

# Discrete Notch signaling requirements in the specification of hematopoietic stem cells

Albert D Kim<sup>1</sup>, Chase H Melick<sup>1</sup>, Wilson K Clements<sup>1,2</sup>, David L Stachura<sup>1</sup>, Martin Distel<sup>1</sup>, Daniela Panáková<sup>3,4</sup>, Calum MacRae<sup>4</sup>, Lindsey A Mork<sup>5</sup>, J Gage Crump<sup>5</sup> & David Traver<sup>1,6,\*</sup>

## Abstract

Hematopoietic stem cells (HSCs) require multiple molecular inputs for proper specification, including activity of the Notch signaling pathway. A requirement for the Notch1 and dispensability of the Notch2 receptor has been demonstrated in mice, but the role of the remaining Notch receptors has not been investigated. Here, we demonstrate that three of the four Notch receptors are independently required for the specification of HSCs in the zebrafish. The orthologues of the murine Notch1 receptor, Notch1a and Notch1b, are each required intrinsically to fate HSCs, just prior to their emergence from aortic hemogenic endothelium. By contrast, the Notch3 receptor is required earlier within the developing somite to regulate HSC emergence in a non-cell-autonomous manner. Epistatic analyses demonstrate that Notch3 function lies downstream of Wnt16, which is required for HSC specification through its regulation of two Notch ligands, *dlc* and *dlid*. Collectively, these findings demonstrate for the first time that multiple Notch signaling inputs are required to specify HSCs and that Notch3 performs a novel role within the somite to regulate the neighboring precursors of hemogenic endothelium.

**Keywords** hematopoietic stem cell; hemogenic endothelium; Notch; somite

**Subject Categories** Development & Differentiation; Signal Transduction; Stem Cells

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## Introduction

The developmental ontogeny of the hematopoietic system is complex and proceeds through four ordered, temporal waves during vertebrate development. The first two generate primitive erythroid and myeloid cells, which arise through the direct specification of mesoderm to rapidly generate cells capable of transporting oxygen

throughout the developing embryo and providing immunity, respectively (Davidson & Zon, 2004; Tober *et al*, 2007; Le Guyader *et al*, 2008; Orkin & Zon, 2008). Next, these primitive waves are followed by specification of two definitive waves, the first giving rise to erythromyeloid progenitors (EMPs), transient precursors that give rise to cells of the erythroid and myeloid pathways (Palis *et al*, 1999; Bertrand *et al*, 2007), and the second to hematopoietic stem cells (HSCs), which have both the ability to self-renew and differentiate into the complete repertoire of mature blood cells for an organism's lifespan. HSC specification is spatially conserved across vertebrate species and involves the transdifferentiation of hemogenic endothelium in the ventral wall of the dorsal aorta (DA) (de Bruijn *et al*, 2000; Zovein *et al*, 2008; Bertrand *et al*, 2010a; Boisset *et al*, 2010; Kissa & Herbomel, 2010). Numerous key studies have demonstrated that HSC specification requires specific molecular inputs from a number of signaling pathways, including Notch.

Notch signaling is a conserved cell-to-cell signaling pathway responsible for a multitude of critical cell-fate decisions during the lifespan of metazoan organisms (Lai, 2004; Kopan & Ilagan, 2009). In mammals and zebrafish, Notch signaling occurs through the interaction of many proteins. First, one of four transmembrane Notch receptors (Notch1, Notch2, Notch3, and Notch4 in mice; Notch1a, Notch1b, Notch2, and Notch3 in zebrafish) on a signal-receiving cell binds to a Notch ligand, termed Jagged and Delta, on a signal-emitting cell (Rebay *et al*, 1991). Ligand-dependent activation of Notch signaling requires cleavage of the Notch receptor, first by members of ADAM TACE metalloproteases at the S2 site (Brou *et al*, 2000; Bozkulak & Weinmaster, 2009), then by  $\gamma$ -secretase at the S3 site to release a Notch intracellular domain (NICD), which translocates to the nucleus (Mumm *et al*, 2000) to modulate transcription of Notch target genes (Kopan & Ilagan, 2009). The specification, lineage commitment, and maintenance of many tissues require the precise regulation of Notch signaling.

Notch signaling is especially important for the initial specification of the adult hematopoietic system during embryogenesis. While Notch signaling is dispensable for the generation of transient,

1 Department of Cellular and Molecular Medicine, University of California at San Diego, La Jolla, CA, USA

2 Department of Hematology, St. Jude Children's Research Hospital, Memphis, TN, USA

3 Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany

4 Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

5 Department of Stem Cell Biology and Regenerative Medicine, University of Southern California Keck School of Medicine, Los Angeles, CA, USA

6 Section of Cell and Developmental Biology, University of California at San Diego, La Jolla, CA, USA

\*Corresponding author. Tel: +1 858 822 4593; E-mail: dtraver@ucsd.edu

embryonic blood cells (Bertrand *et al.*, 2010b), it is absolutely required for the generation of HSCs across vertebrate phyla (Krebs *et al.*, 2000; Kumano *et al.*, 2003; Hadland *et al.*, 2004; Burns *et al.*, 2005; Robert-Moreno *et al.*, 2005). Several Notch pathway mutants that fail to specify the DA have defects in HSCs (Krebs *et al.*, 2000, 2004; Lawson *et al.*, 2001; Duarte *et al.*, 2004), suggesting that the DA is a morphogenetic prerequisite to HSCs. However, several studies have demonstrated that HSC specification can be rescued even in the context of impaired DA formation (Burns *et al.*, 2005; Ren *et al.*, 2010), confounding a clear necessity for a properly formed DA in subsequent HSC formation. Furthermore, mutants in the Notch ligand *Jagged1* are deficient in HSCs but have normal arterial formation, suggesting that HSC formation has unique Notch requirements distinct from those required for arterial fate (Robert-Moreno *et al.*, 2008). Previous work from our laboratory demonstrated that Wnt16 regulates the somitic expression of two Notch ligands, *dlc* and *dld*. While the somitic expression of *dlc* and *dld* was dispensable for DA specification, it was required for the formation of the sclerotome compartment of the somite and subsequent HSC specification (Clements *et al.*, 2011). We reasoned that if Notch signaling performs differential functions in the somites versus the endothelium, then this specificity might be achieved through the discrete use of specific Notch receptors during these different processes.

In this report, we investigated which of the four Notch receptors are required for HSC specification, and when and where each of these requirements is needed. We have determined that Notch1a and Notch1b are autonomously required in the precursors of hemogenic endothelium, whereas Notch3 is dispensable in the endothelium and instead required in the somites to indirectly specify the HSC program. Furthermore, we demonstrate that this novel Notch requirement functions within the Wnt16/Notch signaling pathway that we previously showed is necessary to specify the sclerotome and HSCs.

## Results

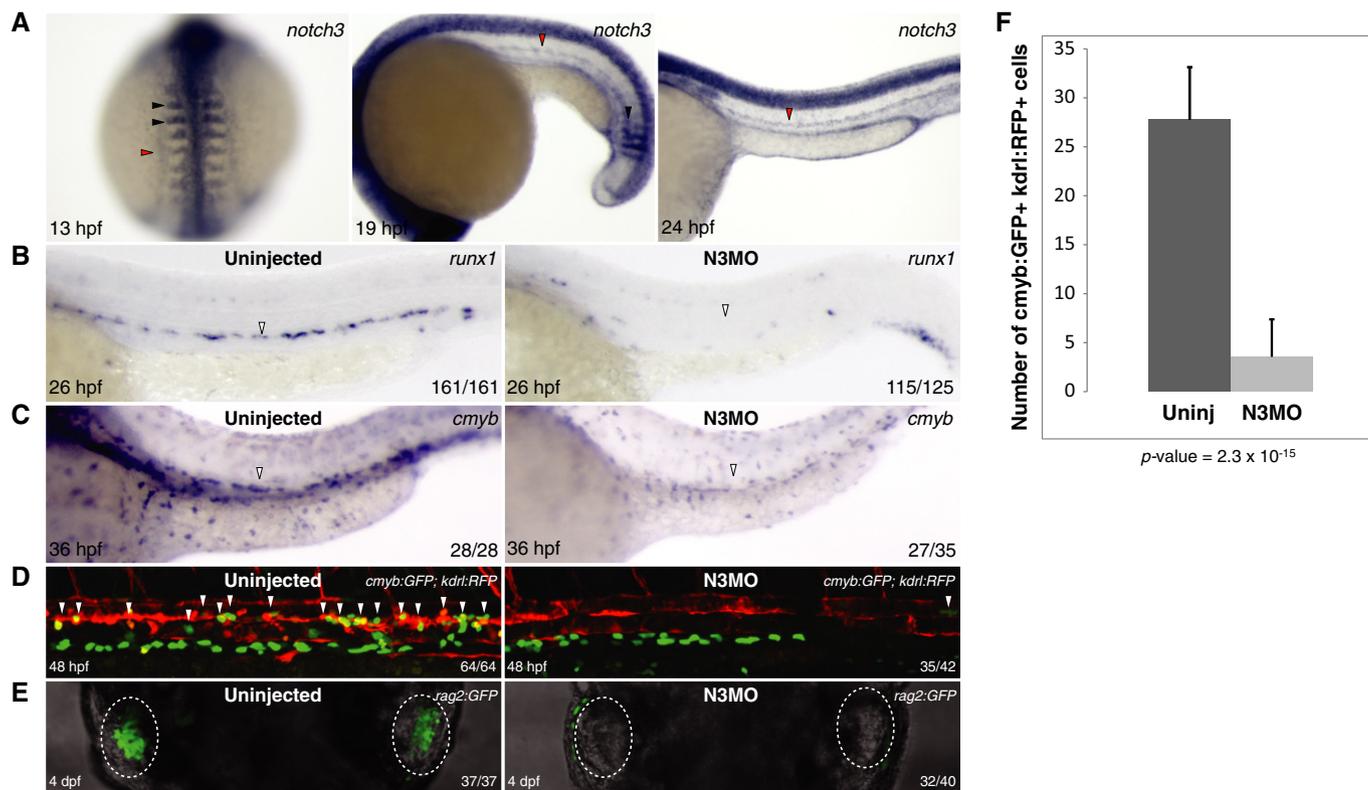
### Notch3 is required for HSC specification

Previous studies have investigated the role of two of the four murine Notch receptors in HSC specification. Notch1 is required cell-autonomously for HSC specification, while Notch2 is dispensable (Kumano *et al.*, 2003; Hadland *et al.*, 2004). However, the role of Notch3 or Notch4 has not previously been explored. First, we characterized the expression pattern of *notch3* in zebrafish embryos at 13, 19, and 24 h post-fertilization (hpf) and identified expression in HSC-related tissues. *Notch3* was expressed widely throughout posterior lateral mesoderm (PLM) and somites at 13 hpf (Fig 1A). At 19 hpf, *notch3* expression was reduced in mature somites but maintained in the 3–5 youngest somites and nascent endothelium. At 24 hpf, *notch3* was largely restricted to the endothelium. To examine whether Notch3 was required for HSC specification, we knocked down its expression with a *notch3* splice-blocking morpholino (Ma & Jiang, 2007). Whole-mount *in situ* hybridization (WISH) analysis of *runx1* and *cmyb*, two early markers of hematopoietic stem and progenitor cells (HSPC) in the DA, were greatly reduced in *notch3* morphants compared to uninjected control embryos (Fig 1B and C). Consistent with these WISH results, confocal imaging of *cmyb:GFP*; *kdr1:RFP* embryos indicated that *cmyb*<sup>+</sup>*kdr1*<sup>+</sup> double-positive HSPCs

(Bertrand *et al.*, 2010a) that normally emerge from hemogenic endothelium were absent at 48 hpf in *notch3* morphants (Fig 1D). Furthermore, *rag2:GFP*<sup>+</sup> T lymphocytes, which are dependent upon upstream HSC precursors, were completely absent at 4 dpf in *notch3* morphants (Fig 1E). Quantification of the number of *cmyb*<sup>+</sup>*kdr1*<sup>+</sup> double-positive HSPCs demonstrated that differences between uninjected and *notch3* morphants are statistically significant (Fig 1F). Together, these results indicate that Notch3 is required for the specification of HSCs.

To investigate whether the reduction of HSPCs in *notch3* morphants was caused by defects in vasculature formation, we examined several markers of endothelium and DA specification by WISH (Fig 2). *Kdr1* expression was reduced in intersomitic vessels and moderately upregulated in trunk endothelium, whereas aortic expression of *efnb2a* and *dlc* was unaffected in *notch3* morphants, indicating that DA formation occurs normally and is not likely an explanation for reduced HSPC number (Fig 2C–E). As our previous work suggested that somite and sclerotome formation is linked to HSPC formation (Clements *et al.*, 2011), we investigated the expression of the somite marker *myod* and the sclerotome-specific markers *foxc1b* and *twist1b* in *notch3* morphants. The somites in *notch3* morphants were specified but exhibited moderate upregulation of *myod* expression in ventral domains (Fig 2F). In contrast, sclerotomal expression of *foxc1b* and *twist1b* was greatly reduced in *notch3* morphants compared to uninjected embryos (Fig 2G and H). We confirmed that the loss of HSPCs and sclerotome observed in *notch3* morphants is specifically due to loss of function of the *notch3* gene and not due to off-target effects from morpholino injection as evidenced by similar defects in *notch3*<sup>fn332</sup> mutants (Quillien *et al.*, 2014) (Supplementary Fig S1). Earlier examination of *notch3* morphants and *notch3*<sup>fn332</sup> mutants at 17 hpf showed reduction of *foxc1b*, *twist1a*, and *twist1b* expression, indicating that sclerotome specification is impaired (Supplementary Fig S2). These results indicate that Notch3 is essential for sclerotome and HSPC specification, but is largely dispensable for DA formation.

We next wished to determine possible roles for the remaining Notch receptors Notch1a, Notch1b, and Notch2 in HSC specification. The expression pattern of *notch1a* and *notch1b* was similar to that of *notch3*, whereas *notch2* was exclusively observed in the somites at these developmental stages (Supplementary Fig S3A–C). We utilized a splice-blocking morpholino for *notch1a* (Ma & Jiang, 2007) and designed splice-blocking morpholinos for *notch1b* and *notch2* (Supplementary Fig S3D and E). Loss of function of *notch1a* and *notch1b*, but not *notch2*, resulted in loss of *runx1* expression in the DA (Supplementary Fig S4A), consistent with the requirement for Notch1 but not Notch2 in murine HSC specification (Kumano *et al.*, 2003). *Notch1* mutant mice have vascular defects including a failure to specify DA (Krebs *et al.*, 2000). In agreement with these findings, we observed variable loss of intersomitic vessels and defective aortic *efnb2a* and *dlc* expression in *notch1a* morphants, whereas *notch1b* morphants had only mild defects in *dlc* (Supplementary Fig S4B–D). *Notch2* morphants displayed loss of intersomitic *kdr1* and *dlc* expression but maintained trunk endothelium and aortic markers. *Notch1a*, *notch1b*, and *notch2* morphants showed normal *myod*<sup>+</sup> somites and *foxc1b*<sup>+</sup>/*twist1b*<sup>+</sup> sclerotome (despite affected somite boundaries in *notch1a* morphants), suggesting that formation of somites does not require these Notch receptors (Supplementary Fig S4E–G). We confirmed that the tissue-specific



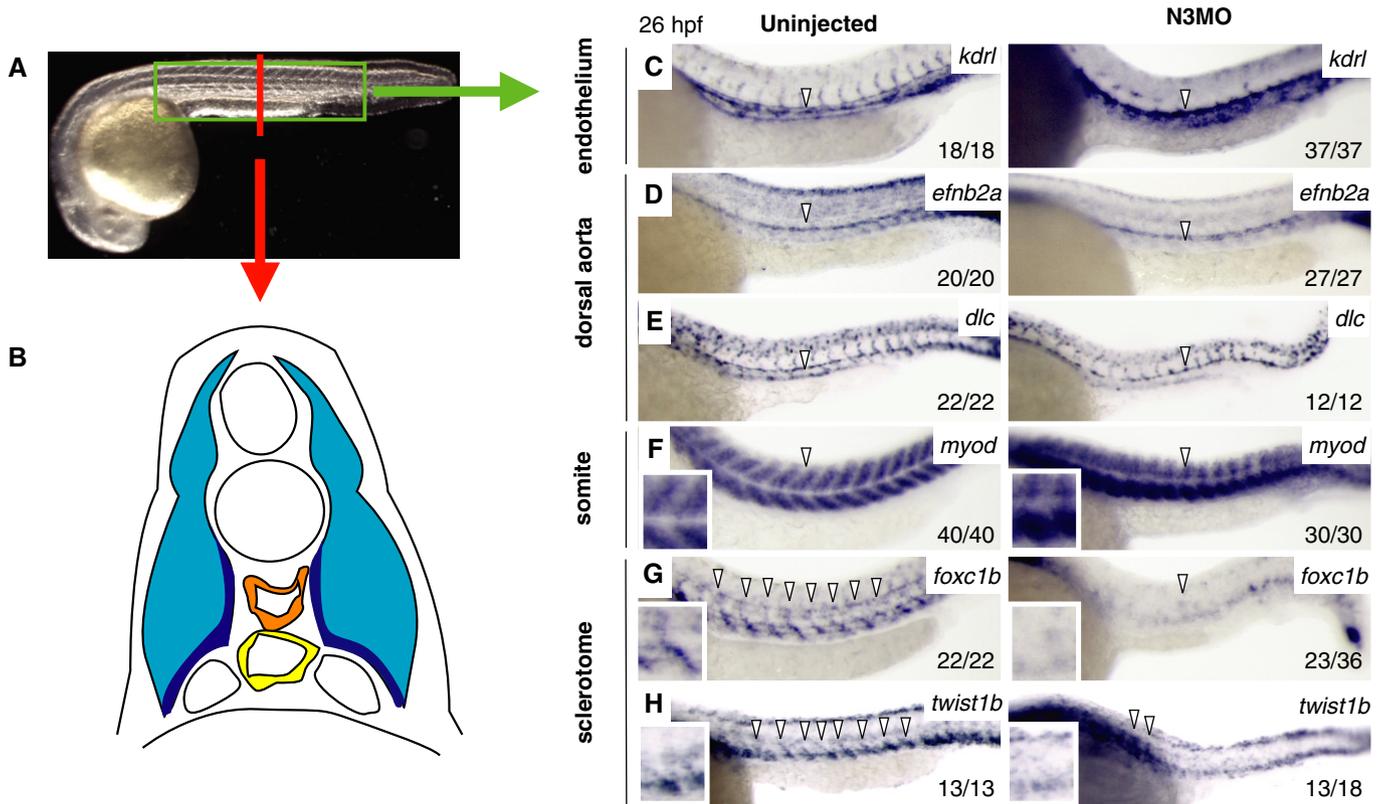
**Figure 1. Notch3 is required for HSPC specification.**

- A** WISH of *notch3* viewed dorsally at 13 hpf (left) and laterally in the trunk at 19 (middle) and 24 hpf (right). Black arrowheads denote somitic expression; red arrowheads denote PLM expression at 13 hpf and endothelial expression at 19 and 24 hpf.
- B, C** WISH of the HSPC marker *runx1* at 26 hpf (**B**) and *cmyb* at 36 hpf (**C**) on uninjected and *notch3* morphants. Arrowheads indicate HSPCs in the DA.
- D, E** Confocal fluorescence microscopy images of transgene reporter expression in *cmyb:GFP; kdr1:RFP* trunk region at 48 hpf (**D**) and *rag2:GFP* at 4 dpf (**E**) transgenics uninjected or with *notch3* morpholino injected. Arrowheads in *cmyb:GFP; kdr1:RFP* embryos indicate double-positive HSPCs, and dotted lines in *rag2:GFP* embryos outline the thymic lobes where GFP<sup>+</sup> lymphoid cells should reside.
- F** Enumeration of *cmyb:GFP*<sup>+</sup>; *kdr1:RFP*<sup>+</sup> cells in the floor of the DA at 48 hpf. Bars represent mean ± SEM of double-positive cells for uninjected (*n* = 12) and *notch3* morphants (*n* = 20). *P* =  $2.3 \times 10^{-15}$ .

defects observed in *notch1a* and *notch2* morphants are not due to off-target morpholino effects as evidenced by similar aortic defects in *runx1* and *efnb2a* expression in *notch1a*<sup>b420</sup> mutants (Gray *et al*, 2001) and loss of intersomitic vessel expression of *dlc* but normal aortic expression of *runx1* in *notch2*<sup>el517</sup> mutants (Supplementary Fig S5A–D). Although singular loss of *notch1b* or *notch3* does not result in a loss of aortic *efnb2a*, a recent study demonstrated that both receptors are required synergistically for aorta specification (Quillien *et al*, 2014). Injection of *notch1b* morpholino #2 from Quillien *et al* resulted in the reduction of HSPCs but did not affect *efnb2a*, validating our observed *notch1b* morphant phenotype. Furthermore, coinjection of *notch1b* morpholino #2 and our *notch3* morpholino resulted in the loss of aortic *efnb2a*, supporting the previous finding that loss of *notch3* alone is tolerated by the aortic program in the presence of functional *notch1b* (Supplementary Fig S5E and F). Consistent with the endothelial-specific effects observed in *notch1a* and *notch1b* morphants, we found coexpression of *notch1a/notch1b* within and around *efnb2a* and *runx1* expression domains in wild-type embryos (Supplementary Fig S6A–D). These data suggest that the role of the paralogous *notch1a* and *notch1b* genes in zebrafish is functionally conserved to that described for the murine *Notch1* gene.

### Notch3 is required non-cell-autonomously for HSC specification

By temporal induction of an NICD transgene, we previously showed that a Notch signal downstream of *wnt16* in the somite was required for HSC specification during a brief permissive window beginning at 14 hpf. This finding helped us determine that this requirement was non-cell-autonomous, since the earliest Notch signaling events in HSC precursors were not detectable until 20–22 hpf (Clements *et al*, 2011). We therefore investigated whether similar induction of NICD could rescue HSCs in *notch3* morphants. To perform these experiments, we utilized *hsp70:gal4; UAS:NICD-myc* double-transgenic animals to temporally control Notch signaling. Expression of NICD-Myc protein was detected by whole-mount immunofluorescence within an hour and up to 24 h after induction as previously reported (Clements *et al*, 2011). Induction of NICD in uninjected embryos did not affect the number of *runx1*<sup>+</sup> HSPCs (unpublished observations). Early induction of NICD at 14 hpf rescued *runx1*<sup>+</sup> HSPCs in *notch3* morphants, while late induction at 20 hpf did not (Fig 3A–C), suggesting that Notch3 may mediate the early, non-cell-autonomous HSC specification requirement. In contrast, *notch1a* and *notch1b*



**Figure 2. Notch3 is dispensable for DA, but required for sclerotome.**

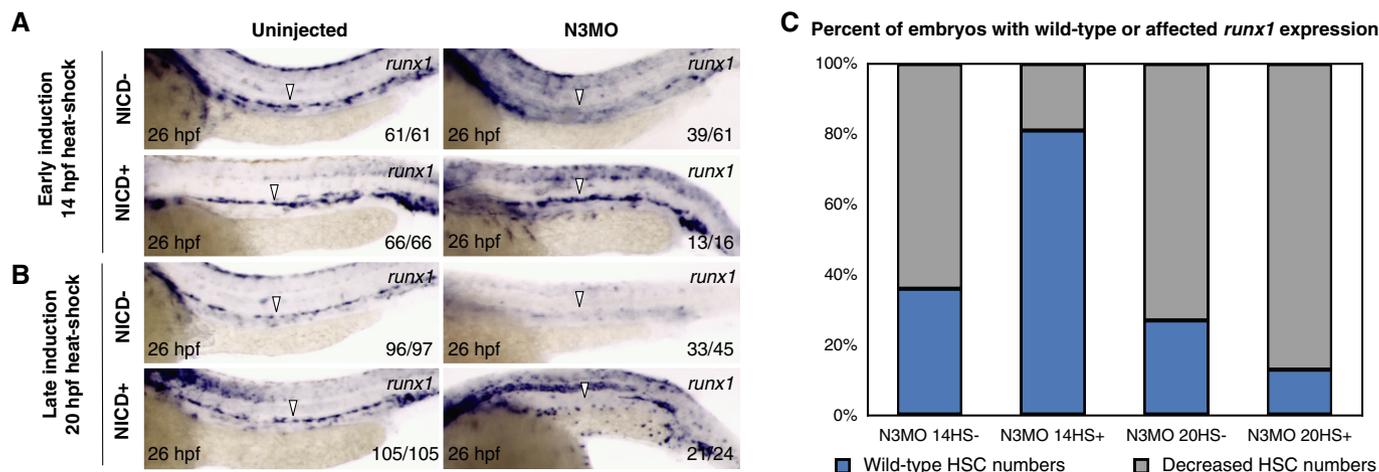
A Brightfield image of a 26 hpf zebrafish.  
 B Cartoon cross section of the embryonic trunk marking somites in light blue, sclerotome in purple, venous endothelium in yellow, and aortic endothelium in orange.  
 C–H WISH of uninjected and *notch3* morphants at 26 hpf for the endothelial marker *kdr1* (C), dorsal aorta markers *efnb2a* (D) and *dlc* (E), the somite marker *myod* (F), and sclerotome markers *foxc1b* (G) and *twist1b* (H). Magnified panels are shown for somitic and sclerotomal markers in lower left corner. Arrowheads indicate tissue-specific gene expression.

morphants were robustly rescued by 14 hpf induction and, more importantly, by 20 hpf induction (Supplementary Fig S7A–D).

The observation that *notch3* morphants had different temporal requirements for NICD-mediated HSC rescue when compared to *notch1a* and *notch1b* morphants suggests that there are at least two temporal windows in which Notch signaling is important for HSC specification. We predicted that global Notch inhibition that spanned 14 hpf would phenocopy loss of *notch3*, while inhibition at 20 hpf would phenocopy loss of either *notch1a* or *notch1b*. To pharmacologically inhibit Notch signaling, we utilized the  $\gamma$ -secretase inhibitor DBZ, treating embryos during 6–15 hpf or 15–26 hpf developmental time windows and subsequently assaying for tissue-specific effects. We observed a reduction in *runx1* intensity in the DA of embryos treated during either drug treatment window, indicating that Notch signaling was required for HSPC specification during both windows (Fig 4A). We observed sclerotome malformation when embryos were treated between 6 and 15 hpf that phenocopied *notch3* morphants, but no effect when embryos were treated between 15 and 26 hpf (compare Figs 4B and 2E). In contrast, the DA transcripts *efnb2a* and *dlc* were severely reduced during the 15–26 hpf window resembling *notch1a* and *notch1b* morphants (compare Fig 4C and D to Supplementary Fig S4C and D), but unaffected by drug treatment during 6–15 hpf. Interestingly, the loss

of *efnb2a* and *dlc* caused by DBZ treatment from 15 to 26 hpf was more dramatic than that observed in *notch1a* or *notch1b* morphants, suggesting that each may have non-redundant requirements or that remaining Notch receptors, likely Notch3 (Supplementary Fig S5F), may partially compensate for the loss of either Notch1a or Notch1b during DA specification. These results indicate that Notch signaling performs transient and non-redundant roles during somitogenesis compared to DA formation that are both essential for HSC production.

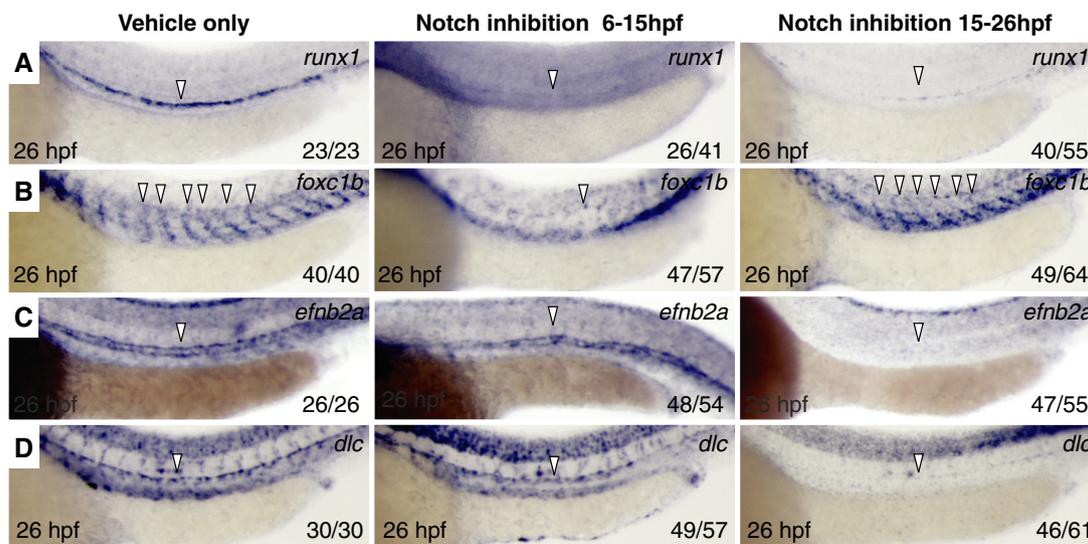
To determine whether spatially restricted expression of NICD was sufficient to rescue HSPCs in *notch3* morphants, we utilized tissue-specific drivers of Gal4. To drive NICD within the HSC lineage, we utilized a *kdr1:miniGAL4* transgenic line whereby expression is targeted to the vasculature, including hemogenic endothelial cells (Supplementary Fig S8A). Since *notch3* is expressed in the sclerotome (Supplementary Fig S8B), and since *notch3* morphants displayed defects in both sclerotome and HSPCs, we asked whether enforced somitic expression of NICD could rescue HSPCs in *notch3* morphants. To perform these experiments, we utilized the *phldb1:gal4-mCherry* transgenic line (Distel et al, 2009), which drives robust expression specifically in the somite (Supplementary Fig S8C–G). Double-transgenic  *$\alpha$ -actin:GFP; phldb1:gal4-mCherry* embryos showed high levels of mCherry in



**Figure 3. Specific temporal activation of Notch signaling is sufficient to rescue HSPCs in *notch3* morphants.**

A, B WISH for *runx1* in 26 hpf *hsp70:gal4*; *UAS:NICD-myc* injected or *notch3* morphant transgenic embryos with heat-shock induction at 14 hpf (A) or 20 hpf (B), with or without enforced NICD expression. Arrowheads indicate the presence or absence of HSPCs at the midline.

C Quantitation of results recording percentages of embryos displaying normal or decreased numbers of *runx1*<sup>+</sup> HSPCs at 26 hpf in *notch3* morphants with heat-shock induction conditions.

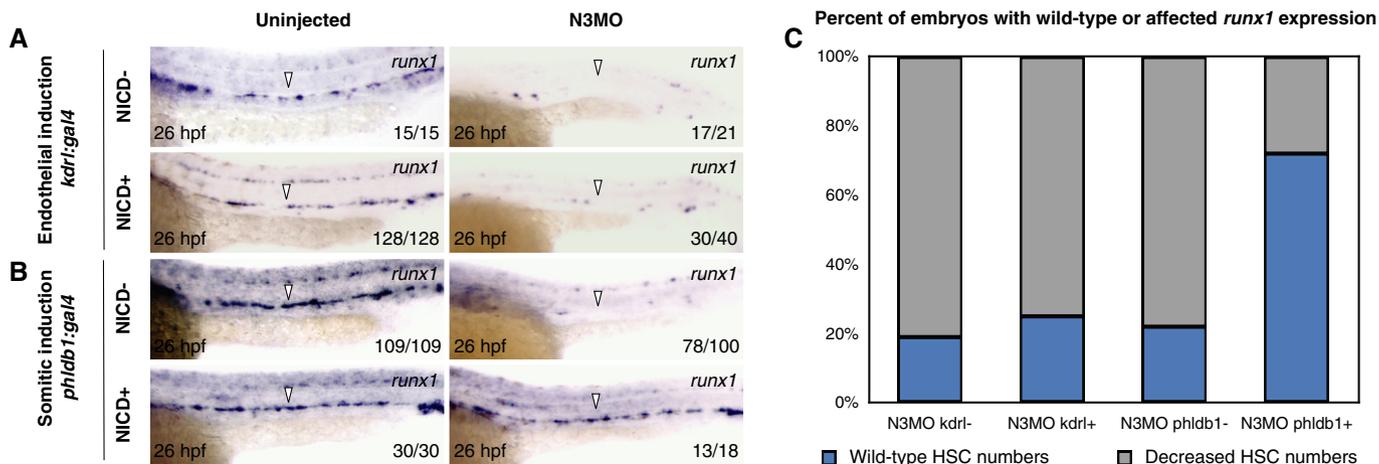


**Figure 4. Notch signaling is required during two distinct time windows for specification of sclerotome and dorsal aorta.**

A–D WISH for *runx1* (A), *foxc1b* (B), *efnb2a* (C), and *dlc* (D) in 26 hpf embryos treated with DMSO vehicle (left), 4  $\mu$ M  $\gamma$ -secretase Notch inhibitor DBZ at 6–15 hpf (middle), or 15–26 hpf (right). Arrowheads indicate tissue-specific expression.

all GFP<sup>+</sup> cells by fluorescence-activated cell sorting (FACS), indicating *phldb1:gal4-mCherry* is expressed widely in somitic tissues (Supplementary Fig S8G). Enforced expression of NICD in the somite rescued *runx1*<sup>+</sup> HSPCs in *notch3* morphants with significantly greater frequency than endothelial-driven NICD did in our analyses (Fig 5A–C). In contrast, HSPCs in *notch1a* and *notch1b* morphants were rescued with the vascular-specific *kdr1:gal4* NICD driver but not with the somite-specific *phldb1:gal4-mCherry* driver in our analyses (Supplementary Fig S9A–D). These findings indicate that *notch1a* and *notch1b* are required for activation of Notch signaling within the endothelium, but not the somites, to specify

HSCs. We next asked whether the induction of NICD sufficient to rescue HSCs in *notch3* or *notch1a* morphants could also rescue the defects observed in the sclerotome and DA, respectively. NICD induction globally at 14 hpf or somitically using the *phldb1:gal4-mCherry* driver in *notch3* morphants also restored expression of *twist1b* in the sclerotome (Supplementary Fig S10A and B). Similarly, global NICD induction at 20 hpf or in vascular cells using the *kdr1:miniGAL4* driver restored expression of *efnb2a* in the DA (Supplementary Fig S10C and D). Together, these results demonstrate that Notch3 is required in the somite at 14 hpf to specify sclerotome and HSPCs and that Notch signaling is then needed



**Figure 5. Specific spatial activation of Notch signaling is sufficient to rescue HSPCs in *notch3* morphants.**

A, B WISH for *runx1* in 26 hpf *kdr1:gal4* (A) or *phldb1:gal4-mcherry* (B) crossed to *UAS:NICD-myc* transgenic embryos either uninjected or injected with *notch3* morpholino, with or without enforced NICD expression. Arrowheads indicate the presence or absence of HSPCs at the midline.  
C Quantitation of results recording percentages of embryos displaying normal or decreased numbers of *runx1*<sup>+</sup> HSPCs at 26 hpf in *notch3* morphants with tissue-specific induction conditions.

again for HSPC fate via subsequent function of Notch1a and Notch1b in the vasculature at 20 hpf.

### Notch3 is required downstream of somitic *dlc*, *dld*, and *wnt16* for HSC specification

Because *wnt16*, *dlc*, *dld* (Clements *et al*, 2011), and *notch3* are each important for sclerotome formation, and because loss of *wnt16* or *notch3* can be rescued only by early NICD induction, we investigated the functional relationship between *dlc*, *dld*, and *notch3* in HSC specification. We tested whether there was synergy between *dlc* or *dld* and *notch3* by combinatorial low-dose knockdown experiments and assessed the severity of affected phenotypes. Heterozygotes from the *dlc* mutant, *beamter* (Julich *et al*, 2005), low-dose (5 ng) *dld* morphants, or low-dose (5 ng) *notch3* morphants each showed a partial loss of *runx1*, *twist1b*, and *foxc1b* expression compared to controls (Fig 6A–D). Combinatorial knockdown of *dlc/notch3* or *dld/notch3*, however, had more severe effects on HSPCs and sclerotome than any of the single knockdown controls, suggesting that *dlc* and *dld* interact with *notch3* (Fig 6E and F). To test whether *notch3* was genetically upstream of *dlc* and *dld* expression in somites, we examined whether *notch3* morphants had defects in somitic *dlc* or *dld* expression. We observed no significant reduction in *dlc* or *dld* in *notch3* morphants compared to uninjected embryos, suggesting that *notch3* is not required to induce *dlc* or *dld* expression (Supplementary Fig S11A and B). In addition, HSPC formation in *notch3* morphants was not rescued by combined *dlc/dld* mRNA injection, a strategy that was able to rescue HSPCs following loss of *wnt16* (Fig 7A–D) (Clements *et al*, 2011). Collectively, these findings suggest that Notch3 function lies downstream of DeltaC/DeltaD function. This hypothesis is supported by the finding that loss of *notch3* inhibited the rescue of HSPCs by *dlc/dld* mRNA in *wnt16* morphants (Fig 7E). These results suggest that Notch3 is necessary to receive signals from DeltaC and/or DeltaD in the sclerotome to

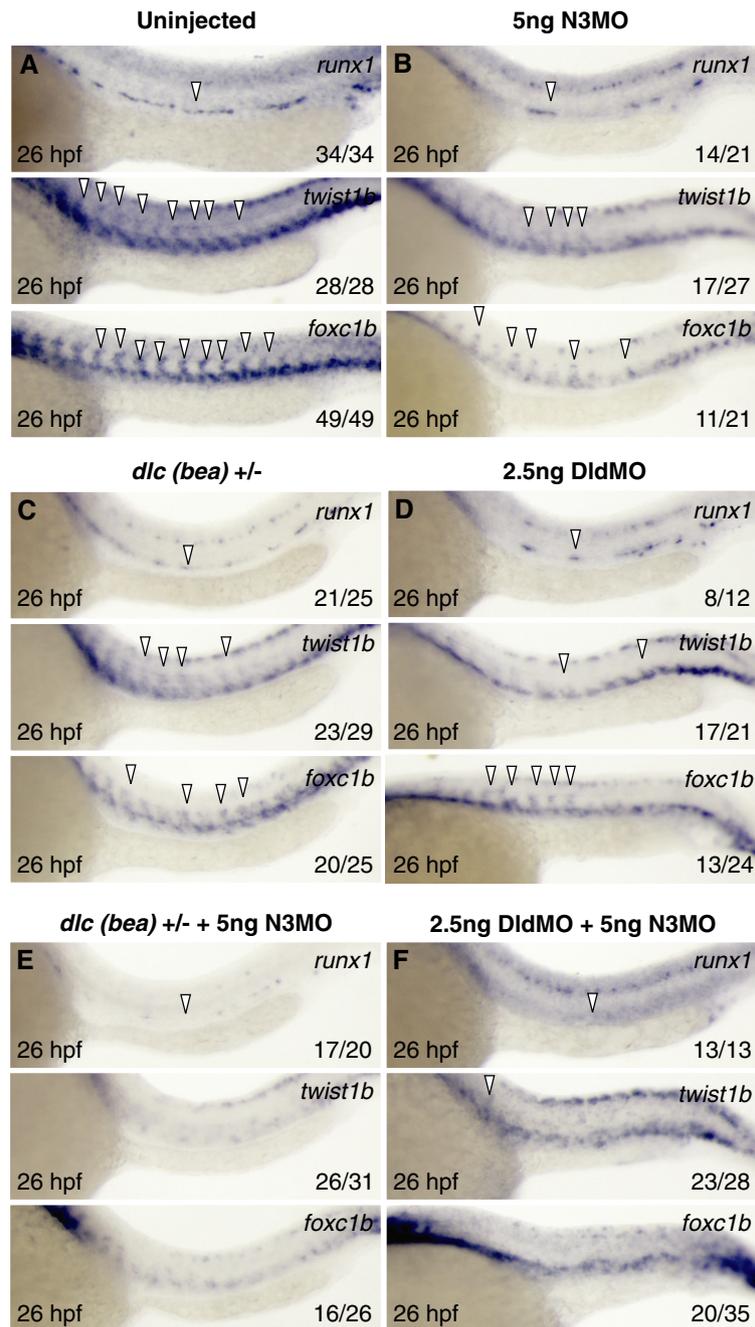
relay further signals to the precursors of HSCs that are required for their proper specification (Supplementary Fig S12).

## Discussion

Previous studies have demonstrated that Notch signaling is required for HSC specification (Kumano *et al*, 2003; Hadland *et al*, 2004; Burns *et al*, 2005; Robert-Moreno *et al*, 2005, 2008; Yoon *et al*, 2008; Bertrand *et al*, 2010b). Of the four murine Notch receptors, Notch1 is required cell-autonomously, while Notch2 is dispensable (Kumano *et al*, 2003; Hadland *et al*, 2004); the roles of the remaining receptors have not been addressed. Here, we demonstrate that Notch3 is required to activate Notch signaling in the somite by 14 hpf and that this activation is required for HSC specification.

Despite the fact that Notch receptors are widely conserved across vertebrate species, there are evolutionary differences in the Notch receptor genes of mammals and zebrafish. One of the most notable is the presence of two Notch1-related homologues, *notch1a* and *notch1b* in zebrafish. Notch1 has high amino acid identity to both Notch1a and Notch1b, and phylogenetic reconstruction analyses have suggested that *notch1a* and *notch1b* arose from a gene duplication event that occurred early during teleost evolution (Westin & Lardelli, 1997; Kortschak *et al*, 2001). Despite Notch1 and Notch2 genes sharing high amino acid similarity, Notch2 is dispensable for HSC specification in the mouse (Kumano *et al*, 2003). We show that Notch2 is also dispensable in zebrafish for HSC generation, indicating that the individual roles of Notch receptors may be conserved across vertebrates. Supporting this hypothesis, our experiments show that the combinatorial actions of Notch1a and Notch1b in zebrafish functionally phenocopy the activity of Notch1 in other vertebrates.

Several lines of evidence indicate that Notch1a and Notch1b have distinct but overlapping roles in hemogenic endothelium. We show that *notch1a* and *notch1b* are both expressed in endothelium

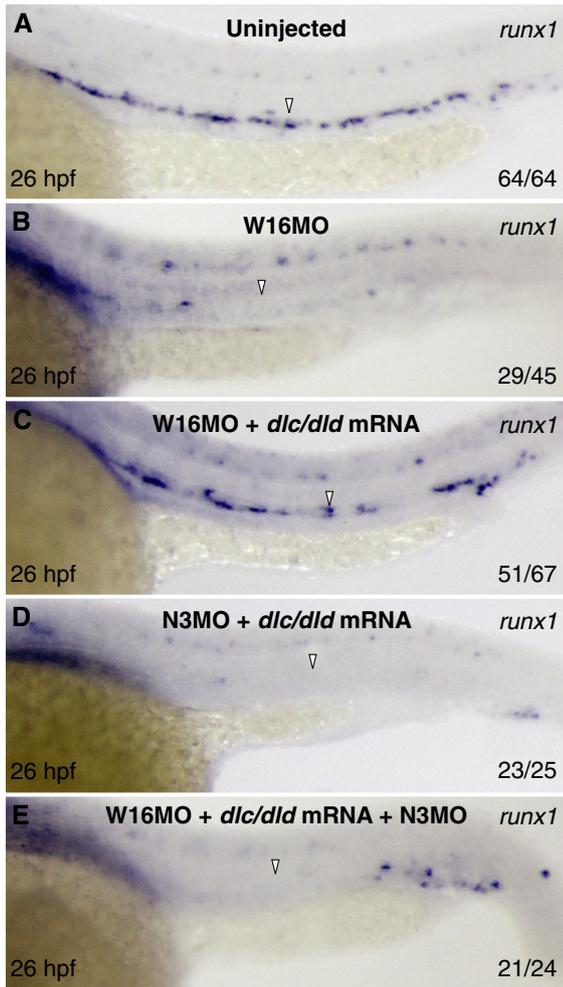


**Figure 6. Notch3 cooperates synergistically with *dld* and *dld* to specify HSPCs.**

A–F WISH for *runx1*, *twist1b*, and *foxc1b* at 26 hpf in uninjected (A), low-dose knockdown of *notch3* (B), heterozygotes for *dld* mutant *bea* (C), low-dose knockdown of *dld* (D), *bea* heterozygotes with low-dose knockdown of *notch3* (E), and combinatorial low-dose knockdown of *dld* and *notch3* (F). Arrowheads indicate tissue-specific expression.

by FISH, but in other tissues, each receptor's expression pattern is more distinct but additively resemble the wider expression pattern of murine Notch1 (Westin & Lardelli, 1997). We show that *notch1a* morphants have reduced HSCs and reduced aortic *efnb2a* and *dld* expression, while *notch1b* morphants had reduced numbers of HSCs and reduced aortic *dld* levels. Additively, these phenotypes resemble *Notch1* mutant mice (Krebs et al, 2004), which suggests that the zebrafish Notch1b receptor has evolved to be more HSC specific.

We also demonstrate that Notch1a and Notch1b have a functional role in endothelial cells; when NICD was specifically expressed in *kdr1<sup>+</sup>* endothelium, *runx1<sup>+</sup>* HSPC formation was rescued in both *notch1a* and *notch1b* morphants. In addition, aortic *efnb2a* expression was restored in *notch1a* morphants. The function of Notch1a and Notch1b receptors is required beginning at approximately 20 hpf, the time at which HSC precursors first experience Notch signaling (Clements et al, 2011). This timing is consistent with our



**Figure 7.** *Wnt16*, *dlc/dld*, and *notch3* function in a linear pathway to specify HSPCs.

A–E Expression of *runx1* at 26 hpf in uninjected (A) or injected with *wnt16*MO (B), *wnt16*MO with 50 pg *dlc/dld* mRNA (C), *notch3*MO with 50 pg *dlc/dld* mRNA (D), and *wnt16*MO/*notch3*MO with 50 pg *dlc/dld* mRNA (E). Arrowheads indicate the presence or absence of HSPCs.

findings that global pharmacological inhibition of Notch between 15 and 26 hpf specifically blocked DA and HSPC specification. Our data demonstrate that like Notch1, Notch1a and Notch1b both perform a cell-autonomous role in HSC specification.

In contrast to Notch1, the role of Notch3 in HSC specification is poorly understood. We investigated whether Notch3 acts cell-autonomously or non-cell-autonomously to specify HSPCs by specific temporal and spatial induction of NICD in *notch3* morphants. In contrast to the rescue of HSCs by induction of NICD at 20 hpf in *notch1a* and *notch1b* morphants, *notch3* morphants could only be rescued by earlier NICD induction at 14 hpf. This suggests that Notch3 is required during a brief permissive window before HSC precursors experience Notch signaling directly. Confirming the requirement for Notch signaling during this permissive window, pharmacological inhibition of Notch signaling during 6–15 hpf showed a strong reduction in HSPC numbers. Our results using tissue-specific drivers of NICD in *notch3* morphants showed

that there were also precise spatial requirements for HSC rescue. Enforced expression of NICD in the somites was sufficient to rescue HSCs in *notch3* morphants, whereas endothelial-specific expression was not, despite the fact that *notch3* is expressed in the DA (Lawson et al, 2002). These results indicate that Notch3 activation is required in the somites, but not in endothelium, to specify HSCs. The temporal and tissue-specific rescue of HSPCs by NICD in *notch3* morphants was also accompanied by the rescue of sclerotome-specific transcripts. These data suggest that the molecular requirements of sclerotome formation are closely linked to HSC specification. Collectively, our data demonstrate that Notch3 is required non-cell-autonomously in the somites to specify HSPCs.

We established that there is a genetic relationship between *dlc/dld* and *notch3* during HSPC specification by combinatorial low-dose knockdown of these genes. Furthermore, the partial knockdown of *dlc* or *dld* was synergistic with a low-dose knockdown of *notch3*, indicating that each of these genes are involved in a linear genetic pathway. In this pathway, *wnt16* is genetically upstream of somitic *dlc/dld* but is dispensible for somitic *notch3* (Clements et al, 2011). However, unlike *wnt16*, *notch3* is not genetically upstream of *dlc/dld* as evidenced by maintenance of *dlc/dld* in the somites of *notch3* morphants. This is bolstered by the finding that reduced numbers of HSCs in *notch3* morphants could not be rescued with coinjection of *dlc/dld* mRNA. Additionally, HSPC rescue in *wnt16* morphants coinjected with *dlc/dld* mRNA was inhibited by the coinjection of the *notch3* morpholino. The simplest explanation is that Dlc and/or Dld directly activates Notch3. Why both ligands might be required to activate Notch3 is unclear. The roles of mammalian homologues Dll3 and Dll1 appear to be conserved with DeltaC and DeltaD, respectively, as evidenced by their conserved expression pattern in somitic tissues and loss of function animals (Hrabe de Angelis et al, 1997; Takahashi et al, 2003; Chapman et al, 2010), though a role in HSC specification has not been described. No studies to our knowledge have demonstrated that Notch3 is an obligate receptor for Dll3 or Dll1. On the contrary, mammalian cell culture experiments have demonstrated that Notch1 and Notch3 can bind a range of Delta and Jagged ligands, suggesting that binding between receptors and ligands is promiscuous (Shimizu et al, 2000). Direct binding has been reported between Dll3 and Notch1; however, this interaction is inhibitory and occurs in *cis*, suggesting that one of the functions of Dll3 is to suppress Notch1 signaling cell-autonomously (Ladi et al, 2005; Chapman et al, 2010). Intriguingly, Dll3 and Dll1 display non-redundant and even counteracting functions in somitogenesis (Takahashi et al, 2003; Ladi et al, 2005; Geffers et al, 2007). Studies in zebrafish may offer an explanation, since both DeltaC and DeltaD within the presomitic mesoderm (PSM) can form heterodimers. Both ligands were observed to be endocytosed together from the plasma membrane, suggesting that both ligands may be required together to effectively activate Notch signaling (Wright et al, 2011). This hypothesis could explain our previous results demonstrating that both *dlc* and *dld* are combinatorially required to restore HSC formation in *wnt16* morphants (Clements et al, 2011). While the lack of a specific Notch3 antibody in zebrafish precludes testing whether or not Notch3 directly interacts with Dlc/Dld by biochemical or histological approaches, our results demonstrate that *wnt16*-induced *dlc/dld* requires the presence of *notch3* to promote HSC emergence.

Notch3 may be required for a specific morphogenetic process and/or activation of another signaling cascade required by HSCs. We have shown that *wnt16*, *dlc*, *dld*, and *notch3* are all required for sclerotome and HSPC formation, but dispensable for DA specification. Notch3 function may be required to specify the sclerotome, which in turn is required to specify HSCs through provision of a relay signal to neighboring PLM cells. Another potential mechanism to explain the link between sclerotome and HSC specification is that sclerotome may give rise to vascular smooth muscle precursors that support the endothelium, as it does in chick and mouse (Pouget *et al*, 2008; Wasteson *et al*, 2008). This is an attractive hypothesis, as Notch signaling is required for the proper emigration of vascular smooth muscle precursors from the somite to the dorsal aorta in the chick embryo (Sato *et al*, 2008). Recent studies performed in embryonic stem cells have confirmed that VSMCs do not directly give rise to hemogenic endothelium, indicating that if VSMCs have a role in HSC specification, it is indirect (Stefanska *et al*, 2014). It is currently unknown if the sclerotome is specifically required for HSC specification, but previous studies have established that somites are, through their production of VEGF, required for HSC formation (Ciau-Uitz *et al*, 2010; Leung *et al*, 2013). Our data elucidate an additional molecular pathway in which the somites are essential for the establishment of HSC fate.

Our study elucidates a previously unappreciated role for *notch3* in the somites that is required for HSC specification, and that the Notch1 orthologues *notch1a* and *notch1b* are both required cell-autonomously in hemogenic endothelium for HSC formation. These data should prove important for future studies focused on the identification of unique targets downstream of each required Notch receptor essential for HSC specification.

## Materials and Methods

### Zebrafish husbandry

Zebrafish strains AB\*, *Tg(UAS:myc-Notch1a-intra)<sup>kca3</sup>* (Scheer & Campos-Ortega, 1999), *Tg(hsp70:l:gal4)<sup>1.5kca4</sup>* (Scheer & Campos-Ortega, 1999), *Tg(actc1b:GFP)<sup>zf13</sup>* (Higashijima *et al*, 1997), *Tg(-80.0myf5:EGFP)<sup>zf37</sup>* (Chen *et al*, 2007), *Tg(rag2:EGFP)<sup>zdf8</sup>* (Langenau *et al*, 2003), *Tg(phldb1:gal4-mCherry)* (Distel *et al*, 2009), *Tg(kdrl:EGFP)<sup>la116</sup>* (Choi *et al*, 2007), *Tg(cmyb:EGFP)zf169* (North *et al*, 2007), *dlc<sup>tit446/tit446</sup>* (from Tübingen 2000 screen), *notch3<sup>fh332</sup>* (Quillien *et al*, 2014), and *notch1a<sup>b420</sup>* (Gray *et al*, 2001) were maintained, injected, and staged as described (Westerfield, 2004) and in accordance with IACUC guidelines. *Tg(kdrl:miniGAL4)* was generated by cloning a 6-kb genomic fragment immediately upstream of the transcription start site from a plasmid carrying *kdrl:R-CFP* (Cross *et al*, 2003) and inserted into pCR8 (Invitrogen). The resulting plasmid was recombined into a Tol2 transgenesis vector *pColdHeart-Gtwy-miniGAL4* (Campbell *et al*, 2007) and coinjected with Tol2 mRNA into 1-cell-stage embryos. A stable transgenic line with a single insertion was established. The *notch2<sup>el517</sup>* mutant was generated using TALENS targeting exon 4 of the *notch2* gene using the following target sequences: left TALEN: 5'-TTGTGTGAACACC ATAGGCT-3'; right TALEN: 5'-TCCGGTGAAGCCAGGTTGGC-3'. TALEN RNAs were synthesized using the mMessage mMachine T7 Ultra kit (Ambion) and injected into 1-cell embryos at 100 ng/μl.

Founders were identified among the injected animals by PCR followed by digestion with *Clal*. The following primers were used for genotyping: *notch2*-F: 5'-GAGCAAGAGGACGCATGTCT-3'; *notch2*-R: 5'-GCTGCGGTAAAATCCCATTA-3'. Stable mutant alleles were isolated in the F1 generation. The *notch2<sup>el517</sup>* allele contains a 104-bp deletion that causes a frameshift and premature stop codon. Heat shocks were performed at the times indicated for 45 min at 37°C as previously described (Burns *et al*, 2005).

### Microinjection of morpholinos, RT-PCR, and mRNA

The following morpholino antisense oligonucleotides were synthesized by Gene Tools, LLC and suspended as 25 mg/ml stocks in DEPC ddH<sub>2</sub>O and diluted to injection strengths: 5 ng *wnt16*-MO, 5 ng *dld*-MO2 (Clements *et al*, 2011), 10 ng *notch1a*-sp MO1, 10 ng *notch3*-sp MO (Ma & Jiang, 2007), 10 ng *notch1b*MO GTCGA-GAATCTTACTTACTTGC, 10 ng *notch2*MO TTCGAATGT-GAAAGTCTTACTGCA, 2.5 ng *notch1b*MO2 (Quillien *et al*, 2014). For RT-PCR, RNA was isolated from groups of 30 uninjected or morpholino-injected embryos at 26 hpf, and cDNA was prepared as previously described (Clements *et al*, 2009). PCR on cDNA was amplified with *notch1b*-sp-F TGCATCTTTCTTCGTGAAAC, *notch1b*-sp-R GGATTGGAAGCAAGGGTTG, *notch2*-sp-F CAAA-TATGGGCCAATTACCC, *notch2*-sp-R GACAGACATGCGTCTCT TGC, b-actin-sp-F AAGATCAAGATCATTGCC, and b-actin-sp-R TTGTCGTTGAAGTTTCTC with Taq polymerase (Invitrogen, Philadelphia, PA) as previously described (Clements *et al*, 2009). Full-length *dlc* and *dld* mRNA was synthesized as described (Clements *et al*, 2011). Injections were performed as described previously (Clements *et al*, 2009). Genotyping of *notch3<sup>fh332</sup>* after phenotypic analysis was performed as described previously (Quillien *et al*, 2014). Genotyping by PCR of *notch2el517* animals was performed with *notch2*-F GAGCAAGAGGACGCATGTCT and *notch2*-R GCTGCGGTAAAATCCCATTA.

### WISH, immunofluorescence, and microscopy

Single enzymatic and double-fluorescence whole-mount *in situ* were performed as previously described (Clements *et al*, 2011). Antisense RNA probes for the following genes were prepared using probes containing digoxigenin or fluorescein-labeled UTP: *runx1*, *kdrl*, *efnb2a*, *dlc*, *myod*, *foxc1b*, *twist1a*, *twist1b*, *notch1a*, *notch1b*, and *notch3* as previously described (Clements *et al*, 2011). Whole-mount immunofluorescence was performed using anti-Myc monoclonal 9E10 antibodies at 1:200 (Covance) and Dylight488 AffiniPure donkey anti-mouse IgG secondary antibodies (Jackson ImmunoResearch Laboratories) at 1:100 as described previously (Clements *et al*, 2011). Fluorescence images of transgenic embryos and embryo samples were imaged using confocal microscopy (Leica, SP5) and processed using Volocity software (Perkin-Elmer) as previously described (Bertrand *et al*, 2010a).

### Pharmacological inhibition of Notch signaling

Dibenzazepine (DBZ)  $\gamma$ -secretase inhibitor (Calbiochem) was dissolved in DMSO at a concentration of 2 mM. Zebrafish embryos were incubated in 3 ml of 4  $\mu$ M DBZ solution in the dark from 6 to 15 or 15 to 26 hpf followed by fixation with 4% PFA.

## Fluorescence-activated cell sorting

*Kdrl:GFP*; *phldb1:gal4-mCherry*, and  $\alpha$ -*actin:GFP*; *phldb1:gal4-mCherry* embryos were collected at 17 hpf and processed for FACS as previously described (Bertrand *et al*, 2007).

**Supplementary information** for this article is available online: <http://emboj.embopress.org>

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## Author contributions

ADK, WKC, and DT designed all experiments. Whole-mount *in situ* hybridization, whole-mount immunofluorescence, and double-fluorescence *in situ* hybridization experiments were performed by ADK and CHM. Cell sorting experiments were performed by DLS and ADK. All other experiments were performed by ADK. DP and CM provided the *Tg(kdrl:miniGAL4)* reporter line. MD provided the *Tg(phldb1:gal4-mCherry)* reporter line. LAM and JGC provided the *notch2<sup>el517</sup>* mutant. The manuscript was written by AK and edited by DLS, WK, and DT with critical input as described in Acknowledgements.

## Conflict of interest

The authors declare that they have no conflict of interest.

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