

CORRECTION

Correction: R-spondin 1 is required for specification of hematopoietic stem cells through Wnt16 and Vegfa signaling pathways (doi: 10.1242/dev.139956)

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There was an error in Development (2018) **145**, dev139956 (doi: 10.1242/dev.139956).

On p. 598, the incorrect sequences for the *wnt16* primers were given.

The correct primer sequences are: *wnt16* forward, 5'-ACCGTTCCAAACGTAAGGTG-3'; *wnt16* reverse, 5'-AGCAATAATTGGGCG-ATTTG-3'.

The online version is correct.

The authors apologise to readers for this mistake.

R-spondin 1 is required for specification of hematopoietic stem cells through Wnt16 and Vegfa signaling pathways

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ABSTRACT

Hematopoietic stem cells (HSCs) are the therapeutic component of bone marrow transplants, but finding immune-compatible donors limits treatment availability and efficacy. Recapitulation of endogenous specification during development is a promising approach to directing HSC specification *in vitro*, but current protocols are not capable of generating authentic HSCs with high efficiency. Across phyla, HSCs arise from hemogenic endothelium in the ventral floor of the dorsal aorta concurrent with arteriovenous specification and intersegmental vessel (ISV) sprouting, processes regulated by Notch and Wnt. We hypothesized that coordination of HSC specification with vessel patterning might involve modulatory regulatory factors such as R-spondin 1 (*Rspo1*), an extracellular protein that enhances β -catenin-dependent Wnt signaling and has previously been shown to regulate ISV patterning. We find that *Rspo1* is required for HSC specification through control of parallel signaling pathways controlling HSC specification: Wnt16/DeltaC/DeltaD and Vegfa/Tgfb1. Our results define *Rspo1* as a key upstream regulator of two crucial pathways necessary for HSC specification.

KEY WORDS: HSCs, Wnt signaling, *Rspo1*, Vegfaa, Zebrafish

INTRODUCTION

Hematopoietic stem cells (HSCs) are tissue-specific stem cells that reside in the bone marrow of mammals and maintain hematopoiesis by production of all mature blood cell types (Clements and Traver, 2013). HSCs are used in treatment of various blood diseases including leukemias and anemias. Autologous or allotransplantation of bone marrow, mobilized or cord blood-derived HSCs is used in treatment, but is currently limited by low numbers of HSCs and a lack of suitable donors. A potential solution is by *in vitro*-directed differentiation of HSCs from induced pluripotent stem cells (iPSCs); however, current protocols are not capable of generating normal transplantable HSCs at high efficiency. A better understanding of embryonic development of HSCs and a fuller elucidation of the necessary signaling pathways might inform attempts at *in vitro* specification and expansion of HSCs.

HSCs arise from hemogenic endothelium in the ventral wall of the dorsal aorta (Chen et al., 2009; Taoudi and Medvinsky, 2007; Zovein et al., 2008). The fact that HSCs arise specifically from arterial endothelium, which is derived from splanchnopleural (in mammals) or lateral plate (in anamniotic vertebrates) mesoderm, suggests that this endothelium receives unique hematopoietic

competence that derives from earlier patterning (Clements and Traver, 2013). Of particular interest, canonical Wnt signaling and downstream master regulators of anterior/posterior regionalization, Caudal type homeobox genes (*Cdx*) and their *Hox* gene targets, are crucial for definitive hematopoietic development (Clements and Traver, 2013). Furthermore, only arterial endothelium produces HSCs, indicating that regulation of arteriovenous specification, which relies on Sonic hedgehog (Shh), Vascular endothelial growth factor A (Vegfa), and Notch signaling must somehow be integrated with HSC programming (Clements and Traver, 2013). Overlapping the time when HSCs bud from the dorsal aorta, the vessel is also maturing through intersegmental vessel (ISV) sprouting (Gering and Patient, 2010). Sprouting, restriction of angiogenic tip cells, and arborization are also regulated by Vegfa, Notch, and Wnt signaling (Corada et al., 2010; Gering and Patient, 2010; Gore et al., 2011; Leslie et al., 2007; Siekmann and Lawson, 2007).

A number of signaling pathways converge on the dorsal aorta, resulting in initiation of the definitive hematopoietic program, which can be observed through expression of the conserved master hematopoietic regulator *runt related transcription factor 1* (*Runx1*) (Kalev-Zylinska et al., 2002; North et al., 2002; Okuda et al., 1996). Notch (Burns et al., 2005; Butko et al., 2016; Clements and Traver, 2013; Gering and Patient, 2010; Lawson et al., 2001), Bone morphogenetic protein (*Bmp*) (Wilkinson et al., 2009), Wnt (Goessling et al., 2009; Luis and Staal, 2009; North et al., 2007; Ruiz-Herguido et al., 2012), Shh (Chen et al., 1996; Gering and Patient, 2005; Lawson et al., 2002; Wilkinson et al., 2009), Vegfa (Burns et al., 2005; Gering and Patient, 2005, 2010; Lawson et al., 2003), and Tgfb1 (Monteiro et al., 2016) all contribute to HSC program initiation.

One of the earliest signaling axes determined to be involved in HSC specification was the Shh/Vegfa/Notch signaling axis (Clements and Traver, 2013; Gering and Patient, 2005; Wilkinson et al., 2012; Williams et al., 2010). Extensive work in mouse and zebrafish supports the idea that endothelial Notch1-mediated signaling is required for HSC specification and expression of the master hematopoietic regulator, *Runx1* (Burns et al., 2005; Hadland et al., 2004; King et al., 2006; Kumano et al., 2003). It is not clear to what extent this role of Notch is distinct from its role in arteriovenous specification (Clements and Traver, 2013). Also, the activation of *Runx1* is not direct, but likely involves intermediate activation of GATA binding protein 2 (*Gata2*) (Butko et al., 2015; King et al., 2006) and perhaps other factors. From work in fish, Notch receptor expression is regulated by both Shh and Vegfa and both are also required for HSC specification (Gering and Patient, 2010; Lawson et al., 2002; Williams et al., 2010). Shh can activate somite expression of *vegfaa* (Lawson et al., 2002). Together, these results lead to a pathway where Shh turns on *vegfaa* in the somites, and this ligand in turn activates Notch receptor display in the endothelium, allowing reception of Notch ligands required for HSC specification. More recently, published work demonstrated that

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Received 17 May 2016; Accepted 22 December 2016

Vegfa induces HSC specification by activating endothelial expression of *tgfb1a* and *tgfb1b*, as well as notochord expression of *tgfb3*, and that these ligands are required to stimulate autocrine endothelial expression of *jag1a*, leading to HSC specification (Monteiro et al., 2016). It thus seems that Vegfa has distinct roles in directing HSC specification.

Interestingly, in vertebrates multiple isoforms of Vegfa are expressed, although the *in vivo* significance of these isoforms has been difficult to access. In *Xenopus*, a short isoform of Vegfa, Vegfa-122 (equivalent to Vegfa-121 in other vertebrates), seems to be responsible for arterial gene expression (Ciau-Uitz et al., 2010; Leung et al., 2013). Conversely, treatments causing a loss of the intermediate isoform Vegfa-170 (equivalent to Vegfa-165 in other vertebrates) cause HSC specification to be specifically disrupted without extensive alterations in arterial patterning (Ciau-Uitz et al., 2010). There are also a growing number of pathways that are able to bypass one or more steps of the ‘canonical’ Shh/Vegfa/Notch arteriovenous/HSC specification process under the right conditions (Ciau-Uitz et al., 2010; Wilkinson et al., 2012).

Further, Wnt signaling is required for HSC specification. Signaling downstream of the Wnt family has been loosely categorized into ‘canonical’ β -catenin-dependent and ‘non-canonical’ β -catenin-independent branches (Niehrs, 2012). We previously determined that non-canonical signaling downstream of Wnt16 is necessary for HSC specification (Clements et al., 2011). Rather than acting as a direct proximal specification signal, Wnt16 seems to work through a series of relay signals that are required both for proper somite patterning and eventual HSC specification (Clements et al., 2011). Wnt16 is required for somitic expression of the Notch ligands *deltaC* (*dlc*) and *deltaD* (*dld*) (Clements et al., 2011), which in turn signal through Notch3 (Kim et al., 2014) to activate expression of unknown specification signals that act on the hemogenic endothelium.

Canonical Wnt signaling is also involved in specification and early maintenance of HSCs, both at the level of establishing hematopoietic tissue competence (Clements and Traver, 2013), and evidently within the hemogenic endothelium and nascent HSCs themselves. Wnt3a is required for both normal numbers and behavior of developing HSCs (Luis and Staal, 2009), and an allelic series of β -catenin mutants indicates that canonical Wnt signaling is required for definitive hematopoietic development (Ruiz-Herguido et al., 2012). Prostaglandin E2 is required for early HSC persistence through a β -catenin-dependent mechanism (Goessling et al., 2009; North et al., 2007). These results demonstrate that HSC development relies on β -catenin-dependent Wnt signaling at multiple points during development.

One factor that modulates Wnt signaling and is crucial in vascular patterning is R-spondin (Kim et al., 2008, 2006; Nam et al., 2006; Wei et al., 2007), which has been shown to enhance low levels of Wnt signaling. R-spondin 1-4 are involved in numerous developmental and physiological processes through augmentation of both canonical and non-canonical Wnt signaling (Glinka et al., 2011; Jin and Yoon, 2012). R-spondins are also implicated in human disease and hold therapeutic potential as stem cell growth factors (Blaydon et al., 2006; Kim et al., 2005, 2006; Zhao et al., 2009). Mutations that alter the function of R-spondin 1 (Rspo1) have been shown to lead to an XX sex reversal, palmoplantar hyperkeratosis, and a predisposition to squamous cell carcinoma of the skin, showing that Rspo1 is essential for sex determination, skin differentiation and skin malignancy (Parma et al., 2006). R-spondins bind several receptors including frizzled (Fzd) (Nam et al., 2006), Lgr4 and Lgr5 (de Lau et al., 2011, 2014; Glinka et al.,

2011), and LRP6 (Wei et al., 2007). In one mechanism, R-spondin causes augmentation of Wnt signaling through the suppression of the Wnt antagonist ZNRF3, causing a decreased turnover of Wnt receptors FZD and LRP6 (Hao et al., 2012). Embryonically, Rspo1 is involved in a variety of events including formation of the vasculature during development (Gore et al., 2011). Gore et al. described Rspo1 as necessary for ISV growth in the developing embryo through collaboration with canonical Wnt signaling and Vegfc/Vegfr3 (Gore et al., 2011).

Many of the signaling pathways involved in arteriovenous specification and angiogenesis in the developing embryo have also been shown to be important in HSC specification. Given the role of Rspo1 in vascular patterning, we speculated it could be involved in HSC specification. We took advantage of strong conservation of hematopoietic patterning and the experimental tractability of the zebrafish to examine HSC specification using established mutants, knockdown, gain-of-function, and visualization approaches in this model system. In this study, we find that Rspo1 is required to activate two key HSC specification pathways: Wnt16/Dlc/Dld and Vegfa/Tgfb.

RESULTS

***rspo1* is widely expressed in the developing embryo**

HSCs are specified from the trunk dorsal aorta and the signaling pathways that regulate them are active there. Previously, *rspo1* expression was described in the head and the dorsal aorta of the embryo (<http://zfin.org/ZDB-PUB-010810-1>; Gore et al., 2011). We wanted to examine *rspo1* expression more widely over the time frame relevant to HSC specification, with particular emphasis on the trunk of the developing zebrafish embryo. Using a probe specific for the 3'UTR of Rspo1 and whole-mount *in situ* hybridization (WISH), *rspo1* was expressed in both the head and the trunk of the developing embryo from 16 h post-fertilization (hpf) through to at least 51 hpf (Fig. S1). Trunk expression is ubiquitous at a low level, with stronger expression in the neural tube and nearby dorsal somite, as well as the ventromedial somite near the developing dorsal aorta. By reverse transcription-PCR, we showed that *rspo1* is maternally expressed and is clearly visible through 36 hpf (Fig. S1).

***rspo1* is required for HSC specification**

Rspo1 was previously shown to control vascular patterning (Gore et al., 2011). As many vascular patterning pathways are also involved in HSC specification, we wanted to examine the possibility that Rspo1 might also be necessary for HSC specification. In a previous study, a role for Rspo1 in regulating primitive (not HSC-derived) erythrocyte development (as marked by *gata1* expression at 18 hpf) was examined, but whether definitive HSC specification was affected was not determined (Gore et al., 2011).

To determine if *rspo1* is required for HSC specification, we performed loss-of-function studies using a previously published splice-blocking morpholino (Rspo1 MO) (Gore et al., 2011) as well as the *rspo1* mutant, *down the tubes* (*dtv*¹³⁵, a gift from B. Weinstein; Gore et al., 2011), which carries an S193L mutation and causes the same vascular patterning defects as morpholino knockdown. We confirmed that expression of *rspo1* is greatly reduced in Rspo1 MO-injected embryos (Fig. S1M, Fig. S2). The primary vessels (dorsal aorta and posterior cardinal vein) in the trunk are maintained, but display the previously described (Gore et al., 2011) stunted intersegmental vessel sprouting morphology (Fig. S3G,H). *rspo1* homozygous mutants can be positively identified by their vascular phenotype and yolk sac extension

defect. Both *Rspo1* MO-injected and *dtty¹³⁵* homozygous mutant embryos, compared with uninjected, wild-type (WT) or heterozygous siblings, had a significant loss of HSCs as indicated by comparative reduction in cells positive for the HSC markers *runx1* at 24 hpf (Fig. 1A-F) and *cmyb* at 36 hpf (Fig. 1G-L). *dtty¹³⁵* and *Rspo1* morphants also failed to develop thymic *rag1⁺* T lymphocytes, which develop from an HSC precursor, from 96 hpf (Fig. 1M-R). These results indicate that *rsपो1* is required for HSC specification.

To examine the specificity of the *Rspo1* loss-of-function phenotype, we examined the surrounding tissues by looking at the expression of several genes marking important anatomical structures in *Rspo1* morphants. Consistent with previous reports (Gore et al., 2011), *Rspo1* morphants have normal primary vasculature [*cadherin-5* (*cdh5*); Fig. S3A,B], dorsal aorta (*notch1b*; Fig. 3J,K; *dlc*; Fig. S3C,D; *efnb2a*; Fig. S3E,F), primitive blood (*gata1*; Fig. S4A,B), somite myotome (*myoda*; Fig. S4C,D), neural crest (*crestin*; Fig. S4E,F), and thymic epithelium (*foxn1*; Fig. S4G,H), indicating that the requirement for *rsपो1* in hematopoietic development is specific, and not the result of failure to generate arterial hemogenic endothelium or more global patterning defects.

Rspo1 regulates *wnt16*, *dlc* and *dld* expression

To determine the cause of HSC specification defects in *Rspo1* loss-of-function animals, we systematically examined the state of signaling inputs known to be required for HSC specification. One pathway known to be involved in HSC specification is the Wnt16/Dlc/Dld pathway (Clements et al., 2011). The expression domains of *wnt16* and *rsपो1* overlap in the somite (Figs S1 and S2), raising the possibility that *Rspo1* might regulate *wnt16* expression, which could explain HSC loss. To determine if there is any alteration in *wnt16* expression in *Rspo1* knockdown animals, we examined the expression of *wnt16* in *Rspo1* MO-injected embryos. *Rspo1* morphants had decreased *wnt16* expression in the dorsoanterior portion of each somite at all time points examined (18, 22 and 24 hpf) as compared with WT embryos (Fig. 2A-I). The region where *wnt16* expression is diminished in *Rspo1* morphants coincides with the endogenous *rsपो1* expression domain in the dorsal somite and nearby neural tube. We confirmed the apparent loss of expression observed by *in situ* hybridization using quantitative real time-PCR (qRT-PCR) (Fig. 2J), which demonstrated that *wnt16* transcription was significantly decreased in embryos at 18, 20 and 24 hpf. Knockdown of *wnt16* using a previously described Wnt16 splice-blocking morpholino (Clements

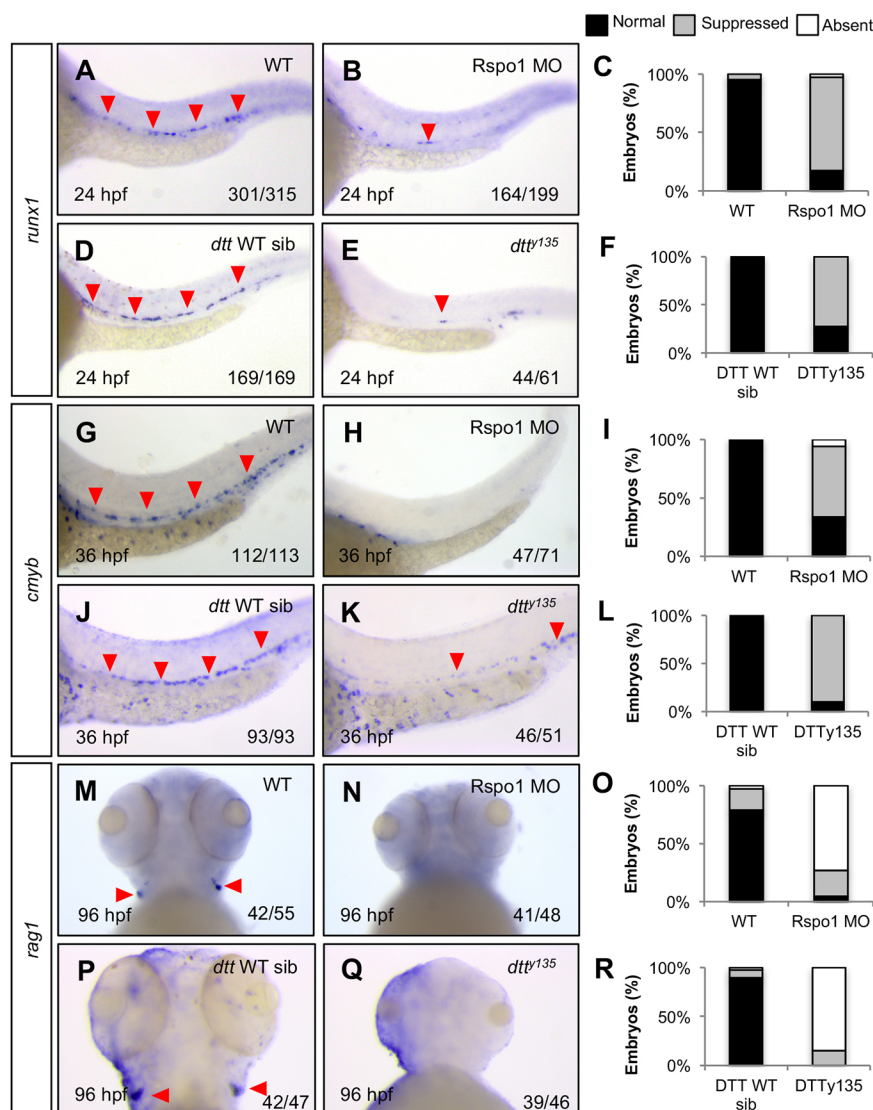
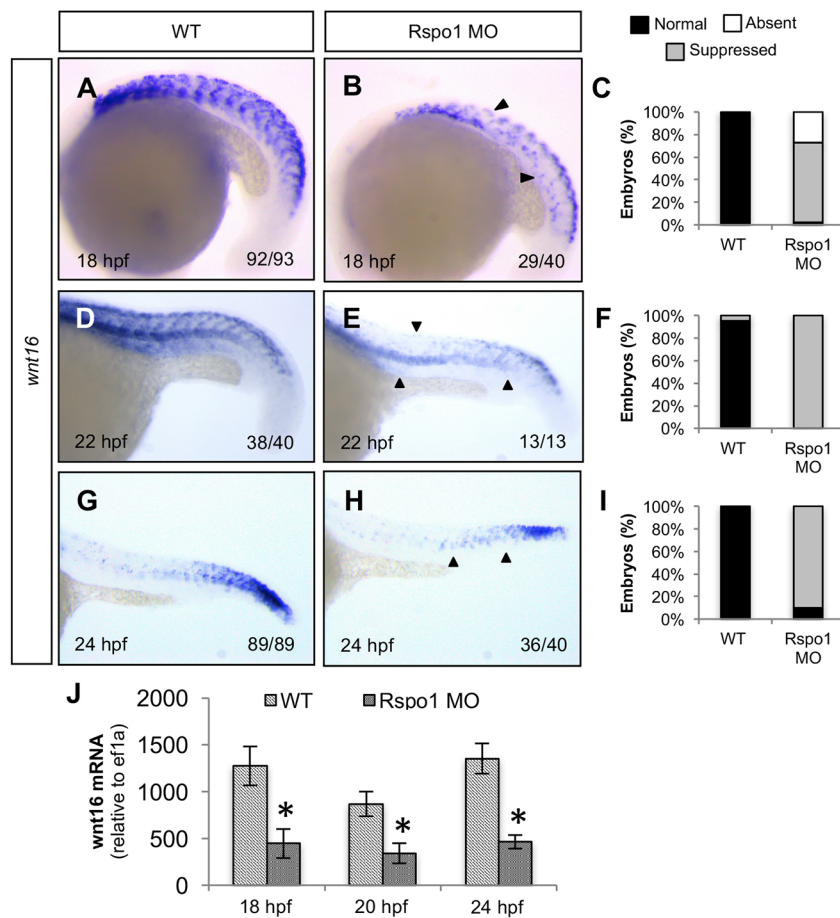


Fig. 1. *Rspo1* is required for HSC specification. The HSC markers *runx1* (A,B,D,E) and *cmyb* (G,H,J,K), which identify HSCs emerging from the dorsal aorta, as well as the T lymphocyte marker *rag1* (M,N,P,Q), found in thymic T cells, were examined at 24 hpf, 36 hpf and 96 hpf as indicated, by whole-mount *in situ* hybridization (WISH) in wild-type (WT; A,G,M), *Rspo1* MO-injected (B,H,N), *Rspo1* wild-type and heterozygous siblings (D,J,P), or *rsपो1 dtty¹³⁵* homozygous mutants (E,K,Q). Red arrowheads indicate HSC precursors in the dorsal aorta (A,B,D,E,G,J,K) or T lymphocytes in the thymus (M,P), when present. Numbers of embryos displaying the depicted phenotype are indicated. (C,F,I,L,O,R) Histograms illustrate percentages of phenotypes. Lateral mid-trunk or ventral head views at 160× magnification.



et al., 2011) did not have a reciprocal effect on *rspo1* expression but did cause the expected decrease in *wnt16* (Fig. S2), *runx1* (Fig. S2), *dlc* (Fig. S5), and *dld* expression (Fig. S5). Our results indicate that Rspo1 regulates expression of *wnt16*, which might explain the observed defects in HSC specification.

Wnt16 activates somitic expression of two Notch family ligands, *dlc* and *dld*, to specify HSCs (Clements et al., 2011). To determine if decreased *wnt16* in Rspo1 leads to the expected decrease in *dlc* and *dld*, we examined their expression in Rspo1 morphants. Rspo1 morphants had a consistent decrease in *dlc* and *dld* expression by both *in situ* hybridization and qRT-PCR (Fig. 3A-F,S,T). As with the loss of *dlc* and *dld* observed in Wnt16 loss-of-function animals, this decrease was confined to the formed somites and not observed in the pre-somitic mesoderm (Clements et al., 2011), which we also confirmed by qRT-PCR analysis of the trunk (Fig. 3S,T). As predicted, Rspo1 morphants did not have altered Notch receptor expression (Fig. 3G-R). These results are consistent with the possibility that failure of HSC specification in Rspo1 loss-of-function animals is the result of abrogation of the Wnt16/Dlc/Dld pathway; however, we cannot exclude the possibility that Rspo1 regulates *dlc* and *dld* directly.

Rspo1 regulates *vegfaa*, *tgfb1a*, and *tgfb1b*

We wanted to examine the state of additional signaling pathways with known importance in HSC specification. Signaling through the Vegfa receptor, Kinase insert domain receptor like (Kdr1; also known as Flk1 and Vegfr2), has previously been shown to be required for HSC specification (Gering and Patient, 2005; Monteiro et al., 2016), and multiple perturbations where *vegfa* expression is altered lead to

HSC defects in *Xenopus* (Ciau-Uitz et al., 2010; Leung et al., 2013). These results indicate that Vegfa signaling is required for HSC specification, although it has been difficult to discriminate this requirement from its role in arteriovenous specification (Lawson et al., 2002), which is likely a precondition for HSC specification. We found that Rspo1 morphants had decreased *vegfaa* expression in somites compared with WT controls by both *in situ* hybridization and qRT-PCR (Fig. 4A-C,S). As Shh signaling regulates *vegfaa* expression (Lawson et al., 2002), we examined the state of Shh target gene expression as well as *shha* itself to determine if decreased *vegfaa* expression is due to defects in Shh signaling. Both *shha* and its target *ptch2* (previously known as *ptc1*) were unaffected in Rspo1 morphants (Fig. 4D-I). Our results indicate that Rspo1 regulation of *vegfaa* is distinct from its regulation by Shh.

Recent work has uncovered a role for *vegfaa* in HSC specification by controlling Tgfb β family autocrine signaling that in turn regulates *jag1a* expression (Monteiro et al., 2016). To determine whether this pathway might be affected in Rspo1 morphants, we examined the expression of *tgfb1a*, *tgfb1b*, and their putative receptor, *tgfbr2*. Expression of both ligands, *tgfb1a* and *tgfb1b*, was decreased in Rspo1 morphants whereas the receptor, *tgfbr2*, was unaffected (Fig. 4J-R). Our results are consistent with the idea that Rspo1 regulates the Vegfa/Tgfb β HSC specification axis.

We previously showed that *vegfaa* expression is not regulated by Wnt16 (Clements et al., 2011), suggesting that Rspo1 regulates two independent signaling inputs required for HSC specification, Wnt16/Dlc/Dld and Vegfa/Tgfb β . To better understand the separability of the Wnt16 and Vegfa pathways, we tested whether Vegfa could rescue *dlc* and *dld* expression in Rspo1 morpholino-injected animals. We

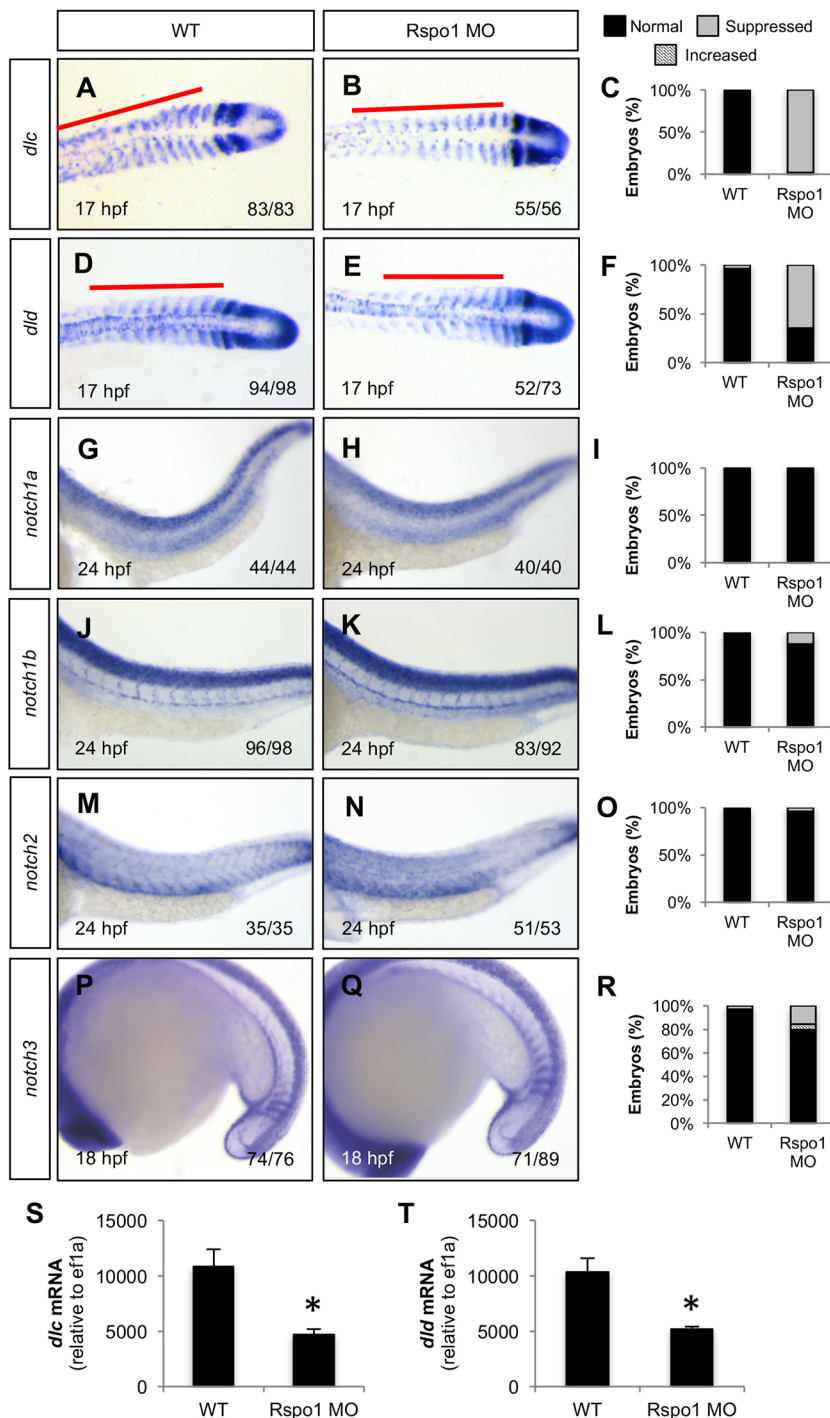


Fig. 3. Notch pathway gene expression in Rspo1 morphants.

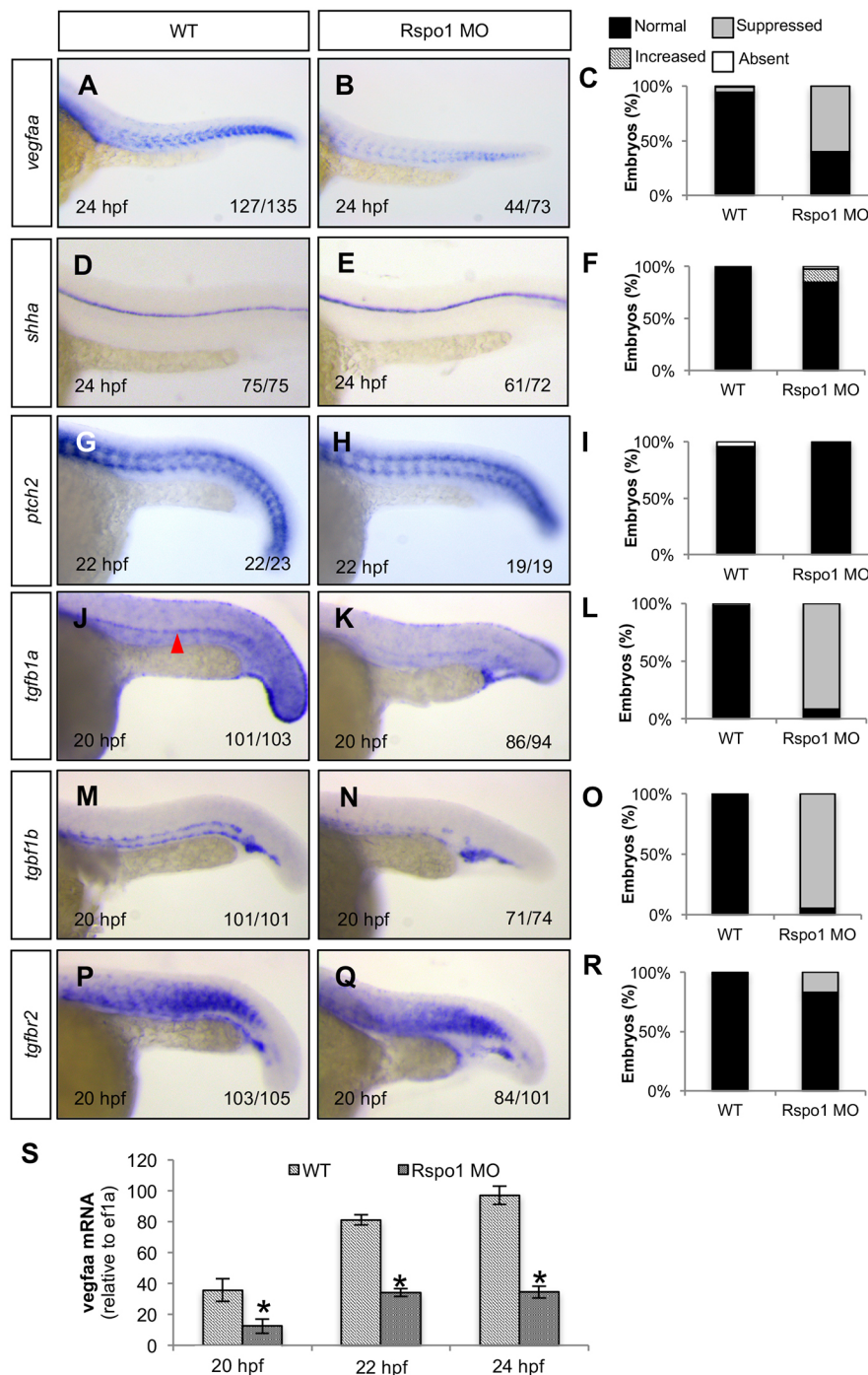
Wild-type (A,D,G,J,M,P) or Rspo1 MO-injected animals (B,E,H,K,N,Q) were examined by WISH for expression of the Notch ligands *dlc* (A,B), *dld* (D,E), or Notch receptors *notch1a* (G,H), *notch1b* (J,K), *notch2* (M,N) and *notch3* (P,Q) at the time points indicated. Compared with wild-type (A,D), *dlc* and *dld* are specifically decreased (B,E) in the formed somites (below red bars), but not the pre-somitic mesoderm. Notch receptor expression is unaffected. Flat mount dorsal views of the trunk or lateral views at 160 \times magnification. Numbers of embryos displaying the depicted phenotype are indicated. (C,F,I,L,O,R) Histograms illustrate percentages of phenotypes. (S,T) Decreased *dlc* and *dld* mRNA in Rspo1 knockdown (Rspo1 MO) was confirmed by qRT-PCR, normalized to expression of *ef1a*, of transcript isolated from dissected embryo trunks (mean \pm s.e.m.). * $P\leq 0.0006$, determined by one-way ANOVA.

found that neither injection of *vegfaa-121*, nor *vegfaa-165* mRNA could rescue *dlc* or *dld* expression (Fig. S6). Similarly *dlc*, *dld* or *wnt16* mRNA could not recover *vegfaa* expression in Rspo1 morphants (Fig. S7). Thus, altered *vegfaa* expression in Rspo1 morphants is independent of changes in the Wnt16/Dlc/Dld pathway, highlighting the separability of these inputs.

Dlc, dld and vegfaa can rescue HSCs in Rspo1 morphants

Our results show that in Rspo1 knockdown animals, expression of *wnt16*, as well as its downstream effectors *dlc* and *dld*, is diminished. Likewise, we observe loss of *vegfaa*, *tgfb1a* and *tgfb1b* expression in Rspo1 morphants. To determine whether either

or both of these alterations might explain the loss of HSCs observed in Rspo1 knockdown animals, we performed a series of rescue experiments. mRNA encoding *dlc* and *dld*, alone or in combination, was injected at the 1-cell stage, and we then investigated HSC specification by examination of the markers, *runx1* and *cmyb*, and the T lymphocyte marker, *rag1*, which provides an additional readout of the state of definitive hematopoietic development. We found that, compared with WT (Fig. 5A-F), *dlc* and *dld* alone could partially restore HSCs (Fig. 5G-L), and that *dlc* and *dld* together could fully restore HSC specification in Rspo1 morphants (Fig. 5M-O). These results indicate that decreased *dlc* and *dld* are responsible for hematopoietic defects observed in Rspo1 morphants.



In complementary experiments, we tested the ability of Vegfa to rescue HSC specification in Rspo1 morphants. Previous reports have suggested isoform specificity of Vegfa in regulating arterial gene expression and hematopoietic induction specifically, where the short form, Vegfa-121, regulates arterial gene expression and intermediate form, Vegfa-165/170, controls HSC specification (Ciau-Uitz et al., 2010; Leung et al., 2013). As arterial gene expression is unaffected in Rspo1 morphants (this study; Gore et al., 2011), we predicted that Vegfa-165 (the intermediate isoform in zebrafish) might be the form specifically affected. Consistent with this hypothesis, we found that injection of *vegfaa-165*, but not *vegfaa-121*, was able to rescue HSC specification (Fig. 5P-U).

DISCUSSION

Our results demonstrate that Rspo1 controls expression of genes in, and thus the activity of, two key HSC specification pathways: Wnt16/Dlc/Dld and Vegfa/Tgfb. Importantly, HSC specification defects can be rescued by supplying mediating factors via mRNA injection in Rspo1 morphants, indicating that alterations in expression of these genes do indeed explain the loss of HSCs in Rspo1 loss-of-function. This is the first description of a requirement for Rspo1 in HSC specification. Our results provide an upstream integration of two pathways crucial for hematopoietic stem cell specification.

Rspo1 amplifies Wnt signaling by allowing Wnt receptors to stay on the cell membrane surface by blocking ubiquitination (Binnerts

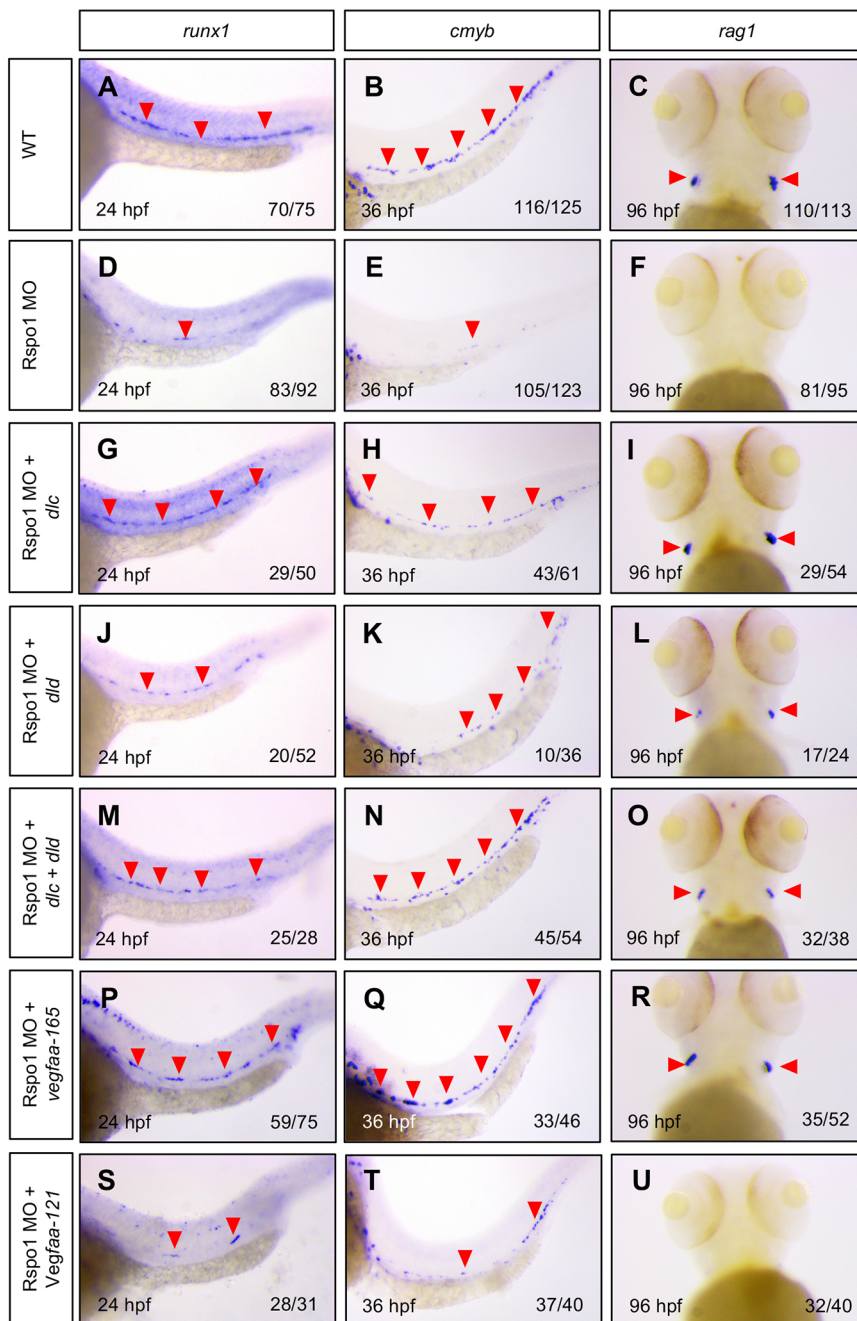


Fig. 5. *Dlc*, *dld* and *vegfaa-165* can rescue HSC specification in *Rspo1* morphants. Expression of the HSC markers *runx1* (A, D, G, J, M, P, S) and *cmyb* (B, E, H, K, N, Q, T), and the T lymphocyte marker *rag1* (C, F, I, L, O, R, U) in the thymus were examined at 24 hpf, 36 hpf, and 96 hpf as indicated, by WISH in wild-type embryos (A-C), or embryos injected with 5 ng *Rspo1* MO (D-F), or embryos injected with 5 ng *Rspo1* MO and mRNAs encoding candidate rescue genes *dlc* (100 ng; G-I), *dld* (50 ng; J-L), *dlc+dld* (50 ng and 25 ng, respectively; M-O), *vegfaa-165* (100 ng; P-R) or *vegfaa-121* (100 ng; S-U). *dlc* and *dld* mRNA were independently able to partially rescue HSCs. Co-injection of *dlc* and *dld* caused a more complete rescue of HSCs. *vegfaa-165*, but not *vegfaa-121*, was able to rescue HSCs. Red arrowheads indicate *runx1*⁺ (A, D, G, J, M, P, S), and *cmyb*⁺ (B, E, H, K, N, Q, T) HSCs, or *rag1*⁺ T lymphocytes (C, I, L, O, R), when visible. Numbers of embryos displaying the depicted phenotype are indicated. Lateral views of the mid trunk at 160× magnification.

et al., 2007; Hao et al., 2012) and recycling of Wnt receptors, or through interruption of the activity of Wnt signaling antagonists (Kim et al., 2008). *Rspo1* has been implicated in both canonical and non-canonical Wnt signaling (Glinka et al., 2011; Jin and Yoon, 2012; Ohkawara et al., 2011). Our data do not define the time at which *Rspo1* is required for its regulation of either pathway. As canonical Wnt signaling directs somitic and lateral plate mesoderm *cdx* and *hox* gene expression, which are required for hematopoietic competence, it is possible that *Rspo1* has a role in this regulation, consistent with its early expression.

During later somitogenesis, *rspo1* is expressed most strongly in the neural tube and dorsal and ventromedial somite (Fig. S1), and in the dorsal aorta (Gore et al., 2011). The fact that *Rspo1* modifies the expression of somite genes [*wnt16* (Fig. 2), *dlc*, *dld* (Fig. 3), and *vegfaa* (Fig. 4)] makes it probable that *Rspo1* is acting within the

somite, likely by modulating canonical Wnt signals known to regulate somite patterning (Geetha-Loganathan et al., 2008). Fig. 6 illustrates a model for *Rspo1* regulation of HSC specification through key stages of development. In our model, *Rspo1* acts to locally amplify signaling by canonical Wnt signal(s), for example *Wnt1*, which is expressed from the neural tube. *Rspo1* is required for optimal expression of *wnt16*, which is expressed in the dorsal somite and regulates downstream Notch ligand expression, *dlc* and *dld*. *rspo1* is also expressed in the ventromedial somite, where it likely potentiates Wnt activation of *vegfaa* expression there. *Vegfaa-165* in turn is required for expression of *tgfb1a*, *tgfb1b*, and downstream *jag1a* expression in the dorsal aorta, ligands recently shown to be necessary for HSC specification (Monteiro et al., 2016). Our results strongly suggest that one or more unknown canonical Wnt ligands regulate *wnt16* and *vegfaa* expression in conjunction with *Rspo1*.

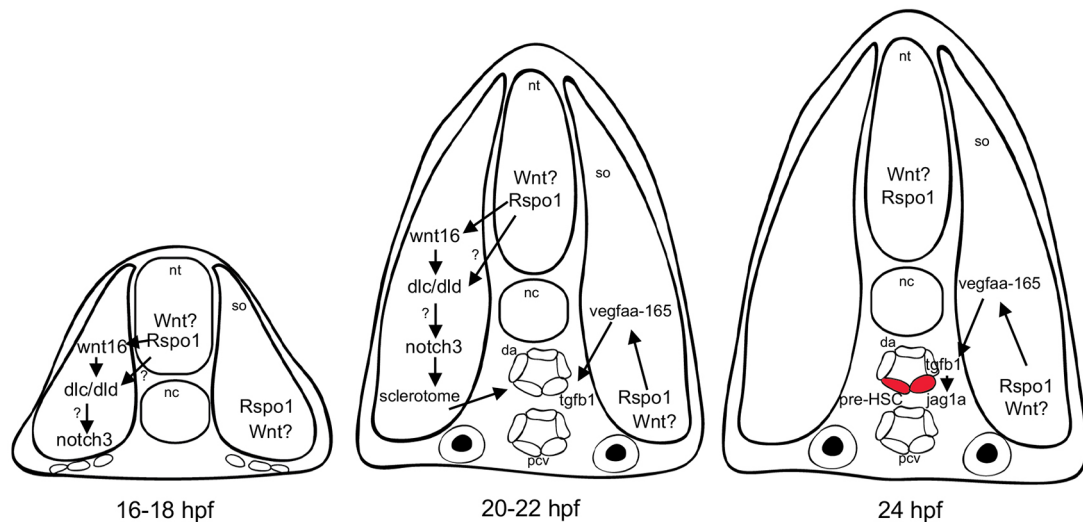


Fig. 6. Model of Rspo1 regulation of the Wnt16/Dlc/Dld and Vegfa/Tgfb HSC specification pathways. Schematic depicting a transverse section through the trunk embryo of a zebrafish embryo at (left) 16-18 hpf prior to formation of the dorsal aorta, (middle) 20-22 hpf just prior to initiation of definitive hematopoiesis, and (right) 24 hpf when HSC precursors (red cells) are first visible in the formed dorsal aorta by expression of the marker *runx1*. Rspo1 regulates expression of key signaling factors in the somite (so), likely by amplifying the signaling activity of undetermined Wnt ligands. *rspo1* is expressed throughout the somite but at higher levels in the neural tube (nt), dorsal and ventromedial somite, and axial vasculature (dorsal aorta, da; posterior cardinal vein, pcv). At 16 hpf *rspo1* is expressed in the dorsal somite and is required for maximal expression of *wnt16*, which acts through relay Notch signals, including *dlc* and *dld*, and additional as-yet-undetermined downstream relay signals. *rspo1* is also expressed in the medial somite where it is required for expression of *vegfa-165*, by directing expression of *tgfb1a* and *tgfb1b* expression in the dorsal aorta. Autocrine Tgfb signaling activates *jag1a* expression required for HSC specification at 24 hpf. nc, notochord.

We previously showed that Wnt16 is necessary for HSC specification (Clements et al., 2011). Wnt16 activates expression of the Notch ligands *dlc* and *dld*, the activity of both of which is required within the somite through Notch3 to optimally specify HSCs, likely through the action of one or more additional downstream relay signals (Kim et al., 2014). Little is known about the signaling regulating *wnt16* expression, although it has previously been shown to be negatively regulated by Shh (Wilkinson et al., 2012) and require *meox* (Nguyen et al., 2014). However, we found no difference in Shh signaling (Fig. 4) or in *meox* expression (data not shown) in Rspo1 morphants. This study confirms and extends our knowledge of the regulation of the Wnt16/Dlc/Dld pathway and begins to define its relationship to signaling through Vegfa/Tgfb.

Vegfa signaling is crucial for vascular development (Carmeliet et al., 1996; Ferrara et al., 1996), and its role in HSC specification is becoming clearer. In *Xenopus*, Vegfa-122 regulates convergence of dorsal aorta angioblast precursors from the lateral plate mesoderm to the midline (Cleaver and Krieg, 1998). Work in *Xenopus* additionally suggests that Vegfa might have isoform-specific roles on vascular patterning with the smaller Vegfa isoform (122 in frogs, 121 in zebrafish) responsible for angioblast migration (Cleaver and Krieg, 1998) and arterial specification, whereas the intermediate Vegfa isoform (170 in frogs, 165 in zebrafish) seems to act more directly to potentiate the HSC program (Ciau-Uitz et al., 2010; Leung et al., 2013). It seems probable that arterial specification is a prerequisite to hematopoietic program development (Clements and Traver, 2013), which does not exclude a subsequent direct role of Vegfa in directing HSC specification. In Rspo1 knockdown animals, we and previous studies find intact arterial patterning (Gore et al., 2011), but loss of HSCs (this work). Hematopoietic defects can be rescued by injection of *vegfaa-165*, but not *vegfaa-121*, further supporting the idea that Vegfa-165 might have a discrete role in HSC specification, independent of arteriovenous specification. The major role of Vegfa in HSC specification was until recently posited to be activation of arterial *notch1b* expression,

which would potentiate reception of the key cell-autonomous Notch signal(s) necessary for HSC specification. Surprisingly, we see no loss of *notch1b* expression, despite a clear decrease in *vegfaa*. Instead we observe decreased expression of key Tgfb family ligands, *tgfb1a* and *tgfb1b*, which have recently been shown to be necessary for HSC specification (Monteiro et al., 2016). Our data fit the idea that Vegfa-165 has a role in HSC specification, independent of induction of *notch1b*, by directing Tgfb ligand expression and downstream display of the Notch ligand, *jag1a*.

A full description of the endogenous processes leading to HSC specification during embryonic development will enhance our understanding of the geography and signaling environment that directs definitive hematopoietic development. A clear understanding of these elements will likely inform attempts to recapitulate normal signaling and derive culture environments that can efficiently replicate *in vivo* specification of HSCs for *in vitro*-directed differentiation of pluripotent cells. Multiple pathways required for HSC specification have now been described, including the Shh/Vegfa/Tgfb/Notch1 and the Wnt16/Dlc/Dld pathways, and it is important to more clearly define how they interact with one another, as well as whether there are facets of the pathways that deviate from expectations, which might suggest the existence of as-yet-unknown regulatory inputs. Here, we describe a previously unknown role for Rspo1, a Wnt amplification factor, in upstream regulation of these pathways, and derive unexpected insight into the proximal signaling events.

MATERIALS AND METHODS

Zebrafish strains and husbandry

Wild-type (WT) zebrafish (*Danio rerio*, WIK and AB lines), the *rspo1* mutant *dt^{v135}* and *Tg(fli1a:EGFP)^{v1}* embryos of both sexes in approximately equal numbers were obtained and maintained in accordance with standard husbandry procedures (Westerfield, 2007) and in compliance with IACUC guidelines and all relevant institutional and national animal welfare laws, guidelines and policies. Mating pairs, ages 3 to 18 months of age, were set up overnight and embryos were collected and

maintained in E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) or 0.03% Instant Ocean (Spectrum Brands).

Cloning, constructs, probes and whole-mount *in situ* hybridization

rspo1 and *rspo1* 3'UTR were amplified from WIK whole-embryo cDNA at 24 h post fertilization (hpf), using primers Rspo1: forward, 5'-GGCGCTGGCAGTGGTCTTCTTCAGCT-3'; reverse, 5'-CCATTCGAACATGATGGAGAACTTCAG-3'; Rspo1 3'UTR: forward, 5'-GGATCCTTCAATCACAGCATCAGGTGGGC-3'; reverse, 5'-CAAGCACAGAATCTTTATACACATACGTC-3', designed based on the NCBI reference sequence NM_001002352, cloned into pCR4-TOPO (Invitrogen) and subcloned into pCS2+ (Turner and Weintraub, 1994). The sequenced clones conform to NM_001002352.1 (*rspo1*) and did not contain any non-silent alterations. *tgfb1a*, *tgfb1b* and *tgfb2* probe fragments were amplified from cDNA using primers *tgfb1a*: forward, 5'-GCATTATGAGGTTGGTTGCTTGG-3'; reverse, 5'-CGCAGTATAACCTCAGCTCCAAGG-3'; *tgfb1b*: forward, 5'-GCAATGTCTGTTGGGATTTGTGC-3'; reverse, 5'-CACCTCTATTGCGGGACAAACCTGC-3'; *tgfb2*: forward: 5'-CAAAGCCAAGCTGA-GACAAAGCC-3'; reverse, 5'-CCTCCATTTGGCTCTAAAGTCCGAG-3', cloned into pCRII (Invitrogen), and verified by sequencing.

Probe synthesis and whole mount *in situ* hybridization was performed as described previously (Clements et al., 2011, 2009) with a minimum of three independent replications. The following probe constructs were used: pBK-CMV *myb* (L. Zon, Boston Children's Hospital and Dana Farber Cancer Institute, Boston, MA, USA), pCRII *crestin* (D. Raible, University of Washington, Seattle, WA, USA), pBS *d1c* (J. Lewis, Cancer Research UK, London, UK), pCS2+ *d1d* (S. Holley, Yale University, New Haven, CT, USA), pBS *efnb2a* (Clements et al., 2011), pBS *gatal* (D. Ransom, National Cancer Institute, Rockville, MD, USA), pCRII *myoda* (Clements et al., 2011), pCR-Script *notch1a* (J. Campos Ortega, Institut für Entwicklungsbiologie, Köln, Germany), pCR-Script *notch1b* (M. Lardelli, University of Adelaide, Adelaide, Australia), pCR-Script *notch2* (B. Appel, University of Colorado, Denver, CO, USA), pCR-Script *notch3* (M. Lardelli), pBS *ptch1* (J. Waxman, Cincinnati Children's Hospital, Cincinnati, OH, USA), pCRII *rag1* (N. Trede, Acetylon Pharmaceuticals, Boston, MA, USA), pCR4 *rspo1* 3'UTR, pCS21 *runx1* (C. Burns, Massachusetts General Hospital, Charlestown, MA, USA), pCS2 *shha* (Clements et al., 2011), pCRII *tgfb1a*, pCRII *tgfb1b*, pCRII *tgfb2*, pCRII *vegfaa-165* (Clements et al., 2011), and pCS2 *wnt16* (Clements et al., 2011). The following constructs were subcloned into pCS2+ from pCRII via *EcoRI*: pCS2+ *vegfaa-165* and pCS2+ *vegfaa-121*.

Transverse *in situ* hybridization sections

Embryos processed by *in situ* hybridization for *rspo1* were embedded in 4.5% low melt agarose. Transverse sections were cut using Leica VT1200 vibratome. Sections were imaged using a Leica M205 FA stereo microscope.

Morpholinos, mRNA and microinjections

Antisense-morpholinos (MOs, Gene Tools, LLC) were diluted to 3 mM stock (25 mg/ml) in DEPC-treated water. The *Rspo1* splice-blocking morpholino (Rspo1 MO, 5'-AGAAACATCAGCACTCACTCCGTC-3') was previously described (Gore et al., 2011). Embryos at the 1-cell stage of development as previously described (Clements et al., 2009) were injected with 5 ng of Rspo1 MO. The *Wnt16* splice-blocking morpholino (*Wnt16* MO2, 5'-GCGTGGAAACTTACATCCAACCTTC-3') was previously described (Clements et al., 2011) and 5 ng was injected at the 1-cell stage of development. Linearized expression constructs pCS2+ *d1c* (Clements et al., 2011), pCS2+ *d1d* (Clements et al., 2011), pCS2+ *vegfaa-165* and pCS2+ *vegfaa-121* were synthesized in house from 5'-G-capped mRNA using mMessage mMachine kit (Ambion) transcription (Clements et al., 2009). For rescue experiments, mRNA was co-injected with 5 ng of Rspo1 MO into 1-cell-stage embryos at concentrations between 25 and 100 pg, as indicated in the text.

Reverse transcription PCR (RT-PCR)

RT-PCR was as previously described (Clements et al., 2011). Briefly, total cellular RNA was isolated from embryos using Trizol reagent and treated

with DNase. cDNA from polyadenylated mRNA was synthesized using SuperScript III first-strand synthesis supermix kit (Invitrogen). PCR for *rspo1* was carried out with custom primers: forward, 5'-GGCGCTGGCACTGGTCTTCTTCAGCT-3'; reverse, 5'-CCATTCGAACATGATGGAG-GAACTTCAG-3'.

Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR was performed following a previously described protocol (Lan et al., 2009). Briefly, uninjected and Rspo1 morpholino-injected embryos were collected. Mid-trunk tissue from 17 hpf, whole 17 hpf, whole 20 hpf, whole 22 hpf, and whole 24 hpf embryos was collected. RNA was isolated from frozen embryos with Trizol. Samples were treated with DNase. cDNA was synthesized using Superscript III immediately following RNA extraction and purification. qRT-PCR was performed with an Applied Biosystems Step One Plus Real-Time PCR System. cDNA was diluted 1:10 and added to 1× Fast SYBR Green Master Mix (Thermo Fisher) plus 300 nM primer in a 20 µl reaction. Samples were run in triplicate and a total of three biological replicates per treatment were analyzed. qRT-PCR data was analyzed using the Step One Plus software. Data shown is relative to *ef1a* expression (mean± s.e.m.). Statistical significance was determined by one-way analysis of variance with a Fisher LSD post-hoc test using StatPlus:Mac Pro software (AnalystSoft). Statistical significance was reached with a *P*-value ≤0.05.

Primers for qRT-PCR were either previously published or designed as previously described (Lan et al., 2009). The following primers were used: *d1c* (Hamada et al., 2014) forward, 5'-GACCGGTGCAGCAGTGACCC-3'; reverse, 5'-TGTGCCCATGAAGCCTGCCG-3'; *d1d* (Hamada et al., 2014) forward, 5'-AGCGACGGCGACAAAAACGGA-3'; reverse, 5'-TGTGGCGTTACACCTCGGTTGC-3'; *ef1a* (McCurley and Callard, 2008) forward, 5'-CTTCTCAGGCTGACTGTGC-3'; reverse, 5'-CCGCTAGCA-TTACCCTCC-3'; *rspo1* (Zhang et al., 2011) forward, 5'-GAAAGGGGC-GTCCTCAGT-3'; reverse, 5'-TGATGGAGGAACCTCAGTGCT-3'; *vegfaa-165* forward, 5'-GCCAAAGGCAGAAGTCAAAG-3'; reverse, 5'-CTTGCAATTCATTTGTGTGA-3'; *wnt16* forward, 5'-ACCGTTCCAAA-CGTAAGGTG-3'; *wnt16* reverse, 5'-AGCAATAATTGGGCGATTTG-3'.

Acknowledgements

We are grateful to Dr Brant Weinstein for gift of the *dtf¹³⁵* fish.

Competing interests

The authors declare no competing or financial interests.

Author contributions

J.R.G. and W.K.C.: Conception and design of research; interpretation of results of experiments; editing and revision of manuscript. J.R.G.: Performed experiments and analyzed data; prepared figures and drafted manuscript. W.K.C.: approved final version of manuscript.

Funding

This work was supported by the National Heart, Lung, and Blood Institute (grant R00HL097150 to W.K.C.) and March of Dimes Foundation Basil O'Connor Starter Scholar Research Award (#5-FY14-42 to W.K.C.). Deposited in PMC for release after 12 months.

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.139956.supplemental>

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Supplemental Figures

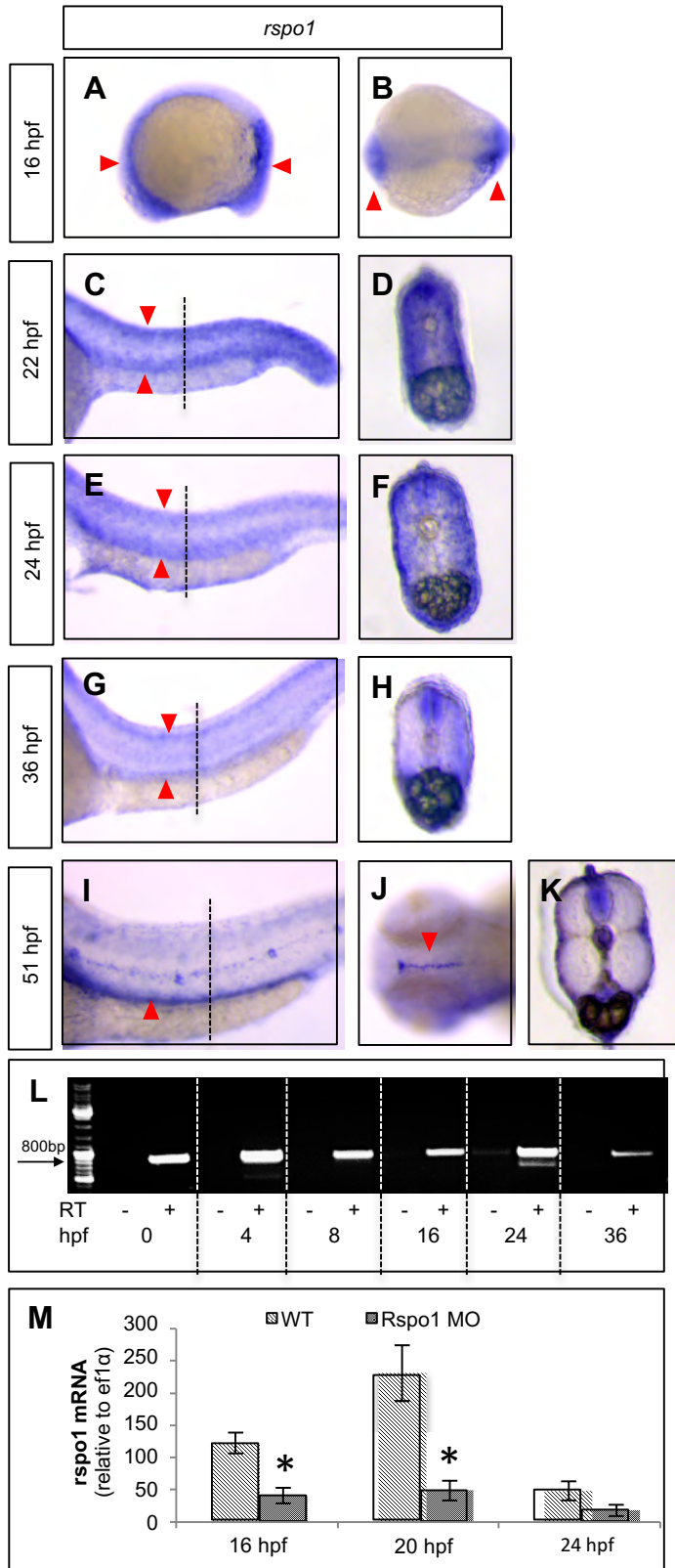


Figure S1. *R-spondin-1 (rspo1)* expression. (A-K) Embryos fixed at the times indicated and processed by WISH for expression of *rspo1* transcript. Lateral (A,C,E,G,I), dorsal (B,J) views or transverse sections (D,F,H,K) are presented, at 70X (A,B) or 160X (C-K) magnification. Black dotted lines indicate the level of transverse sections (C,E,G,I). Red arrowheads identify regions of more abundant *rspo1* expression. *Rspo1* was expressed in the neural tube and in the trunk mesoderm of embryos 22 hpf – 36 hpf (A-H). *Rspo1* expression was limited to the lateral line (I), a cranial ventricular region (J), and an area ventral to the dorsal aorta (K) in 51 hpf embryos. (L) RT-PCR for *rspo1* message in mRNA from whole embryos at the stages indicated. No reverse transcriptase (RT) control lanes are indicated (-). (M) *Rspo1* mRNA levels (mean \pm SEM) relative to *ef1 α* control quantified by q-RT-PCR from uninjected and *Rspo1* MO injected animals. * = statistically significant ($p \leq 0.007$) determined by one-way ANOVA

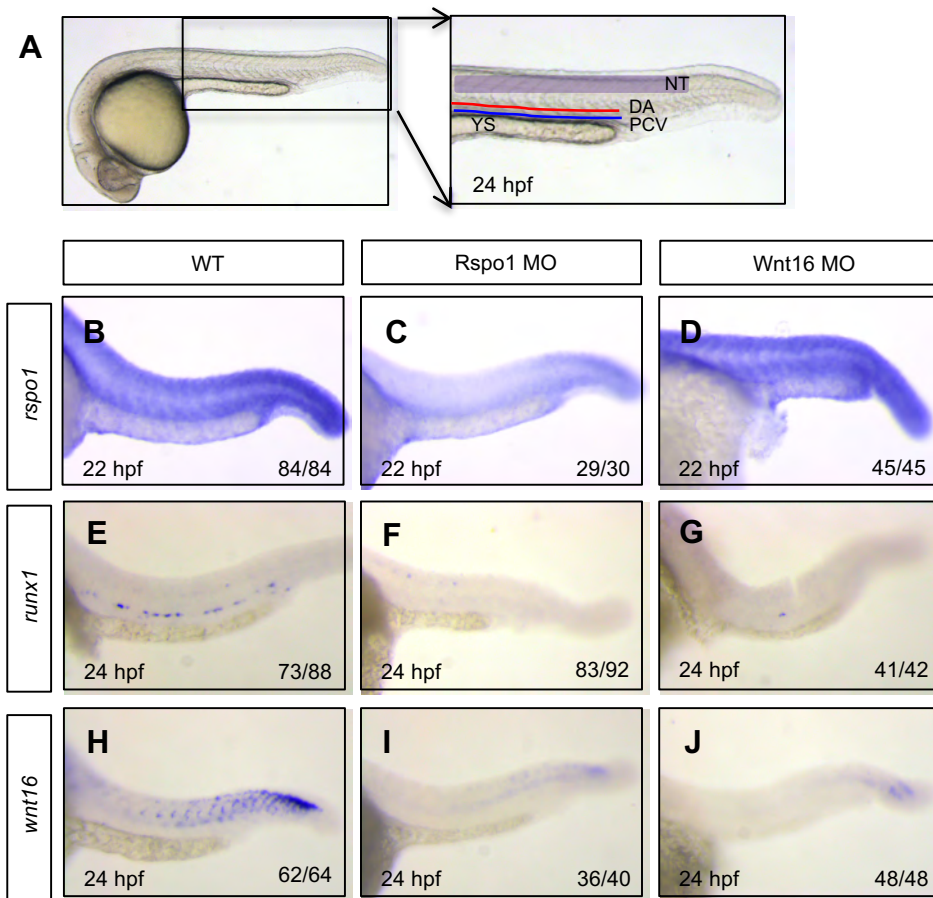


Figure S2. *Rspo1*, *runx1*, *wnt16* levels in *Rspo1* MO or *Wnt16* MO animals.

(A) Image of WT embryo at 24 hpf showing area of images taken and labeled with the location of the neural tube (NT, gray), dorsal aorta (DA, red line), posterior cardinal vein (PCV, blue line), and yolk sac (YS). Representative wild type (WT; B,E,H), *Rspo1* MO (C,F,I), and *Wnt16* MO (D,G,J) embryos processed by WISH for *rspo1* (B-D), *runx1* (E-G), and *wnt16* (H-J) mRNA expression at 22 hpf or 24 hpf as indicated in each image. Lateral views of the trunk at 160x magnification. Numbers of embryos displaying the depicted phenotype are indicated.

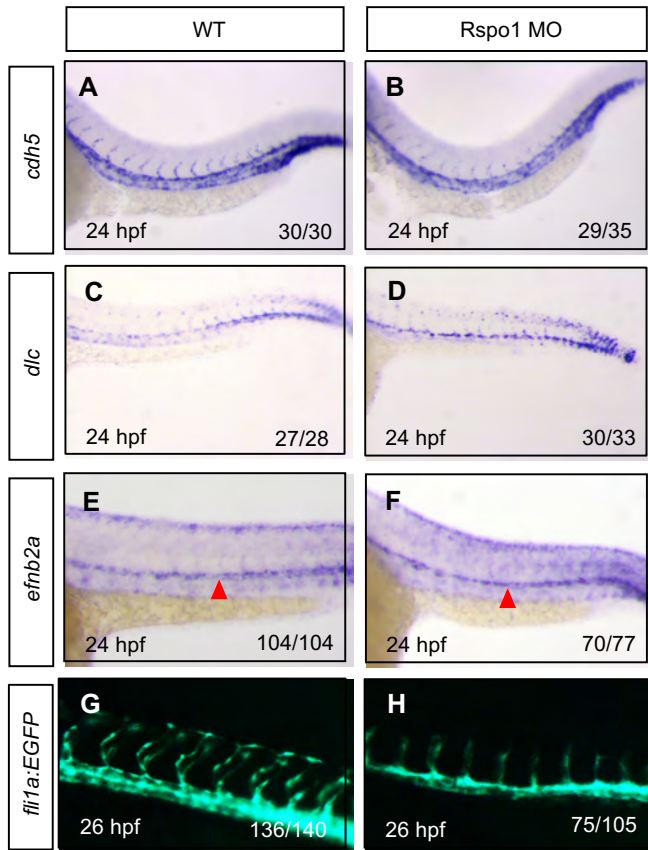


Figure S3. Rspo1 knockdown does not alter primary vascular specification.

Wild-type (A,C,E,G) or Rspo1 MO injected animals (B,D,F,H) were examined by WISH for expression of endothelium (*cdh5*, A,B), arterial identity (*dlc*, C,D and *efnb2a*, E,F), or by fluorescence for vessel patterning (*fli1a:EGFP*, G,H) at the time points indicated, revealing normal patterning. (H) Rspo1 morphants have stunted intersegmental vessel growth at 26 hpf. Numbers of embryos displaying the depicted phenotype are indicated. Magnification 160X.

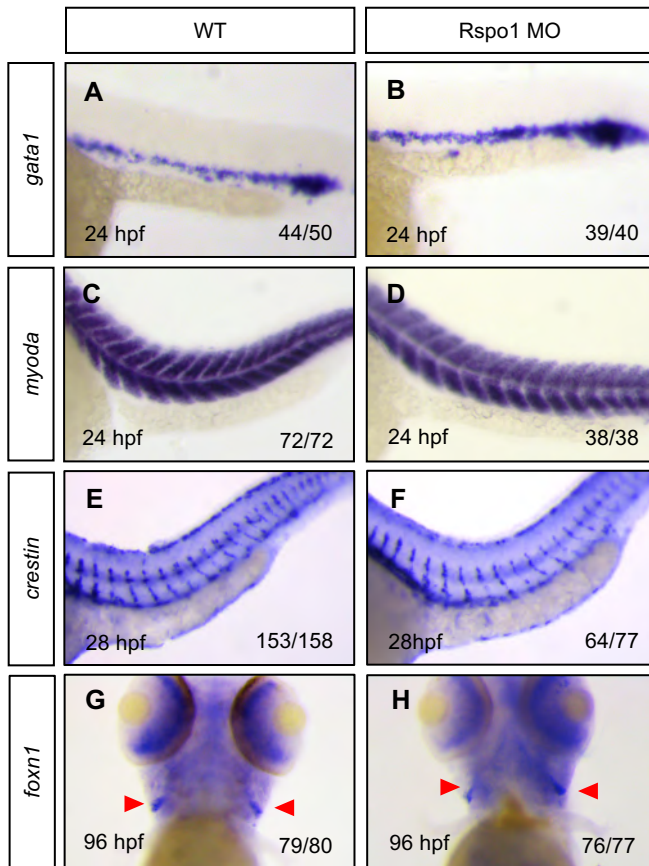


Figure S4. Rspo1 phenotype is specific. Wild-type (A,C,E,G) or Rspo1 MO injected animals (B,D,F,H) were examined by WISH for markers of primitive erythrocytes (*gata1*, A,B), myotome (*myoda*, C,D), neural crest (*crestin*, E,F), or thymic epithelium (*foxn1*, G,H) at the time points indicated, revealing normal patterning. Numbers of embryos displaying the depicted phenotype are indicated. Lateral trunk (A-F) or ventral head views (G,H) are presented. Magnification 160X.

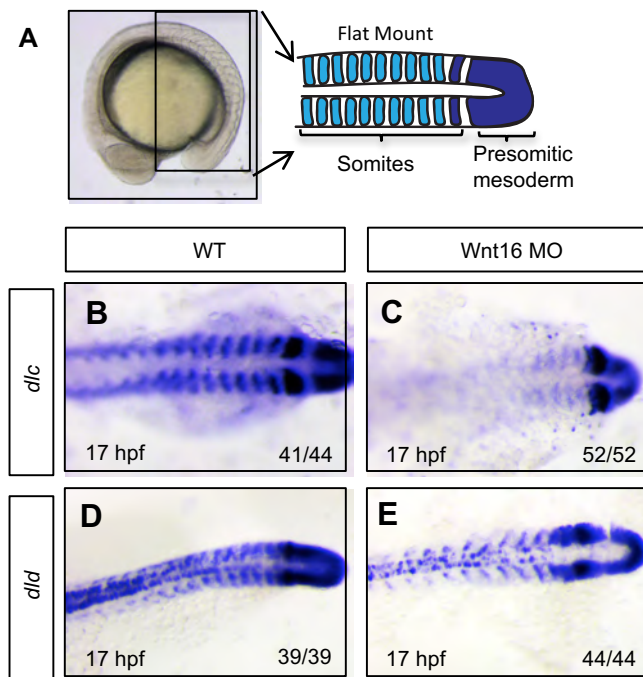


Figure S5. *Dlc* and *dld* expression is decreased in *Wnt16* morphants. (A) Lateral view image of wild-type 17 hpf embryo with box indicating the location of *in situ* images. The cartoon shows a flat mount section of the tail of the embryo with formed somites and the pre-somitic mesoderm indicated. Wild-type (B,D), and *Wnt16* MO (C,E) embryos were examined by WISH for expression *dlc* (B,C) or *dld* (D,E). Numbers of embryos displaying the depicted phenotype are indicated. Magnification 160X.

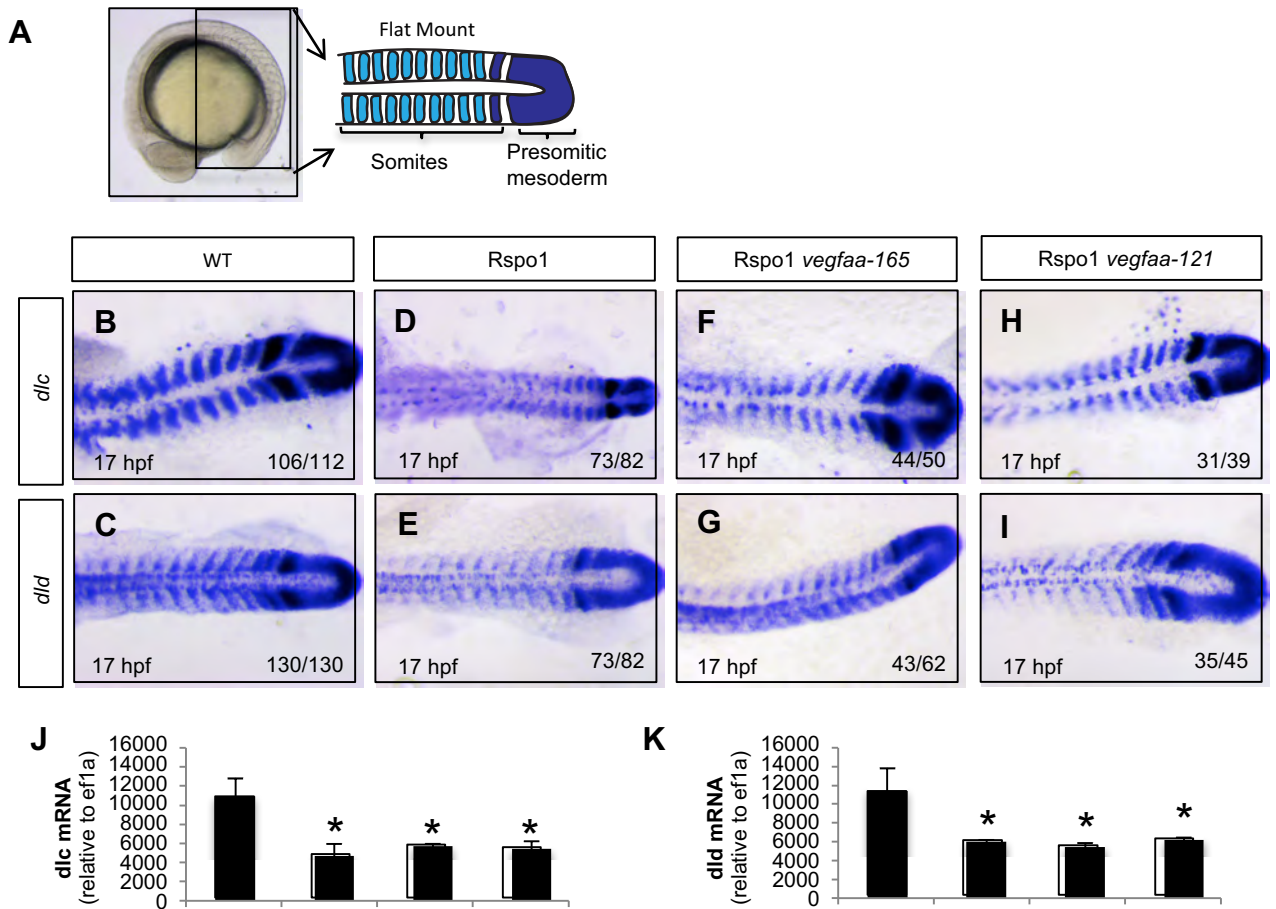


Figure S6. Vegfa does not rescue *dlc* and *dld* expression in Rspo1 morphants.

(A) Lateral view image of wild-type 17 hpf embryo with box indicating the location of *in situ* images. The cartoon shows a flat mount section of the tail of the embryo with formed somites and the pre-somitic mesoderm indicated. Wild-type (B,F), Rspo1 MO (C,G), Rspo1 MO + *vegfaa-165* mRNA (D,H), or Rspo1 MO + *vegfaa-121* mRNA (E,I) were examined by WISH for expression *dlc* (B-E) or *dld* (F-I). Numbers of embryos displaying the depicted phenotype are indicated. Magnification 160X. (J-K) *dlc* and *dld* mRNA (mean±SEM) levels relative to *ef1a* control quantified by qRT-PCR from uninjected (WT), Rspo1 MO Rspo1 MO + *vegfaa-165* mRNA and Rspo1 MO + *vegfaa-121* mRNA injected animals. * significantly different from WT ($p \leq 0.005$) determined by one-way ANOVA.

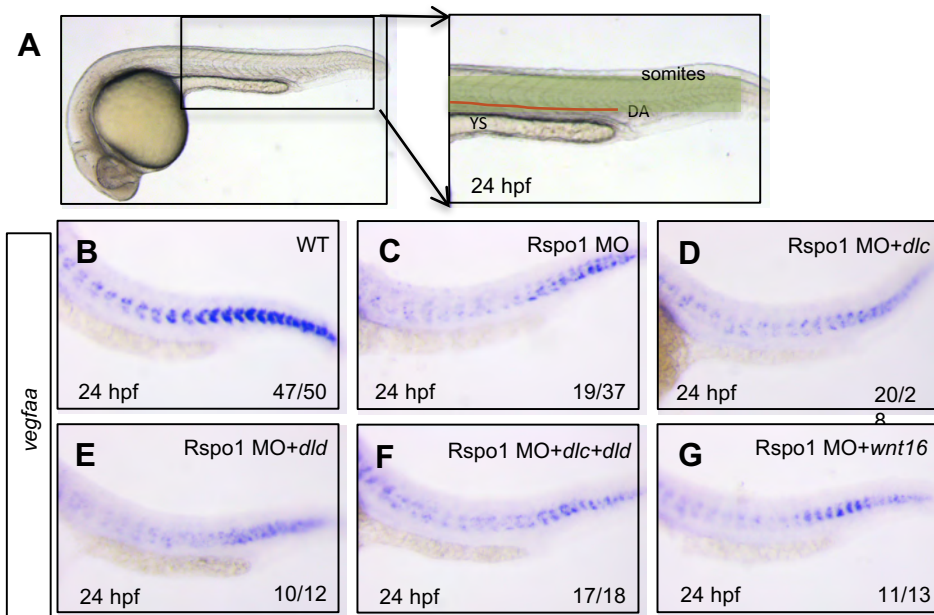


Figure S7. Wnt16, Dlc, and Dld do not rescue *vegfaa* expression in *Rspo1* morphants. (A) Diagram of the location of interest. *Vegfaa* expression is found in the medial somite of the trunk and tail at 24 hpf. *In situ* hybridization for *vegfaa* expression in wild-type (WT; B), *Rspo1* morphants (*Rspo1* MO; C), *Rspo1* MO plus *dlc* mRNA (*Rspo1* MO + *dlc*, D), *Rspo1* MO plus *dld* mRNA (*Rspo1* MO + *dld*, E), *Rspo1* MO plus *dlc* and *dld* mRNA (*Rspo1* MO + *dlc* + *dld*, F), and *Rspo1* MO plus *wnt16* mRNA (*Rspo1* MO + *wnt16*, G). Numbers of embryos displaying the depicted phenotype are indicated. Magnification 160X.