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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

# Jagged 2b induces intercellular signaling within somites to establish hematopoietic stem cell fate in zebrafish

**Yukino Wada[1], Hikaru Tsukatani[2], Chihiro Kuroda[2], Yurika Miyazaki[2], Miku Otoshi[3] and Isao Kobayashi[3,*]**

## ABSTRACT

During development, the somites play a key role in the specification of hematopoietic stem cells (HSCs). In zebrafish, the somitic Notch ligands Delta-c (Dlc) and Dld, both of which are regulated by Wnt16, directly instruct HSC fate in a shared vascular precursor. However, it remains unclear how this signaling cascade is spatially and temporally regulated within somites. Here, we show in zebrafish that an additional somitic Notch ligand, Jagged 2b (Jag2b), induces intercellular signaling to drive *wnt16* expression. Jag2b activated Notch signaling in segmented somites at the early stage of somitogenesis. Loss of *jag2b* led to a reduction in the expression of *wnt16* in the somites and an HSC marker, *runx1*, in the dorsal aorta, whereas overexpression of *jag2b* increased both. However, Notch-activated cells were adjacent to, but did not overlap with, *wnt16*-expressing cells within the somites, suggesting that an additional signaling molecule mediates this intercellular signal transduction. We uncover that Jag2b-driven Notch signaling induces *efna1b* expression, which regulates *wnt16* expression in neighboring somitic cells. Collectively, we provide evidence for previously unidentified spatiotemporal regulatory mechanisms of HSC specification by somites.

**KEY WORDS: Hematopoietic stem cells, Notch, Jagged, Somite, Zebrafish, Ephrin**

## INTRODUCTION

The ontogeny of the hematopoietic system is composed of two waves: the primitive and definitive waves. The primitive wave of hematopoiesis originates from erythroid- and myeloid-restricted progenitors, whereas the definitive wave initiates from multipotent hematopoietic progenitors, including hematopoietic stem cells (HSCs). During embryonic development, HSCs arise from a specific subset of vascular endothelial cells, termed hemogenic endothelial cells (HECs), in the ventral floor of the dorsal aorta (DA) through endothelial-to-hematopoietic transition (EHT) (Boisset et al., 2010; Bertrand et al., 2010; Kissa and Herbomel, 2010). Despite evolutionary divergence, the genetic programs governing hematopoiesis are highly conserved among vertebrates. Some key signaling molecules that regulate HSC formation have been identified using zebrafish and mouse embryos, such as Notch (Kumano et al., 2003; Hadland et al., 2004; Burns et al., 2005; Robert-Moreno et al., 2005; Kim et al., 2014), BMP4 (Wilkinson et al., 2009), FGF (Lee et al., 2014; Pouget et al., 2014) and Wnt (Clements et al., 2011; Grainger et al., 2016; Richter et al., 2018). These signaling molecules directly or indirectly confer HSC programs in a shared vascular precursor, the angioblast, to generate HECs in the DA.

Notch signaling plays a crucial role in HSC specification as well as patterning of the trunk vasculature. Canonical Notch signaling is induced when a membrane-bound Notch ligand (Delta or Jagged) on a signal-sending cell directly interacts with the Notch receptor on the cell surface of the signal-receiving cell. Upon binding to a Notch ligand, the receptor is cleaved by ADAM TACE metalloproteases at the S2 site, followed by further cleavage by γ-secretase at the S3 site. These cleavages of the Notch receptor result in translocation of the Notch intracellular domain (NICD) to the nucleus, activating transcription of Notch target genes (Brou et al., 2000; Bozkulak and Weinmaster, 2009; Mumm et al., 2000; Kopan and Ilagan, 2009). Studies on Notch1-deficient mice and chimeric mice generated from both wild-type and Notch1-deficient cells demonstrated that Notch1 signaling is required for HSC specification in a cell-autonomous manner (Kumano et al., 2003; Hadland et al., 2004).

In zebrafish, HSC specification requires three Notch genes, *notch1a*, *1b* and *3*, which are expressed in the somites and the posterior lateral plate mesoderm (PLPM) (Kim et al., 2014). Kim et al. showed that loss of *notch1a* impairs both HSC and DA formation, whereas loss of *notch1b* or *notch3* results in loss of HSCs but does not affect DA formation. These data suggest that Notch1a is required for both HSC specification and aortic formation, whereas Notch1b and Notch3 are essential for HSC specification only. Unlike Notch1, however, Notch3 non-cell-autonomously regulates HSC specification. Enforced expression of NICD in somitic cells has been shown to rescue HSC formation in *notch3*-deficient zebrafish (Kim et al., 2014), providing evidence that Notch signaling within the somites indirectly regulates HSC specification.

The somitic Notch ligands Delta-c (Dlc) and Dld have been shown to be required for HSC specification in zebrafish. A non-canonical Wnt ligand, Wnt16, regulates the expression of both *dlc* and *dld* in somites (Clements et al., 2011). During somitogenesis, angioblasts emerging in the PLPM migrate axially along the ventral domain of the somites, where Dlc and Dld are expressed. A portion of migrating angioblasts adhere tightly to the somites based on the interaction of junctional adhesion molecules or integrins, and this intimate contact promotes efficient Dlc/Dld signal transduction from the somites to the angioblast (Kobayashi et al., 2014; Rho et al., 2019). Shortly after reaching the midline, angioblasts that received high levels of Notch signaling begin to express *gata2b*, which is essential for EHT and is directly regulated by Notch signaling (Butko et al., 2015; Robert-Moreno et al., 2005). Notch signaling from the somites thus plays an important role in the

[1]Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Ishikawa 920-1192, Japan. [2]Faculty of Natural System, Institute of Science and Engineering, Kanazawa University, Ishikawa 920-1192, Japan. [3]Faculty of Biological Science and Technology, Institute of Science and Engineering, Kanazawa University, Ishikawa 920-1192, Japan.

*Author for correspondence (ikobayashi@se.kanazawa-u.ac.jp)

H.T., 0000-0002-1332-3154; C.K., 0000-0003-2537-1046; Y.M., 0000-0002-0045-9001; I.K., 0000-0002-2750-3244

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establishment of HSC fate in angioblasts. Here, we show in zebrafish that an additional somitic Notch ligand, Jagged 2b (Jag2b), promotes HSC specification by regulating *wnt16* expression. Jag2 signaling has been shown to promote the survival and proliferation of hematopoietic progenitors in the murine bone marrow, and it is also involved in T-cell differentiation in the thymus (Tsai et al., 2000; Van de Walle et al., 2011). However, the role of Jag2 in HSC development has not been established in mammals or zebrafish. In the zebrafish embryo, we detected expression of *jag2b* in the somites as early as 11 h post-fertilization (hpf), when *wnt16* was not yet expressed. Loss of *jag2b* resulted in a loss of *wnt16* expression in the somites and a loss of HSCs in the DA, whereas *wnt16*-expressing cells were adjacent to Notch-activated cells in the somites. We found that Ephrin A1b mediates this intercellular signaling within the somites. In summary, we have identified signal transduction mechanisms of the Notch pathway that regulate HSC specification.

## RESULTS

### Somitic Jag2b regulates HSC formation

To investigate the expression pattern of *jag2b* in the zebrafish embryo, we first performed whole-mount *in situ* hybridization using *jag2b*-specific probes. Expression of *jag2b* was predominantly detected in the nervous system, somites, and intermediate mesoderm, which gives rise to the kidney. The expression of *jag2b* in the somites shifted anteroposteriorly during somitogenesis: it was first detected in segmented somites at 11 hpf [3 somite-stage (ss)], posteriorly elongated by 16 hpf (14 ss), and downregulated in anterior somites after 18 hpf (18 ss) (Fig. 1A-H). In addition, *jag2b* expression was also altered laterally within the somites, particularly at 13-14 hpf (8-10 ss) (Fig. 1D,E). Histological analysis revealed that *jag2b* expression at 13 hpf was detected throughout the somite, including both the lateral and adaxial domains of the somite (Fig. 1I), which later form fast and slow muscle fibers, respectively (Stickney et al., 2000). At 15 hpf (12 ss), however, expression was restricted to the adaxial domain (Fig. 1J), suggesting that *jag2b* expression is axially downregulated within the somites during somitogenesis. At 24 hpf, *jag2b* expression was detected in the ventral domain of the neural tube, lateral line, pronephros, and somites in the tail region (Fig. 1H,K).

To investigate the role of Jag2b in HSC formation in the zebrafish embryo, we utilized a gene knockdown method based on the CRISPR/Cas9 system, in which injection of four single guide RNAs (sgRNAs) redundantly targeting a single gene results in the recapitulation of null phenotypes (Wu et al., 2018). Four different sgRNAs targeting the *jag2b* gene were co-injected with *Cas9* mRNAs in one-cell-stage embryos. The efficiency of *jag2b* knockdown was examined by quantitative reverse transcription PCR (qRT-PCR) using primer sets that recognize two different sgRNA target sites. We observed >96% reduction in *jag2b* expression in *jag2b* sgRNA-injected embryos (*jag2b*$^{sgRNA}$ embryos) (Fig. S1A,B), indicating that *jag2b* function is largely blocked in *jag2b*$^{sgRNA}$ embryos. Expression of an HSC marker gene, *runx1*, was detected in the DA of wild-type embryos, whereas this was largely reduced in *jag2b*$^{sgRNA}$ embryos (Fig. 2A). Morpholino knockdown of *jag2b* also showed decreased expression of *runx1* (Fig. S1C,D) and another HSC marker, *cmyb*, in the DA (Fig. 2B). Unexpectedly, however, genetic mutants of *jag2b* did not recapitulate the expression pattern of *runx1* in *jag2b*$^{sgRNA}$ embryos or *jag2b* morphants (Fig. S2A-E), suggesting that genetic compensation may occur in *jag2b* mutant embryos (Rossi et al., 2015). Indeed, we observed upregulation of *jag1a* and *jag1b* in *jag2b* mutant embryos, whereas the expression of these two genes was unchanged in *jag2b*$^{sgRNA}$ embryos or *jag2b* morphants (Fig. S2F,G). To visualize developing HSCs, we utilized a gene trap line, *gSAIzGFFM1770A*, which contains *Gal4FF* in exon 3 of *gata2b* (hereafter denoted as *gata2b-Gal4FF*) (Kawakami et al., 2010). When combining *gata2b-Gal4FF* with *UAS:GFP* and *fli1:lifeact-mCherry* lines, GFP-expressing cells could be continuously detected in the ventral floor of the DA at 26 hpf, suggesting that the *gata2b-Gal4FF*; *UAS:GFP* line precisely recapitulates endogenous *gata2b* expression. Compared with wild-type embryos, the number of *gata2b*$^{+}$ cells was significantly lower in *jag2b*$^{sgRNA}$ embryos (Fig. 2C).

Given that hematopoietic and vascular endothelial cells develop in close proximity to each other within the PLPM, we next investigated whether loss of *jag2b* affects vascular formation or primitive hematopoiesis. Expression of *fli1* (a pan-endothelial marker), *efnb2a* (a DA marker) and *gata1a* (an erythrocyte marker) was intact in *jag2b*$^{sgRNA}$ embryos (Fig. 2D-F). Furthermore, expression of the PLPM markers *fli1* and *npas4l* was also unchanged in *jag2b*$^{sgRNA}$ embryos at 14 hpf (Fig. S3A,B), suggesting that loss of *jag2b* specifically affects HSC formation. To investigate further the role of Jag2b in HSC formation, *jag2b* was

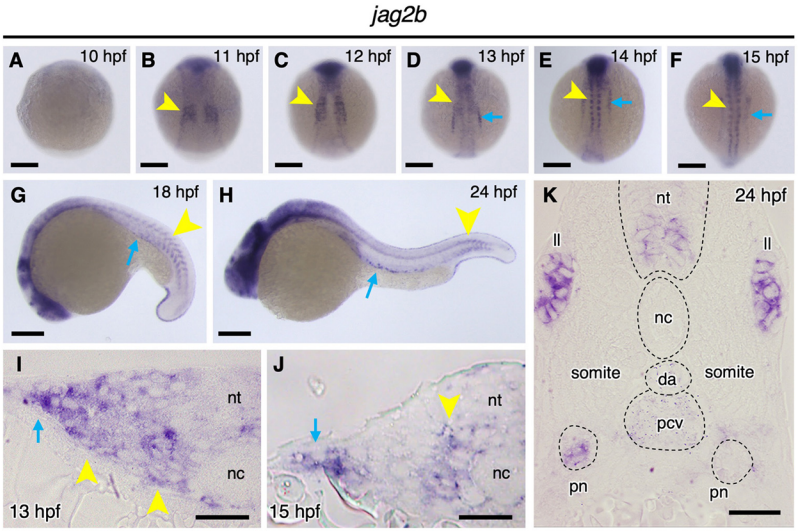


**Fig. 1. The expression domain of *jag2b* shifts spatiotemporally during somitogenesis.** (A-H) Expression of *jag2b* in wild-type embryos at 10-15, 18 and 24 hpf. (I-K) Expression of *jag2b* in the somites at 13, 15 and 24 hpf. Yellow arrowheads and blue arrows in I and B-J indicate expression domains in somites and intermediate mesoderm, respectively. Dashed lines in K outline the neural tube (nt), notochord (nc), dorsal aorta (da), posterior cardinal vein (pcv) and pronephros (pn). II, lateral line. Scale bars: 200 µm (A-H); 20 µm (I-K).

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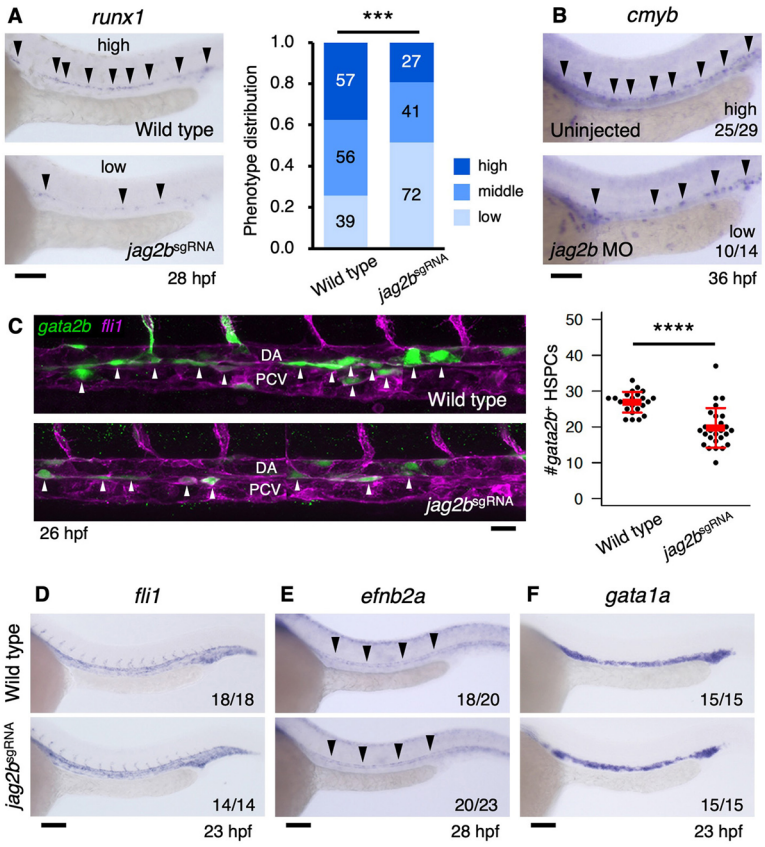


**Fig. 2. *jag2b*$^{sgRNA}$ embryos show a loss of HSCs.**
(A) Representative examples of *runx1* expression in the DA of wild-type and *jag2b*$^{sgRNA}$ embryos. Graph shows the distribution of the 'high', 'middle' or 'low' *runx1* expression phenotypes. ***$P$<0.001. (B) Expression of *cmyb* in the DA of an uninjected embryo and an embryo injected with *jag2b* MO. (C) *gata2b*-positive cells in wild-type and *jag2b*$^{sgRNA}$ embryos. White arrowheads indicate *gata2b*-positive cells in the ventral floor of the DA. Graph shows mean±s.d.; ****$P$<0.000001. HSPCs, hematopoietic stem/progenitor cells. (D-F) Expression of *fli1* (a pan-endothelial marker), *efnb2a* (a DA marker) and *gata1a* (an erythroid marker) in wild-type and *jag2b*$^{sgRNA}$ embryos. Black arrowheads in A-C indicate expression in the DA. Scale bars: 100 µm (A,B,D-F); 20 µm (C).

overexpressed in wild-type embryos by injection of *jag2b* mRNA. Expression of *runx1* in the DA was upregulated by injection of 50 pg *jag2b* mRNA, whereas it was downregulated by injection of 150 pg mRNA (Fig. S3C). These data suggest that Jag2b promotes HSC formation in the zebrafish embryo, although extremely high doses disrupt this process.

### Jag2b regulates HSC formation through *wnt16* expression in somites

Because Jag2b is a Notch ligand expressed predominantly in the somites, we next examined the spatiotemporal distribution of Notch-activating cells in the somites using a Notch reporter line, *Tp1:GFP*, which expresses GFP under the control of Notch-responsive elements (Parsons et al., 2009). Expression of *Tp1:GFP* was weakly detected in the somites at 11 hpf and upregulated by 13 hpf (Fig. 3A-F), a time window when *jag2b* was entirely expressed within the somites (Fig. 1B-D,I). Histological analysis revealed that the majority of *Tp1:GFP*-expressing cells were adjacent to *jag2b*-expressing cells at 13 hpf (Fig. 3G). Moreover, *Tp1:GFP* expression was reduced in the somites of *jag2b*$^{sgRNA}$ embryos compared with wild-type embryos (Fig. 3H), suggesting that Notch signaling in the somites is activated by Jag2b. To test whether ectopic activation of Notch signaling in the somites is sufficient to rescue HSCs in *jag2b*$^{sgRNA}$ embryos, expression of a dominant activator of the Notch pathway, NICD, was forced in the somites using a double-transgenic line, *phldb1:Gal4*; *UAS:NICD*, which expresses NICD under the control of somite-specific *phldb1* enhancers (Burns et al., 2005; Kim et al., 2014). The expression of *runx1* in the DA was partially restored in NICD (+) *jag2b*$^{sgRNA}$ embryos (Fig. 3I), indicating that Jag2b-driven Notch signaling in the somites regulates HSC formation in the zebrafish embryo.

Given the requirement of Jag2b-driven Notch signaling in the somites for HSC formation, the expression of some somite markers was investigated in Jag2b-deficient embryos. Expression of both *desma* (a somite marker) and *nkx3-1* (a sclerotome marker) was unaffected in *jag2b*$^{sgRNA}$ embryos at 15 hpf (Fig. 4A,B), indicating that loss of *jag2b* does not affect somite and sclerotome formation. Although the expression of *notch1a* and *1b* was slightly upregulated, the expression of *notch3* in the somites was unchanged in *jag2b* morphants (Fig. S4A-C). However, we observed a reduction in *wnt16* expression in the somites of both *jag2b*$^{sgRNA}$ embryos and *jag2b* morphants (Fig. 4C, Fig. S4D). Consistent with this, loss of *jag2b* also reduced both *dlc* and *dld* expression in the somites (Fig. 4D,E, Fig. S4E,F), which are the downstream target genes of Wnt16 (Clements et al., 2011). Conversely, injection of 50 pg *jag2b* mRNAs in wild-type embryos increased the expression of *wnt16*, *dlc* and *dld* in the somites (Fig. S4G-I). To test whether forced expression of *dlc* and *dld* can rescue HSC formation in Jag2b-deficient embryos, as has been shown in *wnt16* morphants (Clements et al., 2011), *dlc* and *dld* mRNAs were co-injected with *jag2b* MO into embryos. The expression of *runx1* in the DA was restored by co-injection of *dlc* and *dld* mRNA in *jag2b* morphants (Fig. S4J). These data suggest that Jag2b-driven Notch signaling regulates HSC formation by regulating the Wnt16–Dlc/Dld signaling axis in the somites.

Expression of *wnt16* was first detected at 14 hpf in anterior somites and was posteriorly elongated during somitogenesis (Fig. 4F-J). To determine whether Notch-activated cells do indeed express *wnt16*, the expression pattern of *wnt16* was examined in the somites of *Tp1:GFP* embryos. Interestingly, histological analysis revealed that the majority of *wnt16*-expressing cells were adjacent to, but not merged with, *Tp1:GFP*-expressing cells in the medial region of the somites at 15 hpf (Fig. 4K,L). The distribution of

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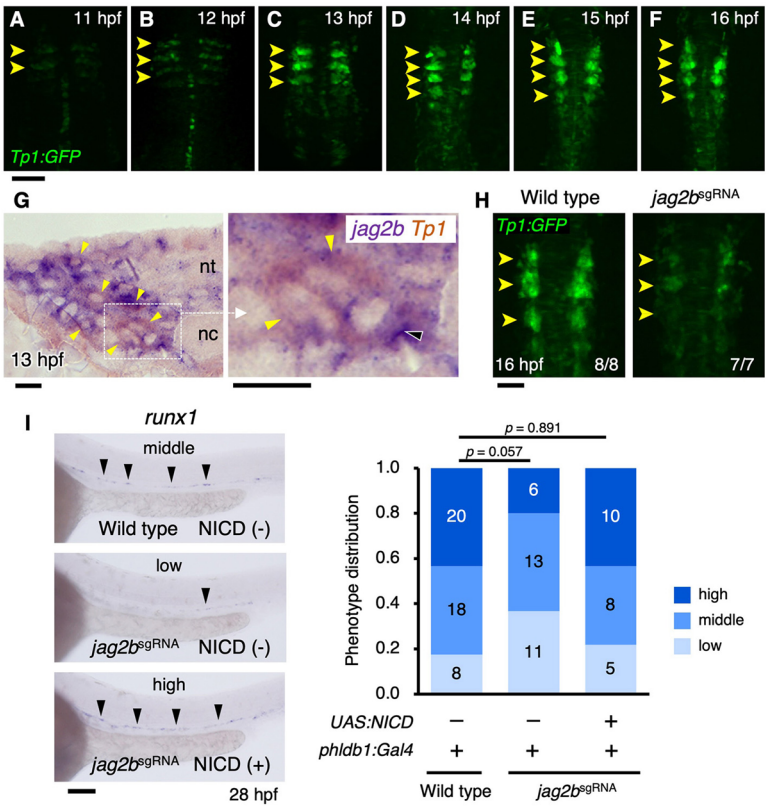


**Fig. 3. Jag2b activates Notch signaling in somites.** (A-F) Time course of *Tp1:GFP* (a Notch reporter) expression in the somites of wild-type embryos. Dorsal views of the embryo are shown. Yellow arrowheads indicate *Tp1:GFP* expression in the somites. (G) Expression of *jag2b* and *Tp1:GFP* in the somites at 13 hpf. Black and yellow arrowheads indicate *jag2b*- and *Tp1:GFP*-expressing cells, respectively. A high-magnification view of the boxed region is shown on the right. (H) Expression of *Tp1:GFP* in the somites of wild-type and *jag2b*$^{sgRNA}$ embryos. Yellow arrowheads indicate *Tp1:GFP*-expressing somites. (I) Expression of *runx1* in the DA of wild-type and *jag2b*$^{sgRNA}$ embryos expressing (+) or not expressing (–) NICD under the control of *phldb1:Gal4* induction. Black arrowheads indicate *runx1* expression in the DA. Graph shows the distribution of embryos exhibiting 'high', 'middle' or 'low' *runx1* expression phenotypes. Scale bars: 100 µm (A-F,I); 10 µm (G); 50 µm (H).

*wnt16*- and *Tp1:GFP*-expressing cells at 15 hpf was similar to that of *jag2b*- and *Tp1:GFP*-expressing cells at 13 hpf; the two cell types were intermittently distributed to neighbor each other in the somites (Fig. 3G). These data suggest that Jag2b-driven Notch signaling indirectly regulates *wnt16* expression, and raise the possibility that additional paracrine/juxtacrine signaling may mediate this intercellular signal transduction.

### Ephrin A1b mediates Jag2b–Wnt16 signaling in somites

Ephrin–Eph signaling is transduced between neighboring cells and is known to be involved in the formation of somite boundaries (Durbin et al., 1998; Davy and Soriano, 2007; Watanabe et al., 2009; Watanabe and Takahashi, 2010). In zebrafish, two Ephrin genes, *efna1b* and *efnb2a* (encoding Ephrin A1b and B2a, respectively), have been shown to be expressed in somites (Durbin et al., 1998). As Ephrin signaling fits our model of intercellular signaling in the somites, we next investigated whether *efna1b* or *efnb2a* are regulated by Jag2b. Expression of *efna1b* was reduced in the somites of *jag2b*$^{sgRNA}$ embryos and *jag2b* morphants, although the expression of *efnb2a* was unchanged (Fig. 5A,B, Fig. S5A,B). Injection of *jag2b* mRNAs conversely increased the expression of *efna1b*, but not *efnb2a*, in the somites (Fig. S5C,D), suggesting that *efna1b* expression in somites is regulated by Jag2b. Expression of *efna1b* in the somites was first detected at 13 hpf and increased by 14 hpf (Fig. 5C-G), a time window when Notch signaling is highly active in somites (Fig. 3C,D). Moreover, *efna1b* and *Tp1:GFP* expression mostly overlapped in the somites at 14 hpf (Fig. 5H-J). Double-fluorescence whole-mount *in situ* hybridization using probes for *efna1b* and *wnt16* revealed that *efna1b*-expressing cells were laterally and anteroposteriorly adjacent to *wnt16*-expressing cells at 15 hpf (Fig. 5K). Similar to *jag2b* expression, *efna1b* expression shifted predominantly to the adaxial region of the somite at 16 hpf, whereas *wnt16* expression was localized to the dorsomedial region of the somite (Fig. S5E,F). To determine whether Ephrin A1b is involved in HSC formation, four different sgRNAs targeting the *efna1b* gene were co-injected with *Cas9* mRNA into embryos (Fig. S6A,B). Embryos injected with *efna1b* sgRNAs (*efna1b*$^{sgRNA}$) showed a reduction in *runx1* expression in the DA and *wnt16*, *dlc* and *dld* expression in the somites. In contrast, *jag2b*, *desma* and *nkx3-1* expression in the somite remained intact (Fig. 5L,M, Fig. S6C-G). Consistent with these observations, a genetic mutant line of *efna1b*, *efna1b*$^{kz5}$, which contains a premature stop codon in exon 2 of *efna1b*, also showed reduced *runx1* expression in the DA and *wnt16*, *dlc* and *dld* expression in the somites (Fig. S7A-F). These data suggest that loss of *efna1b* phenocopies loss of *jag2b*.

In order to confirm the role of Ephrin A1b in *wnt16* regulation, *efna1b* mRNAs were injected into embryos. Overexpression of *efna1b* in wild-type embryos increased *wnt16* expression in the somites and *runx1* expression in the DA (Fig. 6A,B). Co-injection of *efna1b* mRNAs together with *jag2b* sgRNAs and *Cas9* mRNAs resulted in recovery of *runx1* expression in the DA (Fig. 6C). Furthermore, co-injection of *wnt16* mRNAs together with *efna1b* sgRNAs and *Cas9* mRNAs rescued *runx1* expression in the DA (Fig. 6D). Taken together, these data strongly suggest that Jag2b-driven Notch signaling regulates *efna1b* expression in the somite to modulate the Wnt16–Dlc/Dld signaling axis, which is required for HSC specification.

## DISCUSSION

In the present study, we have shown in the zebrafish embryo that Jag2b regulates HSC specification by triggering intercellular signal transduction in the somites. Expression of *jag2b* in the somites is initiated as early as 11 hpf (3 ss), activating Notch signaling in the segmented somite at 11-13 hpf (3-8 ss). Jag2b-driven Notch signaling then regulates *efna1b* expression at 13-14 hpf (8-10 ss).

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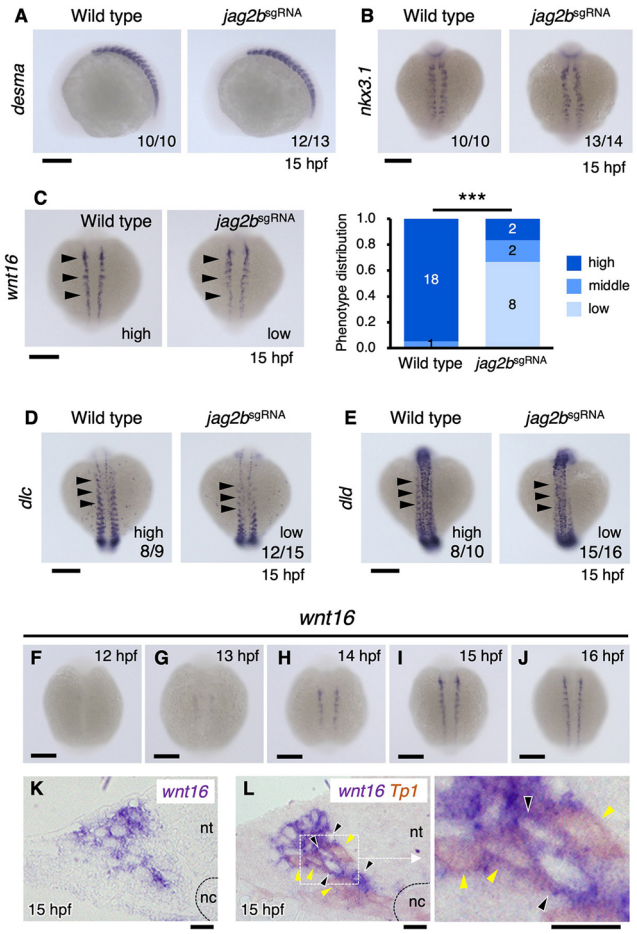


**Fig. 4. *jag2b*$^{sgRNA}$ embryos showed reduced *wnt16* expression in the somites.** (A,B) Expression of *desma* (a somite marker) and *nkx3-1* (a sclerotome marker) in wild-type and *jag2b*$^{sgRNA}$ embryos. (C) Expression of *wnt16* in wild-type and *jag2b*$^{sgRNA}$ embryos. Graph shows the distribution of embryos exhibiting 'high', 'middle' or 'low' *runx1* expression phenotypes. ***$P$<0.001. (D,E) Expression of *dlc* and *dld* in wild-type and *jag2b*$^{sgRNA}$ embryos. Black arrowheads denote *dlc* or *dld* expression in the somite. (F-J) Time course of *wnt16* expression in wild-type embryos. (K,L) Expression of *wnt16* or both *wnt16* and *Tp1:GFP* in the somites at 15 hpf. A high-magnification view of the boxed region in L is shown to the right. Black and yellow arrowheads indicate *wnt16*- and *Tp1:GFP*-expressing cells, respectively. Scale bars: 200 µm (A-J); 10 µm (K,L).

Ephrin A1b transduces signals to neighboring cells to drive *wnt16* expression at 14-15 hpf (10-12 ss), which modulates the expression of *dlc* and *dld* to establish HSC fate in angioblasts during axial migration at 15-17 hpf (12-16 ss) (Clements et al., 2011; Kobayashi et al., 2014). Thus, our data clearly demonstrate spatiotemporal regulatory mechanisms of intercellular signal transduction in the somites that are required for HSC specification (Fig. 7).

In zebrafish, R-spondin 1 (Rspo1) has been implicated in the regulation of *wnt16* expression. R-spondins have been shown to regulate various developmental and physiological processes through enhancement of both canonical and non-canonical Wnt signaling (Glinka et al., 2011; Jin and Yoon, 2012). Loss of *rspo1* in the zebrafish embryo has been shown to result in reduced expression of *wnt16* in the somites and impaired HSC formation in the DA. The HSC formation defect in *rspo1*-deficient embryos could be rescued by injection of *dlc* and/or *dld* mRNAs, suggesting that Rspo1 regulates HSC formation via the Wnt16–Dlc/Dld signaling axis (Genthe and Clements, 2017). However, as *rspo1* is broadly expressed in the zebrafish embryo, it is unclear how Rspo1 spatiotemporally regulates *wnt16* expression in the somites. Our data demonstrate that *wnt16* expression was initiated shortly after Notch-activated cells were detected in the somites (14-15 hpf). During this time window, *wnt16* expression was induced by neighboring Notch-activated cells via Ephrin A1b signaling. Loss of either *jag2b* or *efna1b* decreased *wnt16* expression in the somites, but overexpression of either one increased *wnt16* expression. These observations suggest that spatiotemporal expression of *wnt16* is predominantly regulated by Jag2b-driven Notch signaling, and Rspo1 may boost *wnt16* expression in the somite.

The expression domain of *jag2b* in the somite is dynamically altered at early somitogenesis, and this spatiotemporal pattern of *jag2b* reflects the distribution of *efna1b*- and *wnt16*-epxressing cells. At 11-13 hpf, *jag2b* was expressed entirely in the anterior somites, which in turn activated Notch signaling in neighboring cells at 12-13 hpf. At 14 hpf, however, *jag2b* expression was restricted to the adaxial domain of somites. Similarly, *efna1b* and *wnt16* expression was also detected in the entire domain of the somite at 13-14 hpf and 14-15 hpf, respectively, whereas they were later detected mainly in the adaxial to dorsomedial domain. Given that HSC fate is established by receiving Dlc–Dld signaling during angioblast migration along the ventral surface of the sclerotome (15-17 hpf) (Kobayashi et al., 2014), *dlc* and *dld* expression in the sclerotome could be induced by Wnt16 at 14-15 hpf, a time window when *wnt16* is entirely expressed in the somites. Collectively, these observations suggest that, although the expression of *jag2b*, *efna1b* and *wnt16* is shifted during somitogenesis, each requisite signal for HSC specification is transmitted to neighboring somitic cells within a very narrow timeframe (~1 h).

Ephrin–Eph signaling plays pivotal roles in neural tube patterning (Gale and Yancopoulos, 1997; Stolfi et al., 2011), neural crest cell migration (Smith et al., 1997; Wang and Anderson, 1997; Santiago and Erickson, 2002; Davy and Soriano, 2007) and somite boundary formation (Durbin et al., 1998; Davy and Soriano, 2007; Watanabe et al., 2009; Watanabe and Takahashi, 2010). In mammals, Eph B4-expressing HSCs interact with Ephrin B2-expressing mesenchymal stromal cells, and this interaction triggers detachment of HSCs from stromal cells, leading to erythroid differentiation of HSCs (Foo et al., 2006; Suenobu et al., 2002). Within the presomitic mesoderm, when an Eph A4-expressing cell is adjacent to an Ephrin B2-expressing cell, these cells repel each other, resulting in boundary formation followed by epithelialization at this somite boundary (Watanabe et al., 2009; Watanabe and Takahashi, 2010). In zebrafish, blocking Eph signaling by injecting mRNAs encoding a dominant-negative form of EphA4 resulted in abnormal boundary formation in somites. Similar phenotypes were also observed with overexpression of soluble Ephrin A1b or B2a, both of which can bind to EphA4 to block its signaling (Durbin et al., 1998). However, we did not observe somite defects in *efna1b*$^{sgRNA}$ or *efna1b*$^{kz5}$ embryos, as evidenced by the expression of *desma* and *nkx3-1*. These observations suggest that, although Ephrin A1b can potentially bind to EphA4, Ephrin A1b is dispensable for boundary formation in somites. Expression of *efna1b* and *epha4* was detected not only in the somite boundary but also in the middle region of the somite where no boundary formed (Durbin et al., 1998). Clements et al. reported that loss of *wnt16* led to reduced expression of sclerotome maker genes at 22 hpf, a time point after sclerotome migration adjacent to the neural tube and notochord (Clements et al., 2011). In contrast, we observed intact expression of the sclerotome marker *nkx3-1* in *jag2b*$^{sgRNA}$ and *efna1b*$^{sgRNA}$

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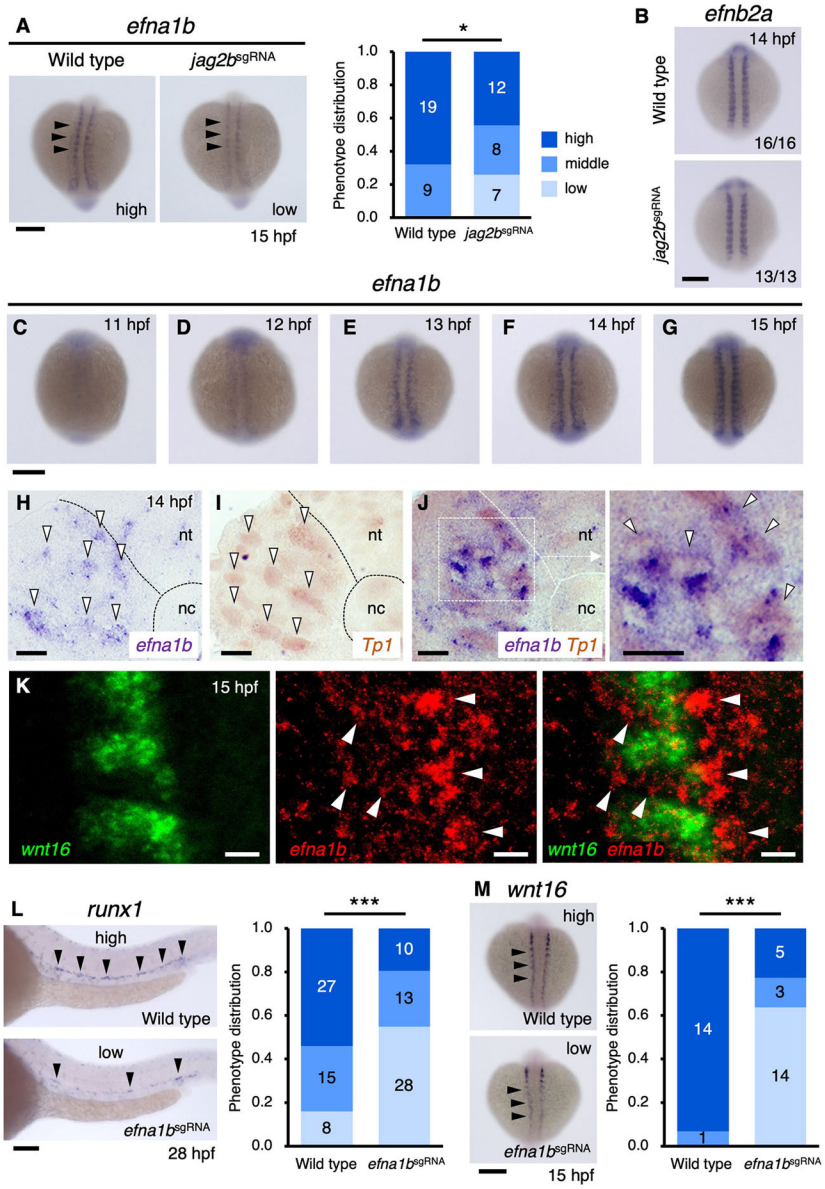


**Fig. 5. Jag2b regulates *efna1b* expression in the somites.** (A) Expression of *efna1b* in wild-type and *jag2b*$^{sgRNA}$ embryos. Black arrowheads indicate *efna1b* expression in the somites. Graph shows the distribution of embryos exhibiting 'high', 'middle' or 'low' *runx1* or *wnt16* expression phenotypes. (B) Expression of *efnb2a* in the somites of wild-type or *jag2b*$^{sgRNA}$ embryos. (C-G) Time course of *efna1b* expression in wild-type embryos. (H-J) Expression of *efna1b*, *Tp1:GFP* or both in the somites at 14 hpf. White arrowheads indicate cells expressing *efna1b*, *Tp1:GFP* or both. A high-magnification view of the boxed region in J is shown to the right. (K) Expression of *wnt16* (green) and *efna1b* (red) in the somites at 15 hpf. Dorsal views of the embryo are shown. White arrowheads indicate instances of an *efna1b*-expressing cell adjacent to a *wnt16*-expressing cell. (L,M) Expression of *runx1* in the DA and *wnt16* in the somite of wild-type and *efna1b*$^{sgRNA}$ embryos. Black arrowheads indicate *runx1* expression in the DA (L) and *wnt16* expression in the somites (M). Graphs show the distribution of embryos exhibiting 'high', 'middle' or 'low' *runx1* or *wnt16* expression phenotypes. *$P$<0.05; ***$P$<0.001. Scale bars: 200 µm (A-G,M); 10 µm (H-J); 20 µm (K); 100 µm (L).

embryos at 15 hpf despite a reduction in *wnt16* expression. Because *wnt16* expression begins at 14 hpf, it is likely that early sclerotome formation at the ventromedial surface of the somite is independent of *wnt16* expression. Previous cell-lineage analysis in zebrafish demonstrated that somitic cells within the newly segmented somite undergo rearrangement relative to their neighbors prior to whole-somite rotation (Hollway et al., 2007), indicating that somitic cells rearrange their positions dynamically during somitogenesis. In the intestinal epithelium, Ephrin–Eph signaling in epithelial cells drives Wnt signaling to modulate the spatial distribution of cells in the crypt-villus axis (Batlle et al., 2002, 2005). Although further studies are needed to determine whether Ephrin A1b controls somitic cell rearrangements, Ephrin A1b is a crucial mediator of Jag2b-triggered intercellular communication, representing a previously unappreciated cellular mechanism in HSC development.

There are at least five Notch ligands that are involved in establishing HSC programs in the zebrafish embryo. Dlc and Dld are involved in early HSC specification during angioblast migration (Clements et al., 2011; Kobayashi et al., 2014), and we have shown here that the expression of these Notch ligand genes is regulated in part by Jag2b-driven Notch signaling in the somites. It has been shown that Delta-like 4 (Dll4) is required for specification of the arterial endothelium; however, knockdown of *dll4* has also been shown to lead to loss of HSCs, suggesting that arterial programs are required before formation of HECs (Bonkhofer et al., 2019). Within the aortic floor, Jag1a expressed by arterial endothelial cells activates Notch in neighboring endothelial cells to establish HSC programs (Espín-Palazón et al., 2014; Monteiro et al., 2016). Thus, multiple waves of Notch signaling provided by various cell types and ligands are involved in HSC development. Further studies of Notch-related genes will elucidate the spatiotemporal regulatory mechanisms of HSC development.

## MATERIALS AND METHODS

### Zebrafish husbandry

Zebrafish strains AB*, *jag2b*$^{hu3425}$, *jag2b*$^{kz6}$, *jag2b*$^{kz7}$, *efna1b*$^{kz5}$, *Tg(Tp1:GFP)*$^{um14}$ (Parsons et al., 2009), *gSAIzGFFM1770A* (*gata2b-Gal4FF*), *Tg(UAS:GFP)* (Asakawa et al., 2008), *Tg(fli1:lifeact-mCherry)*$^{ncv7}$

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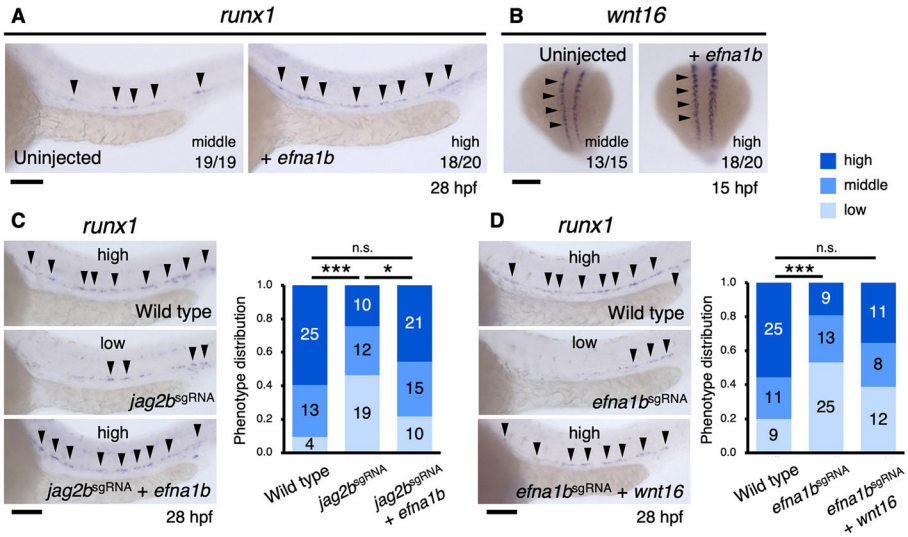


**Fig. 6. Ephrin A1b regulates HSC specification by regulating *wnt16* expression.**
(A,B) Expression of *runx1* in the DA or *wnt16* in the somites of uninjected embryos or embryos injected with *efna1b* mRNA. (C,D) Expression of *runx1* in the DA of wild-type and *jag2b*$^{sgRNA}$ uninjected embryos or embryos injected with *efna1b* mRNA (C) or *efna1b*$^{sgRNA}$ uninjected embryos or embryos injected with *wnt16* mRNA (D). Black arrowheads indicate *runx1* expression in the DA (A,C,D) and *wnt16* expression in the somites (B). Graphs in C and D show the distribution of embryos exhibiting 'high', 'middle' or 'low' *runx1* expression phenotypes. *$P$<0.05; ***$P$<0.001; n.s., not significant. Scale bars: 100 µm (A,C,D); 200 µm (B).

(Wakayama et al., 2015), *Et(phldb1:Gal4-mCherry)* (Distel et al., 2009) and *Tg(UAS:NICD)*$^{kca3}$ (Burns et al., 2005) were raised in a circulating aquarium system (AQUA) at 28.5°C on a 14 h/10 h light/dark cycle and maintained in accordance with guidelines from the Committee on Animal Experimentation of Kanazawa University.

### CRISPR/Cas9

Previously designed guide RNA (gRNA) sequences were utilized (Wu et al., 2018) and are listed in Table S1. sgRNAs were synthesized as previously described (Kobayashi-Sun et al., 2020). Briefly, a single-strand DNA oligo containing the gRNA target and T7 promoter sequence was annealed with the gRNA scaffold primer, followed by synthesizing the double-strand DNA with MightyAmp DNA polymerase (Thermo Fisher Scientific). sgRNAs were then synthesized by *in vitro* transcription using the MEGAshortscript T7 Transcription Kit (Thermo Fisher Scientific) and purified with the mirVana miRNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. For knockdown experiments, embryos were injected with a mixture of four different sgRNAs (200 ng/µl each) and *Cas9* mRNA (100 ng/µl) at the one-cell stage. For generation of a mutant line, embryos injected with a single sgRNA and *Cas9* mRNA were raised to adulthood to obtain the F1 generation. Fish from the F1 generation were screened using primers that flanked the target region, and mutant alleles were identified by sequencing. The gRNA scaffold primer and genotyping primers are listed in Table S1.

### Whole-mount *in situ* hybridization and immunohistochemistry

cDNAs were cloned by reverse transcription-polymerase chain reaction (RT-PCR) using the specific primers listed in Table S1 and ligated into the pCRII-TOPO vector (Invitrogen). Digoxigenin (DIG)- and fluorescein-labeled RNA probes were prepared by *in vitro* transcription with linearized constructs using the DIG RNA Labeling Kit (SP6/T7; Sigma-Aldrich) and Fluorescein RNA Labeling Mix with T7 polymerase (Sigma-Aldrich), respectively. For permeabilization, embryos fixed with 4% paraformaldehyde in PBS were treated with proteinase K (10 µg/ml) (Sigma-Aldrich) for 30 s to 15 min at room temperature or acetone for 7 min at −20°C, refixed with 4% paraformaldehyde, and washed twice with 0.1% Tween-20 (Sigma-Aldrich) in PBS (PBST). Hybridization was then performed using DIG-labeled antisense RNA probes diluted in hybridization buffer [50% formamide, 5× standard saline citrate (SSC), 0.1% Tween-20, 500 µg/ml torula RNA, 50 µg/ml heparin] for 3 days at 65°C. For detection of DIG-labeled RNA probes, embryos were blocked in 0.2% bovine serum albumin (Sigma-Aldrich) in PBST and incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG antibody (Sigma-Aldrich, 1093274) at 1:5000. After washing with PBST, embryos were developed using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Sigma-Aldrich) in staining buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20). For double-fluorescence *in situ* hybridization, embryos were simultaneously hybridized with DIG-labeled *efna1b* and fluorescein-labeled *wnt16* probes, followed by

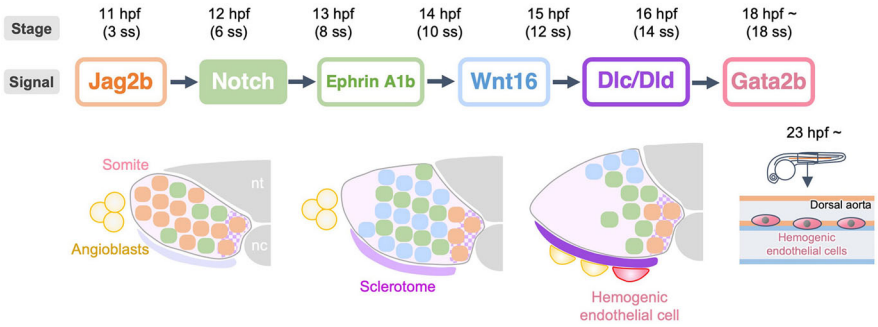


**Fig. 7. Schematic of intercellular signaling in the somites.** Expression of *jag2b* is initiated at 11 hpf in the somites, and Jag2b protein activate Notch signaling in adjacent somitic cells (~13 hpf). Notch-activated cells then express *efna1b* (~13 hpf ), and Ephrin A1b protein transduces signals to adjacent somitic cells to drive *wnt16* expression (~14 hpf ). Wnt16 regulates *dlc* and *dld* expression in the ventral domain of the somite (sclerotome), where angioblasts receive Dlc/Dld signaling to become hemogenic endothelial cells (~16 hpf ). Hemogenic endothelial cells express *gata2b* (~18 hpf ) and arise in the ventral floor of the DA (~23 hpf ). The cross-hatched region within the somite denotes the adaxial domain where *jag2b*-expressing slow muscle precursors are located. Colors of cells relate to the protein signal scheme above. nc, notochord; nt, neural tube.

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staining with horseradish peroxidase (HRP)-conjugated anti-DIG antibody (Sigma-Aldrich, 11207733910) at 1:500. Embryos were developed using the TSA Plus Cyanine 3 System (Perkin Elmer). After quenching with 0.2% $H_2O_2$ in methanol, embryos were stained with HRP-conjugated anti-fluorescein antibody (Sigma-Aldrich, 11426346910) at 1:100, followed by development with the TSA Plus Fluorescein System (Perkin Elmer). For immunohistochemistry, embryos were embedded in paraffin and sectioned at 3 µm thickness. Deparaffinized tissue sections were incubated with Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween-20) at 90°C for 90 min for antigen retrieval. Sections were then blocked with 0.2% bovine serum albumin in PBS for 60 min at room temperature and stained with chicken anti-GFP (Aves Labs, GFP-1020) at 1:1000 at 4°C overnight, followed by staining with anti-chicken IgY HRP-conjugated secondary antibody (Abcam, ab6877) at 1:1000 at room temperature for 1 h. Sections were developed with 3, 3'-diaminobenzidine (DAB) substrate solution (Wako Chemicals) for 1-2 min. After washing, sections were mounted with mounting medium (Mount-Quick Aqueous, Daido Sangyo). For whole-mount immunohistochemistry, fixed embryos were blocked with 2% blocking reagent, and incubated overnight at 4°C with chicken anti-GFP and/or rabbit anti-RFP (Abcam, ab34771) at 1:1000. After washing with PBST, embryos were incubated overnight at 4°C with goat anti-chicken IgY Alexa Fluor 488-conjugated (Abcam, ab150173) and/or donkey anti-rabbit IgG Alexa Fluor 647-conjugated (Abcam, ab150115) at 1:1000.

### qRT-PCR

Total RNA was extracted from embryos using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix (Toyobo). qRT-PCR assays were performed using TB Green Premix Ex Taq II (Takara Bio) on a ViiA 7 Real-Time PCR System following the manufacturer's instructions (Thermo Fisher Scientific). Expression of *ef1a* was used for normalization. Primers used for qRT-PCR are listed in Table S1.

### Microscopy

For fluorescence imaging, embryos were mounted in a glass-bottomed dish filled with 0.6% low-gelling agarose (Sigma-Aldrich) in E3 medium and imaged using a FV10i confocal microscope and Fluoview FV10i-SW software (v2.1.1) (Olympus). Visible light imaging was captured using an Axiozoom V16 microscope (Zeiss) with a TrueChrome II digital camera (BioTools) and TCapture software (ver. 4.3.0.602) (Tucsen Photonics) or an Axioplan2 microscope (Zeiss) with a Moticam Pro 285B digital camera and Motic Images Plus 2.3S software (v2.3.4) (Shimazu).

### Morpholino and mRNA injection

The morpholino oligo (MO; GeneTools) sequence against *jag2b* is as follows: TCCTGATACAATTCCACATGCCGCC (Lorent et al., 2004). Capped mRNAs were synthesized from linearized *pCS2+* constructs using the mMessage mMachine SP6 kit (Thermo Fisher Scientific). Embryos were injected at the one-cell stage with 1 nl of MOs and/or mRNAs at the following concentrations: *jag2b* MO, 400 µM; *Cas9* mRNA, 100 pg/nl; *jag2b* mRNA, 50-150 pg/nl; *dlc* mRNA, 50 pg/nl; *dld* mRNA, 50 pg/nl; *efna1b* mRNA, 100 pg/nl; *wnt16* mRNA, 100 pg/nl.

### Quantification and statistical analyses

Data were analyzed for statistical significance after at least two repeated experiments. To quantify the expression levels of each tested gene, individual embryos were classified into three (high, middle or low) or two (high or low) categories based on the signal intensity. Statistical differences of phenotypic distributions between groups were determined using Pearson's $\chi^2$ test. The statistical difference in count data between groups was determined by unpaired two-tailed Student's *t*-test. Values of $P<0.05$ were considered statistically significant.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: I.K.; Methodology: Y.W., H.T., C.K., M.O., I.K.; Validation: Y.W., H.T., C.K., Y.M., I.K.; Formal analysis: Y.W., I.K.; Investigation: Y.W., H.T., C.K., Y.M., M.O.; Data curation: Y.W., I.K.; Writing - original draft: I.K.; Writing - review & editing: Y.W.; Visualization: I.K.; Supervision: I.K.; Project administration: I.K.; Funding acquisition: I.K.

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