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A Crucial Interaction between Embryonic Red Blood Cell Progenitors and Paraxial Mesoderm Revealed in *spadetail* Embryos

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Summary

Zebrafish embryonic red blood cells (RBCs) develop in trunk intermediate mesoderm (IM), and early macrophages develop in the head, suggesting that local microenvironmental cues regulate differentiation of these two blood lineages. s*padetail* (*spt*) mutant embryos, which lack trunk paraxial mesoderm (PM) due to a cell-autonomous defect in *tbx16*, fail to produce embryonic RBCs but retain head macrophage development. In *spt* mutants, initial hematopoietic gene expression is absent in trunk IM, although endothelial and pronephric expression is retained, suggesting that early blood progenitor development is specifically disrupted. Using cell transplantation, we reveal that *spt* is required cell autonomously for early hematopoietic gene expression in trunk IM. Further, we uncover an interaction between embryonic trunk PM and blood progenitors that is essential for RBC development. Importantly, our data identify a hematopoietic microenvironment that allows embryonic RBC production in the zebrafish.

Introduction

The differentiation of embryonic (primitive) blood cells in zebrafish initiates within two separate intraembryonic environments. The first two blood lineages detected in zebrafish are a transient group of embryonic red blood cells (RBCs) that derive solely from blood progenitors in the trunk (Kimmel et al., 1990; Lieschke et al., 2002) and early macrophage cells that originate from progenitors located in the head (Figure 1A; Herbomel et al., 1999). This spatial restriction of embryonic RBC and early macrophage differentiation may reflect a role for the local environment in specifying these different fates. During adult (definitive) hematopoiesis in mammalian systems, signals from the microenvironment, the so-called "stem cell niche," are thought to regulate hematopoietic stem cells (HSCs), the self-renewing progenitors of all blood lineages (Lemischka and Moore, 2003).

In the zebrafish embryo, RBCs derive from intermediate mesoderm (IM), a bilateral tissue extending along the anterior/posterior axis between trunk paraxial mesoderm (PM) and lateral plate mesoderm (LPM). Zebrafish hematopoietic tissue has been referred to as LPM; however, morphology (Al-Adhami and Kunz, 1977) and molecular markers suggest that IM and LPM are separate tissues. In addition to RBCs, IM gives rise to endothelial and pronephric lineages (Lieschke et al., 2002; Serluca and Fishman, 2001). At mid-segmentation stages, trunk blood and endothelial cells migrate to the midline, forming a single structure, the Intermediate Cell Mass, from which RBCs enter circulation at 24 hpf (hours post fertilization; Detrich et al., 1995; Gering et al., 1998).

Zebrafish homologs of vertebrate hematopoietic regulators include those important for the development of early blood progenitors, such as *scl*, *lmo2*, *hhex*, and *gata2* (Detrich et al., 1995; Liao et al., 1998; Liao et al., 2000; Thompson et al., 1998), as well as those involved in specification and terminal differentiation of the RBC lineage such as *gata1*, *biklf/klf4*, *jak2a*, and embryonic *globin* genes (Brownlie et al., 2003; Detrich et al., 1995; Oates et al., 1999; Quinkertz and Campos-Ortega, 1999). Sequential expression of these key genes in zebrafish hematopoietic domains is similar to that observed in tetrapods (Minko et al., 2003; Silver and Palis, 1997), and is associated with progression of developing blood cells toward lineage restriction (Amatruda and Zon, 1999).

In *Xenopus*, mouse, and chick, developing embryonic blood and endothelial cells require signals from adjacent tissues (Baron, 2003). Although non-cell-autonomous requirements for zebrafish hematopoiesis have been suggested (Liao et al., 2002; Parker and Stainier, 1999), a tissue supplying cues to IM has not yet been identified. One potential source is suggested by zebrafish *spadetail* (*spt*) embryos in which RBC loss is associated with an absence of trunk PM (Kimmel et al., 1989; Thompson et al., 1998). *spt* embryos have a lesion in the T-box transcription factor, *tbx16* (Griffin et al., 1998; Ruvinsky et al., 1998), which is normally required cell autonomously during gastrulation for proper localization of trunk PM precursors (Ho and Kane, 1990). Loss of *spt/tbx16* results in mislocation of these cells to the tail, leaving the trunk severely deficient in PM (Kimmel et al., 1989). The cause of RBC loss in *spt* embryos is unclear, although it does not appear to result from a general blood defect, as early macrophages develop normally in the *spt/tbx16* head and later myeloid cells are detected (Lieschke et al., 2002). Previous investigation of hematopoietic and endothelial gene expression in *spt* embryos (Thompson et al., 1998; Oates et al., 1999) suggested that *spt* functions after blood progenitor specification by affecting transition to RBC fate, possibly at the level of an HSC (Amatruda and Zon, 1999).

To further define the *spt* blood defect, we examined early events in the hematopoietic program, finding a severe loss of both blood progenitor and RBC gene expression. Using reciprocal cell transplantation between wt and *spt* embryos, we discovered not only that *spt/tbx16* function is required cell autonomously within IM during early hematopoiesis, but that an interaction

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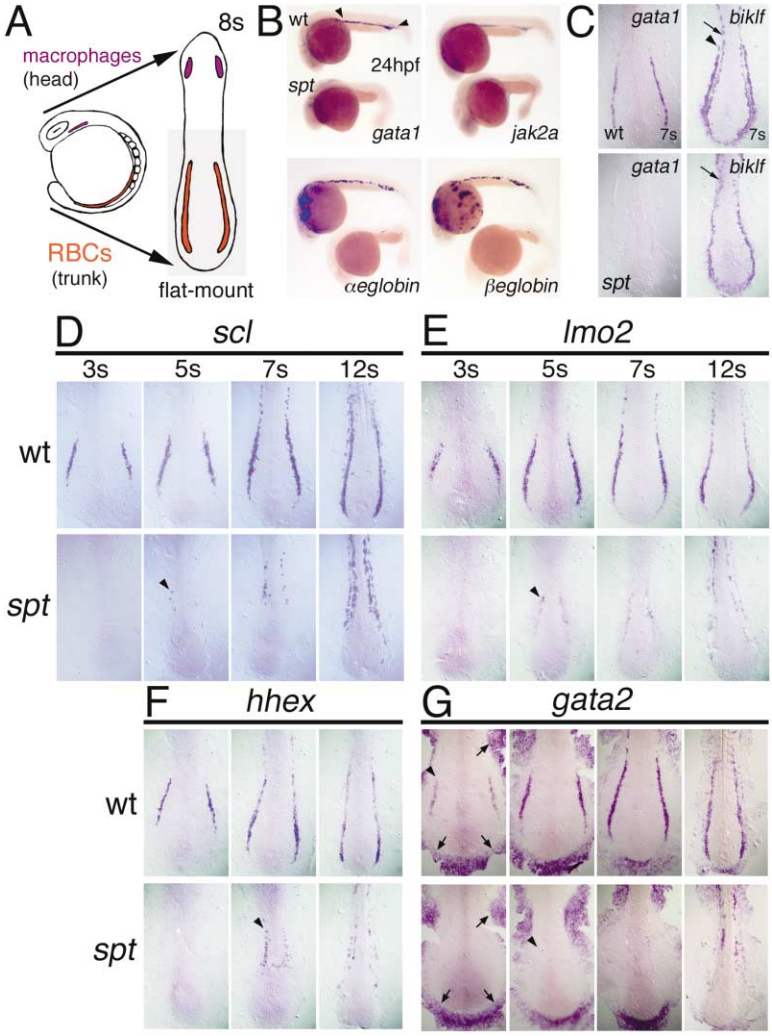

Figure 1. RBC Gene Expression Is Absent in *spt* Embryos

(A) Regions producing early macrophages (head, purple) and RBCs (trunk, red) in 8s embryos. Flat-mount in dorsal view, anterior at top. Gray shading indicates trunk and tailbud area shown in Figures 1–5.

(B) *gata1* (arrowheads), *jak2a*, α*e1globin*, and β*e1globin* expression in 24 hpf wild-type (wt) and *spt* embryos.

(C–G) Trunk and tailbud regions. (C) *gata1* and *biklf/klf4* hematopoietic expression (arrowhead) at 7s is absent in *spt*. Ectodermal *biklf/klf4* expression remains (arrows). (D–G) *scl* (D), *lmo2* (E), *hhex* (F), and *gata2* (G) expression is initially absent in *spt*, appears at 5s–7s (arrowheads). Nonneural ectodermal *gata2* expression in *spt* (arrows).

with trunk PM is additionally required for RBC lineage-specific expression in IM. Our data demonstrate a novel role for trunk PM in establishing a critical environment for differentiation of the zebrafish embryonic RBC lineage.

Results

Hematopoietic Gene Expression Defects in *spt* Embryos

Circulating embryonic RBCs are absent in *spt* embryos along with expression of RBC markers, *gata1* and *jak2a*, at 24 hpf (Figure 1B; Kimmel et al., 1989; Oates et al., 1999; Thompson et al., 1998). Consistent with these previous findings, embryonic α*e1globin* and β*e1globin* expression is also lost at 24 hpf (Figure 1B). To investigate the onset of RBC lineage differentiation in *spt* embryos, we assayed early *gata1* expression, as it is the first RBC-specific marker and is required for blood progenitors to differentiate RBCs (Lyons et al., 2002). *gata1* is detected by the 6 somite-stage (6s; 12 hpf) in wt trunk IM posterior to somite 6 (Detrich et al., 1995) but is not initiated in *spt* embryos (Figure 1C). Morpholino knockdown suggests a role for *biklf/klf4* expression in the maintenance of *gata1* expression (Kawahara and Dawid, 2001). At 7s (12.5 hpf), when *biklf/klf4* hematopoietic expression is distinct from overlying ectodermal expression in wt embryos, we detect no expression in *spt* trunk IM (Figure 1C). Thus, the hematopoietic program in *spt* embryos is blocked prior to expression of the earliest RBC markers, strongly suggesting that RBC differentiation is not initiated.

Prior to RBC lineage marker expression, genes critical for early events in the vertebrate hematopoietic program are expressed in zebrafish trunk IM starting at 2s (10.5 hpf). We looked for this early gene expression in *spt* IM to determine if loss of RBCs might result from defects in blood progenitor formation. *scl*, *lmo2*, and *hhex* are expressed within developing blood and endothelial progenitors in zebrafish and are thought to function in the specification of hemangioblasts, bipotent cells hypothesized to produce both blood and endothelial cells (Gering et al., 1998, 2003; Liao et al., 1998; Liao et al., 2000; Thompson et al., 1998). Mammalian data suggest that *gata2* acts by regulating the proliferation and survival of HSCs, the progeny of the hemangioblast (Tsai and Orkin, 1997). At 3s (11 hpf), there was a complete loss of *scl, lmo2*, and *gata2* expression in *spt* trunk IM, and

hhex likewise failed to initiate at 5s (11.5 hpf; Figures 1D–1G). However, from 5s to 7s, expression of these genes gradually appeared in a small number of *spt* trunk IM cells (Figures 1D–1G). By 12s, the number of expressing cells increased markedly, but had not reached wt levels (Figures 1D–1G). Therefore, gene expression associated with two potential early hematopoietic events, hemangioblast specification and HSC development, is defective in *spt* IM, suggesting that an early defect in blood progenitor formation underlies RBC loss in *spt* embryos.

The earliest described marker of the zebrafish trunk hematopoietic IM is the putative nuclear protein *draculin* (*dra*), whose function in hematopoiesis has not yet been demonstrated (Herbomel et al., 1999). In gastrulating wt embryos, *dra* is expressed in ventrolateral mesodermal cells of the hypoblast (Herbomel et al., 1999) in a domain that separates from PM marked by *her1* (Müller et al., 1996) by Bud stage, at which time *dra* expression appears largely restricted to IM (10 hpf; Figure 2A). We found *dra* expression reduced throughout the hypoblast of the *spt* gastrula at 70% epiboly (8 hpf), after which expression decreased until no longer detected in the trunk at Bud or later during segmentation stages (Figure 2A). In wt, *dra* expression is retained in developing RBCs and early macrophages (Herbomel et al., 1999).

Consistent with previously described expression of the hematopoietic regulator, *pu1/spi1* in the *spt* head blood domain (Lieschke et al., 2002), we found head expression of *scl*, *lmo2*, *gata2*, and *hhex* also unperturbed (data not shown), suggesting that *spt* embryonic blood defects are limited to the trunk. In summary, we have shown that *spt* trunk IM exhibits a combination of delayed, severely reduced, or lost hematopoietic gene expression, including *dra*, the earliest known marker of these cells. As the *spt* mutation specifically affects trunk blood, we next asked whether this reflects either a general loss of trunk IM or an overall delay in differentiation of its derivatives.

IM and LPM Marker Expression in the *spt* Trunk

Coexpression of blood, pronephros, and endothelial markers in wt zebrafish embryos (Davidson et al., 2003; Gering et al., 1998, 2003), and fate mapping studies (Lieschke et al., 2002; Serluca and Fishman, 2001), indicate that these tissues develop closely within IM, perhaps originating from common progenitors. *fli1* is expressed in wt trunk IM by Bud stage and is initially coexpressed with *gata2*, but is later maintained only in endothelial cells (Brown et al., 2000; Thompson et al., 1998). We find *fli1* expressed in *spt* IM with the same time course as in wt embryos; however, as detected at stages 3s to 12s, the *fli1* expression domain in *spt* embryos is widened (Figure 2B). Expression of another endothelial marker *flk1* is detected in *spt* IM at 12s (Figure 2D), in a pattern similar to *scl* (Figure 1D), suggesting that *scl* expression in *spt* IM at 12s is confined to a population of developing endothelial cells. *pax2.1* expression in pronephric precursors (Majumdar et al., 2000) also occurs along a normal time course in *spt* IM and, like *fli1*, appears broader in comparison to wt embryos (Figure 2C). *pax2.1* expression in *spt* embryos at 12s is disordered, consistent with previous reports of disorganized pronephric and endothelial structures in older *spt* embryos (Kimmel et al., 1989; Thompson et al., 1998). Additionally, the anterior limit of *pax2.1* expression in *spt* IM is similar to wt, suggesting that significant alteration in IM anterior-posterior identity has not occurred. Disruption of the earliest hematopoietic markers in *spt* trunk IM, and later RBC loss, is therefore not due to a general failure to differentiate IM or to a delay in IM development, suggesting that the defect is specific to hematopoietic IM.

We find hematopoietic IM distinct from LPM marked by *dhand* expression (Figure 2E; Angelo et al., 2000). By 6s in wt embryos, *scl*, *fli1*, and *dhand* are already expressed in restricted mediolateral domains (Figures 2F and 2G). In *spt* embryos at 6s, LPM and IM as marked by *dhand* and *fli1* remain distinct (Figure 2G). Thus, *spt* embryos appear to maintain a correct mediolateral organization of trunk mesoderm.

Finally, in the mouse, *gata1*$^{-/-}$ proerythroblasts undergo apoptosis (Weiss and Orkin, 1995), raising the possibility that *spt* RBC progenitors have died. Acridine orange staining revealed no difference between *spt* and wt IM at early segmentation stages through 24 hpf (data not shown).

spt/*tbx16* Is Required Cell Autonomously for RBC Formation

To test if RBC loss in *spt* embryos is caused by a requirement for *spt* within developing RBCs, we transplanted combinations of differently labeled wt and *spt* donor cells at pregastrula stages into unlabeled wt host embryos (Figure 3A). At 27 hpf, we found that if cells from two wt donors contributed to IM, RBCs with both labels were found in circulation (n = 13/17 embryos; Figure 3B). In contrast, if wt and *spt* donor cells contributed to IM, only wt donor cells were detected as RBCs (n = 4/4 embryos; Figure 3C). Importantly, both wt and *spt* donor cells formed pronephric and endothelial structures (Figure 3D). These results demonstrate that *spt* cells, while able to differentiate as IM (Kimmel et al., 1989; Thompson et al., 1998), are unable to form RBCs even when placed within the appropriate wt environment. *spt* function is therefore required cell autonomously for the formation of RBCs.

spt/*tbx16* Is Required Cell Autonomously for Early Hematopoietic Expression

To establish if *spt* plays a cell-autonomous role during early RBC development, we transplanted *spt* cells into wt host embryos at pregastrula stages, then assayed for *gata1* and *dra* expression at 8s and Bud stage, respectively (Table 1; Figures 3E–3I). When *spt* donor cells formed IM, no *gata1* expression was detected in these cells, although the surrounding wt host cells expressed *gata1* (n = 0/18 events, Table 1; Figures 3F and 3G). Similarly, at Bud stage when *spt* cells were located within the *dra* expression domain in a wt host, the *spt* cells did not express *dra* (n = 0/5 embryos, Table 1; Figure 3I). As expected, wt cells transplanted into wt hosts expressed *gata1* and *dra* (*gata1* n = 21/25 events, *dra* n = 8/8 embryos, Table 1; Figures 3E and 3H). Therefore, *spt* is required cell autonomously for expression

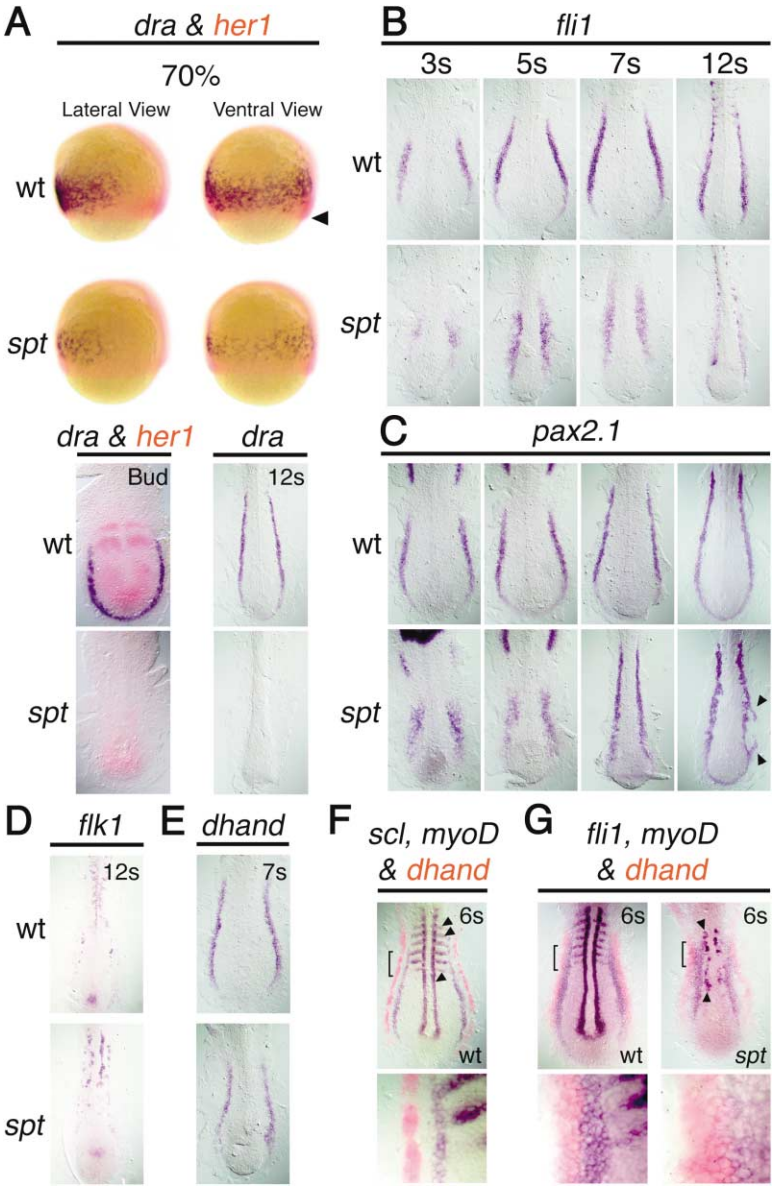

Figure 2. IM and LPM Gene Expression in *spt* Embryos

(A) *dra* (blue) and *her1* (red) expression at 70% epiboly (lateral view, dorsal to right), tailbud (Bud), and 12s. *dra* expression domain in wt separates from *her1* expressing PM (arrowhead, 70%). *dra* is reduced in *spt* at 70% and absent at Bud through 12s.

(B and C) *fli1* and *pax2.1* expression in wt and *spt* at 3s–12s.

(D) Endothelial *flk1* expression in wt and *spt* at 12s. (E) LPM *dhand* expression in wt and *spt* at 7s. (F) Co-in situ hybridization in 6s wt for *scl* (blue), *dhand* (red), and *myoD* (blue, arrowheads). (G) Co-in situ hybridization in 6s wt and *spt* embryos for *fli1* (blue), *dhand* (red), *myoD* (blue, arrowheads). Bracketed areas enlarged.

of *gata1*, a gene critical to RBC differentiation, and *dra*, a marker of early blood progenitors.

Interaction between IM and PM Is Required for *gata1* Expression

To test if *spt* function in IM is sufficient to rescue RBC formation or if environmental cues normally required for RBC formation are lacking in *spt* mutants, we transplanted wt cells at pregastrula stages into *spt* embryos. This manipulation resulted in *spt* hosts at 8s that contained regions in which wt donor cells formed different combinations of trunk tissues, which we identified using both position and morphology. We assayed for *gata1* expression within trunk regions of *spt* host embryos that contained either wt donor-derived PM alone, or IM alone, or a combination of apposed IM and PM. All data were collected from host trunk regions within normal anterior and posterior limits of *gata1* expression at 8s. Transplanted wt cells that formed large regions of trunk PM, including somites, in the PM-deficient *spt* host were not sufficient to rescue expression of *gata1* in adjacent *spt* IM (n = 0/38 events, Table 1; Figure 4B). Wt donor-derived nervous system and notochord likewise did not rescue *gata1* expression in *spt* IM (Table 1), consistent with our finding that *spt* function is required within IM cells for RBC formation.

In regions of *spt* host embryos in which wt donor cells contributed only to trunk IM (Figures 4C and 4E), wt cells, like the surrounding *spt* host IM, failed to express *gata1* (n = 0/10 events, Table 1). This indicates that cell-autonomous *spt* function in IM is not sufficient to rescue RBC formation in *spt* embryos and suggests that an environmental hematopoietic factor(s), ordinarily present in wt embryos could be additionally absent in *spt* embryos. Here we show that *gata1* expression depends upon an interaction occurring between the IM and trunk PM. In regions of *spt* host embryos in which wt donor

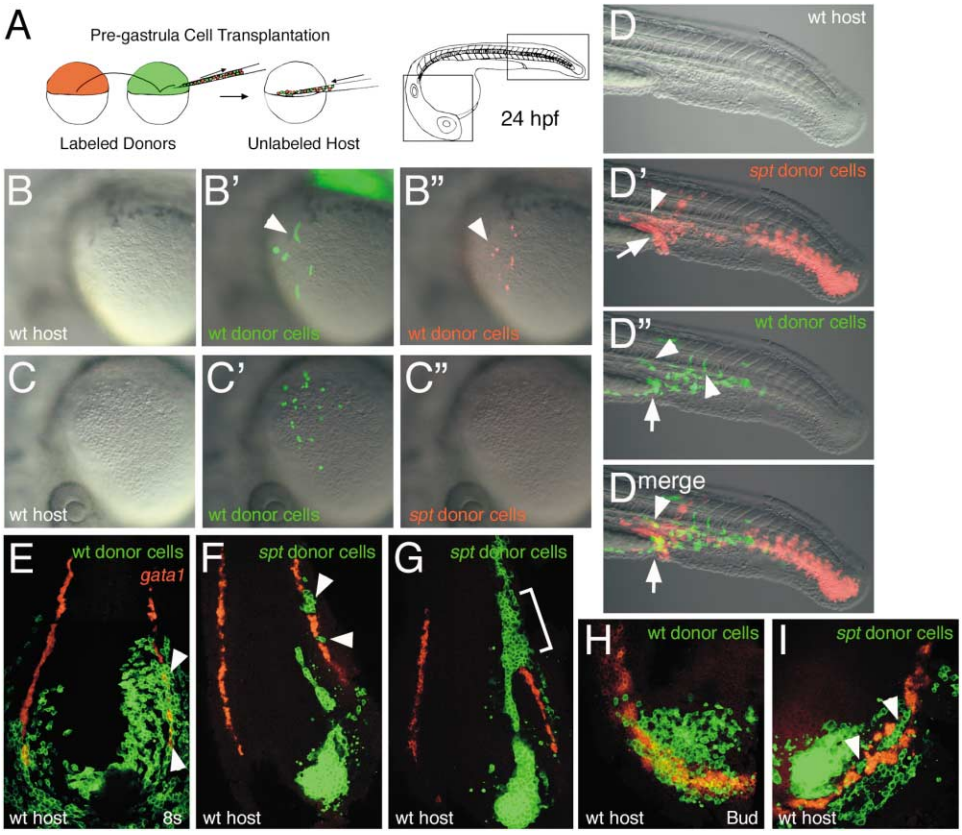

Figure 3. *spt/tbx16* Is Required Cell Autonomously for Production of RBCs

(A) Pregastrulation cells from labeled donors cotransplanted into unlabeled wt hosts. Boxed areas of 24 hpf wt as shown below.

(B and C) Donor RBCs circulating across yolk in live 27 hpf wt host. (B) Control, RBCs derive from both wt donors (arrowheads [B′ and B″]). (C) RBCs derive from the wt donor cells (green [C′]), but not *spt* (red [C″]).

(D) Posterior of embryo in (B). *spt* (red [D′ and D^{Merge}]) and wt (green [D″ and D^{Merge}]) donor cells form vasculature (arrowheads) and pronephros (arrows).

(E–G) Composite confocal sections through *gata1* expression domain (red) in wt host embryos at 8s. Donor cells in green. (E) Control, wt donor-derived IM expresses *gata1* (arrowheads). (F–G) *spt* donor-derived IM cells do not express *gata1* (arrowheads, bracket).

(H and I) Confocal sections through *dra* expression domain (red) in half of posterior wt host at Bud. (H) Control, wt donor cells (green) express *dra*. (I) *spt* donor cells (green) fail to express *dra* (arrowheads).

cells formed a combination of IM and adjacent trunk PM, strong *gata1* expression was now observed in wt IM cells (n = 12/19 events, Table 1; Figures 4C–4E). In all cases in which wt donor cells contributed to nonaxial trunk mesodermal tissue, *gata1* expression was only detected in wt donor-derived IM that was apposed to PM formed by wt donor cells (n = 12/67 events, Table 1). This rescue is in contrast to the above results in which wt donor-derived PM alone or IM alone was not sufficient for *gata1* expression. Our cell-transplantation data thus reveal a novel interaction between hematopoietic IM and trunk PM for embryonic RBC lineage formation.

Rescue of *dra* Expression

To determine if an interaction with PM is also required for *dra* expression in wt donor cell IM, we assayed rescue throughout the trunk and tailbud IM at 8s (Table 1; Figure 5). Similar to *gata1*, we saw no *dra* expression in wt donor cells that formed IM alone in the trunk of *spt* hosts (n = 0/8 events, Table 1; Figures 5B and 5C). Expression of *dra* in the trunk was only seen in cases where wt donor-derived IM was adjacent to wt PM (n = 32/34 events, Table 1; Figures 5B–5D). Therefore, in trunk IM at 8s, *dra* expression also requires both functional *spt* and an interaction with wt PM. In contrast to the results seen in the trunk, wt donor-derived IM in the tailbud of *spt* hosts was observed to express *dra* in the absence of nearby wt PM (n = 8/8 events, Table 1; Figure 5D), although potentially in contact with large numbers of *spt* PM precursor cells present in the host tailbud. Wt PM alone failed to rescue *dra* expression in trunk and tailbud *spt* host IM (Figures 5B and 5C).

Wt Trunk PM Is Not Sufficient to Rescue RBC Formation in *spt* Embryos

As we have shown, in *spt* trunk IM there is a late appearance of limited *scl, lmo2, hhex*, and *gata2* expression (Figure 1). Although we were unable to detect circulating RBCs from *spt* IM cells in wt host embryos (Figure 3), the possibility remains that wt PM might induce *spt* IM cells to express RBC markers later than 8s. We therefore tested the ability of wt PM to rescue expression of

Table 1. Cell-Transplantation Experiments

Cell Transplant	Situation Scored	Donor-Derived Trunk Tissue				
		CNS	Noto	PM Alone	IM Alone	IM+PM
A. Cell Transplants Analyzed for *gata1* Expression at 8s						
wt → wt (n^t = 140)	hosts (n^h [%])	35 (25)	76 (54)	50 (36)	22 (16)	36 (26)
	events (n^e)	35	78	54	25	38
	gata1 pos. IM (n^{pos} [%])	35 (100)	76 (97)	51 (94)	21 (84)	34 (90)
wt → *spt* (n^t = 56)	hosts (n^h [%])	12 (21)	7 (13)	33 (59)	7 (13)	15 (27)
	events (n^e)	12	7	38	10	19
	gata1 pos. IM (n^{pos} [%])	0	0	0	0	12 (63)
spt → wt (n^t = 23)	hosts (n^h [%])	6 (26)	10 (43)	0	13 (57)	0
	events (n^e)	6	10	0	18	0
	gata1 pos. IM (n^{pos} [%])	6 (100)	10 (100)	0	0	0
B. Cell Transplants Analyzed for *dra* Expression at 8s						
wt → wt (n^t = 59)	hosts (n^h [%])	21 (36)	32 (54)	24 (41)	14 (24)	18 (31)
	events (n^e)	21	32	24	19	19
	dra pos. IM (n^{pos} [%])	21 (100)	32 (100)	23 (96)	18 (95)	17 (90)
wt → *spt* (n^t = 40)	hosts (n^h [%])	3 (8)	9 (23)	18 (45)	9 (23)	22 (55)
	events (n^e)	3	9	22	16	34
	dra pos. IM (n^{pos} [%])	0	0	0	8 (50)[a]	32 (94)
C. Cell Transplants Analyzed for *dra* Expression at Bud						
		Non-Axial Mesoderm				
wt → wt (n^t = 33)	hosts (n^h [%])	24 (73)				
	dra pos. IM (n^d [%])	8 (30)				
spt → wt (n^t = 19)	hosts (n^h [%])	10 (53)[b]				
	dra pos. IM (n^d [%])	0				

Donor-derived tissues are: Central nervous system (CNS); Notochord (Noto); PM without adjacent donor IM (PM alone); IM without adjacent donor PM (IM alone); and a combination of apposed IM and PM (IM+PM). An event is a contiguous region of donor cell contribution to a particular tissue(s).

n^t = total hosts examined; n^h = hosts containing given donor-derived tissue type (% = n^h/n^t); n^e = events of donor cell contribution to a given tissue(s); n^{pos} = events of *gata1* or *dra* expression in host IM at 8s (when donor cells contribute to CNS, Noto, or PM alone) or in donor IM (when donor cells contribute to IM alone, or IM+PM) (% = n^{pos}/n^e); n^d = host containing *dra* positive donor cells (% = n^d/n^h)

[a] Positive expression found only in donor cells in tailbud region.

[b] 5 host embryos contained *spt* donor cells within the wt host *dra* domain

αe1globin in *spt* IM at 24 hpf. Wt host embryos containing wt donor-derived PM showed normal αe1globin expression (n = 5; Figures 6A and 6B), indicating no disruption by the pregastrula transplantation. In *spt* host embryos, wt PM was not sufficient to rescue αe1globin expression in *spt* host IM (n = 12 embryos; Figures 6C and 6D), nor were wt donor cells in other nonhematopoietic regions (data not shown). Wt cells in the *spt* host hematopoietic region expressed αe1globin (n = 4 embryos; Figures 6C and 6D), but, consistent with our earlier results, wt PM was in the vicinity of these cells. Dependence of expression upon PM is unclear, however, as the spatial relationship between the αe1globin-positive cells and wt donor-derived PM may not reflect the arrangement at the onset of hematopoiesis, and differentiated *spt* trunk PM appears by 24 hpf (Kimmel et al., 1989). In conclusion, despite limited, late expression of hematopoietic genes, *spt* IM does not respond at stages examined here to an interaction with wt PM.

Discussion

spadetail (*spt*) exhibits a severe loss of both trunk PM and RBCs (Kimmel et al., 1990; Thompson et al., 1998). We demonstrate that RBC loss in *spt/tbx16* mutants is the consequence of a novel 2-fold requirement for *spt/tbx16* function (Figure 7): (1) *spt/tbx16* function is required cell autonomously within RBCs and (2) *spt/tbx16* function is also needed within PM to position it in the trunk, thereby creating the proper environment for RBC development in neighboring IM. Thus, the previously proven cell-autonomous requirement for *spt/tbx16* in trunk PM during convergence (Ho and Kane, 1990) can now also be described as a non-cell-autonomous requirement for blood development.

Interaction between IM and Trunk PM

In zebrafish, RBC-producing IM is closely associated with prospective PM from gastrulation onward. At Bud stage, a distinct division into apposed regions of IM and PM is observed using molecular markers (Figure 2). Transplanted wt cells that form IM in the normally PM-deficient trunk of a *spt* host do not express *gata1* at 8s unless adjacent to wt trunk PM (Figure 4; Table 1), indicating that these two tissues must interact at some point prior to *gata1* onset. Considering that *gata1* expression is a key first step toward RBC lineage differentiation, this IM/PM interaction is essential to form embryonic RBCs (Figure 7).

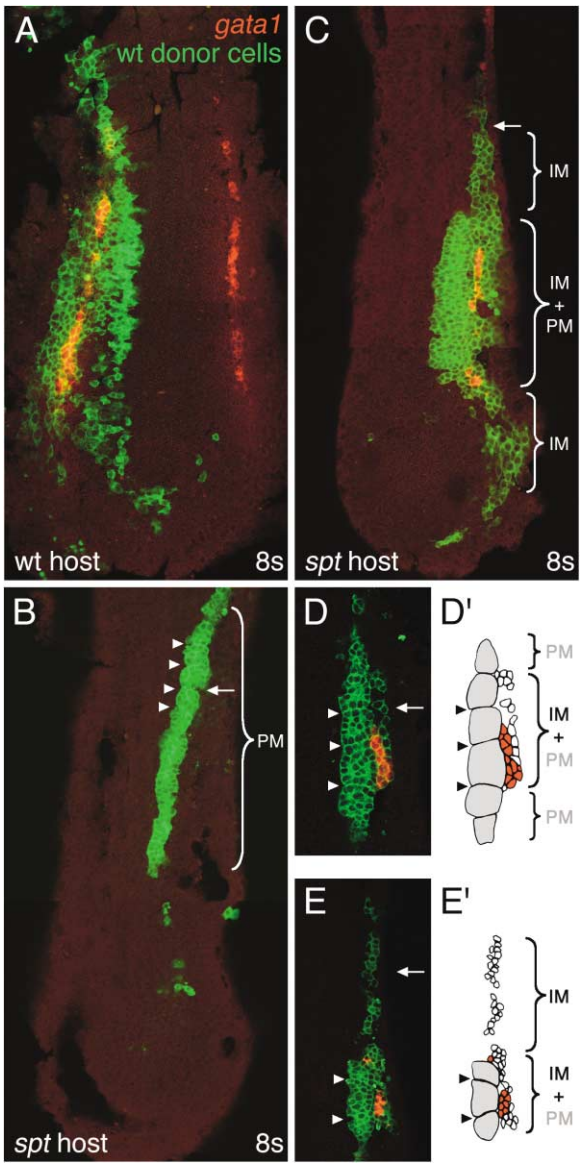

Figure 4. PM Is Required for *gata1* Expression in Adjacent IM

(A–E) Composite confocal sections at level of *gata1* expressing IM (red). Transplanted wt cells (green) formed the following trunk tissues in hosts: PM without adjacent donor-derived IM (PM); IM without adjacent donor-derived PM (IM); and a combination of apposed IM and PM (IM+PM). Somite boundaries marked by arrowheads. (A) Control, wt cells express *gata1* in a wt host. (B–E) *spt* hosts containing wt donor cells. Expected anterior limit of *gata1* expression marked (arrows). (B) Trunk PM fails to rescue *gata1* expression in *spt* host. (C) IM alone does not express *gata1*. IM cells adjacent to PM express *gata1*. (D and E) IM and IM+PM in *spt* hosts. (D′ and E′) Illustration of wt IM cells (outline) and wt PM somitic structures (gray blocks).

Expression of the early hematopoietic marker *dra* is also dependent on PM in the trunk at 8s; however, we found *dra* expression in transplanted wt IM cells located in *spt* host tailbuds despite the absence of wt PM (Figure 5D). We offer two possible explanations for this occurrence. First, a requirement for PM might be supplied by *spt* cells that differentiate normally as PM in the tail (Kimmel et al., 1989), and/or by mislocated *spt* trunk PM cells. Second, posterior cells within an embryo are less developed; therefore *dra*-expressing cells in the tailbud may be progenitors undergoing early hematopoiesis independent of PM. Consistent with a less stringent requirement for PM, we find *dra* expression is easier to rescue than *gata1* (63% events *gata1*, 94% events *dra* (IM+PM), Table 1).

Environmental Influences on Vertebrate Blood Development

Previous studies of zebrafish hematopoietic mutants support a role for environmental cues in embryonic blood development. *bloodless* (*bls*) embryos have a severe loss of embryonic *gata1* expression; however, *bls* cells express *gata1* in wt hosts at 24 hpf, indicating a non-cell-autonomous role for *bls* (Liao et al., 2002). In *cloche* mutants, loss of both blood and endothelial cells arises from a cell-autonomous defect; however, early RBC differentiation in *cloche* depends solely on an unidentified non-cell-autonomous contribution (Parker and Stainier, 1999). Parker and Stainier (1999) found that wt cells in a *cloche* host expressed *gata1* only in association with a large group of nonexpressing donor cells, which they hypothesized to be supplying an endothelial-derived signal. In contrast, we found that rescue of *gata1* and *dra* expression in transplanted wt cells in a *spt* trunk is always associated with PM, independent of IM clone size (Figures 4 and 5).

Visceral endoderm in the mouse and chick, and ectoderm in *Xenopus*, are sources of signals received by embryonic blood progenitors (Baron, 2003). Our results suggest that nonautonomy of embryonic blood formation may be conserved across vertebrates. However, the source and molecule fulfilling this nonautonomous requirement may vary. Unlike mouse and chick, endoderm is not required in the zebrafish, as *casanova* mutants, which fail to form endoderm, still make RBCs (Parker and Stainier, 1999). Ectodermal-derived signals in zebrafish hematopoiesis have yet to be demonstrated, although PM may relay cues from ectoderm. Interestingly, IM/PM communication is thought to occur in chick and *Xenopus* during pronephric differentiation (Mauch et al., 2000; Seufert et al., 1999).

What might be the factor(s) from zebrafish PM? Based on the necessity for apposition of *gata1*-expressing IM cells and PM, such a factor is restricted to act at short range. In mouse, visceral endoderm signals appear to instruct blood and endothelial development within the underlying blood islands, and rely on activity of visceral endoderm-derived Indian hedgehog and Vascular endothelial growth factor (VEGF) signaling (Baron, 2003). Zebrafish VEGF is expressed in PM during segmentation stages (Liang et al., 1998), and putative VEGF receptors *flk1* and *flt1* are expressed in IM (Liao et al., 1997; Thompson et al., 1998), suggesting a potential for hematopoietic function similar to mouse. Indeed, overexpression of zebrafish *VEGF* mRNA isoforms induces *scl* and *gata1* expression prematurely and ectopically (Liang et al., 2001). However, morpholino knock-down of *vegfA* (Nasevicius et al., 2000) or loss of the *flk1* VEGF receptor (Habeck et al., 2002) does not compromise blood production. Hedgehog (Hh) signaling also seems not to be

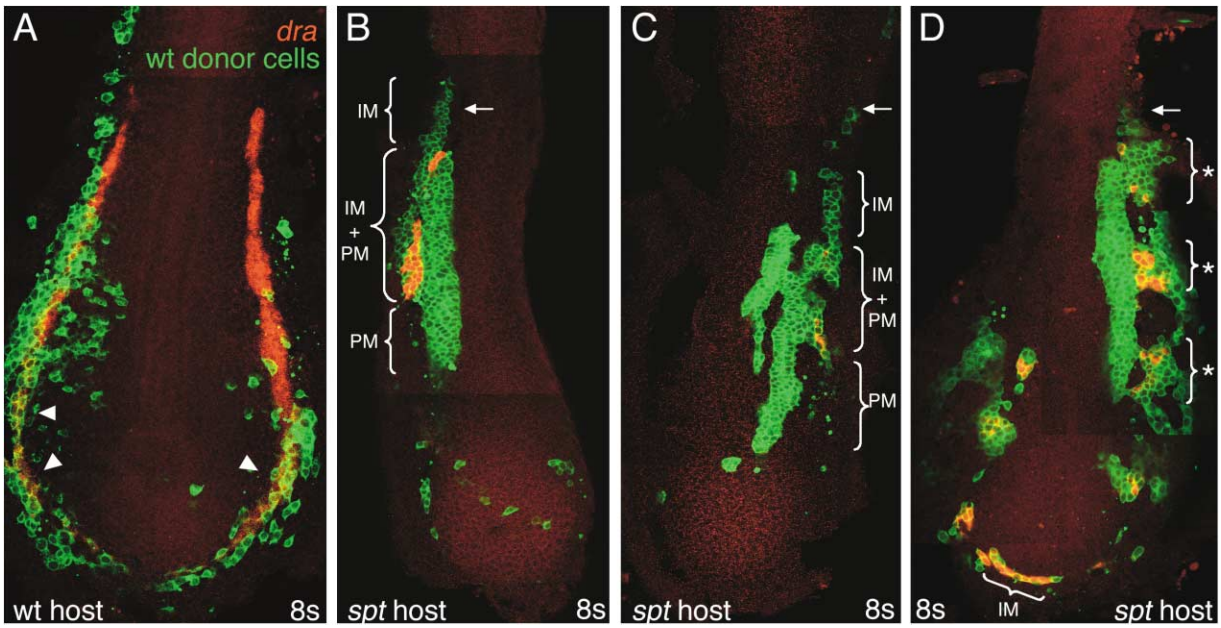

Figure 5. PM Is Required for Anterior Trunk Expression of *dra* at 8s

(A–D) Composite confocal sections at the level of *dra* expression in 8s hosts. Transplanted wt cells (green) and *dra* expression (red). See Figure 4 for description of PM, IM, and IM + PM wt donor-derived tissue. (A) Control, wt cells express *dra* in a wt host (arrowheads). (B–D) Wt IM in *spt* trunks does not express *dra*. Wt trunk IM adjacent to wt PM (* in [D]) expresses *dra*. Wt PM is unable to induce *dra* expression in *spt* host. (D) Wt IM alone (arrows) in *spt* host tailbud region expresses *dra*.

required for zebrafish hematopoiesis, since neither *shh*, *ihh*, or *ehh* ligands are expressed in PM (Currie and Ingham, 1996; Krauss et al., 1993), nor are *patched1*, *patched2*, or *smoothened* receptors expressed in IM (Chen et al., 2001; Lewis et al., 1999), and loss of Hh signaling has not been reported to cause blood defects (Chen et al., 2001). Thus, known homologs of genes involved in mouse hematopoietic induction do not seem to be obvious candidates for mediating zebrafish IM/PM communication.

During *Xenopus* gastrulation, BMP-4 is required for an interaction between ectoderm and underlying mesoderm to produce blood islands (Kikkawa et al., 2001). Walters et al. (2002) suggest that these ectodermal signals regulate lineage differentiation of previously specified blood progenitors; however, data from Walmsley et al. (2002) support an action earlier in hematopoiesis. Zebrafish BMP signaling mutants are severely dorsalized and fail to form RBCs along with other posterior structures such as pronephros (Schier, 2001), making an independent role for BMP signaling in RBC development unclear. Transplanted *swirl/bmp2b* and *somitabun/smad5* mutant cells form RBCs in a wt host (Hild et al., 1999; Kishimoto et al., 1997; Nguyen et al., 1998), indicating BMP signaling through these components is not required within zebrafish RBCs. Likewise, a direct role for Wnts and FGFs, as reported during *Xenopus* and mammalian hematopoiesis (de Haan et al., 2003; Kumano and Smith, 2000; Reya et al., 2003), has not been described for zebrafish. Current evidence therefore does not support direct involvement of known hematopoietic inducers either during the IM/PM interaction or through *spt* cell-autonomous function.

spt/tbx16 Function in the IM

spt regulates expression of *paraxial protocadherin*, a cell-adhesion molecule implicated in PM convergence during early gastrulation (Yamamoto et al., 1998). Given this morphogenetic role, which might reflect a general mechanism for T-box genes in development (Ahn et al., 2002), one hypothesis is that *spt* is cell-autonomously involved in hematopoiesis though control of IM cell movements. Indeed, IM convergence defects during gastrulation may result in broadened *fli1* and *pax2.1* domains (Figures 2B and 2C). However, convergence defects in trunk mesoderm alone do not appear to be sufficient to curtail hematopoiesis (Marlow et al., 2004; A.C.O., unpublished data).

As *spt* is cell-autonomously required for early *dra*, and *gata1* expression (Figure 3), it is possible that *spt* acts during events that lead to specification of blood precursors, such as the proposed hemangioblast and HSC (Figure 7). Prior to gastrulation, *spt* is expressed in presumptive mesoderm within the marginal zone (Kimmel et al., 1990); however, later expression in the trunk is found mainly in posterior PM (Griffin et al., 1998; Ruvinsky et al., 1998). When *scl* and *gata1* are first expressed in IM, Spt protein is detected only within the most posterior trunk and tail mesoderm and does not mirror blood expression during segmentation stages (Amacher et al., 2002; L.A.R., unpublished data). Thus, timing of *spt* expression within IM supports the hypothesis that *spt* acts prior to expression of hematopoietic regulators such as *scl*, *lmo2*, *hhex*, *gata2*, and *gata1*. Indeed, expression of these genes may bypass a prior cell-autonomous requirement for *spt*, as overexpression of zebrafish *hhex*

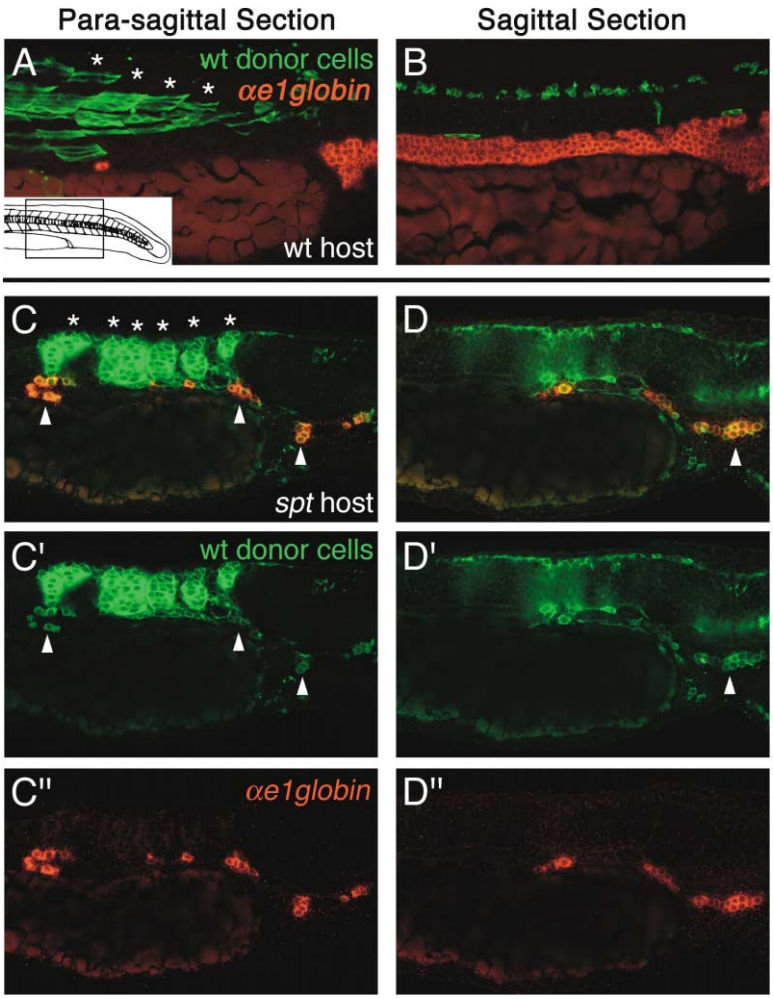

Figure 6. Wt Trunk PM Fails to Rescue RBC Production from *spt* Host

(A–D) Left are confocal sections of host trunk at PM level (para-sagittal sections, anterior to left). Right panels are sections through midline hematopoietic region (sagittal section) of left embryo. Transplanted wt cells in green, and α*e1globin* in red. (A and B) Control, wt cells incorporated into PM somites of 24 hpf wt host (asterisks in [A]). (C and D) 24 hpf *spt* host containing wt trunk somites (asterisks in [C]). α*e1globin* expression is not rescued in *spt* host cells, but is expressed in wt cells (arrowheads). Expression (C″ and D″) restricted to wt cells (C′ and D′).

can ectopically induce *flk1* and *gata1* expression in nonmesodermal regions (Liao et al., 2000). The IM/PM interaction may act later than early blood genes given that while co-overexpression of *scl* and *lmo2* induces ectopic *fli1* in nonaxial mesoderm along the entire axis, *gata1* expression can be induced only within IM (Gering et al., 2003). Gering et al. (2003) suggest that this limited *gata1* induction results from a requirement for endogenous cofactors localized within IM. Our data suggest that this might additionally be due to a need for close proximity to trunk PM.

Comparison of key hematopoietic gene expression in wt and *spt* embryos (Figure 1) suggests that these genes normally exhibit two phases of expression. The first phase of *scl*, *lmo2*, *gata2*, and *hhex* expression may be blood specific and initiates in wt, but not *spt*, between stages 2s to 5s in IM posterior to somite 6. The second phase begins after 5s in anterior trunk IM at the level

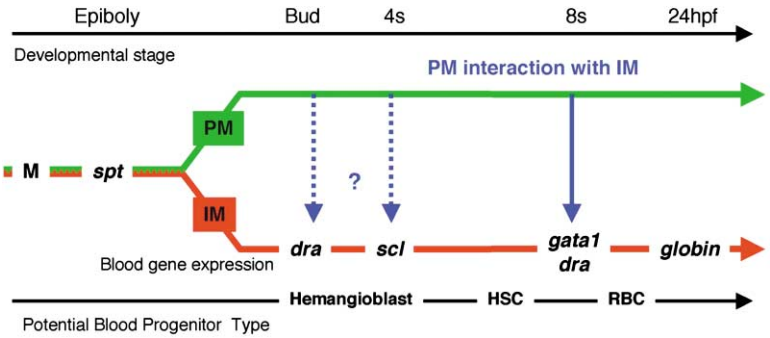

Figure 7. Model of *spt/tbx16* Cell-Autonomous Function and IM/PM Interaction during Zebrafish Embryonic RBC Production

PM (green) and IM (red) lineages develop closely throughout epiboly and are molecularly observed to be separate populations by Bud stage. *spt* functions cell autonomously in trunk PM for gastrulation movements. *spt* also functions cell autonomously in trunk hematopoietic IM for expression of *dra* at Bud and *gata1* at 8s, and for ultimate production of RBCs by 24 hpf. PM supplies an interaction (blue arrows) that is crucial to RBC development. PM must interact with IM at or prior to the onset of *gata1* expression (solid blue arrow). This interaction may be required for expression of early hematopoietic markers, such as *dra* and *scl*, and formation of early blood progenitors (dashed blue arrows).

of somites 1 to 5; this "anterior expansion" has been previously described for *scl*, *lmo2*, and *hhex* (Davidson et al., 2003; Gering et al., 2003; Liao et al., 2000). In wt embryos, endothelial cells, but not blood, derive from the anterior expression domain (Lieschke et al., 2002). Strikingly, the appearance of scattered *scl*-, *lmo2*-, *gata2*-, and *hhex*-expressing IM cells in *spt* embryos at stages 5s to 7s correlates with the onset of wt anterior expression. Previous reports have interpreted these *scl*-expressing cells in *spt* embryos as arrested HSCs (Oates et al., 1999), but our findings suggest that late expression of these genes in *spt* mutants reflects a normal progression of the endothelial program. In wt posterior IM, a late-occurring wave of endothelial-specific expression would normally be masked by gene expression associated with developing blood.

In summary, our data suggest an important role for the hematopoietic microenvironment in zebrafish embryonic RBC production. The observed spatial separation of embryonic RBC (trunk) and early macrophage blood lineages (head) in zebrafish is suggestive of a possible instructive role for trunk PM at the level of lineage choices made by HSCs (Figure 7). Future experiments apposing trunk PM and early macrophage blood progenitors will test the ability of trunk PM to instruct a RBC fate.

Experimental Procedures

Embryo Collection

Wild-type (wt) embryos from *AB and TL (Johnson and Zon, 1999) and commercial lines (Princeton, NJ; Chicago, IL). *spadetail* ^{b104} (*spt*) homozygote embryos collected from heterozygote spawning and wt siblings used as staging references (Kimmel et al., 1995). Embryos cultured at 28.5°C in embryo medium (Westerfield, 2000), dechorionated manually, and fixed 2 days in 4% PFA in PBS at 4°C and stored in MeOH at −20°C.

Pregastrula Cell Transplantation

Donor embryos microinjected at 1–2 cell stages with 5% fixable fluorescein-conjugated dextran (40 kDa, Molecular Probes) in 0.2 M KCl. Cell transplantation previously described by Ho and Kane (1990). Donor cells (10–50) from high-sphere staged embryos were transplanted into the margin of similarly staged hosts (Kimmel et al., 1990). Hosts cultured in EM + 0.2% penicillin-streptomycin (Bio-Whittaker). *spt* homozygote donors identified at segmentation stages. Rates of host death and damage varied with clutch health 5%–20%.

In Situ Hybridization

FITC or DIG (Roche) labeled antisense riboprobes synthesized (Promega) for: *gata1, gata2* (Detrich et al., 1995); *jak2a* (Oates et al., 1999); α*e1globin,* β*e1globin* (Brownlie et al., 2003); *biklf/klf4* (Oates et al., 2001); *scl* (Liao et al., 1998); *lmo2, fli1, flk1* (Thompson et al., 1998); *hhex* (Liao et al., 2000); *dra* (Herbomel et al., 1999); *pax2.1* (Krauss et al., 1991); *dhand* (Angelo et al., 2000); *myoD* (Weinberg et al., 1996). Single and double in situ hybridization performed as described (Prince et al., 1998).

Cell Transplantation Analysis and Imaging

Fixed hosts screened for fluorescently labeled donor cells on a Leica MZFLIII fluorescent dissection microscope. Hosts containing donor-derived IM, or other tissues requiring close inspection, were de-yolked, flat-mounted, and analyzed on a Zeiss Axiovert confocal (LSM510). Aided by knowledge of PM, IM, and LPM gene expression patterns, we determined donor-derived cell types using the following criteria: PM was identified by proximity to notochord, presence of epithelial boundaries, multi-cell-layer thickness, and compact mesenchymal cell shape; IM was identified by position relative to both PM and hematopoietic gene positive cells in the same host, and by a less compact distribution, larger cell size, more ventral position, and fewer cell layers compared to PM. A transplant "event" as recorded in Table 1 is defined as a contiguous region in which wt donor cells formed a specific tissue(s). One or two events for a particular tissue(s) usually found per host. Gene expression visualized fluorescently from Fast Red precipitate detected with rhodamine filters.

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References

Ahn, D.G., Kourakis, M.J., Rohde, L.A., Silver, L.M., and Ho, R.K. (2002). T-box gene tbx5 is essential for formation of the pectoral limb bud. Nature *417*, 754–758.

Al-Adhami, M.A., and Kunz, Y.W. (1977). Ontogenesis of haematopoietic sites in *Brachydanio rerio* (Hamilton-Buchanan)(Teleostei). Dev. Growth Differ. *19*, 171–179.

Amacher, S.L., Draper, B.W., Summers, B.R., and Kimmel, C.B. (2002). The zebrafish T-box genes no tail and spadetail are required for development of trunk and tail mesoderm and medial floor plate. Development *129*, 3311–3323.

Amatruda, J.F., and Zon, L.I. (1999). Dissecting hematopoiesis and disease using the zebrafish. Dev. Biol. *216*, 1–15.

Angelo, S., Lohr, J., Lee, K.H., Ticho, B.S., Breitbart, R.E., Hill, S., Yost, H.J., and Srivastava, D. (2000). Conservation of sequence and expression of Xenopus and zebrafish dHAND during cardiac, branchial arch and lateral mesoderm development. Mech. Dev. *95*, 231–237.

Baron, M.H. (2003). Embryonic origins of mammalian hematopoiesis. Exp. Hematol. *31*, 1160–1169.

Brown, L.A., Rodaway, A.R., Schilling, T.F., Jowett, T., Ingham, P.W., Patient, R.K., and Sharrocks, A.D. (2000). Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. Mech. Dev. *90*, 237–252.

Brownlie, A., Hersey, C., Oates, A.C., Paw, B.H., Falick, A.M., Witkowska, H.E., Flint, J., Higgs, D., Jessen, J., Bahary, N., et al. (2003). Characterization of embryonic globin genes of the zebrafish. Dev. Biol. *255*, 48–61.

Chen, W., Burgess, S., and Hopkins, N. (2001). Analysis of the zebrafish smoothened mutant reveals conserved and divergent functions of hedgehog activity. Development *128*, 2385–2396.

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

Davidson, A.J., Ernst, P., Wang, Y., Dekens, M.P., Kingsley, P.D., Palis, J., Korsmeyer, S.J., Daley, G.Q., and Zon, L.I. (2003). cdx4 mutants fail to specify blood progenitors and can be rescued by multiple hox genes. Nature *425*, 300–306.

de Haan, G., Weersing, E., Dontje, B., van Os, R., Bystrykh, L.V., Vellenga, E., and Miller, G. (2003). In vitro generation of long-term repopulating hematopoietic stem cells by fibroblast growth factor-1. Dev. Cell *4*, 241–251.

Detrich, H.W., 3rd, Kieran, M.W., Chan, F.Y., Barone, L.M., Yee, K., Rundstadler, J.A., Pratt, S., Ransom, D., and Zon, L.I. (1995). Intraembryonic hematopoietic cell migration during vertebrate development. Proc. Natl. Acad. Sci. USA *92*, 10713–10717.

Gering, M., Rodaway, A.R., Gottgens, B., Patient, R.K., and Green, A.R. (1998). The SCL gene specifies haemangioblast development from early mesoderm. EMBO J. *17*, 4029–4045.

Gering, M., Yamada, Y., Rabbitts, T.H., and Patient, R.K. (2003). Lmo2 and Scl/Tal1 convert non-axial mesoderm into haemangioblasts which differentiate into endothelial cells in the absence of Gata1. Development *130*, 6187–6199.

Griffin, K.J., Amacher, S.L., Kimmel, C.B., and Kimelman, D. (1998). Molecular identification of spadetail: regulation of zebrafish trunk and tail mesoderm formation by T-box genes. Development *125*, 3379–3388.

Habeck, H., Odenthal, J., Walderich, B., Maischein, H., and Schulte-Merker, S. (2002). Analysis of a zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. Curr. Biol. *12*, 1405–1412.

Herbomel, P., Thisse, B., and Thisse, C. (1999). Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development *126*, 3735–3745.

Hild, M., Dick, A., Rauch, G.J., Meier, A., Bouwmeester, T., Haffter, P., and Hammerschmidt, M. (1999). The *smad5* mutation *somitabun* blocks Bmp2b signaling during early dorsoventral patterning of the zebrafish embryo. Development *126*, 2149–2159.

Ho, R.K., and Kane, D.A. (1990). Cell-autonomous action of zebrafish spt-1 mutation in specific mesodermal precursors. Nature *348*, 728–730.

Johnson, S.L., and Zon, L.I. (1999). Genetic backgrounds and some standard stocks and strains used in zebrafish developmental biology and genetics. Methods Cell Biol. *60*, 357–359.

Kawahara, A., and Dawid, I.B. (2001). Critical role of biklf in erythroid cell differentiation in zebrafish. Curr. Biol. *11*, 1353–1357.

Kikkawa, M., Yamazaki, M., Izutsu, Y., and Maeno, M. (2001). Two-step induction of primitive erythrocytes in *Xenopus laevis* embryos: signals from the vegetal endoderm and the overlying ectoderm. Int. J. Dev. Biol. *45*, 387–396.

Kimmel, C.B., Kane, D.A., Walker, C., Warga, R.M., and Rothman, M.B. (1989). A mutation that changes cell movement and cell fate in the zebrafish embryo. Nature *337*, 358–362.

Kimmel, C.B., Warga, R.M., and Schilling, T.F. (1990). Origin and organization of the zebrafish fate map. Development *108*, 581–594.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. Dev. Dyn. *203*, 253–310.

Kishimoto, Y., Lee, K.H., Zon, L., Hammerschmidt, M., and Schulte-Merker, S. (1997). The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. Development *124*, 4457–4466.

Krauss, S., Johansen, T., Korzh, V., and Fjose, A. (1991). Expression of the zebrafish paired box gene pax[zf-b] during early neurogenesis. Development *113*, 1193–1206.

Krauss, S., Concordet, J.P., and Ingham, P.W. (1993). A functionally conserved homolog of the *Drosophila* segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. Cell *75*, 1431–1444.

Kumano, G., and Smith, W.C. (2000). FGF signaling restricts the primary blood islands to ventral mesoderm. Dev. Biol. *228*, 304–314.

Lemischka, I.R., and Moore, K.A. (2003). Stem cells; Interactive niches. Nature *425*. 778–779.

Lewis, K.E., Concordet, J.P., and Ingham, P.W. (1999). Characterisation of a second patched gene in the zebrafish *Danio rerio* and the differential response of patched genes to Hedgehog signalling. Dev. Biol. *208*, 14–29.

Liang, D., Xu, X., Chin, A.J., Balasubramaniyan, N.V., Teo, M.A., Lam, T.J., Weinberg, E.S., and Ge, R. (1998). Cloning and characterization of vascular endothelial growth factor (VEGF) from zebrafish, *Danio rerio*. Biochim. Biophys. Acta *1397*, 14–20.

Liang, D., Chang, J.R., Chin, A.J., Smith, A., Kelly, C., Weinberg, E.S., and Ge, R. (2001). The role of vascular endothelial growth factor (VEGF) in vasculogenesis, angiogenesis, and hematopoiesis in zebrafish development. Mech. Dev. *108*, 29–43.

Liao, W., Bisgrove, B.W., Sawyer, H., Hug, B., Bell, B., Peters, K., Grunwald, D.J., and Stainier, D.Y. (1997). The zebrafish gene cloche acts upstream of a flk-1 homologue to regulate endothelial cell differentiation. Development *124*, 381–389.

Liao, E.C., Paw, B.H., Oates, A.C., Pratt, S.J., Postlethwait, J.H., and Zon, L.I. (1998). SCL/Tal-1 transcription factor acts downstream of cloche to specify hematopoietic and vascular progenitors in zebrafish. Genes Dev. *12*, 621–626.

Liao, W., Ho, C.Y., Yan, Y.L., Postlethwait, J., and Stainier, D.Y. (2000). Hhex and scl function in parallel to regulate early endothelial and blood differentiation in zebrafish. Development *127*, 4303–4313.

Liao, E.C., Trede, N.S., Ransom, D., Zapata, A., Kieran, M., and Zon, L.I. (2002). Non-cell autonomous requirement for the bloodless gene in primitive hematopoiesis of zebrafish. Development *129*, 649–659.

Lieschke, G.J., Oates, A.C., Paw, B.H., Thompson, M.A., Hall, N.E., Ward, A.C., Ho, R.K., Zon, L.I., and Layton, J.E. (2002). Zebrafish SPI-1 (PU.1) marks a site of myeloid development independent of primitive erythropoiesis: implications for axial patterning. Dev. Biol. *246*, 274–295.

Lyons, S.E., Lawson, N.D., Lei, L., Bennett, P.E., Weinstein, B.M., and Liu, P.P. (2002). A nonsense mutation in zebrafish gata1 causes the bloodless phenotype in vlad tepes. Proc. Natl. Acad. Sci. USA *99*, 5454–5459.

Majumdar, A., Lun, K., Brand, M., and Drummond, I.A. (2000). Zebrafish no isthmus reveals a role for pax2.1 in tubule differentiation and patterning events in the pronephric primordia. Development *127*, 2089–2098.

Marlow, F., Gonzalez, E.M., Yin, C., Rojo, C., and Solnica-Krezel, L. (2004). No tail co-operates with non-canonical Wnt signaling to regulate posterior body morphogenesis in zebrafish. Development *131*, 203–216.

Mauch, T.J., Yang, G., Wright, M., Smith, D., and Schoenwolf, G.C. (2000). Signals from trunk paraxial mesoderm induce pronephros formation in chick intermediate mesoderm. Dev. Biol. *220*, 62–75.

Minko, K., Bollerot, K., Drevon, C., Hallais, M.F., and Jaffredo, T. (2003). From mesoderm to blood islands: patterns of key molecules during yolk sac erythropoiesis. Gene Expr. Patterns *3*, 261–272.

Müller, M., v. Weizsäcker, E., and Campos-Ortega, J.A. (1996). Expression domains of a zebrafish homologue of the *Drosophila* pair-rule gene *hairy* correspond to primordia of alternating somites. Development *122*, 2071–2078.

Nasevicius, A., Larson, J., and Ekker, S.C. (2000). Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant. Yeast *17*, 294–301.

Nguyen, V.H., Schmid, B., Trout, J., Connors, S.A., Ekker, M., and Mullins, M.C. (1998). Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a bmp2b/swirl pathway of genes. Dev. Biol. *199*, 93–110.

Oates, A.C., Brownlie, A., Pratt, S.J., Irvine, D.V., Liao, E.C., Paw, B.H., Dorian, K.J., Johnson, S.L., Postlethwait, J.H., Zon, L.I., and Wilks, A.F. (1999). Gene duplication of zebrafish JAK2 homologs is accompanied by divergent embryonic expression patterns: only jak2a is expressed during erythropoiesis. Blood *94*, 2622–2636.

Oates, A.C., Pratt, S.J., Vail, B., Yan, Y., Ho, R.K., Johnson, S.L., Postlethwait, J.H., and Zon, L.I. (2001). The zebrafish *klf* gene family. Blood *98*, 1792–1801.

Parker, L., and Stainier, D.Y. (1999). Cell-autonomous and non-autonomous requirements for the zebrafish gene cloche in hematopoiesis. Development *126*, 2643–2651.

Prince, V.E., Moens, C.B., Kimmel, C.B., and Ho, R.K. (1998). Zebrafish hox genes: expression in the hindbrain region of wild-type and mutants of the segmentation gene, *valentino*. Development *125*, 393–406.

Quinkertz, A., and Campos-Ortega, J.A. (1999). A new beta-globin gene from the zebrafish, betaE1, and its pattern of transcription during embryogenesis. Dev. Genes Evol. *209*, 126–131.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L. (2003). A role for Wnt

signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409–414.

Ruvinsky, I., Silver, L.M., and Ho, R.K. (1998). Characterization of the zebrafish tbx16 gene and evolution of the vertebrate T-box family. Dev. Genes Evol. *208*, 94–99.

Schier, A.F. (2001). Axis formation and patterning in zebrafish. Curr. Opin. Genet. Dev. *11*, 393–404.

Serluca, F.C., and Fishman, M.C. (2001). Pre-pattern in the pronephric kidney field of zebrafish. Development *128*, 2233–2241.

Seufert, D.W., Brennan, H.C., DeGuire, J., Jones, E.A., and Vize, P.D. (1999). Developmental basis of pronephric defects in Xenopus body plan phenotypes. Dev. Biol. *215*, 233–242.

Silver, L., and Palis, J. (1997). Initiation of murine embryonic erythropoiesis: a spatial analysis. Blood *89*, 1154–1164.

Thompson, M.A., Ransom, D.G., Pratt, S.J., MacLennan, H., Kieran, M.W., Detrich, H.W., 3rd, Vail, B., Huber, T.L., Paw, B., Brownlie, A.J., et al. (1998). The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. Dev. Biol. *197*, 248–269.

Tsai, F.Y., and Orkin, S.H. (1997). Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. Blood *89*, 3636–3643.

Walmsley, M., Ciau-Uitz, A., and Patient, R. (2002). Adult and embryonic blood and endothelium derive from distinct precursor populations which are differentially programmed by BMP in Xenopus. Development *129*, 5683–5695.

Walters, M.J., Wayman, G.A., Notis, J.C., Goodman, R.H., Soderling, T.R., and Christian, J.L. (2002). Calmodulin-dependent protein kinase IV mediated antagonism of BMP signaling regulates lineage and survival of hematopoietic progenitors. Development *129*, 1455–1466.

Weinberg, E.S., Allende, M.L., Kelly, C.S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O.G., Grunwald, D.J., and Riggleman, B. (1996). Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. Development *122*, 271–280.

Weiss, M.J., and Orkin, S.H. (1995). Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. Proc. Natl. Acad. Sci. USA *92*, 9623–9627.

Westerfield, M. (2000). The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). (Eugene, OR: University of Oregon).

Yamamoto, A., Amacher, S.L., Kim, S.H., Geissert, D., Kimmel, C.B., and De Robertis, E.M. (1998). Zebrafish paraxial protocadherin is a downstream target of spadetail involved in morphogenesis of gastrula mesoderm. Development *125*, 3389–3397.