Schematic drawing of the C-terminal Myc-tagged or intracellular HA-tagged Delta1 deletion constructs is shown. DSL, Delta/Serrate/LAG-2 domain; EGF-repeat, epidermal growth factor repeat domain; TM, transmembrane domain; PDZ-BD, PDZ-binding domain. (D) Multiple sequence alignment of the membrane-proximal region for Delta family proteins using Clustal W. Identical amino acids to mDelta1 are black boxed. TM, transmembrane; MPR, membrane-proximal region. The letter in front of Delta indicates the species of origin for the sequences as follows: h, human; m, mouse; z, zebrafish; d, *Drosophila*. (E) MPR-A is important for filopodia formation. Efficiency of filopodia formation was scored using a series of deletion constructs for Delta1 TMICD. (A–C and E) Efficiency of filopodia formation was scored as outlined in Fig. 1. More than 100 cells per sample were counted in duplicate transfections. The values shown are means ± SD (%).

cells adopted a neural fate [18]. Nevertheless, ectopic neuronal cells were observed less frequently in *mib* mutants than in TMICD injected embryos (35%, $n = 17$) Therefore, ectopic positioning of neurons is most likely due to changes in their migratory behavior and not due to an increase in neuron numbers caused by inhibition of Notch signaling. Collectively, these data suggest that indepen-

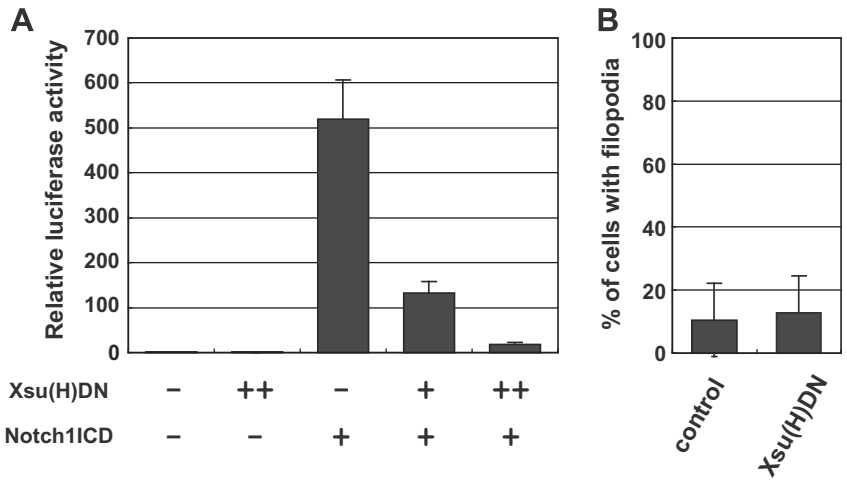

Fig. 3. Inhibition of Notch signaling does not promote filopodia formation. (A) Xsu(H)DN suppresses Notch signaling. Neuro2a cells were transfected with 0.1 μg 8× HES-luciferase reporter plasmid, 0.1 μg of Notch1ICD (pEFBOSneo-RAMIC) and varying amounts of Xsu(H)DN (+0.02 μg; ++0.1 μg). Error bars represent the means ± SD of three independent experiments. (B) Xsu(H)DN does not promote filopodia formation. Efficiency of filopodia formation was scored as outlined in Fig. 1. More than 100 cells per sample were counted in duplicate transfections. The values shown are means ± SD (%).

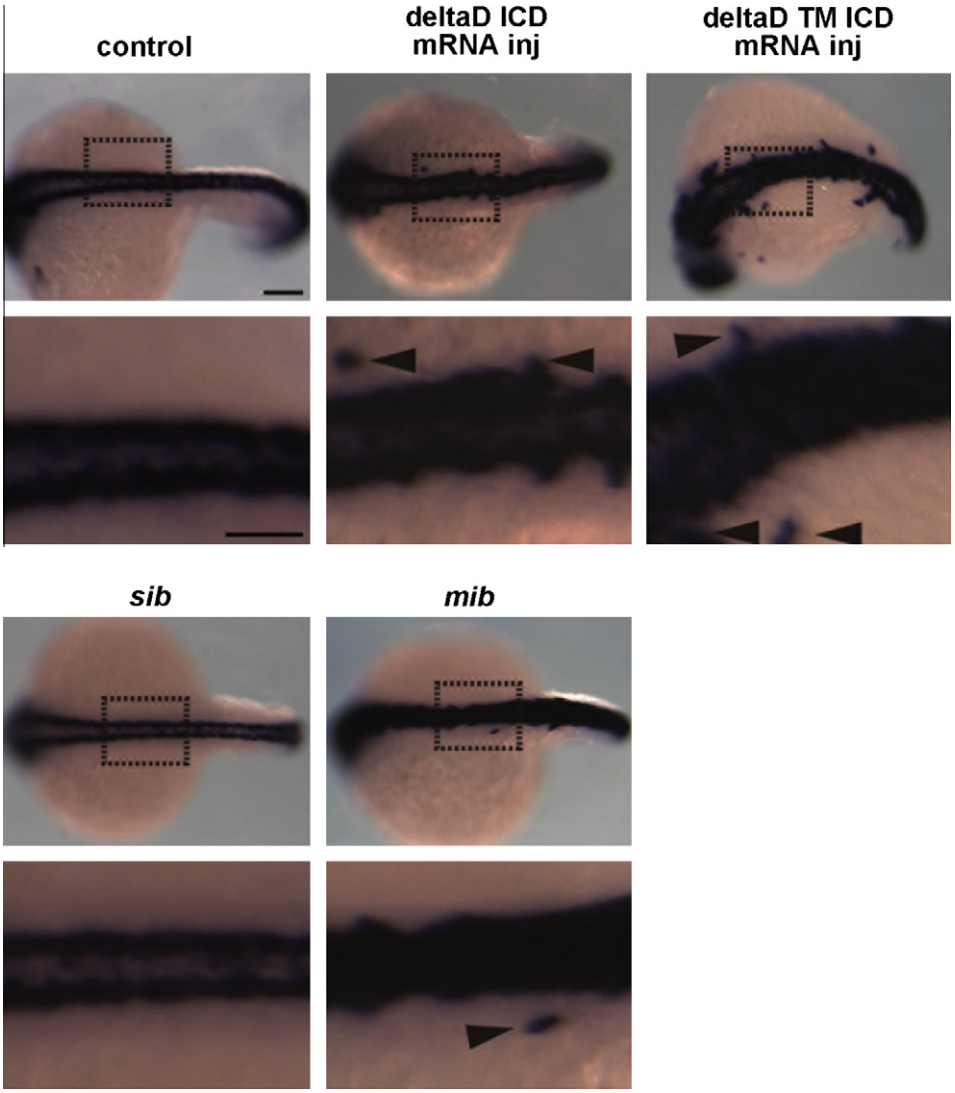

Fig. 4. Membrane tethered intracellular domain of DeltaD is involved in neuronal migration independent of Notch signaling. Some *elavl3*-positive neurons are ectopically positioned in deltaD TMICD mRNA-injected embryos but less in deltaD ICD mRNA-injected embryos or *mib* mutants. Expression of *elavl3* at 20 h post-fertilization (hpf). Bottom panels show enlarged images of boxed regions in upper panels. Arrowheads indicate mislocalized *elavl3*-positive cells. control, uninjected control. *sib*, sibling control.

dently of Notch signaling, the filopodial inducing activity of Delta is involved in cell motility *in vivo*.

4. Discussion

4.1. Delta1 regulates actin reorganization independently of Notch signaling

Previous reports have determined that Delta associates with the actin cytoskeleton in keratinocytes and promotes filopodial lengthening in *Drosophila* [3,15], suggesting a functional link between Delta proteins and actin organization. Additionally, Notch activation influences actin organization in the *Drosophila* wing [26]. Furthermore, during axogenesis in *Drosophila*, a genetic interaction was identified between Notch and Abl kinase, a protein that recruits Rho family GTPases and is thus expected to play a role in actin assembly [27]. Delta3 does not alter actin organization (this study) and does not activate Notch signaling [28]; therefore, Delta1 may regulate actin dynamics by activating Notch signaling. However, this hypothesis is not likely because a membrane-tethered form of Delta1 that lacks the extracellular domain essential for binding to Notch has the same actin reorganizing activity as full-length Delta1.

As the ligand of Notch, Delta promotes Notch activity; however, Delta proteins also exert an inhibitory influence on Notch activity when they are expressed in Notch-positive cells [22,23]. Therefore, the actin reorganizing activity of Delta1 may be mediated through inhibition of Notch activity. However, this hypothesis is also unlikely because this inhibitory effect on Notch depends on the extracellular domain of Delta1 [24], and inhibition of Notch signaling by a dominant negative form of su(H) does not induce filopodia formation in neuro2a cells. Taken together, our data argue that Delta1 regulates the actin cytoskeleton to form filopodia independently of Notch signaling.

4.2. Mode of Delta1 action on the formation of filopodial actin filaments

The mechanism of induction of filopodia protrusions by Delta proteins remains unknown; however, Estrach et al. identified the Delta1 interacting proteins Syntenin and Filamin A, which may mediate filopodial formation by Delta1 [9]. They showed that Syntenin mediates Delta1-induced cell adhesion by enabling Delta1 to remain at the cell surface [9]. Moreover, as in the case of Delta1, overexpression of Syntenin enhances filopodia formation in several cell lines, suggesting a functional connection between Delta1 and Syntenin in actin rearrangement [29–31]. Filamin is an actin-binding protein that links intracellular and membrane bound proteins to actin [32]. Filamin binds RalA, a Ras-related small GTPase, and an active form of RalA stimulates actin-rich filopodia formation. RalA does not induce filopodia development in Filamin-deficient cells, suggesting that Filamin is necessary for RalA-induced filopodia [33]. These studies suggest that Syntenin and Filamin may be involved in Delta1-mediated filopodia formation. The interaction between Delta1 and Syntenin depends on the Delta1 PDZ-binding domain; however, the mechanism by which Delta1 interacts with Filamin has not been determined. The membrane-proximal domain of Delta1, an important region for filopodia induction, may link Delta1 to Filamin. Future studies are necessary to determine if Delta1 interacts with these other proteins to regulate actin dynamics.

4.3. Delta is involved in neuronal cell migration independent of Notch activation

Previously, Notch-independent involvement of Delta in cell migration was reported in mouse fibroblast cells and zebrafish spinal cord neurons [5,6]. These studies suggest that the C-terminal PDZ-binding domain of Delta is essential to promote cell adhesion and inhibit cell motility. Interestingly, the aberrant migratory behavior reported by Wright et al. differs from the behavior observed in deltaD TMICD mRNA-injected embryos. For example, neurons become abnormally motile toward the midline in embryos expressing DeltaD that lacks a PDZ-binding domain [6]; however, in deltaD TMICD mRNA-injected embryos, the neurons remain in lateral regions. It is currently unknown how the overexpression of deltaD TMICD affects cell behavior *in vivo*; however, the mechanism does not involve Notch signaling activity and cell adhesion through the extracellular domain of Delta. These data suggest the presence of multiple modes of cell movement regulated by Notch-independent Delta activity. Cellular motility requires actin reorganization to form peripheral lamellipodial and filopodial protrusions. These data along with the knowledge that Delta1 protein lacking the PDZ-binding domain, but not the membrane-proximal region, retains actin reorganization activity similar to full-length Delta1 suggest that the membrane-proximal region of Delta1 may play a role in neuronal migration via actin regulation.

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