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Mib-Jag1-Notch signalling regulates patterning and structural roles of the notochord by controlling cell-fate decisions

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SUMMARY

In the developing embryo, cell-cell signalling is necessary for tissue patterning and structural organization. During midline development, the notochord plays roles in the patterning of its surrounding tissues while forming the axial structure; however, how these patterning and structural roles are coordinated remains elusive. Here, we identify a mechanism by which Notch signalling regulates the patterning activities and structural integrity of the notochord. We found that Mind bomb (Mib) ubiquitylates Jagged 1 (Jag1) and is essential in the signal-emitting cells for Jag1 to activate Notch signalling. In zebrafish, loss- and gain-of-function analyses showed that Mib-Jag1-Notch signalling favours the development of non-vacuolated cells at the expense of vacuolated cells in the notochord. This leads to changes in the peri-notochordal basement membrane formation and patterning surrounding the muscle pioneer cells. These data reveal a previously unrecognized mechanism regulating the patterning and structural roles of the notochord by Mib-Jag1-Notch signalling-mediated cell-fate determination.

KEY WORDS: Mind bomb, Notch signalling, Notochord, Basement membrane, Muscle patterning, Zebrafish

INTRODUCTION

The structural complexity of an organism is accomplished during its development from the fertilized egg to the adult. Cell-cell communication is crucial for the structural organization of tissues during embryogenesis. The signals between cells are necessarily transient and are controlled spatiotemporally (Joubin and Stern, 2001; Meinhardt, 2008). Groups of regionally restricted cells that have an organizing activity to pattern their surrounding tissues are generated, but their activity must be suppressed as the cells give rise to parts of the functional tissue structures of the body. How this patterning activity and structural organization are controlled is a fundamental question in developmental biology.

The notochord is a midline structure derived from the organizer. In zebrafish, the organizer gives rise to three different germ layers: the floor plate, notochord and hypochord (Latimer et al., 2002). Until the segmentation stage, the notochord secretes inductive factors, Hedgehog family proteins, to pattern its adjacent tissues, including neurons and the myotome (Currie and Ingham, 1996; Ericson et al., 1996; Lewis and Eisen, 2001). From around the end of the segmentation stage, the notochord differentiates to form large vacuoles and a thick extracellular sheath (also called the peri-notochordal basement membrane, PBM), which are essential for the axial structure (Stemple, 2005). The establishment of the notochord structure is accompanied by a reduction in hedgehog expression and patterning activity. Recent studies have suggested that the formation of the extracellular sheath is involved in notochord differentiation (including the suppression of Hedgehog expression), in addition to supporting the embryo structurally (Parsons et al., 2002; Coutinho et al., 2004). However, how the structural construction and the patterning activity are coordinated remains largely unknown.

Notch signalling is an evolutionarily conserved signalling pathway that is involved in a variety of cell-fate decisions during development (Artavanis-Tsakonas et al., 1999; Lai, 2004). In midline development, the activation of Notch signalling by Delta drives the development of the hypochord and floor plate cells at the expense of the notochord (Appel et al., 1999; Latimer et al., 2002; López et al., 2005). The ability of Notch to determine these cell fates, however, is restricted to before the gastrulation stage (López et al., 2003; Latimer and Appel, 2006). After the gastrulation stage, genes encoding another class of Notch ligands, the Jagged genes, and the Notch genes are expressed in the notochord, but their functions in notochord development have been unknown (Zecchin et al., 2005; Oates et al., 2005; Sprague et al., 2006).

Mind bomb (Mib, also known as Mib1) is a ubiquitin ligase that is essential for Delta to send its signal to Notch (Itoh et al., 2003). Midline defects are reported in *mib* mutants, which show a decrease in the number of floor plate and hypochord cells, and a reciprocal increase in notochord cells (Appel et al., 1999). These observed changes in the midline of *mib* mutants are thought to be due to defects in Delta-Notch signalling (Appel et al., 1999). Mib might also be involved in the maintenance of notochord structural integrity, because morphological changes of the notochord cells in *mib* mutants have been observed (Stemple et al., 1996). Recently,

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several studies showed that Mib enhances the activity of Jagged as well as of Delta (Koo et al., 2005; Wang and Struhl, 2005; Lai et al., 2005; Le Borgne et al., 2005). These findings raised the questions of whether Mib-mediated Delta or Jagged-induced Notch signalling is involved in the structural construction of the notochord, and whether Mib-mediated Notch signalling is associated with the patterning activity of the notochord.

In this study, we identified Jagged 1 (Jag1) as a Mib-interacting protein and showed that Mib is essential for Jag1 to send its signal to Notch. Strikingly, we found that the Mib-mediated activation of Notch signalling through Jag1 regulates cell-fate determination in the notochord-lineage cells (vacuolated cells versus non-vacuolated epithelial-like cells). This cell-fate choice plays an important role in notochord structural integrity, because it determines the formation of vacuoles and the PBM. Furthermore, we found that the Mib-meditated Jagged-Notch signalling is involved in the muscle-patterning activity of the notochord via the regulation of Hedgehog expression. Our findings support the possibility that Hedgehog expression is regulated through the formation of the PBM, which has been suggested to promote notochord differentiation. These findings provide new insights into how the patterning activity and the structural organization of a tissue are coordinated during development.

MATERIALS AND METHODS

Yeast two-hybrid screening

Yeast two-hybrid screening was carried out as previously described (Broder et al., 1998). In brief, cdc25-2 cells were first transformed with the pMet 425-Myc-Ras(61) ΔF-zebrafish mib_m132 bait, and then with a human fetal brain cDNA library (Stratagene, CA, USA). Transformants were grown on selectable minimal glucose plates for 5 days at 25°C, then replica plated onto minimal galactose plates, and incubated at 37°C for 5-7 days. Positive colonies exhibiting efficient growth on galactose plates at 37°C were isolated and tested for galactose and methionine-dependent growth at 37°C. The library plasmids were recovered and further analyzed by DNA sequencing and retransformation of the cdc25-2 cells to test the specificity of the interaction.

Ubiquitylation assay

Ubiquitylated Jag1a, Jag1b or DeltaD was detected as previously described (Itoh et al., 2003). Briefly, COS7 cells were transiently transfected with 2 μg of each plasmid DNA construct (Jag1a-HA, Jag1b-HA, DeltaD-HA, Myc-Mib, Myc-MibC1001S, and Flag-Ub) per 10-cm dish using Fugene6 (Roche, Switzerland). The lysates were immunoprecipitated with an anti-HA antibody (HA11, Covance, CA, USA) and then blotted with an anti-Flag antibody (M2, Sigma, MO, USA).

RNA interference

Jag1 or Notch1 transfectant cells were established by retrovirus-mediated transfection of the murine *Jag1* or *Notch1* gene into NIH-3T3 cells (designated Jag1-3T3 and Notch1-3T3 cells, respectively). Synthetic siRNA oligonucleotides were purchased from B-Bridge International. The transfection of siRNAs was performed using RNAiMAX (Invitrogen, CA, USA). The control siRNA was the siTrio negative control siRNA (B-Bridge, CA, USA) or the AllStars negative control siRNA (Qiagen, Germany). The 21-nucleotide siRNA sequences for Mib were: #1, 5′-GGATAAAGATGGAGATCGATT-3′; and #2, 5′-GCAAAATGTC-ATAAGGAAATT-3′. The Jag1-3T3 or Notch1-3T3 cells were transfected with control or Mib siRNA (2 nM or 10 nM). Forty-eight hours after the transfection, the expression of Jag1 and Mib was analyzed by western blotting with anti-Flag (M2, Sigma, CA, USA) and anti-Mib (monoclonal Armenian Hamster clone 3A3-4A; generated against the bacterially expressed N-terminal domain of mouse Mib) as primary antibodies, and anti-mouse and anti-Armenian Hamster immunoglobulin-horseradish peroxidase (Jackson ImmunoResearch, PA, USA), respectively, as secondary antibodies.

Reporter gene assays

The assay for the effect of Mib on Jag1-expressing cells was performed as follows. MIG- (control) or Flag-tagged Jag1-expressing cells (Jag1-3T3, 1.2×10^6) were transfected with control or two different Mib siRNAs (2 nM or 10 nM) and, 24 hours later, NIH3T3Notch1-expressing NIH3T3 (Notch1-3T3, 1.2×10^6) cells were transfected with the TP1 plasmid (a Notch reporter plasmid, 0.5 μg) and pRL-EF (5 ng). Forty-eight hours after the siRNA transfection, the Notch1-3T3 cells were collected and plated in 24-well plates (5×10^4). These cells were then co-cultured with the MIG- (control) or Jag1-3T3 cells (5×10^4) in 24-well plates for 40 hours, and the Firefly and *Renilla* luciferase activities were determined with the Promega Dual luciferase assay system (Madison, WI, USA). The pRL-EF plasmid, which expresses *Renilla* luciferase under the control of the EF-1α promoter, was used to normalize the transfection efficiency of the luciferase reporters. The assay for the effect of Mib on Notch1-expressing cells was performed as follows. Notch1-3T3 cells were transfected with control or two different Mib siRNAs (10 nM), and 24 hours later, the cells were further transfected with the TP1 plasmid and pRL-EF. Forty-eight hours after the siRNA transfection, the Notch1-3T3 cells were collected and split into three equal groups of 5×10^4 cells. These groups were then co-cultured with MIG- (control) or Jag1-3T3 cells (5×10^4) in 24-well plates for 48 hours, and the luciferase activity was determined as described above. The values shown are the average of experiments repeated three times, with each transfection performed in duplicate per experiment.

Flow cytometry

Jag1-3T3 cells were monitored by the co-expression of GFP. The Jag1-3T3 cells were treated with the Mib siRNAs (10 nM). After 48 hours, the cells were incubated with an anti-Jag1 mAb (Moriyama et al., 2008) or with Armenian Hamster IgG (IgG1, k, A19-3; BD Bioscience, San Jose, CA), followed by biotinylated anti-Armenian Hamster IgG (eBioscience, CA, USA) and phycoerythrin (PE)-conjugated streptavidin (BD Biosciences), and analyzed by FACSCalibur (BD Biosciences, CA, USA) using CellQuest software (BD Biosciences, CA, USA).

Surface biotinylation assay

Jag1-3T3 cells were washed three times with ice-cold PBS buffer. The cells were then incubated at 4°C for 15 minutes in a biotinylation buffer [0.25 mg/ml EZ-link sulfo-NHS-LC-biotin (Pierce) in PBS]. After removal of the biotinylation buffer, the cells were lysed. The lysates were immunoprecipitated with an anti-Flag antibody. The surface-biotinylated Jag1 protein was visualized using horseradish peroxidase-conjugated NeutrAvidin (Pierce, IL, USA) by western blot.

Endocytosis assay

Jag1-3T3 cells were washed three times with ice-cold PBS buffer. The cells were then incubated at 4°C for 40 minutes in a biotinylation buffer [0.25 mg/ml EZ-link sulfo-NHS-SS-biotin (Pierce) in HBBS]. After removal of the biotinylation buffer, the cells were incubated at 37°C for 20 minutes. The remaining cell-surface biotin was stripped by three 25-minute incubations with stripping buffer (50 mM MesNa, 50 mM Tris pH 8.3, 100 mM NaCl, 1 mM EDTA, 0.2% BSA) on ice, and then the cells were lysed in extraction buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 12.5 mM b-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, 20 μM aprotinin, 1% Triton X-100). The enzyme-linked immunosorbent assay (ELISA) was used to quantify biotinylated Jag1 and total Jag1 in extracts using anti-Flag (M2, Sigma, CA, USA) and anti-Jag1 (H-114, Santa Cruz, CA, USA) antibodies, and horseradish peroxidase-conjugated NeutrAvidin (Pierce, IL, USA).

Fish maintenance and mutants

The zebrafish were raised and maintained under standard conditions. The *mib ta52b* mutant has been previously described (Itoh et al., 2003). The 214A-GFP fish line was created by crossing SAGFF214A with UAS:GFP fish and selected by GFP expression in the notochord. SAGFF214A fish were obtained by injection with the T2KSAGFF construct (Asakawa et al., 2008). The insertion of SAGFF214A was analyzed by inverse PCR and mapped within the NADH-cytochrome b5 reductase 2 gene on

chromosome 25. The *jag1b* mutant will be published elsewhere (Zuniga et al., 2010). The *jag1b* lesion creates an early stop codon [a single base change at 887 (G to A), and an amino acid change at 211 (W to Stop)].

Heat-shock experiments

For the Notch gain-of-function analyses, embryos were obtained from matings between Tg(UAS:myc-Notch1a-intra) and Tg(hsp70:Gal4) carriers (Scheer et al., 2002). Notch1aICD (ICD, intracellular domain) expression was induced by heat shock at 39°C for 30 minutes at the 3 somite stage (ss), and then the embryos were incubated at 28.5°C until fixation. After in situ hybridization, the embryos were digested by proteinase K and genotyped by PCR, as previously described (Scheer et al., 2002).

Whole-mount in situ hybridization, antibody staining, BODIPY labelling and electron microscopy

Whole-mount in situ hybridization was performed as described previously, except a different blocking buffer was used (5% sheep serum, 1% BMB, 0.1% Tween 20, 1% DMSO, 150 mM NaCl, 100 mM maleic acid, pH 7.5) (Thisse and Thisse, 2008). All the probes have been previously published: *jag1a* (Zecchin et al., 2005), *jag1* (Zecchin et al., 2005), *her4* (Takke et al., 1999), *her9* (Latimer et al., 2005), *ihhb* (Currie and Ingham, 1996), *shha* (Krauss et al., 1993), and *ntl* (Schulte-Merker et al., 1992). Whole-mount antibody staining was performed using the following antibodies: anti-Eng (4D9, DSHB), anti-Collagen type II (II-II6B3, DSHB), anti-GFP (Living Colors A.v. Peptide Antibody, Clontech, CA, USA), anti-KDEL (10C3, Stressgen), anti-γ-tubulin (GTU-88, Sigma, MO, USA), rabbit anti-laminin (L9393, Sigma, MO, USA), and Rhodamine-phalloidin (Invitrogen, CA, USA). A mouse monoclonal Ntl antibody (clone 3) was generated against bacterially expressed zebrafish Ntl protein. A rabbit polyclonal anti-Jag1b antibody was raised against a mixture of two peptides (CKDYEGKNSIIAKIRTHN and CNRDLETAQSLNRMEYIV). Alexa-555 anti-mouse IgG or Alexa-488 rabbit IgG (Invitrogen, CA, USA), and Histofine Simple Stain MAX PO MULTI (Nichirei Biosciences, Tokyo, Japan) were used as the secondary antibodies. For double staining with anti-collagen type II and KDEL, Alexa-488 anti-mouse IgG1 and Alexa-555 anti-mouse IgG2a were used. Embryos were stained in 50 μM BODIPY-FL-C5-Ceramide or BODIPY TR methyl ester (Invitrogen, CA, USA) in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) for 6 hours. Electron microscopy was performed according to previously reported procedures (Shimizu et al., 2005).

Plasmid, mRNA, and morpholino antisense oligonucleotide injection

Flh-GFP plasmid (Gamse et al., 2003) (25 pg) was injected into fertilized eggs, and the GFP-positive cells were observed by time-lapse microscopy with an AxioCamHRc using the time-lapse option of AxioVision (Zeiss, Germany). For the microinjection of mRNA, nls-mCherry in the pCS2P+ plasmid was linearized and transcribed with SP6 RNA polymerase using the mMessage mMachine Kit (Ambion, TX, USA). The sequences of the morpholinos (Gene Tools, OR, USA) were:

*jag1a*_splMO, 5′-AAGCCAAACCCGCACATACCCGCAT-3′;
*jag1b*_atgMO, 5′-CTGAACTCCGTCGCAGAATCATGCC-3′;
*jag1b*_splMO, 5′-AAATCAAGACTCACCGTCGTCCGCA-3′;
*su(H)*MO, 5′-CAAACTTCCCTGTCACAACAGGCGC-3′;
*her9*MO, 5′-CTCCATATTATCGGCTGGCATGATC-3′.

Two to five nanograms of oligonucleotides were injected into one- to two-cell-stage embryos.

RT-PCR and sequencing of the Jagged mRNA structure in splicing morpholino-injected embryos

Total RNA was extracted from morpholino-injected embryos, and the cDNAs were synthesized by reverse transcriptase polymerase chain reaction (RT-PCR). The PCR primer combinations were as follows:

*jag1a*_142up (PA1), 5′-GAGAGGCGTGTGGCGGCTGAAGTGGT-AGTT-3′;
*jag1a*_533low (PA2), 5′-GATCCGGGTTTTGTCGTTTGTTTGGT-CA-3′;
*jag1b*_174up (PB1), 5′-CCGTGAGGCGGCTACAAACTTCTGA-GTCTC-3′;
*jag1b*_571low (PB2), 5′-CGGCCACGCAAAACTGAAGGGTAAAA-CAAT-3′.

The PCR products were subcloned into pGEMT-easy and sequenced using the ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, CA, USA).

RESULTS

Mind bomb is required for Jagged 1-mediated Notch activation

We previously identified Mib ubiquitin ligase as an important Notch regulator (Itoh et al., 2003). We showed that Mib ubiquitylates Delta proteins, a class of Notch ligands, and that this ubiquitylation is important for the activation of Notch signalling by Delta-expressing cells. However, zebrafish *mib* mutants show a more severe phenotype than those of any known Delta mutants, which suggests that Mib might have other substrates besides Delta. Therefore, we performed yeast two-hybrid screening using Mib as bait, and isolated a cDNA fragment encoding Jag1. Because Mib is a ubiquitin ligase, we next examined whether Jagged protein is ubiquitylated by Mib. Two homologues of Jag1 (Jag1a and Jag1b) have been identified in zebrafish. Wild-type Mib ubiquitylated Jag1a and Jag1b proteins, as it did DeltaD, but the Mib mutant C1001S, which lacks ubiquitin ligase activity, did not ubiquitylate Jag1a or Jag1b (Fig. 1A). Therefore, Mib ubiquitylates Jag1 in a ubiquitin ligase activity-dependent manner.

The ubiquitylation of Delta by Mib regulates Delta's signalling activity (Itoh et al., 2003). If Mib also regulates the signalling activity of Jag1 by ubiquitylation, then a reduction in Mib activity in Jag1-expressing cells should affect Jag1-induced Notch activation in a non-autonomous manner. We tested this possibility by using a co-culture assay consisting of NIH3T3 cells ectopically expressing Jag1 or Notch1. We observed that the knockdown of Mib by two independent siRNAs in the Jag1-expressing cells reduced Notch reporter gene activity, whereas knocking down Mib in Notch1-expressing cells did not affect it (Fig. 1B,C; see also Fig. S1A,B in the supplementary material). Thus, Mib is required for the signalling activity of Jag1 in signal-emitting cells, but not for activation of the Notch signal in signal-receiving cells. We previously reported that Mib is required for the endocytosis of Delta, which enables it to activate Notch in adjacent cells (Itoh et al., 2003). Therefore, we next examined whether the endocytosis of Jag1 is affected by Mib knockdown. Unexpectedly, Mib knockdown did not significantly affect the cell-surface expression or bulk endocytosis rate of Jag1 (Fig. 1D,E; see also Fig. S1C in the supplementary material), suggesting that most of the Jag1 internalization is Mib independent, and is not correlated with the Jag1 signalling activity.

Zebrafish Jagged 1 expression in the notochord is controlled by Notch signalling

To explore the function of the Mib-mediated Jag1 activity in vivo, we next examined the expression of the *jag1a* and *jag1b* genes in zebrafish embryos. We found that *jag1a* and *jag1b* were expressed in the notochord in similar but not identical pattern (Fig. 2A,B). The expression of *jag1a* was patchy, whereas that of *jag1b* was continuous in the notochord at 10 ss [14 hours post-fertilization (hpf)]. From around this time point, their expression started to fade from the anterior region of the notochord, but remained in the tail region at 20 ss (arrowheads in Fig. 2A,B). These expression patterns suggested that not all the cells within the developing notochord cells express the *jag1* genes to the same extent.

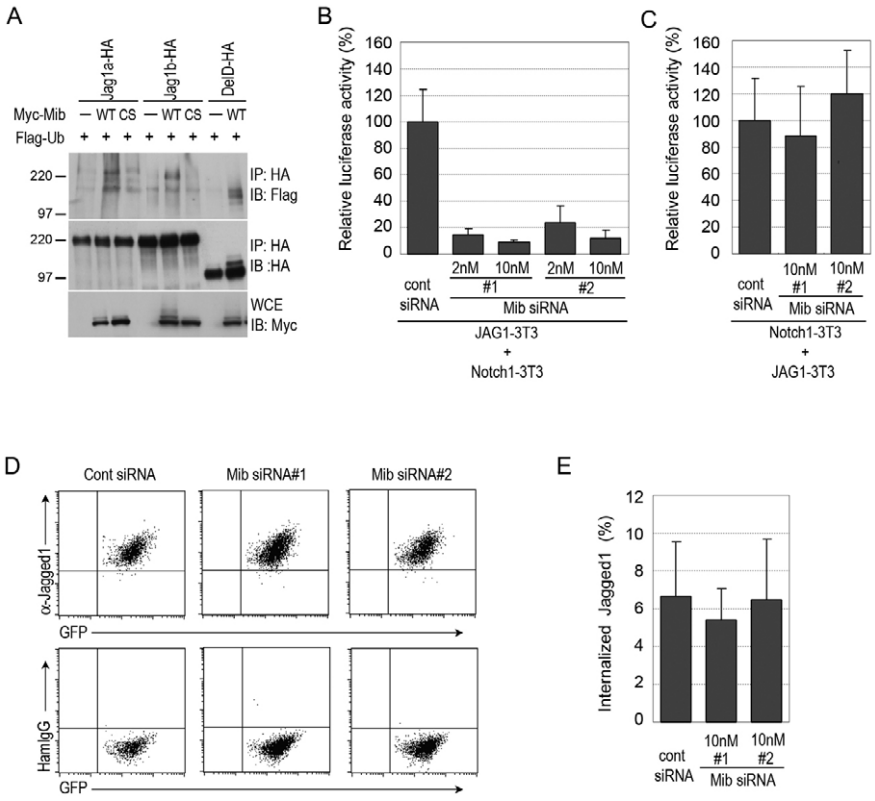

Fig. 1. Mind bomb ubiquitylates Jagged 1 and is required for Jagged 1-induced Notch activation. (**A**) Jag1a and Jag1b were ubiquitylated by Mib. COS7 cells were transfected with HA-tagged Jag1a (Jag1a-HA), HA-tagged Jag1b (Jag1b-HA), HA-tagged DeltaD (DelD-HA), Myc-tagged wild-type Mib (WT), C1001S Mib (CS) and Flag-ubiquitin (Ub), as indicated. Cell extracts were then subjected to immunoprecipitation with an anti-HA antibody. The immunoprecipitated complexes were immunoblotted with anti-Flag (top) and anti-HA (top and middle) antibodies. Whole cell extracts (WCEs) were immunoblotted with an anti-Myc antibody (bottom). Molecular weight marker sizes in kDa are indicated on the left. (**B**) Mib was required for Jag1-induced Notch activation in Jag1-expressing cells. Jag1-3T3 cells were transfected with each Mib siRNA and then co-cultured with Notch1-3T3 cells. Notch activity was measured in Notch1-3T3 cells transfected with TP1, a Notch reporter plasmid. (**C**) Mib was not required for Jag1-induced Notch activation in Notch1-expressing cells. Notch1-3T3 cells were transfected with each Mib siRNA and the TP1 reporter plasmid, and then co-cultured with Jag1-3T3 cells. Error bars in B,C represent the mean ± s.d. of three independent experiments. (**D**) The expression of Jag1 on the cell surface was not altered by Mib knockdown. GFP-positive Jag1-3T3 cells were transfected with Mib siRNAs and, after 48 hours, the expression of Jag1 was examined by flow cytometry with an anti-Jag1 antibody. Hamster IgG was used as a control. (**E**) The Jag1 endocytosis rate was not dramatically altered by Mib knockdown. The surface proteins of Jag1-3T3 cells were labelled with Sulfo-NHS-SS-Biotin and incubated for 20 minutes at 37°C. The remaining cell-surface biotin was then stripped, and WCEs were prepared. Internalized biotinylated Jag1 and total Jag1 was measured by an ELISA. The values shown are mean ± s.d. (%).

We next investigated whether Notch signalling influences the expression of the *jag1* genes in the notochord. In *mib* mutants, which are deficient in Notch signalling, *jag1a* and *jag1b* expression was increased and continuous throughout the notochord cells at 10 ss (Fig. 2C,D). Conversely, the activation of Notch at 3 ss by the heat-shock treatment of transgenic embryos carrying hsp70:Gal4; UAS:Notch1aICD reduced the expression of *jag1a* and *jag1b* at 10 ss (Fig. 2E,F). These data suggested that the activation of Notch signalling regulates the expression of the two *jag1* genes in the notochord cells. Given that *notch2* and *notch3* are expressed in the notochord cells when the two *jag1* genes are expressed, it is possible that Notch signalling regulates notochord cell function through target gene activation (Oates et al., 2005; Sprague et al., 2006). Furthermore, negative feedback loops between Notch and Jagged might regulate the fates of adjacent cells and control the pattern of notochord differentiation, as Notch activation in a cell is dependent upon the level of ligand expression in its neighbours.

Jagged-Notch signalling defects cause morphological changes in vacuolated notochord cells

To determine the function of *jag1a* and *jag1b* during notochord development, we used antisense morpholino oligonucleotides (MOs) to knock down Jag1a and Jag1b protein levels. *jag1a* and *jag1b* splMOs were designed to target the splice donor sites of the first exon-intron boundary of each gene. The injection of either *jag1a* splMO or *jag1b* splMO caused a reduction in the wild-type transcript and produced short transcript forms (see Fig. S2A-C in the supplementary material). DNA sequencing of these transcripts revealed that these splice-blocking MOs activated cryptic splice donor sites and potentially produced truncated Jag1a and Jag1b proteins (see Fig. S2A,B in the supplementary material). We also used a second *jag1b* morpholino (*jag1b* atgMO) to block the translation of Jag1b. Injection of the *jag1b* atgMO efficiently reduced the level of Jag1b protein (see Fig. S2D in the

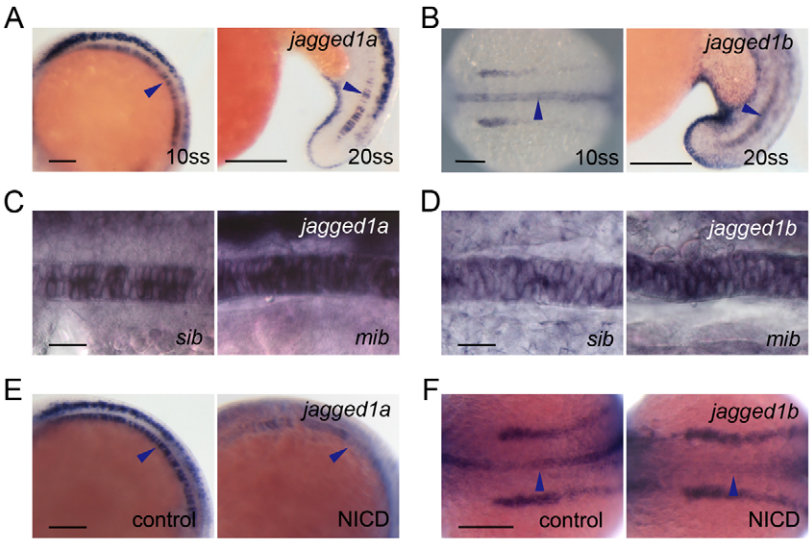

Fig. 2. The two zebrafish *jagged 1* homologues are expressed in spatially and temporally restricted patterns in the developing notochord, and their expression is controlled by Notch signalling. (**A**,**B**) Expression of *jag1a* (A) and *jag1b* (B) in the notochord cells at 10 and 20, ss. (**C**,**D**) *jag1a* (C) and *jag1b* (D) expression was increased in the *mib* mutants at 10 ss. *sib*, sibling control. (**E**,**F**) *jag1a* (E) and *jag1b* (F) expression was decreased at 10 ss in the notochord of embryos with increased Notch activity. Control siblings (control) or double transgenic Tg(UAS:myc-Notch1a-intra);Tg(hsp70:Gal4) embryos (NICD) were heat shocked at 3 ss. Arrowheads show expression in the notochord. (A-E) Lateral view with anterior to the left, except for 10 ss in B. 10 ss in B and F is a dorsal view with anterior to the left. Scale bars: 100 μm in A,B,E,F; 20 μm in C,D.

supplementary material). We also used a *jag1b* mutant line that produced a truncated protein lacking residues 212 to the end (residue 1213).

The notochord is derived from the embryonic shield (the early gastrula organizer). During gastrulation, chordamesoderm is formed from the shield by mediolateral cell intercalation. Later, the chordamesoderm differentiates and each cell forms a large vacuole by 2 days post-fertilization (dpf). At around 34 hpf, the control MO-injected embryos had large disc-shaped vacuolated notochord cells (Fig. 3A). Although the impairment of either *jag1a* or *jag1b* gene function alone caused only mild or no notochord morphological defects, a more drastic morphological change in the notochord cell shape was observed by the double knockdown of *jag1a* and *jag1b* (co-injection with *jag1a* splMO and *jag1b* atgMO, hereafter referred to as *jag1a/1b* MOs, 97%, *n*=73; *jag1a* splMO in *jag1b* mutants, 100%, *n*=4). A similar morphological change in the notochord was observed in the *mib* mutants, as reported previously [see figure 3A in Stemple et al. (Stemple et al., 1996)]. We further examined whether this notochord phenotype was due to a defect in Notch signalling by knocking down two Su(H) proteins, [Su(H)1 and Su(H)2), which act downstream of Notch signalling (Zhang, C. et al., 2007)]. The injection of a *su(H)* MO that can inhibit both *su(H)1* and *su(H)2* gene function caused a similar morphological change in the notochord cells (Fig. 3A, 95%, *n*=38). By contrast, the knockdown of DeltaA and DeltaD, which are required for hypochord differentiation (Latimer et al., 2002), did not affect the gross morphology of the notochord (Fig. 3A). We next examined whether defects in mediolateral intercalation at earlier stages could have caused the morphological change in vacuolated cells in the *jag1a/1b* knockdown embryos. However, mediolateral intercalation was not dramatically impaired in *jag1a/1b* knockdown embryos at 10ss (see Fig. S3 in the supplementary material). Together, these data showed that Mib-Jag1-Notch signalling is required to establish the normal vacuolated notochord cell shape, but is not involved in the regulation of notochord cell behaviour.

Jagged 1a and Jagged 1b regulate cell fates during notochord development

Given that Jagged-Notch signalling does not control the notochord cell morphology before vacuolation, we next examined whether the *jag1* deficiency causes excess notochord cells to develop. Earlier studies showed that Notch activation induces hypochord and floor-plate development at the expense of the notochord, and that excess notochord cells develop in *deltaA* and *mib* mutant embryos (Appel et al., 1999; Latimer et al., 2002; López et al., 2003). To determine the total number of notochord cells, the nuclei in the notochord were labelled either by injecting nuclear localized-mCherry mRNA or by an anti-Ntl antibody (Fig. 3C). We counted the vacuolated cells by using a transgenic fish line, 214A-GFP, which expresses GFP selectively in the vacuolated cells (Fig. 3C; see also Fig. S4 in the supplementary material). We found that the total number of nuclei was not significantly different between the control and the *jag1a/1b* knockdown embryos, but the embryos injected with *jag1a/1b* MOs had more vacuolated cells than did control embryos (Fig. 3E). These results suggested that the increased number of vacuolated cells by *jag1a/1b* knockdown is not due to excess notochord cells.

We noticed that there were many non-vacuolated cells in the notochord of control embryos (red arrowheads in Fig. 3C), but fewer in the *jag1a/1b* knockdown embryos (Fig. 3D). By contrast, the vacuolated cells, whose nuclei appeared yellow, were increased in *jag1a/1b* knockdown embryos (green arrows in Fig. 3C,D). These findings raised the possibility that the two *Jag1* genes regulate the cell fate choice between vacuolated cells and non-vacuolated cells. A fate-mapping study previously showed that notochord precursor cells develop into two morphologically distinguishable cell types: large vacuolated structural cells and thin non-vacuolated epithelial cells, which are designated as sheath cells (Melby et al., 1996). We confirmed this finding by tracking cells with GFP expressed under the promoter of the *floating head* gene (Flh-GFP), which allows the selective visualization of notochord cells (Fig. 3F; see also Fig. S5 and Movie 1 in the supplementary material) (Gamse et al., 2003; Kida et al., 2007). The epithelial-type cells in the notochord continued to be observed at least until 3 dpf, when vacuolization was complete (see Fig. S5 in the supplementary material). Therefore, we next examined the proportion of these two types of notochord cells by following the Flh-GFP-positive notochord cells. We found that the *jag1a/1b* knockdown cells in the notochord were less likely than control cells to become non-vacuolated epithelial cells (Fig. 3G). Furthermore, Notch activation at 3 ss suppressed the formation of the large vacuolated cells, and most of the cells within the notochord became non-vacuolated cells, as observed by diffuse cytosolic staining with

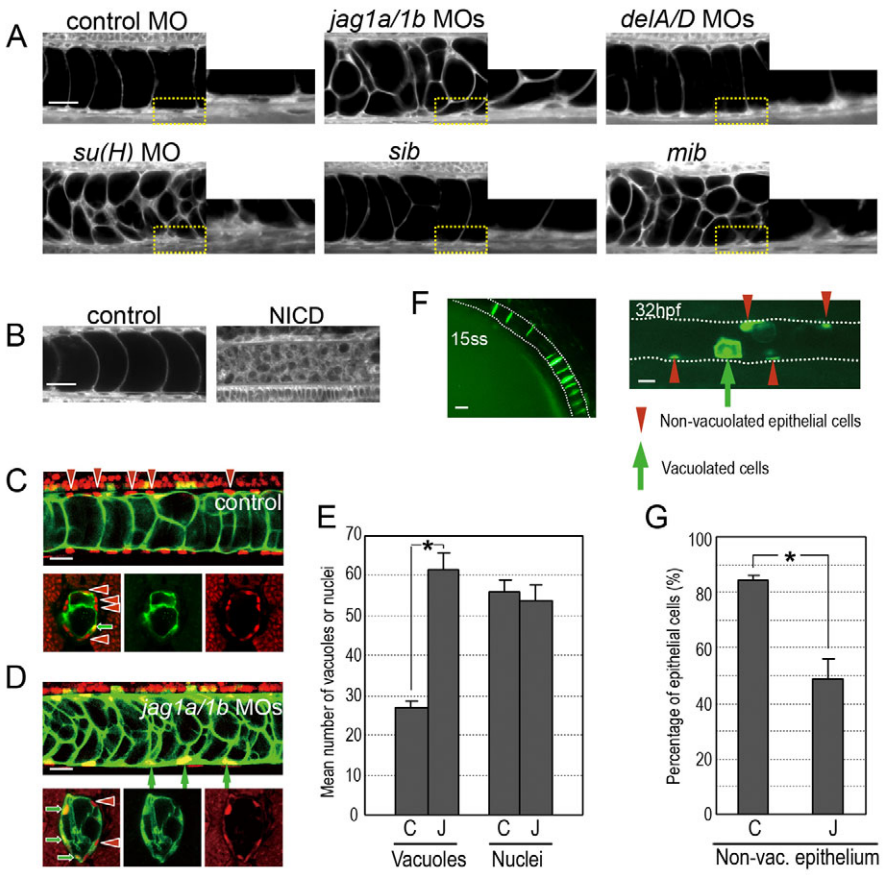

Fig. 3. Jagged 1a and Jagged 1b regulate cell fates during notochord development. (**A**) Defects in Mib-Jag1-Notch but not DeltaA/D-Notch signalling led to increased vacuoles in the notochord. Enlarged views of the yellow-boxed areas are shown in each right panel. (**B**) Notch activation reduced the vacuolated cells in the notochord. Control siblings (control) or double transgenic Tg(UAS:myc-Notch1a-intra);Tg(hsp70:Gal4) embryos (NICD) were heat shocked at 3 ss. A and B are side views of the BODIPY TR methyl ester-stained notochord cells in embryos at around 34 hpf. (**C**,**D**) Vacuolated cells were increased at the expense of non-vacuolated cells in *jag1a/1b* knockdown embryos. Nuclei were visualized by nuclear-localized mCherry (red, upper panels in C,D) or by Ntl antibody (red, lower panels in C,D). Vacuolated cells were revealed by using a transgenic fish line (214A-GFP), in which the vacuolated cells are labelled with GFP (green). Red arrowheads indicate non-vacuolated cell nuclei. Green arrows indicate vacuolated cell nuclei. (**E**) Quantification of the mean number of vacuoles and nuclei. The vacuoles and nuclei were counted within the trunk region of the notochord (230 μm in width, as in the upper images of C,D). Control MO (C, *n*=20), *jag1a/1b* MOs (J, *n*=22). (**F**) Vacuolated cells and non-vacuolated epithelial cells are derived from notochord precursor cells. The GFP-positive cells at 15 ss were followed until 32 hpf in an embryo injected with Flh-GFP plasmid. (**G**) Quantification of the proportion of non-vacuolated cells. GFP-positive vacuolated and GFP-negative non-vacuolated cells were counted in embryos co-injected with Flh-GFP plasmid and the control MO (C, *n*=6) or *jag1a/1b* MOs (J, *n*=8). Asterisks in F and G indicate statistically significant differences relative to the control ($P<0.005$). Error bars indicate s.e.m. Scale bars: 20 μm in A-D,F.

BODIPY TR methyl ester (Fig. 3B). In support of these findings, we observed that the number of BODIPY TR-stained epithelial cells were reduced in *jag1a/1b*-, *su(H)*- and *mib*-deficient embryos, but not in *deltaA/D*-knockdown embryos (Fig. 3A). Collectively, these data suggest that Jag1 signalling regulates the vacuolated versus non-vacuolated epithelial cell-fate decision during notochord development.

Jagged and Delta, two Notch ligands, regulate midline cell fate differently

The above data suggested that Jag1 signalling acts as a cell-fate switch from vacuolated to non-vacuolated cells within the notochord, rather than as a switch from notochord to other cell types. By contrast, signalling from another family of ligands, DeltaA, C and D, in zebrafish acts as a switch from notochord to hypochord (Latimer et al., 2002). These observations led us to investigate how the two different classes of ligands, Jagged and Delta, regulate their downstream signalling pathways. In hypochord precursor cells, *her4*, one of the downstream target genes of Notch signalling, is expressed. We observed that the expression of *her4* in the hypochord precursors was reduced in *deltaA/D* double-knockdown embryos, as previously reported (81%, *n*=16) (Latimer et al., 2002). *her4* expression was also decreased in *mib* mutants, but not in *jag1a/1b* knockdown embryos (100%, *n*=13, and 100%, *n*=21, respectively, in Fig. 4A). These findings suggested that the activation of Mib-mediated Delta but not Jag1 signalling favours the development of hypochord cells through the induction of *her4* expression.

Conversely, no Notch target genes expressed in the notochord have been reported. Therefore, we sought to identify such genes among the Hairy-related (Her) genes, which are Notch target genes. We found that *her9* was expressed in the notochord at least from 6 ss to 18 ss, but its expression gradually decreased at later stages (Fig. 4B; see Fig. S6 in the supplementary material). The

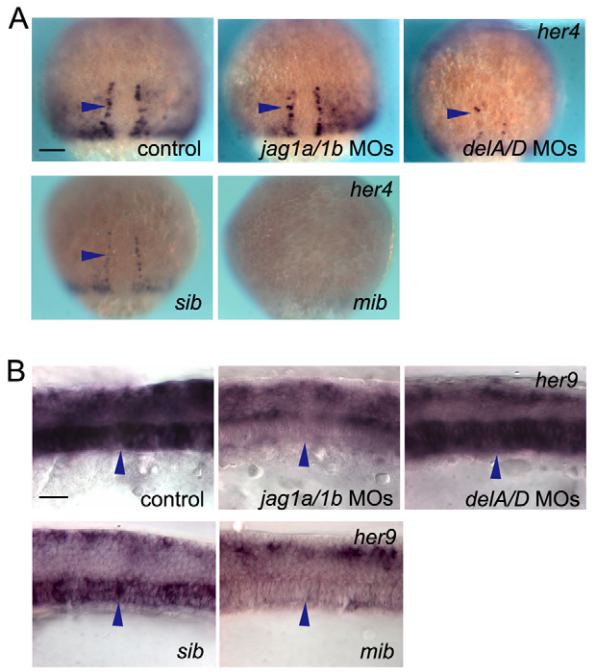

Fig. 4. Jagged 1-Notch signalling regulates *her9* but not *her4* expression, independent of Delta-Notch signalling. (**A**) *her4* expression was reduced in the hypochord precursor cells of *deltaA/D* (*delA/D*) knockdown embryos and *mib* mutants, but not in those of *jag1a/1b* (*jag1a/1b*) knockdown embryos. Arrowheads indicate the hypochord precursor cells. Dorsal views at 80% epibody are shown. (**B**) *her9* expression was decreased in the notochord of *jag1a/1b* knockdown and *mib* mutant embryos, but not in that of *deltaA/D* knockdown embryos. Arrowheads indicate notochord cells. *sib*, sibling control. Side views at 10 ss are shown. Scale bars: 100 μm in A; 20 μm in B.

spatiotemporal *her9* expression overlapped with *jag1a/1b* expression in the notochord. Thus, we tested whether *her9* is one of the downstream target genes of Jag1-Notch signalling in the notochord. Indeed, we found that *her9* expression was downregulated in the notochord of *jag1a/1b* knockdown embryos (Fig. 4B; 100%, *n*=55). Its expression in the notochord was also lost in the *mib* mutant (Fig. 4B) and in *su(H)* knockdown embryos (data not shown; 97%, *n*=42). By contrast, *deltaA/D* knockdown did not alter *her9* expression (Fig. 4B; 97%, *n*=34). These data indicated that Mib regulates both Delta- and Jag1-induced Notch activation, and that Delta-Notch and Jagged-Notch signalling independently regulate *her4* and *her9* gene expression, respectively. These regulations might contribute to the differential cell-fate switches controlled by the signalling pathways of the two ligand families during midline development.

Jagged 1-Notch signalling is required for notochord basement membrane formation

As Jag1-Notch signalling regulates the cell-fate determination between vacuolated cells and non-vacuolated cells, we next investigated whether altering the cell fate affected the structural function of the notochord. Vacuoles play a structural role in generating turgor in the notochord, whereas the functional role of the epithelial cells is not clearly understood. We therefore examined the cellular ultrastructure of the epithelial cells by electron microscopy. We found that the cytoplasm of the non-vacuolated epithelial cells, but not of the vacuolated cells, contained abundant rough endoplasmic reticulum (ER) at 2 dpf (Fig. 5A). To confirm this observation, we used an ER marker, an anti-KDEL antibody, which recognizes the ER chaperones BiP and GRP94 (Fig. 5B). In combination with the 214A-GFP transgenic line, anti-KDEL staining was observed in most of the GFP-negative cells, but not in the strongly GFP-positive vacuolated cells (arrowheads and arrows, respectively, in Fig. 5B). Furthermore, *jag1a/1b*-knockdown embryos and *mib* mutants, but not *deltaA/D*-knockdown embryos, had a reduced number of the cells with abundant ER, as detected by the anti-KDEL antibody (Fig. 5D; see also Fig. S7 in the supplementary material; *jag1a/1b*, 90%, *n*=21; *mib*, 100%, *n*=4). An increase in rough ER might correlate with the ability of a cell to secrete the extracellular matrix proteins that are required for peri-notochordal basement membrane (PBM; also called sheath) formation (Fig. 5A). Therefore, we next examined PBM formation in Jag1-Notch signalling knockdown embryos.

The PBM is composed of three layers: the outer, medial and inner layers (Fig. 5C). In the *jag1a/1b* knockdown embryos, the medial layer was relatively thin compared with that of control embryos; however, the outer and inner layers were not dramatically affected (Fig. 5C). The medial and inner layers are thought to consist of collagen and laminin, respectively (see Fig. S8 in the supplementary material) (Stemple, 2005). Accordingly, type II collagen was decreased in the *jag1a/1b* knockdown embryos (Fig. 5D; *jag1a* splMO in *jag1b* mutants, 81%, *n*=21; *jag1a/1b* MOs, 93%, *n*=32). However, the laminin protein in the PBM was not affected (see Fig. S9 in the supplementary material). This could be explained by the observation that the laminin-rich inner layer is supplied by its surrounding tissues (Coutinho et al., 2004). Similar observations were made in the *mib* mutants and *su(H)* morpholino-injected embryos (see Fig. S9 in the supplementary material). Conversely, Notch activation at 3 ss increased the anti-KDEL-positive cells and collagen protein in the PBM (Fig. 5E; 74%, *n*=42). Altogether, these data suggest that Jag1-Notch signalling plays an important role in PBM formation by promoting cell-fate commitment to the non-vacuolated epithelial cells, which are active in collagen secretion.

Jagged 1-Notch signalling is required for regulating Hedgehog signalling and muscle patterning

Besides its structural functions, the notochord is important as a signalling source for patterning its surrounding tissues (Stemple, 2005). Therefore, we next examined whether Jag1-Notch signalling is involved in the patterning activity of the notochord. Hedgehog family members are secreted from the notochord and are known to be responsible for its patterning activity. As the notochord differentiates, the expression of *Indian hedgehog homolog b* (*ihhb*) and *sonic hedgehog* (*shh*) gradually decreases in the notochord cells (Currie and Ingham, 1996). In *jag1a/1b* knockdown embryos, however, their expression was sustained at high levels in the notochord cells (Fig. 6A; see also Fig. S10 in the supplementary material; 95%, *n*=59, 67%, *n*=12, respectively), and the expression of *ihhb* was not downregulated in *mib* mutants either (Fig. 6B, 95%, *n*=21). The determination of the correct number of muscle cells to generate the different muscle cell types is dependent on Hh signalling levels (Currie and Ingham, 1996; Wolff et al., 2003). When the Hh signalling level is high, the numbers of Engrailed-positive muscle pioneers (MPs) and medial fast fibres (MFFs) increase, whereas a low level of Hh signalling reduces their numbers (Wolff et al., 2003). Accordingly, the MPs and MFFs

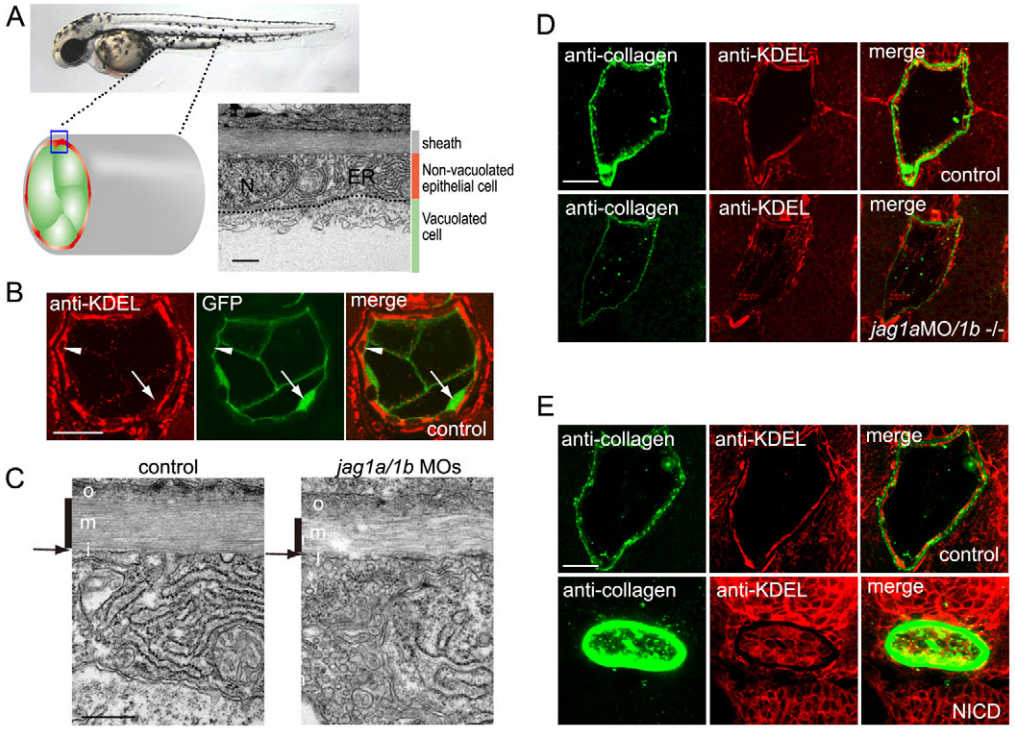

Fig. 5. Jagged 1a/1b is required for notochord extracellular matrix sheath formation. (**A**) Non-vacuolated epithelial cells contained abundant rough ER compared with vacuolated cells. N, nuclei; ER, endoplasmic reticulum. Transmission electron micrographs (TEMs) of transverse sections of a wild-type embryo on day 2. (**B**) Non-vacuolated cells were rich in rough ER. ER was stained by an anti-KDEL antibody (red), and vacuolated cells were revealed by the 214A-GFP transgenic line (green). Arrowheads indicate anti-KDEL-positive non-vacuolated cells. Arrows indicate GFP-positive vacuolated cells. (**C**) The medial layer of the peri-notochordal basement membrane was thinner in the *jag1a/1b* knockdown embryos than in wild-type embryos. TEM of transverse sections. o, outer; m, medial; i, inner. Black vertical lines indicate the medial layer of the sheath. Arrows indicate the thin inner layer. (**D**) *jag1a/1b* knockdown resulted in reduced type II collagen deposition and fewer cells with abundant rough ER in the notochord. (**E**) Notch activation increased type II collagen deposition and ER-positive cells in the notochord. Control siblings (control) or double transgenic Tg(UAS:myc-Notch1a-intra);Tg(hsp70:Gal4) embryos (NICD) were heat shocked at 3 ss. A-E are transverse sections of day 2 embryos. Scale bars: 0.5 μm in A,C; 20 μm in B,D,E.

were increased in the *jag1a/1b* knockdown embryos and *mib* mutants, as detected by an anti-Engrailed antibody (Fig. 6D,E; 82%, *n*=33, 100%, *n*=16, respectively). Conversely, the activation of Notch at 3 ss reduced *ihhb* expression and the number of MPs and MFFs (Fig. 6C,F; 87%, *n*=13, 94%, *n*=51, respectively). These results suggested that Mib-Jag1-Notch signalling plays an important role in preventing the excessive production of MPs and MFFs, by reducing Hh signalling.

DISCUSSION

Based on our findings, we propose a model in which Mib-Jag1-Notch signalling regulates the patterning and structural roles of the notochord by affecting cell-fate decisions (Fig. 7). Jag1-Notch and Delta-Notch signalling function at different stages of midline development. The dorsal organizer gives rise to the notochord and hypochord, and these two cell fates are controlled by Mib-mediated Delta-Notch signalling. At a later stage, Mib-mediated Jag1-Notch signalling is involved in the cell-fate choice of the developing notochord. The activation of Notch signalling favours the development of non-vacuolated cells at the expense of vacuolated cells. The non-vacuolated cells are responsible for secreting extracellular matrix proteins to form the thick peri-notochordal basement membrane. The formation of both vacuoles and peri-notochordal basement membrane is essential for the notochord to serve as the axial skeleton of the embryo. Furthermore, Mib-mediated Jag1-Notch signalling contributes to the patterning function of the notochord, which modulates the levels of Hedgehog to generate diverse muscle cell types.

Mind bomb is a positive regulator of Notch activation by Jagged

Previous reports have shown that Mib regulates two families of the canonical Notch ligands, Delta and Jagged (Serrate) in mice and *Drosophila* (Koo et al., 2005; Wang and Struhl, 2005; Lai et al., 2005; Le Borgne et al., 2005; Pitsouli and Delidakis, 2005). Our results show that zebrafish Mib also ubiquitylates and activates Jag1 in vitro and in vivo. The data from our co-culture reporter assay indicated that Mib is required for the signal-sending activity of Jag1, but not for the signal-receiving activity of the Notch receptor. The Mib ubiquitin ligase activity is associated with enhanced Notch ligand ubiquitylation, endocytosis, and signalling; however, there has been no direct evidence that Mib directly binds and ubiquitylates Notch ligand proteins, or that the Mib-dependent ubiquitylation of Notch ligands provides the signalling activity (Wang and Struhl, 2005). Our results suggest that Mib directly ubiquitylates Jag1, because our yeast two-hybrid screening identified a specific interaction between Mib and Jag1.

Nicholas et al. reported that Delta endocytosis is necessary for Notch activation, using a mammalian cultured cell line (Nichols et al., 2007). However, no direct evidence for the involvement of

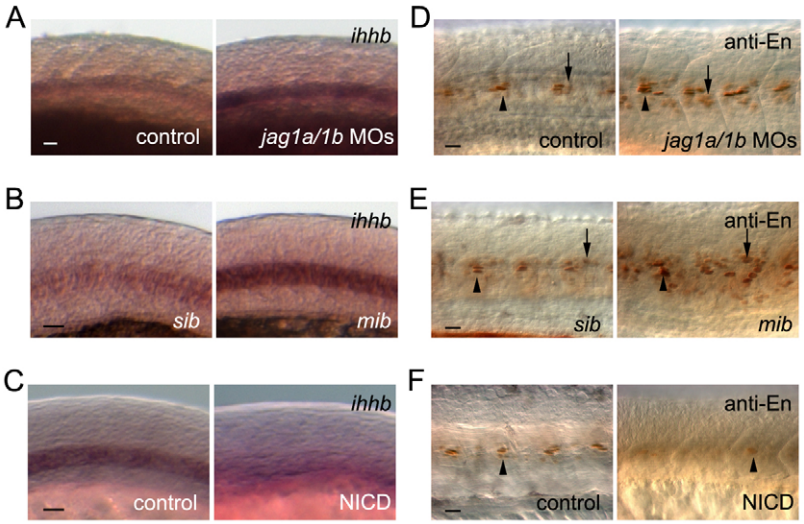

Fig. 6. Mind bomb-Jagged-Notch signalling regulates muscle cell identities by altering *ihhb* expression. (**A-C**) *ihhb* expression was increased in *jag1a/1b* (*jag1a/1b*)-knockdown embryos and *mib* mutants, whereas Notch activation decreased *ihhb* expression at 18 ss. (**D-F**) Engrailed-positive (anti-En) MPs and MEFs were increased in *jag1a/1b* knockdown and *mib* mutant embryos, whereas Notch activation decreased the Engrailed-positive cells at 24 hpf. (B,E) *sib*, sibling control. (C,F) Control siblings (control) or double transgenic Tg(UAS:myc-Notch1a-intra);Tg(hsp70:Gal4) embryos (NICD) were heat-shocked at 3 ss. MPs and MFFs are indicated by arrowheads and arrows, respectively. Scale bars: 20 μm in A-F.

Jagged endocytosis in Notch activation has been reported. Mib knockdown did not cause a reduction in the surface Jag1 protein expression. Furthermore, the bulk endocytosis of Jag1 protein in the absence of Notch receptor binding was not altered in Mib knockdown cells. These observations raised the possibility that either the Mib ubiquitin ligase activity is absent when Jag1 does not bind Notch (i.e. Mib ubiquitylates Jag1 only when Jag1 interacts with Notch), or that endocytosis of Jagged per se is not required, but its ubiquitylation is required for the localization of Jagged to microdomains with an appropriate environment to support the ability of Jag1 to activate Notch signalling, as was suggested in the case of Delta by Heuss et al. (Heuss et al., 2008). Further studies should clarify the mechanism by which Mib activates Jag1 through ubiquitylation.

Midline cell-fate determination is regulated differently by Delta-Notch and Jagged-Notch signalling

In this study, we show a previously unrecognized role of Jag1-mediated Notch signalling in cell-fate determination in notochord cells. Jag1-Notch signalling acts differently from Delta-Notch signalling in the following respects. First, different midline fates are determined: Delta-Notch signalling determines notochord versus hypochord (Latimer et al., 2002; Latimer et al., 2006), whereas Jag1-Notch signalling determines vacuolated versus non-vacuolated cells within the notochord. Second, the timing of the decision-making is different: Delta-Notch signalling acts during gastrulation (Latimer et al., 2002; Latimer and Appel, 2006), whereas Jag1-Notch signalling acts at later stages, i.e. segmentation. Third, the target genes are different: Delta-Notch signalling induces *her4* (Latimer et al., 2002; Latimer and Appel, 2006; López et al., 2003), whereas Jag1-Notch signalling induces *her9*.

The *her9* expression is transient and uneven in the notochord, suggesting that *her9* is involved in the cell-fate choice regulated by Jagged-Notch signalling. However, the knockdown of *her9* alone did not alter the notochord cell fate (data not shown). This may be due to the involvement of other effectors working in parallel with *her9*. The expression of *her4* and *her9* is exclusively controlled by the Delta-Notch and Jag1-Notch interaction, respectively, as *her4* but not *her9* expression was reduced in Delta-knockdown embryos, and *her9* but not *her4* expression was reduced in Jag1-knockdown embryos. By contrast, the expression of both *her4* and *her9* was reduced in the *mib* mutants. These observations, together with the

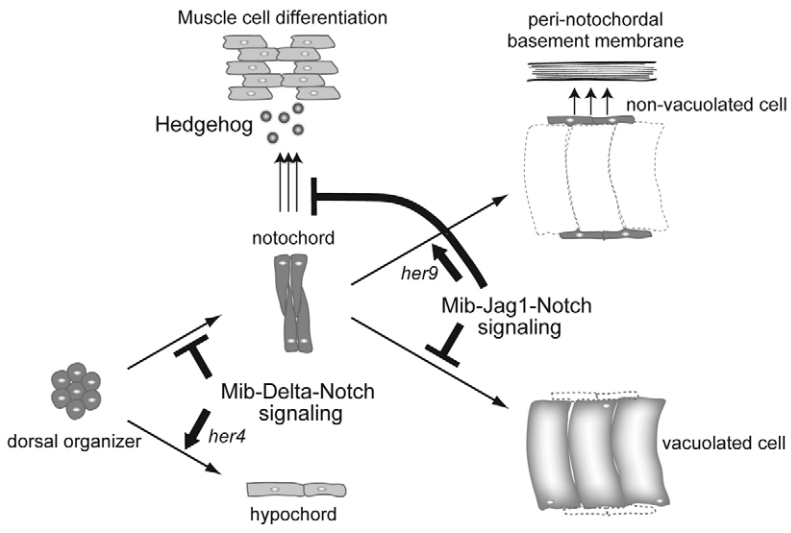

Fig. 7. Model of how Mind bomb-Jagged 1-Notch signalling regulates the patterning and structural roles of the notochord by affecting cell-fate decisions. Mib-Delta-Notch signalling acts during the gastrula stage and determines notochord versus hypochord, possibly through *her4* activation. Mib-Jag1-Notch signalling acts at later stages, and determines vacuolated versus non-vacuolated cell fate in the notochord, possibly through *her9* activation. This cell-fate choice affects peri-notochordal basement membrane formation and muscle-patterning activity.

notochord cell-fate change in *mib* mutants, are consistent with previous findings that Mib regulates both the Delta and the Jagged ligands (Koo et al., 2005; Wang and Struhl, 2005; Lai et al., 2005; Le Borgne et al., 2005; Pitsouli and Delidakis, 2005). Collectively, Delta-Notch and Jagged-Notch signalling determine midline cell fates at different developmental stages and by inducing distinct target genes. Although the two ligand families have distinct roles in midline cell fate determination in vivo, it is unclear whether they differ functionally in inducing different target genes. Previous studies showed that both Delta1 and Jag1 similarly prevent the differentiation of C2C12 myoblast cells into myotubes in vitro (Kuroda et al., 1999; Lindsell et al., 1995). If this is the case, a different ligand system might not be necessary; rather, different target genes might be induced because of intrinsic differences in the progenitor cell types in which Notch signaling is activated.

Both the patterning and the structural roles of the notochord are controlled by Jagged 1-Notch signalling

The notochord serves two important roles: (1) to pattern its surrounding tissues, such as the muscle and the neural tube; and (2) to provide a structural element as the axial skeleton. Given that Jagged-Notch signalling regulates the fates of the notochord cells during development, these two roles of the notochord might be linked to the differentiation of the two cell types. The increase in vacuolated cells and the upregulation of Hedgehog expression in Jagged-Notch signalling-deficient embryos might suggest that vacuolated cells but not non-vacuolated cells are responsible for Hedgehog expression, which plays an essential role in the patterning activity. However, this is unlikely to be the case, because the *ihhb* and *shh* expression levels in the notochord gradually fade as the vacuolated and non-vacuolated cells differentiate during normal notochord development. Previous reports have shown that an increased persistent expression of *ihhb* is correlated with a failure of notochord differentiation. In addition, the peri-notochordal basement membrane is essential for proper notochord differentiation (Parsons et al., 2002; Coutinho et al., 2004). Therefore, Jagged-Notch signalling might indirectly regulate the patterning role of the notochord by altering notochord differentiation via its effects on basement membrane formation. However, although a temporally controlled, forced Notch activation decreased *ihhb* expression, the same treatment did not reduce the expression of other differentiation markers, such as *shh* or *ntl* (data not shown). These data could suggest another possibility, that Jagged-Notch signalling directly controls *ihhb* expression by activating downstream target genes, such as *hes*-related repressor genes.

The establishment of both the large vacuolated cells and the thick basement membrane is essential for normal notochord structure. Our findings suggest for the first time that these two important features are associated with the development of two different types of cells, vacuolated- and non-vacuolated cells. Furthermore, the cell-fate decision between these two types of cells is controlled by Mib-Jagged-Notch signalling. Stemple et al. reported that the notochord cells in *mib* mutants become vacuolated, but are spherical instead of the normal scalloped shape, and thus suggested that *mib* is involved in the maintenance of the structural integrity of the notochord in zebrafish (Stemple et al., 1996). Our results revealed that the number of vacuolated cells is increased in Mib-Jagged-Notch signalling-defective embryos because of a cell-fate change of the notochord cells. The spherical shape of the notochord cells might be a consequence of an increased number of vacuolated cells being packed into a limited space.

Later in development, the notochord is replaced by vertebral bodies (centra). Previous studies suggest that the notochord plays a key role in the formation of vertebral bodies in fish (Grotmol et al., 2003; Fleming et al., 2004). Alagille syndrome (AGS) is an autosomal-dominant disorder caused by a haploinsufficiency of JAG1 (Oda et al., 1997). One of the characteristic features of AGS is butterfly vertebra (incomplete formation of the vertebral body) (Sanderson et al., 2002). Given that Jag1-Notch signalling regulates notochord development in zebrafish, the vertebral abnormality in AGS could be caused by aberrant cell-fate determination during notochord development. Further studies are needed to explore this possibility, and will also help to clarify whether the notochord has a conserved role in the instruction of vertebral body formation in all vertebrates.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References

Appel, B., Fritz, A., Westerfield, M., Grunwald, D. J., Eisen, J. S. and Riley, B. B. (1999). Delta-mediated specification of midline cell fates in zebrafish embryos. *Curr. Biol.* **9**, 247-256.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Asakawa, K., Suster, M. L., Mizusawa, K., Nagayoshi, S., Kotani, T., Urasaki, A., Kishimoto, Y., Hibi, M. and Kawakami, K. (2008). Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. *Proc. Natl. Acad. Sci. USA* **105**, 1255-1260.

Broder, Y. C., Katz, S. and Aronheim, A. (1998). The ras recruitment system, a novel approach to the study of protein-protein interactions. *Curr. Biol.* **8**, 1121-1124.

Coutinho, P., Parsons, M. J., Thomas, K. A., Hirst, E. M., Saude, L., Campos, I., Williams, P. H. and Stemple, D. L. (2004). Differential requirements for COPI transport during vertebrate early development. *Dev. Cell* **7**, 547-558.

Currie, P. D. and Ingham, P. W. (1996). Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. *Nature* **382**, 452-455.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Fleming, A., Keynes, R. and Tannahill, D. (2004). A central role for the notochord in vertebral patterning. *Development* **131**, 873-880.

Gamse, J. T., Thisse, C., Thisse, B. and Halpern, M. E. (2003). The parapineal mediates left-right asymmetry in the zebrafish diencephalon. *Development* **130**, 1059-1068.

Grotmol, S., Kryvi, H., Keynes, R., Krossoy, C., Nordvik, K. and Totland, G. K. (2006). Stepwise enforcement of the notochord and its intersection with the myoseptum: an evolutionary path leading to development of the vertebra? *J. Anat.* **209**, 339-357.

Heuss, S. F., Ndiaye-Lobry, D., Six, E. M., Israel, A. and Logeat, F. (2008). The intracellular region of Notch ligands Dll1 and Dll3 regulates their trafficking and signaling activity. *Proc. Natl. Acad. Sci. USA* **105**, 11212-11217.

Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L. et al. (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev. Cell* **4**, 67-82.

Joubin, K. and Stern, C. D. (2001). Formation and maintenance of the organizer among the vertebrates. *Int. J. Dev. Biol.* **45**, 165-175.

Kida, Y. S., Sato, T., Miyasaka, K. Y., Suto, A. and Ogura, T. (2007). Daam1 regulates the endocytosis of EphB during the convergent extension of the zebrafish notochord. *Proc. Natl. Acad. Sci. USA* **104**, 6708-6713.
Koo, B. K., Lim, H. S., Song, R., Yoon, M. J., Yoon, K. J., Moon, J. S., Kim, Y. W., Kwon, M. C., Yoo, K. W., Kong, M. P. et al. (2005). Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. *Development* **132**, 3459-3470.
Krauss, S., Concordet, J. P. and Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-1444.
Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H. and Honjo, T. (1999). Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. *J. Biol. Chem.* **274**, 7238-7244.
Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-973.
Lai, E. C., Roegiers, F., Qin, X., Jan, Y. N. and Rubin, G. M. (2005). The ubiquitin ligase Drosophila Mind bomb promotes Notch signaling by regulating the localization and activity of Serrate and Delta. *Development* **132**, 2319-2332.
Latimer, A. J. and Appel, B. (2006). Notch signaling regulates midline cell specification and proliferation in zebrafish. *Dev. Biol.* **298**, 392-402.
Latimer, A. J., Dong, X., Markov, Y. and Appel, B. (2002). Delta-Notch signaling induces hypochord development in zebrafish. *Development* **129**, 2555-2563.
Latimer, A. J., Shin, J. and Appel, B. (2005). her9 promotes floor plate development in zebrafish. *Dev. Dyn.* **232**, 1098-1104.
Le Borgne, R., Remaud, S., Hamel, S. and Schweisguth, F. (2005). Two distinct E3 ubiquitin ligases have complementary functions in the regulation of delta and serrate signaling in Drosophila. *PLoS Biol.* **3**, e96.
Lewis, K. E. and Eisen, J. S. (2001). Hedgehog signaling is required for primary motoneuron induction in zebrafish. *Development* **128**, 3485-3495.
Lindsell, C., Shawber, C., Boulter, J. and Weinmaster, G. (1995). Jagged: a mammalian ligand that activates Notch1. *Cell* **80**, 909-917.
López, S. L., Paganelli, A. R., Siri, M. V., Ocana, O. H., Franco, P. G. and Carrasco, A. E. (2003). Notch activates sonic hedgehog and both are involved in the specification of dorsal midline cell-fates in Xenopus. *Development* **130**, 2225-2238.
López, S. L., Rosato-Siri, M. V., Franco, P. G., Paganelli, A. R. and Carrasco, A. E. (2005). The Notch-target gene hairy2a impedes the involution of notochordal cells by promoting floor plate fates in Xenopus embryos. *Development* **132**, 1035-1046.
Meinhardt, H. (2008). Models of biological pattern formation: from elementary steps to the organization of embryonic axes. *Curr. Top. Dev. Biol.* **81**, 1-63.
Melby, A. E., Warga, R. M. and Kimmel, C. B. (1996). Specification of cell fates at the dorsal margin of the zebrafish gastrula. *Development* **122**, 2225-2237.
Moriyama, Y., Sekine, C., Koyanagi, A., Koyama, N., Ogata, H., Chiba, S., Hirose, S., Okumura, K. and Yagita, H. (2008). Delta-like 1 is essential for the maintenance of marginal zone B cells in normal mice but not in autoimmune mice. *Int. Immunol.* **20**, 763-773.
Nichols, J. T., Miyamoto, A., Olsen, S. L., D'Souza, B., Yao, C. and Weinmaster, G. (2007). DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. *J. Cell Biol.* **176**, 445-458.
Oates, A. C., Rohde, L. A. and Ho, R. K. (2005). Generation of segment polarity in the paraxial mesoderm of the zebrafish through a T-box-dependent inductive event. *Dev. Biol.* **283**, 204-214.
Oda, T., Elkahloun, A. G., Pike, B. L., Okajima, K., Krantz, I. D., Genin, A., Piccoli, D. A., Meltzer, P. S., Spinner, N. B., Collins, F. S. et al. (1997). Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat. Genet.* **16**, 235-242.
Parsons, M. J., Pollard, S. M., Saude, L., Feldman, B., Coutinho, P., Hirst, E. M. and Stemple, D. L. (2002). Zebrafish mutants identify an essential role for laminins in notochord formation. *Development* **129**, 3137-3146.
Pitsouli, C. and Delidakis, C. (2005). The interplay between DSL proteins and ubiquitin ligases in Notch signaling. *Development* **132**, 4041-4050.
Sanderson, E., Newman, V., Haigh, S. F., Baker, A. and Sidhu, P. S. (2002). Vertebral anomalies in children with Alagille syndrome: an analysis of 50 consecutive patients. *Pediatr. Radiol.* **32**, 114-119.
Scheer, N., Riedl, I., Warren, J. T., Kuwada, J. Y. and Campos-Ortega, J. A. (2002). A quantitative analysis of the kinetics of Gal4 activator and effector gene expression in the zebrafish. *Mech. Dev.* **112**, 9-14.
Schulte-Merker, S., Ho, R. K., Herrmann, B. G. and Nusslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**, 1021-1032.
Shimizu, T., Yabe, T., Muraoka, O., Yonemura, S., Aramaki, S., Hatta, K., Bae, Y. K., Nojima, H. and Hibi, M. (2005). E-cadherin is required for gastrulation cell movements in zebrafish. *Mech. Dev.* **122**, 747-763.
Sprague, J., Bayraktaroglu, L., Clements, D., Conlin, T., Fashena, D., Frazer, K., Haendel, M., Howe, D. G., Mani, P., Ramachandran, S. et al. (2006). The Zebrafish Information Network: the zebrafish model organism database. *Nucleic Acids Res.* **34**, D581-D585.
Stemple, D. L. (2005). Structure and function of the notochord: an essential organ for chordate development. *Development* **132**, 2503-2512.
Stemple, D. L., Solnica-Krezel, L., Zwartkruis, F., Neuhauss, S. C., Schier, A. F., Malicki, J., Stainier, D. Y., Abdelilah, S., Rangini, Z., Mountcastle-Shah, E. et al. (1996). Mutations affecting development of the notochord in zebrafish. *Development* **123**, 117-128.
Takke, C., Dornseifer, P., v. Weizsacker, E. and Campos-Ortega, J. A. (1999). her4, a zebrafish homologue of the Drosophila neurogenic gene E(spl), is a target of NOTCH signalling. *Development* **126**, 1811-1821.
Thisse, C. and Thisse, B. (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat. Protoc.* **3**, 59-69.
Wang, W. and Struhl, G. (2005). Distinct roles for Mind bomb, Neuralized and Epsin in mediating DSL endocytosis and signaling in Drosophila. *Development* **132**, 2883-2894.
Wolff, C., Roy, S. and Ingham, P. W. (2003). Multiple muscle cell identities induced by distinct levels and timing of hedgehog activity in the zebrafish embryo. *Curr. Biol.* **13**, 1169-1181.
Zhang, C., Li, Q., Lim, C. H., Qiu, X. and Jiang, Y. J. (2007). The characterization of zebrafish antimorphic mib alleles reveals that Mib and Mind bomb-2 (Mib2) function redundantly. *Dev. Biol.* **305**, 14-27.
Zuniga, E., Stellabotte, F. and Crump, J. (2010). Jagged-Notch signaling ensures dorsal skeletal identity in the vertebrate face. *Development* **137**, 1843.
Zecchin, E., Conigliaro, A., Tiso, N., Argenton, F. and Bortolussi, M. (2005). Expression analysis of jagged genes in zebrafish embryos. *Dev. Dyn.* **233**, 638-645.