UNIVERSITY OF CALIFORNIA SAN DIEGO

Identifying Notch Receptor-Ligand Interactions Required to Establish Hemogenic Endothelium

A Thesis submitted in partial satisfaction of the requirements for the degree

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in

Biology

by

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Professor Mark Ginsberg
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The Thesis of Priscilla Marie Sellers is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

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by

Priscilla Marie Sellers

Master of Science in Biology

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Professor David Traver, Chair

Hematopoietic stem cells (HSCs) originate from a specialized subset of arterial endothelial cells termed hemogenic endothelium (HE) within the dorsal aorta (DA) during early embryonic development. Notch signaling is known to be required for the specification of HSCs. However, little is still known about the specific Notch receptor-ligand interactions that are required for the specification of the HE. Previous work has shown that Notch ligands Dlc, Dld, and the Notch3 receptor, are required non-cell-

autonomously in the somites to specify HSCs. However, whether Notch3 functions exclusively in the somites to regulate HE specification, and through which ligand(s), is debatable. By contrast, the Notch1b receptor has been shown to function only in a cell-autonomous manner for HE specification, but the Notch ligand(s) that are required to signal through Notch1b are still unknown. Here, we confirm the non-cell-autonomous requirement for Notch3 in the somites to regulate HSC specification. Combinatorial low-dose knockdown experiments also revealed that *notch1b*, *dld*, and *dlc* function synergistically in a linear genetic pathway to establish HE.

INTRODUCTION

Hematopoietic stem cells (HSCs) are multipotent, self-renewing, and responsible for the life-long production of all blood cell types categorized as red blood cells, white blood cells, and platelets (Dean, 2005). HSCs have the potential to cure many different diseases that affect the blood or blood-forming organs. HSCs were also the first stem cells used as therapy for blood diseases in humans through the transplantation of bone marrow (Till and McCulloch, 1961). However, bone marrow transplants pose numerous risks to receiving patients such as: graft-versus-host disease, graft failure, infections, infertility, new cancers, and possibly death (Mayo Foundation for Medical Education and Research, 2019). Another major challenge in bone marrow transplantations is finding matching donors, especially for ethnic minority patients (Esposito, 2016). Induced pluripotent stem cells (iPSCs) generated from adult human somatic cells offer a prospective solution to these challenges as iPSCs can theoretically differentiate into various types of cells under the right conditions (Takahashi et al., 2007). iPSCs derived from somatic cells taken directly from a patient and reprogrammed into HSCs could eliminate the aforementioned challenges of bone marrow transplantation. However, *in vitro* generation of HSCs from iPSCs comes with its own set of challenges, and successful generation of HSCs from iPSCs has not yet been achieved due to insufficient knowledge of the endogenous molecular mechanisms governing their development *in vivo*.

During early embryonic development in all vertebrates, HSCs emerge from a specialized subset of arterial endothelium known as hemogenic endothelium (HE).

Through a process termed as endothelial-to-hematopoietic transition (EHT), HE undergoes a transformation in which they lose endothelial fate and gain blood stem cell fate (Kissa and Herbomel, 2010; Bertrand et al., 2010). During EHT in zebrafish, HE cells bud from the floor of the DA toward the aorta-gonad-mesonphros (AGM) region, until they eventually detach into the sub-aortic space and enter circulation through the neighboring axial vein to colonize subsequent hematopoietic organs. (Kissa and Herbomel, 2010; Bertrand et al., 2010). HE specification requires many molecular and environmental cues from numerous signaling pathways, one of which is the Notch signaling pathway.

Notch signaling is a cell-cell signaling pathway that governs many cell-fate decisions throughout the lifespan of multi-cellular organisms (Lai, 2004; Kopan and Ilagan, 2009). In zebrafish, there are four Notch receptors (Notch1a, Notch1b, Notch2 and Notch3) and eight Notch ligands, termed Delta (Dla, Dlb, Dlc, Dld, Dll4) and Jagged (Jag1a, Jag1b, Jag2b), that have been shown to be required to activate the Notch Signaling pathway. Initiation of the Notch pathway occurs when a signal sending cell expressing a Notch ligand makes direct contact with a signal receiving cell expressing a Notch receptor. Ubiquitination of the Notch ligand by the E3 ubiquitin ligase Mindbomb (Chen and Corliss, 2004; Itoh et al., 2003) and Neutralized (Deblandre et al., 2001; Yeh et al., 2001; Pavlopoulos et al., 2001; Lai et al., 2001) promotes endocytosis of the receptor-bound ligand. The endocytosis process then creates tension on the receptor allowing for cleavage of both the Notch extracellular domain and the Notch intracellular domain (NICD), which then releases the NICD into the cytoplasm (Kao et

al., 1998). The NICD then translocates to the nucleus where it binds to Notch transcriptional partner RBPjK and recruits other coactivators resulting in the transcription of direct Notch target genes (Castel et al., 2013; Kao et al., 1998).

Notch signaling has been shown to be a requirement for the successful development of HSCs across vertebrate phyla. Studies in mice have demonstrated that global knockdown of the Notch signaling pathway by means of mutating the Notch-DNA binding co-factor RBPjk, results in a loss of hematopoietic transcription factors required for HSC development (Robert-Moreno et. al., 2005). Similar results have been shown in zebrafish studies, where global knockdown of the Notch signaling pathway by generating E3 ubiquitin ligase Mindbomb mutants results in the loss of Runt-related transcription factor (Runx1) (Burns et al., 2005). Runx1 is a key transcription factor required for HSCs development (Kissa and Herbomel, 2010; Swiers et al., 2010; Chen et al., 2009; Lancrin et al., 2009; North et al., 2002; Okuda et al., 1996) and is a common genetic marker used to visualize HSC specification. While it is well known that Notch signaling is required for the specification of HE, the specific receptors and ligands governing these signaling events remains unclear. With numerous receptor-ligand combinations possible, identifying distinct receptor-ligand interactions required for HE specification, as well as when and where they are required, is no small feat.

Notch signaling has been shown to be required cell-autonomously within the aortic endothelium for the specification of HSCs (Kumano et al., 2003; Hadland et al., 2004). Earlier studies suggested a link between the specification of arterial endothelium and hemogenic endothelium considering several Notch pathway mutants that failed to

specify the DA also failed to specify HSCs (Krebs et al., 2000, 2004; Lawson et al., 2001). However, more recent studies in single Notch ligand mutants show that it is possible to have normal arterial development but lose HSC development (Robert-Moreno et al., et al. 2008), suggesting that HE specification requires unique Notch inputs distinct from those required for arterial specification.

Previous work done in our laboratory uncovered an unknown requirement for Notch signaling in the somites that is essential for HSC specification (Clements et al., 2011). Somites derived from paraxial mesoderm, are embryonic blocks of mesoderm made up of different compartments, located on either side of the embryonic spine (Burgess et al., 1995). Our laboratory found that Wnt16 controls the expression of Notch ligands *dlc* and *dld* specifically in the somites (Clements et al., 2011). Somitic expression of *dlc* and *dld* are combinatorially required up to 14 hpf for HSC specification but dispensable for arterial specification. Since this time-point is prior to the formation of the DA, it was determined that *dlc* and *dld* are functioning non-cell-autonomously in the somites to specify HE. Furthermore, somitic *dlc* and *dld* are required to specify the ventral compartment of the somite, called the sclerotome, suggesting that proper specification of the sclerotome is required for HSC specification (Clements et al., 2011). These findings laid the groundwork to uncovering a novel relay signal between the somites and the endothelial precursors of HE.

Other work done in our laboratory revealed how specification of the sclerotome compartment in the somites is connected to the emergence of HE. A second Notch signaling event occurs between 14-18 hpf and is believed to be a key relay signal from

the somite to migrating arterial precursor cells that is required to establish hemogenic fate (Kobayashi et al., 2014). Migrating PLM cells have been shown to make direct contact with somitic cells expressing *dlc* and *dld* between 14-18 hpf (Kobayashi et al., 2014). This direct contact between migrating PLM cells and somites, mediated by junctional adhesion molecules, provides cell-autonomous Notch signaling, and confers HE fate to some endothelial precursors. Loss of function of adhesion molecules on migrating PLM cells results in complete loss of HSCs, but overexpression of *dlc* as well as *dld* is sufficient to rescue HE specification, although the rescue was greater with overexpression of *dld* (Kobayashi et al., 2014). Expression patterns of *dlc* show that it is expressed throughout the somite, meanwhile *dld* expression is concentrated in the sclerotome compartment only. Taken together, we reasoned that Dld is the better candidate for delivering the relay signal to aortic HE precursors, though it has not yet been confirmed. In this study, we investigate the role

More recent analysis of the tissue-specific requirements for each of the four zebrafish Notch receptors indicates that Notch activation must occur in both the somites, and in the endothelium, in order for proper HE specification to occur (Kim et al., 2014). Previous studies done in our lab by Kim and colleagues (2014) have shown that Notch3 is only required within the developing somite to specify HE, but not within HE precursors themselves; suggesting that Notch3 is the only receptor functioning in a non-cell-autonomous manner. However, we felt that the non-cell-autonomous requirement for Notch3 needed further investigation. Nonetheless, since *notch3* morphants display similar defects in sclerotome formation (Kim et al., 2014), similar to

those seen in embryos after Wnt16-dependent loss of somitic *dlc* and *dld* (Clements et al., 2011), this suggests that the non-cell-autonomous Notch requirement is most likely mediated by signaling through the Notch3 receptor with dlc and/or dld. Furthermore, previous combinatorial knockdown experiments of *notch3/dlc* and *notch3/dld* show a significant decrease in HE specification, however, combined knockdown of *notch3/dlc* had a greater impact on sclerotome specification (Kim et al., 2014). We hypothesize that Dlc is the ligand signaling to Notch3 mediating the specification of the sclerotome, while Dld is the ligand being presented to migrating PLM.

Notch1a and Notch1b have been shown to be required cell-autonomously within the endothelial precursors of HE to establish hemogenic fate. Although both *notch1a* and *notch1b* morphants are deficient in HSCs, only *notch1a* morphants displayed loss of arterial specification. We reasoned that Notch1a is likely required for arterial specification and development subsequently promoting successful HSC development. Although *Notch1b* morphants are deficient of HSCs, they display normal arterial specification have normal development of the sclerotome compartment. Taken together, we hypothesize that Notch1b is the receptor presented on migrating PLM cells that receives a Notch relay signal from the somites through Dld, activating Notch signaling within the endothelial precursor cells to confer HE fate.

In this report, we utilized morpholino antisense oligonucleotide injections in wild type and transgenic zebrafish embryos (Corey et al., 2001; Ma et al., 2007) to uncover the specific Notch receptor-ligand interactions required for HE specification. We confirmed previous reports that Notch3 only functions non-cell-autonomously

within the somites to specify HE. We have also determined that *notch1b* functions synergistically with *dlc* and *dld* to confer hematopoietic fate to endothelial precursors.

MATERIALS AND METHODS

Zebrafish strains

Adult fish were maintained in accordance with the guidelines provided by the UCSD IACUC. Adult fish were raised in a circulating aquarium system (Aquaneering) at 28°C. The following zebrafish strains were used: wildtype AB*, transgenic lines Tg(*Tbx6:Gal4-GFPnls*), Tg(*UAS:NICD*).

Whole-mount In Situ Hybridization (WISH)

Embryos were incubated at 28°C and treated with 0.003% 1-phenyl-2-thiourea (PTU) to prevent pigmentation. Embryos were dechorionated using pronase and fixed at 26 hpf in fresh 4% paraformaldehyde (PFA) in PBS at 4 °C overnight. Fixed embryos were washed briefly in PBS and dehydrated in ethanol. Embryos were rehydrated in PBS–0.1% Tween 20 (PBST). Rehydrated embryo samples were then incubated acetone for 5.5 min for 26 hpf embryos. After acetone treatment, samples were washed in PBST and re-fixed in 4% PFA in PBS for 20 min at room temperature. After washes in PBST, embryos were prehybridized at 70 °C for 1 h in hybridization buffer (50% formamide, 5x SSC, 500 µg ml−1 torula (yeast) tRNA, 50 µg ml−1 heparin, 0.1% Tween 20, 9 mM citric acid (pH 6.5)). Samples were then hybridized overnight in hybridization buffer including digoxigenin (DIG)-labelled RNA probe. After hybridization, experimental samples were washed stepwise at 70 °C for 30 min each in hybridization buffer, 50% hybridization buffer in 2x SSC mix, 2x SSC, and 0.2x SSC. Samples were incubated in PBST with 2% heat-inactivated goat serum and 2 mg ml−1 bovine serum albumin

(block solution) for 1 h and then incubated overnight at 4 °C in block solution with diluted DIG-antibodies (1:5,000) conjugated with alkaline phosphatase (AP) (Roche). Samples were washed in PBST 7 times for 15 min each wash. To visualize WISH signal, samples were washed two times in AP reaction buffer (100 mM Tris, pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20) for 5 min each and then incubated in the AP reaction buffer with NBT/BCIP substrate (nitro-blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) (Roche).

Microinjection of morpholinos

Embryos were injected at the one to two-cell stage with antisense morpholino oligonucleotides (MOs) solution diluted in water. The following morpholinos were synthesized by Gene Tools, LLC and suspended as 25 ng/ul stocks in DEPC ddH20:

notch3-sp MO AAGGATCAGTCATCTTACCTTCGCT

notch1b-MO7 MO GTCGAGAATCTTATCACTTACTTGC

dlc-MO1 AGCCATCTTTGCCTTCTTGTCTGCT

dld-MO1 AAACAGCTATCATTAGTCGTCCCAT

Morpholinos (MOs) were used at the following concentrations: 10ng *notch3*-sp MO, 7.5ng *notch1b*-MO7, 7ng *dld*-MO1, 0.75ng *Dlc*-MO1.

PCR genotyping

Fixed, WISH-processed individual embryos had DNA isolated in lysis buffer (10 mM Tris, pH 8.3, 50 mM KCl, 0.3% Tween-20, 0.3% NP-40), 94 °C, 20 min, held at 4 °C to allow addition of proteinase K to a final concentration of 1 mg ml−1, 3 hrs at 55 °C, 20 min at 94 °C. Presence of the UAS:NICD transgene was assessed by PCR using the primers UAS:NICD-F 5'-CATCGCGTCTCAGCCTCAC-3', UAS:NICD-R 5'-CGGAATCGTTTATTGGTGTCG-3' (Tm 55 °C, extension time 45 s, 35 cycles).

RESULTS

Notch3 functions non-cell autonomously to specify HSCs

Previous studies have indicated that Notch3 is required only within the somites in a non-cell-autonomous manner to specify HE (Kim et al., 2014). To confirm these reports, we tested whether somitic induction of NICD in *notch3* morphants was sufficient to rescue HE specification. Here, we utilized the somite-specific *tbx6:gal4-GFP* transgenic line to induce NICD expression exclusively in somitic tissue. Adult *tbx6:gal4-GFP* transgenic animals were mated with adult *UAS:NICD* transgenic animals. Offspring embryos were harvested and a portion of the embryos were injected with 10ng of Notch3 morpholino. Since the *tbx6* line used also has a nuclear GFP reporter, we were able to sort embryos that were tbx6^{+}. We therefore sorted all injected and uninjected GFP^{+} embryos since we were interested in those expressing *tbx6* to drive the transcriptional activator gal4 that binds to UAS enhancer sequences that would in turn activate transcription of NICD. Once sorted, all embryos were then fixed at 26 hpf since this is a timepoint after HE specification, but before HSC emergence, and when *runx1* expression is present in HE. We then performed whole-mount in situ hybridization (WISH) analysis for *runx1* in all embryos. All embryos were then imaged and expression levels of *runx1* were qualitatively measured. DNA was extracted from all embryos and then genotyped for *UAS:NICD*. As was expected, we observed that uninjected GFP^{+} embryos, with and without NICD induction, have normal *runx1* expression in the AGM region at 26 hpf (Fig. 1A). GFP^{+} *notch3* morphants without NICD induction show significant loss of *runx1* in the AGM (Fig. 1A and B) when compared to uninjected embryos. In contrast, GFP^{+} *notch3* morphants with NICD

induction show normal *runx1* expression in the AGM (Fig. 1A and B) similar to uninjected embryos. These results indicate that rescuing Notch signaling in *notch3* morphants only within the somites is sufficient to rescue HE specification, confirming previous reports that Notch3 is required non-cell-autonomously in the somites for proper HE development.

Notch1b **acts synergistically with** ***dlc*** **and** ***dld*** **in the same pathway to specify HE**

While previous studies have implicated Notch1b as the receptor on PLM cells receiving a Notch relay signal from the somites, weather Notch1b actually functions in the somite-PLM signaling pathway to specify HSCs, and through which somitic ligand, is still unknown. To investigate the role of Notch1b in this signaling pathway, we performed low-dose morpholino knockdown experiments to determine if *notch1b* acts synergistically with *dld* or *dlc* to specify HE. We first set out to find concentrations for each morpholino that would produce a hemogenic phenotype. We injected a series of different concentrations for each morpholino, Notch1b, Dld, and Dlc, into wild type embryos. We then fixed all embryos at 26 hpf and performed WISH analysis for *runx1* expression in the AGM. Once we found an injectable concentration that was not lethal but yielded a loss of HSCs, we then halved those concentrations and injected wild type embryos with the lower doses of Notch1b (7.5ng), Dld (7ng), and Dlc (0.75ng) morpholinos. WISH analysis for *runx1* confirmed that single low-dose injections of Notch1b, Dlc, and Dld morpholinos into wild type embryos had no effect on *runx1* expression at 26 hpf (Fig. 2B-D) when compared to uninjected wild type embryos (Fig. 2A). We then proceeded with combinatorial injections of Notch1b with Dld, and Notch1b and Dlc morpholinos. In contrast to individual low-

dose injections, when a low-dose of Notch1b morpholino was co-injected with a low-dose of Dld morpholino, half of the injected wild type embryos lost *runx1* expression (Fig. 2E), which is a significant difference when compared to uninjected wild type embryos (Fig. 2A). Similar losses of *runx1* expression were also seen in embryos co-injected with low-doses of Notch1b and Dlc morpholinos (Fig. 2F) when compared to uninjected wild type embryos. Next, we investigated if the combinatorial knock-down of *notch1b*/*dld* and *notch1b*/*dlc* had any effect on arterial specification. We performed the same injections on wild type embryos using the same low-dose concentrations for each morpholino, Notch1b (7.5ng), Dld (7ng), and Dlc (0.75ng). WISH analysis for arterial marker *efnb2a* showed that single low-dose injections of Notch1b, Dlc, and Dld morpholinos into wild type embryos had no effect on *efnb2a* expression at 26 hpf (Fig. 2B-D) when compared to uninjected wild type embryos (Fig. 2A). Similarly, when a low-dose of Notch1b morpholino was co-injected with a low-dose of Dld morpholino, there was no effect on *efnb2a* expression (Fig. 2E) when compared to uninjected wild type embryos (Fig. 2A). Embryos co-injected with low-doses of Notch1b and Dlc morpholino also displayed normal *efn2ba* expression (Fig. 2F) when compared to uninjected wild type embryos (Fig. 2A). These results suggest that *notch1b*, *dld*, and *dlc* function synergistically in a linear genetic pathway, but fall short of confirming that Dld is the specific ligand for Notch1b in the specification of HE.

DISCUSSION

It has been well established that Notch signaling is required across vertebrate phyla for the specification of HSCs (Robert-Moreno et. al., 2005; Burns et al., 2005). It was long believed that specification occurred within HSC precursors in a strictly cell-autonomous manner mediated by the murine Notch1 receptor (Kumano et al., 2003). However, previous work done in our laboratory uncovered an environmental requirement for Notch signaling in the somites that is essential for proper specification of HE beginning at 14 hpf, a timepoint prior to the formation of the vasculature from which they arise (Clements et al., 2011). Although *notch3* is expressed throughout the PLM and somites at 13 hpf, several lines of evidence indicate that Notch3 is only required within the somites to specify HE in a non-cell-autonomous manner (Kim et al., 2014). A critical piece of evidence supporting the non-cell-autonomous requirement for Notch3 was achieved from a previous experiment, in which the loss of HSCs in *notch3* morphants was rescued using somite-specific promoter *phldb1* to induce NICD expression (Kim et al., 2014). However, through fluorescence-activated cell sorting (FACS) analysis, a recent study found that in 14 hpf double transgenic *fli1a:GFP;phldb1:mCherry* embryos, there was a significant population of double positive cells (Kobayashi et al., 2014). Considering *fli1a* is a common marker for PLM, this suggests that *phldb1* is also expressed in some HSC precursors. Therefore, the *phldb1* driver could be directly activating Notch signaling in the PLM to rescue HSCs in *notch3* morphants, thus bypassing a need for somitic Notch signaling to specify HE precursors. We felt this was an important experiment to revisit since the current model of non-cell-autonomous Notch signaling is heavily based on this rescue experiment. We

chose to repeat the experiment in question using the *tbx6* promoter, since current studies being done in our laboratory have shown *tbx6* to be more restricted to the paraxial mesoderm, which gives rise to somites. We observed that NICD induction exclusively in the somites using the *tbx6* promoter was sufficient to rescue the loss of HSCs in *notch3* morphants. Therefore, our results further support that Notch3 is only required non-cell-autonomously in the somites for the specification of HE.

The next goal of this project was to identify the ligand that could be providing the Notch relay signal from the somites to PLM cells. Low-dose synergy experiments previously done in our laboratory have shown that *notch3*, *dlc* and *dld* all function in a genetic pathway to specify the sclerotome and subsequently HSCs, although *notch3* and *dlc* were shown to have a greater synergistic effect on sclerotome formation (Kim et al., 2014). We therefore hypothesized that Dlc is likely the ligand for Notch3 to specify the sclerotome compartment. In a previous study (Kobayashi et al., 2014), when junctional adhesion molecules establishing the intercellular contact between somites and PLM cells were disrupted, overexpression of *dld* was shown to have a higher level of HSC rescue than overexpression of *dlc*, which lead us to hypothesize that Dld was the ligand being presented on the sclerotome to signal to Notch1b on migrating PLM.

Here we showed that partial knockdown of *dld* with *notch1b* have synergistic effects on HSC specification. This result supports our original hypothesis that Notch1b is signaling with Dld to specify HE. However, we were not expecting to see that partial knockdown of *dlc* and *notch1b* would have similar synergistic effects on HSC specification. These results suggest that *notch1b*, *dld*, and *dlc* function synergistically in a

genetic pathway, however, more studies are required to confirm if Dld is the specific ligand for Notch1b in the specification of HE.

Despite the unexpected results from the partial knockdown of *notch1b* with *dlc*, we don't believe our results discredit our hypotheses, since any level of knockdown of ligands upstream can have an effect on ligands downstream. This would indicate that by knocking down *dlc* upstream, this could result in less signaling to Notch3 in the somite. Less Notch signaling in the somite could in turn negatively affect the amount of *dld* expression in the sclerotome and ultimately result in less Notch signaling to PLM cells. We could further test this theory by combinatorially injecting low-doses of Notch3 and Dlc morpholino into wild type embryos, then look at *dld* expression levels in the sclerotome to see if *dld* is being affecting downstream of *dlc* and *notch3* at 17 hpf, since this is the approximate time point when Dld would be required on the sclerotome to signal to Notch1b on migrating PLM. If *dld* expression was being affected by knockdown of notch signaling in the somites, this would further support that *dld* is downstream of *dlc* and *notch3*, and that Dld is likely the ligand being presented on the sclerotome signaling with Notch1b.

There could be another explanation as to why both *dlc* and *dld* were observed to have a synergistic effect with *notch1b*. Previous studies in zebrafish have observed that both Dlc and Dld can form heterodimers within the presomitic mesoderm (Wright et al., 2011). This could explain why multiple studies done in our laboratory have shown that *dlc* and *dld* are combinatorially required to rescue HSCs in *wnt16* morphants (Clements et al., 2011, Kim et al., 2014). However, whether Dlc and Dld function as heterodimers during the hematopoietic specification process has not yet been studied.

We also looked at the expression of arterial marker *efnb2a* at 26 hpf after combinatorial low-dose knockdown of *dld*/*notch1b* and *dlc/notch1b*. Expression levels of *efnb2a* appear to be unaffected and no gross defects were observed in the DA. This suggests that Dlc and Dld interactions with Notch1b are not required for arterial specification. These results also suggest that the loss of HE specification observed after combinatorial low-dose knockdown of *notch1b/dlc* and *notch1b/dld,* is most likely not the result of a compromised DA.

Overall, the findings of this project helped to confirm a non-cell-autonomous requirement for Notch3 in the somites. It also laid the foundation for future experiments that could further support our model that Dlc signals to Notch3 to specify the sclerotome (Fig. 3A), and that Notch1b is the receptor presented on PLM cells receiving a Notch relay signal through Dld presented on the sclerotome (Fig. 3B).

FIGURES

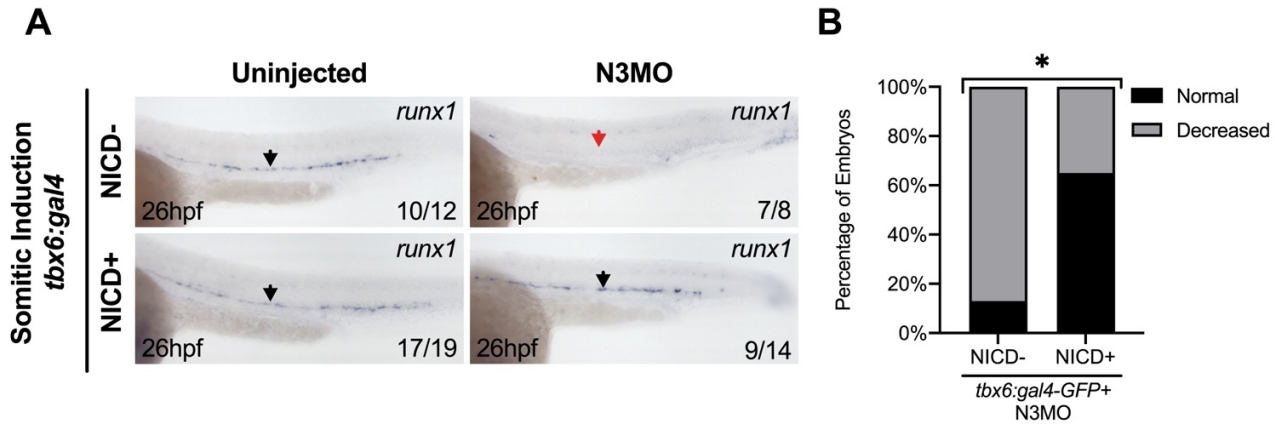

Figure 1. Somite-induced activation of Notch signaling is sufficient to rescue HSCs in *notch3* morphants. (A) WISH for HSC marker *runx1* at 26 hpf in *tbx6:gal4-GFP*;*UAS:NICD* transgenic zebrafish embryos either uninjected or injected with Notch3 morpholino, with or without somite-induced NICD expression. Black arrowheads indicate the presence of HSCs and red arrowheads indicate the absence of HSCs in the aorta-gonad-mesonphros (AGM) regions. Fractions indicate the number of genotyped embryos that displayed the pictured phenotype. (B) Percentages of embryos showing normal (black) or decreased (grey) expression of *runx1* in the AGM regions at 26 hpf in *tbx6+ notch3* morphants. Statistical analysis was performed by means of the Fisher's exact test *$p < 0.05$.

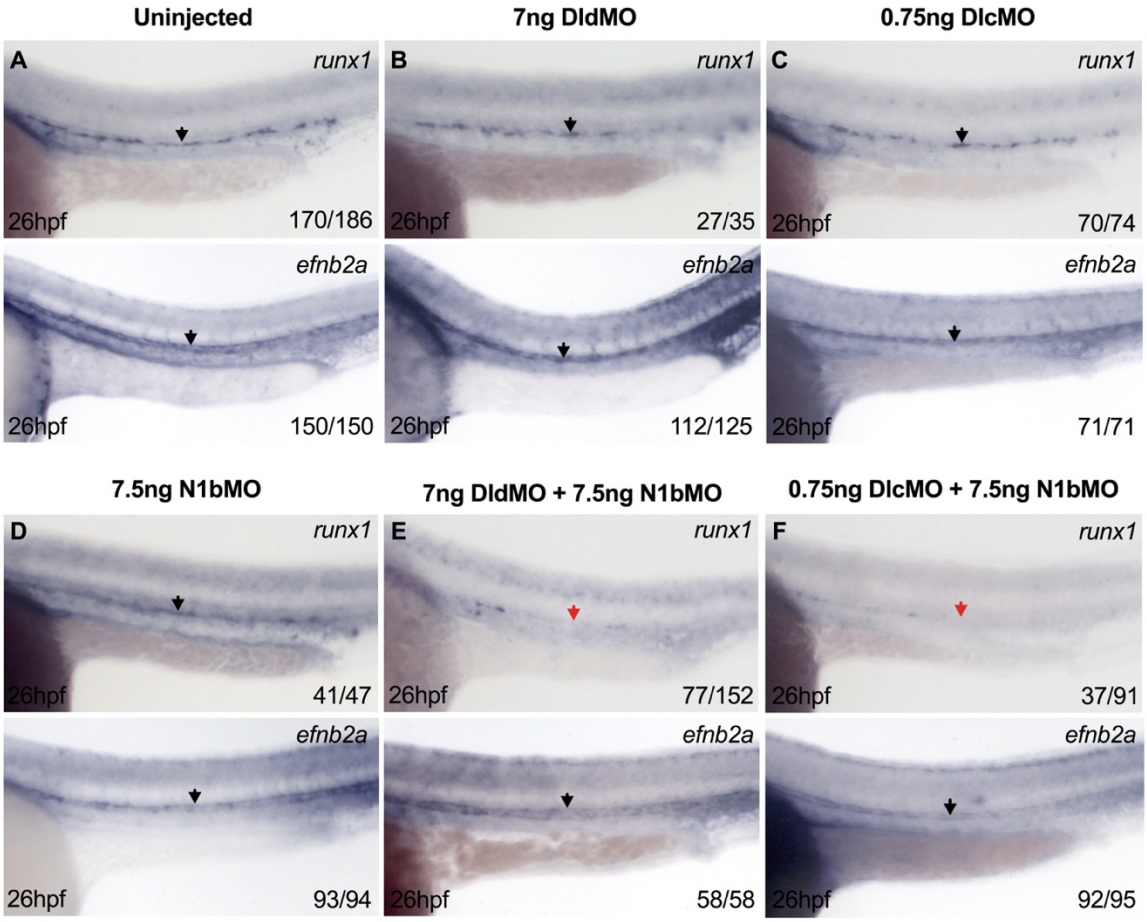

Figure 2. *Notch1b* acts synergistically with *dlc* and *dld* in the same pathway to specify HE. (A-F) WISH for HE marker *runx1* and arterial marker *efnb2a* at 26 hpf in uninjected (A), low-dose knockdown of *dld* (B), low-dose knockdown of *dlc* (C), low-dose knockdown of *notch1b* (D), combinatorial low-dose knockdown of *dld* and *notch1b* (E), and combinatorial low-dose knockdown of *dlc* and *notch1b* (F). Black arrowheads indicate the presence of tissue-specific expression and red arrowheads indicate the absence of tissue-specific expression. Fractions indicate the number of embryos that displayed the pictured phenotype.

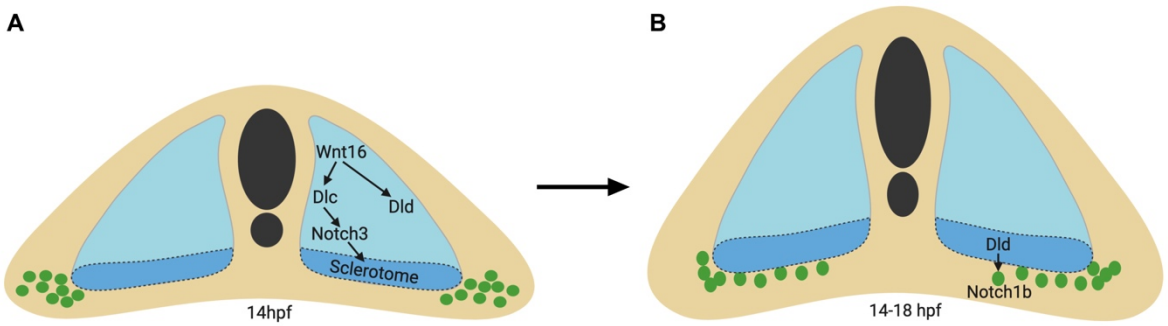

Figure 3. Model of Notch receptor-ligand interactions required for the specification of HE
A schematic model of the Notch receptor-ligand interactions required to establish HE. (A) *Wnt16* regulates the somitic expression of Notch ligands *dlc* and *dld* required for HE specification. Dlc activates somitic Notch3 receptor up to 14 hpf to specify the sclerotome compartment. (B) Dld is presented on the sclerotome and signals to Notch1b on PLM cells between 14-18 hpf.

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