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Distinct Regulation of the Anterior and Posterior *myeloperoxidase* Expression by Etv2 and Gata1 during Primitive Granulopoiesis in Zebrafish

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Abstract

Neutrophilic granulocytes are the most abundant type of myeloid cells and form an essential part of the innate immune system. In vertebrates the first neutrophils are thought to originate during primitive hematopoiesis, which precedes hematopoietic stem cell formation. In zebrafish embryos, it has been suggested that primitive neutrophils may originate in two distinct sites, the anterior (ALPM) and posterior lateral plate mesoderm (PLPM). An ETS-family transcription factor Etsrp/Etv2/ER71 has been implicated in vasculogenesis and hematopoiesis in multiple vertebrates. However, its role during neutrophil development is not well understood. Here we demonstrate using zebrafish embryos that Etv2 has a specific cell-autonomous function during primitive neutropoiesis in the anterior lateral plate mesoderm (ALPM) but has little effect on erythropoiesis or the posterior lateral plate mesoderm (PLPM) expression of neutrophil marker myeloperoxidase *mpo*/*mpx*. Our results argue that ALPM-derived neutrophils originate from *etv2*-expressing cells which downregulate *etv2* during neutropoiesis. We further show that Scl functions downstream of Etv2 in anterior neutropoiesis. Additionally, we demonstrate that *mpx* expression within the PLPM overlaps with *gata1* expression, potentially marking the cells with a dual myelo-erythroid potential. Intriguingly, initiation of *mpx* expression in the PLPM is dependent on *gata1* but not *etv2* function. Our results demonstrate that *mpx* expression is controlled differently in the ALPM and PLPM regions and describe novel roles for *etv2* and *gata1* during primitive neutropoiesis.

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Keywords

etv2; etsrp; er71; gata1; neutrophil; primitive hematopoiesis; zebrafish; granulocytes; myeloid

Introduction

Neutrophils are the most abundant type of leukocyte in the body, and are an important component of the rapid non-specific innate immune response. Defects in the formation and differentiation of myeloid cells can lead to different disorders such as leukemia and neutropenia (Hall and Crosier, 2010; Lakshman and Finn, 2001). During embryogenesis, the first myeloid cells in different vertebrates such as mouse and zebrafish are thought to originate during primitive hematopoiesis, prior to the emergence of hematopoietic stem cells (Galloway and Zon, 2003; Xu et al., 2012). However, the relationships between the factors that control different cell fate decisions during primitive hematopoiesis are not well understood.

Zebrafish have recently emerged as an advantageous model to study hematopoietic development. Their large progeny numbers, external development and early transparency allows for *in vivo* observation of early hematopoietic processes (Bradbury, 2004). Zebrafish possess all of the major hematopoietic lineages that exist in mammalian species, and the genetic pathways directing differentiation of these blood cell types are highly conserved (Burns et al., 2009; Davidson and Zon, 2004). Similar to mammalian embryos, hematopoiesis in the zebrafish occurs in two successive, distinct waves: primitive and definitive hematopoiesis. Primitive hematopoiesis occurs in two anatomically separate regions, the anterior lateral plate mesoderm (ALPM) and the posterior lateral plate mesoderm (PLPM) (Galloway and Zon, 2003). Cells of the ALPM and PLPM co-express hematopoietic and vascular markers, such as *scl*, *lmo2*, *gata2*, *fli1* and *etv2* (Liao et al., 1998; Sumanas et al., 2005; Thompson et al., 1998). These cells have the potential to give rise to both blood and vascular endothelial cells, and are termed “hemangioblasts” (Vogeli et al., 2006). The ALPM forms the rostral blood island (RBI) and gives rise to macrophages, neutrophils, vascular endothelial and endocardial cells. The PLPM produces the intermediate cell mass (ICM) and generates erythroid, vascular endothelial cells and potentially neutrophils (Davidson and Zon, 2004; Hsia and Zon, 2005; Jin et al., 2012; Warga et al., 2009).

There has been some controversy regarding the origin of the earliest primitive neutrophils in a zebrafish embryo. Fate mapping approaches in two independent studies have demonstrated that the ALPM region gives rise to both macrophages and neutrophils (Jin et al., 2012; Le Guyader et al., 2008). However, it was also argued that neutrophils come exclusively from the PLPM region (Warga et al., 2009). During later embryonic stages neutrophils originate during the process of definitive hematopoiesis from hematopoietic stem cells (HSCs) or a recently identified population of erythromyeloid progenitors (EMPs) located within the posterior blood island (PBI) (Bertrand et al., 2007; Le Guyader et al., 2008; Murayama et al., 2006).

One of the most critical factors that regulate differentiation of multiple hematopoietic cell types is *stem cell leukemia (scl/tal1)*. *scl* has been shown to be required for both erythroid and myeloid development and is expressed in the ALPM and PLPM starting at the 2-somite stage where it initiates hematopoietic and vascular endothelial progenitor (angioblast) development (Dooley et al., 2005; Liao et al., 1998; Patterson et al., 2005). In zebrafish, *scl* knockdown embryos fail to form erythrocytes, macrophages and neutrophils (Dooley et al., 2005; Patterson et al., 2005). In mice, *scl* is expressed in the blood islands of the yolk sac, and knockout embryos are embryonic lethal due to defects in primitive erythropoiesis (Robb et al., 1995; Shivdasani et al., 1995). Subsets of *scl*-expressing cells in both the ALPM and PLPM acquire a myeloid fate by expressing *pu.1*, an ETS-family transcription factor essential for myeloid development (Bennett et al., 2001; Lieschke et al., 2002). *pu.1* knockdown zebrafish embryos show absence of neutrophils and macrophages, which could be rescued by injection of murine *pu.1* mRNA (Rhodes et al., 2005). In mice, *pu.1* is expressed exclusively in hematopoietic cells, and knockout mice lack monocytes, T and B lymphocytes, macrophages and neutrophils, however still possess erythroid cells (McKercher et al., 1996; Scott et al., 1994).

In all vertebrates, one of the most specific markers for neutrophil precursors is *myeloperoxidase (mpx / mpo)*. In zebrafish, *mpx* expression is first initiated within the PLPM by the 20-somite stage (Bennett et al., 2001; Le Guyader et al., 2008; Lieschke et al., 2001; Warga et al., 2009). In the ICM, a subset of cells transiently co-express *pu.1* and *mpx* right before 24 hpf, although not all *pu.1* positive cells express *mpx*, and vice versa (Bennett et al., 2001; Herbomel et al., 1999; Lieschke et al., 2001). Shortly thereafter, a separate pool of *mpx* expressing cells is observed in the ALPM region by 22-24 hpf. Fate-mapping studies have argued that both ALPM and PLPM-derived cells contribute to neutropoiesis suggesting that *mpx* expression in both regions labels neutrophil progenitors (Jin et al., 2012; Le Guyader et al., 2008; Warga et al., 2009).

Another important hematopoietic transcription factor, *gata1*, is first expressed at the 5-somite stage in the PLPM, downstream of *scl* (Detrich et al., 1995*). gata1*-expressing cells give rise to erythroid cells. Zebrafish *gata1* knockdown embryos display a loss of erythroid cells and an increase in neutrophils and macrophages at the embryonic stages past 24 hpf, after blood circulation has been established (Galloway et al., 2005; Rhodes et al., 2005). Mice null for *gata1* die during gestation due to failure of erythrocyte precursors to differentiation into red blood cells (Fujiwara et al., 1996). Studies in mouse and zebrafish demonstrate that *gata1* and *pu.1* both repress expression of each other (Rhodes et al., 2005; Zhang et al., 1999).

Ets1-Related Protein (Etsrp/Etv2/ER71) was recently identified as a hemangioblast and vascular endothelial-cell-specific transcription factor being necessary and sufficient for vascular endothelial and myeloid cell development (Pham et al., 2007; Sumanas et al., 2008; Sumanas and Lin, 2006). *etv2* is expressed within the putative vascular endothelial precursors in the zebrafish embryo starting at the 1-somite stage. Knockdown of *etv2* causes a dramatic inhibition of vascular endothelial cell differentiation and loss of macrophages and neutrophils (Sumanas et al., 2008). Vascular endothelial and macrophage-specific *scl* expression is downregulated in the ALPM and PLPM region in *etv2* morpholino (MO)

knockdown embryos (morphants), while erythroid-specific *scl* expression in the PLPM is largely unaffected. Similar to zebrafish embryos, *er71/etv2* knockout mouse embryos fail to undergo yolk sac vasculogenesis and exhibit defects in hematopoiesis (Ferdous et al., 2009; Lee et al., 2008). While previous studies have suggested that neutrophils in zebrafish are both ALPM and PLPM derived, it is currently not known if similar genetic pathways regulate formation of both pools of neutrophils and if Etv2 knockdown affects both populations of neutrophil progenitors. Most previous studies have focused on analysis of neutropoiesis after 24 hpf when the cells derived from both neutrophil populations are mixed (Dooley et al., 2005; Galloway et al., 2005; Jin et al., 2012; Sumanas et al., 2008)

In this study, we focused on the transcriptional regulation of neutropoiesis in the ALPM and PLPM regions. We demonstrate that *etv2* has a critical cell-autonomous role during the primitive neutropoiesis in the ALPM region, but does not have a major role in initiating *mpx* expression in the PLPM. We show that *scl* functions downstream of *etv2* in initiating neutropoiesis in the ALPM. We also demonstrate that *gata1* initially has a supportive role during initiating *mpx* expression in the PLPM region, which later switches to an inhibitory role. Because genetic pathways that regulate early hematopoiesis are highly evolutionarily conserved, our findings will also promote understanding of neutropoiesis and hematopoiesis in mammalian embryos and may ultimately advance medical treatments for hematopoietic-related disorders.

Materials and Method

Zebrafish Strains

Embryos were raised and maintained at 28.5 ^{o}C under standard laboratory conditions (Westerfield, 2007). The following lines were used for experimental procedures: *Tg(mpx:GFP)^{uwm1}* (Mathias et al., 2006), *Tg(etv2:GFP)^{ci1}* (Proulx et al., 2010), *etv2^{y11}* (Pham et al., 2007) and *Tg(etv2:Kaede)^{ci6}* (Kohli et al., 2013). The *etsrp/etv2^{y11}* line was crossed into a *Tg(fli1a:GFP)^{y1}* (Lawson and Weinstein, 2002) background, and mutants were either identified prior to 24 hpf by lack of *fli1a:GFP* expression in the anterior head vasculature and in the endocardial cells fusing at the midline, or later at 24 hpf by lack of intersomitic vessels. *etv2:Kaede* embryos were exposed to UV light from a DAPI filter, photoconverting the Kaede protein from green to red, to allow for detection of *mpx:GFP/ etv2:Kaede* positive cells.

Whole Mount In Situ Hybridization

In situ hybridization (ISH) was performed as previously described (Jowett, 1999). For the two-color in situ hybridization, the same protocol was followed except that embryos were incubated with 2 probes, a digoxigenin-UTP labeled probe, developed with NBT/BCIP and a fluorescein-UTP labeled probe, developed with Vector Red (Vector Laboratories). The following riboprobes were used: *etsrp/etv2* (Sumanas et al., 2005),*mpx* (Bennett et al., 2001), *scl* (Liao et al., 1998), *pu.1* (Lieschke et al., 2002), *gata1* (Detrich et al., 1995), *hbae3* (Brownlie et al., 2003), *lyz* (Liu and Wen, 2002), and *irf8* (Li et al., 2011).

Immunohistochemistry following Whole Mount In Situ Hybridization

Embryos were fixed at the appropriate stage in 4% paraformaldehyde for 3-4 hours at 4°C, and then dehydrated and stored at −20°C until ISH was performed. On the last day of the ISH protocol, after development of probe, embryos were rinsed with PBT and then washed with acetone for 30 minutes at -20°C, followed by a 1 minute wash with water and 3-5 minute washes with 0.1% PBT. Embryos were blocked with MAB (100mM maleic acid, 150mM NaCl) and 2% blocking reagent (Roche Applied Science) for 1 hour at room temperature, and incubated overnight at 4°C in a 1:200 dilution of anti-GFP-Alexa488 (Invitrogen #A21311) in MAB and blocking buffer. Following six 15 minute washes in TNT (0.1M Tris-HCl, pH 7.5, 150mM NaCl, 0.1% Tween-20), a secondary antibody was used to amplify the GFP signal. Embryos were incubated in a 1:200 dilution of goat anti-rabbit-Alexa488 (Invitrogen #A11008) in MAB or TNT overnight at 4°C. Embryos were rinsed 3-4 times in TNT before imaging.

Real-Time RT-PCR

Embryos were injected at the one- to two-cell stage with 25–50 pg of circular *etsrp*-XeX as previously described (Sumanas et al., 2008) in addition to *scl* MO. Batches of 20 injected and control uninjected embryos were frozen on dry ice at the tail bud stage. Total RNA was purified using the RNAquous-4PCR kit (Ambion). cDNA was synthesized using Superscript III reverse transcriptase and oligo-dT primer (Invitrogen). Real-time PCR was performed using Chromo4 thermal cycler (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). The following PCR profile was used: 95°C 5 min; 95°C 1 min, 58°C 1 min, 72°C 1 min, detection at 82°C for 10 sec; steps 2 through 5 repeated 44 times. Relative cDNA amounts for *mpx* were calculated using the Opticon 3 software (Bio-Rad) and normalized to the expression of *elongation factor 1* α (*EF1α*). Primers for measuring *mpx* expression span an intron-exon boundary to detect cDNA instead of genomic DNA. The following primer sequences were used. *mpx*-Forward: CTG CGG GAC CTT ACT AAT GAT GG; *mpx*-Reverse: CCT GGA TAT GGT CCA AGG TGT C; EF1α-Forward: TCA CCC TGG GAG TGA AAC AGC; EF1α-Reverse: ACT TGC AGG CGA TGT GAG CAG

Morpholino Knockdown

Morpholinos (MO) were injected at the one- to two-cell stage against the following mRNA sequences: Translation blocking MOs against *etv2* (7.5 ng MO1 + 7.5 ng MO2)(Sumanas and Lin, 2006), *gata1* (10.8 ng)(Rhodes et al., 2005), and *tnnt2a* (4ng)(Sehnert et al., 2002), a splice-site blocking MO against *scl* (12.5 ng)(Dooley et al., 2005), and a combination of translation (11 ng) and splice site blocking (0.7 ng) MOs against *pu.1* (Clay et al., 2007).

DNA/RNA Injections

To overexpress Etv2, a DNA construct driven under the Xenopus EF1α promoter (referred to as the XeX-*etv2* construct) was injected at a concentration of 25 pg/embryo (Sumanas et al., 2008). To overexpress *scl*, the sense RNA for s*cl* (Liao et al., 1998)was obtained by digesting *scl*-pCS2+ with NotI and transcribing with SP6 RNA polymerase mMessage Machine Kit (Ambion) and injected 3nl at a concentration of 133 pg/nl.

Cell Transplantation

Donor embryos (*mpx*:GFP) were injected with a mixture of *etv2* DNA (55 pg) and Tetramethylrhodamine isothiocyanate (TRITC) dextran at a concentration of 1 mg/ml (Invitrogen, Mw=2MDa) into the blastomere at the 1-cell stage. Donor and recipient embryos (*mpx*:GFP) were dechorionated with 2% Pronase (20 mg/ml) and then transferred to embryo media. Twenty to 50 cells were transplanted at the sphere to 30% epiboly stages using a CellTram Air (Eppendorf). Embryos were observed at 24-27 hpf for presence of TRITC-labeled donor cells and *mpx:*GFP fluorescence.

Imaging

Embryos were whole-mounted in 2% methylcellulose or 1% low melt agarose on glass slides. Images were captured using 20x/0.5 NA objective or 10x/0.3 NA objective on an AxioImager Z1 (Zeiss) compound microscope with Axiocam ICC3 color camera (Zeiss) and Axiocam MMR black and white camera (Zeiss). Images in different focal planes were combined using Extended Focus module within Axiovision software (Zeiss). Image levels were adjusted using Adobe Photoshop CS2 and CS6 to increase the contrast. For fluorescent in situ hybridization imaging, embryos were mounted in 0.6% low melting point agar with an overlay of embryo media. Confocal images were acquired using a Nikon D-Eclipse C1 (Nikon Instruments Inc., USA) equipped with a Plan Aprochromat 20X/0.75 NA microscope objective (Nikon Instruments Inc., USA) or an Apo LWD 40X/1.15 NA microscope objective (Nikon Instruments Inc., USA). NBT/BCIP fluorescence was imaged as previously described,(Trinh le et al., 2007) with an excitation wavelength of 647 nm and an emission wavelength of 745 nm. Vector Red fluorescence was imaged with an excitation wavelength of 561 nm and an emission wavelength of 685 nm. Images in different focal planes were combined and brightness and contrast levels were adjusted using Imaris software (Bitplane).

Results

Etv2 is required for primitive neutropoiesis in the ALPM

Previous studies have implicated a role for Etv2 during neutropoiesis, as *etv2*-MO injected embryos show a reduction in the neutrophil marker *mpx* expression at 24 hpf (Sumanas et al., 2008). However, it was not clear if both ALPM and PLPM-derived neutrophils were affected in *etv2* morphants. To investigate the role for Etv2 during neutropoiesis in greater detail, we analyzed *mpx* expression in the previously identified $etv2^{y11}$ mutants (Pham et al., 2007). At the 20-somite stage, when *mpx* expression is first evident in the PLPM, *etv2* morphants displayed slight reduction in both *mpx* expression level and the number of *mpx*-expressing cells (Fig. 1A-C). At 24 hpf, two separate ALPM and PLPM -derived *mpx*-positive cell populations were apparent in wild-type (wt) embryos (Fig. 1D,F). In contrast, the anterior *mpx*-positive cell population was absent in $etv2^{y11}$ mutant embryos while *mpx*-positive cells in the trunk and tail region were not significantly affected (Fig. 1C-G). After 24 hpf, circulation is initiated in zebrafish embryos and both populations are likely to undergo some mixing. At 30 hpf, the level of *mpx* expression and the number of *mpx* expressing cells were greatly reduced in both the anterior and posterior regions of $etv2^{y11}$ mutant embryos as compared with their wild-type siblings (Fig. 1C,H-K). Additional

markers for the anterior myeloid population *cebpa* and a neutrophil-specific marker l*ysozyme C (lyz)* (Liu and Wen, 2002; Lyons et al., 2001; Meijer et al., 2008; Pase et al., 2012) were nearly completely absent in *etv2*^{y11} mutant embryos at 24 hpf (Fig. 1N-Q). In contrast, and consistent with our previous *etv2* MO knockdown studies (Sumanas and Lin, 2006), erythroid specific *gata1* expression remained largely unaffected in the ICM region of *etv2*^{y11} mutants at 24 hpf (Fig. 1L,M). Thus, Etv2 is required for myelopoiesis in the ALPM and but has little effect in initiating erythropoiesis and *mpx* expression in the ICM prior to the initiation of circulation.

To test if Etv2 was sufficient to initiate *mpx* expression, an Etv2 DNA overexpression construct was injected and embryos were collected for in situ hybridization. *mpx* expression was ectopically induced at the 20-somite stage after *etv2* injection (Fig. 1R,S). Similar induction of GFP expression was also observed in the *mpx*:GFP reporter line (Fig. 1T,U). These findings demonstrate that Etv2 is both required and sufficient to induce *mpx* expression during primitive neutropoiesis.

Neutrophils in the ALPM are derived from *etv2*-positive progenitors

Previous work has demonstrated *etv2* expression in vascular endothelial progenitors (Sumanas and Lin, 2006). However, it is not clear if *etv2* is expressed in any hematopoietic progenitors, including neutrophil precursors. To test if *mpx* and *etv2* expression overlapped during primitive hematopoiesis, double in situ hybridization was performed. It was apparent that *mpx* expression largely did not overlap with *etv2* expression in the ICM region at the 20-somite stage (Fig. 2A-C). In contrast, posterior *mpx* expression overlapped with the hematopoietic master regulator *scl* (Fig. 2D-F), the erythroid precursor marker *gata1* (Fig. 2G-I), and the erythroid cell marker *alpha embryonic hemoglobin hbae3* (Fig. 2J-L). These results suggest that *mpx* may not specifically mark neutrophils in the ICM, and instead *mpx* may be transiently expressed in the erythroid population (see Discussion).

Because *etv2* expression initiates significantly earlier than that of *mpx*, it is possible that neutrophils are derived from *etv2*-positive precursors, which may downregulate *etv2* expression upon differentiation. In order to determine if *mpx*-expressing neutrophils had at one time expressed *etv2*, we utilized an *etv2:GFP* transgenic line (Proulx et al., 2010). GFP has a long half-life, therefore its expression can be used as a lineage tracer to mark the progeny of *etv2*-expressing cells. As observed by a combined GFP immunofluorescence and *mpx* in situ hybridization assay, only 10.8% of *mpx*-expressing cells in the ICM region also showed *etv2*:GFP-fluorescence (out of the total of 417 *mpx* cells counted in 13 embryos) (Fig. 2M-O). Similar results were obtained when using the *etv2:Kaede* transgenic line (Kohli et al., 2013) to detect co-expression of *mpx:GFP* and *etv2:Kaede* in neutrophils. *etv2:Kaede* was photoconverted prior to imaging to completely convert the Kaede protein from green to red. Only rarely was overlap of *mpx*:GFP and *etv2*:Kaede observed in the posterior region (Fig. 2P-R). These results argue that the majority of *mpx*-positive cells in the ICM region are not derived from *etv2*-positive cells. *etv2* expression in the ALPM region as observed by ISH at 24 hpf is restricted to vascular endothelial cells and is not apparent in myeloid cells or neutrophils (Sumanas et al., 2008; Sumanas and Lin, 2006). However, in contrast to the ICM, 64% of *mpx*-positive cells located over the yolk (out of 89 total cells

analyzed in 5 embryos), were also positive for *etv2*:GFP expression (Fig. 2S-U). As shown in the earlier studies, these cells originate in the ALPM region (Le Guyader et al., 2008). These data suggest that the majority of anterior *mpx* cells originate from *etv2*-expressing cells.

To demonstrate further that *etv2*-expressing cells give rise to *mpx*-positive precursors, we utilized cell transplantation to generate mosaic embryos. In a control experiment, 20-50 cells were transplanted at the sphere stage from *mpx:GFP* donor embryos into *mpx:GFP* recipient embryos close to the animal pole. This region normally contributes to ectodermal tissues and is not expected to give rise to myeloid progenitors (Kimmel et al., 1990). Indeed, none of the donor cells showed *mpx*:GFP expression in more than 20 embryos analyzed at 26 hpf (Fig. 3A-C). In contrast, when *mpx*:GFP donor embryos were injected with *etv2* DNA, 83% of the recipient embryos (20 out of 24) had *mpx*:GFP positive donor cells (Fig. 3D-F). This argues that *etv2*-overexpressing cells can autonomously induce *mpx* expression.

Scl functions downstream of Etv2 in the anterior neutropoiesis

Scl has been previously implicated in the formation of all hematopoietic lineages, including myelopoiesis in the ALPM (Dooley et al., 2005; Patterson et al., 2005). Our previous work has shown that *etv2* is required for *scl* expression in the ALPM (Sumanas et al., 2008). To determine if *scl* functions downstream of *etv2* in initiating anterior *mpx* expression, we tested if *scl* overexpression would rescue *mpx* expression in *etv2* knockdown embryos. Indeed, injection of *scl* RNA effectively restored anterior *mpx* expression in *etv2* MO-injected embryos (Fig. 4A-D).

To determine if *scl* was required for precocious induction of *mpx* expression by *etv2*, qPCR analysis was performed at the 12-somite stage, when endogenous *mpx* expression is not yet observed. While *etv2* DNA overexpression resulted in strong precocious induction of *mpx* expression, this induction was absent in the embryos coinjected with *etv2* DNA and a previously characterized scl MO (Dooley et al., 2005) (Fig. 4E). These results demonstrate that *scl* functions downstream of *etv2* during neutropoiesis in the ALPM.

scl and gata1, but not *pu.1* are required for posterior *mpx* expression

Our data argue that Etv2 has only a minor effect on *mpx* expression in the ICM. Therefore we examined the role of other transcription factors that have been previously implicated in hematopoiesis. Previous studies have demonstrated a requirement for *scl* and *pu.1* in neutropoiesis (Dooley et al., 2005; Rhodes et al., 2005). However, the analysis focused largely on embryonic stages past 24 hpf after neutrophils have already entered circulation. To determine if *scl* and *pu.1* have similar requirements in the initiation of *mpx* expression within the ICM region, we performed functional knockdown using previously validated *scl* and *pu.1* MOs (Clay et al., 2007; Dooley et al., 2005). Expression of *mpx* was absent in *scl* knockdown embryos and was largely unaffected in *pu.1* knockdown embryos at the 22-somite stage (Fig. 5B,D, F). As expected, expression of erythroid specific marker *hemoglobin alpha embryonic 3 (hbae3)* was absent only in *scl* morphants and not affected in *pu.1* morphants (Fig. 5A,C, E). To confirm the efficacy of the *pu.1* MO, we examined anterior *mpx* expression at 24 hpf. Anterior *mpx* expression was strongly reduced or absent

in *pu.1* knockdown embryos as previously shown (Fig. 5K,L)(Rhodes et al., 2005), whereas the posterior domain was largely unaffected (Fig. 5M,N). Additionally, the expression of the myeloid marker *lplastin* was strongly reduced in *pu.1* morphant embryos (data not shown).

gata1, which is an important transcription factor for erythroid development, has previously been shown to repress *pu.1* expression during hematopoiesis (Rhodes et al., 2005). Previous studies have demonstrated that *mpx* expression is greatly increased at 32 hpf in *gata1* knockdown embryos, arguing that erythroid precursors assume myeloid fates in the absence of *gata1* (Rhodes et al., 2005). Interestingly, reduced *mpx* expression was observed in *gata1* morphants at 22 hpf (Galloway et al., 2005). We observed a similar loss of *mpx* expression in *gata1* morphants at the 20 somite stage (Fig. 5H, J). As expected, *hbae3* expression was strongly reduced by *gata1* knockdown (Fig. 5G, I). This result suggested an early requirement for *gata1* in initiating *mpx* expression in the ICM, which is different from its later role in suppressing myelopoiesis.

gata1 is required for early initiation of *mpx* expression in the ICM, however later acts to repress the neutrophil fate

To better understand how *mpx* expression in the ICM region is affected in *gata1* morphants, we used a time-course approach to assay for *mpx* and *pu.1* expression after *gata1* MO knockdown. Although *pu.1* is initially expressed in the PLPM region, by the 20-somite stage its expression is downregulated and is no longer apparent in wild-type embryos (Fig. 6A) (Lieschke et al., 2002). In agreement with the previous studies (Galloway et al., 2005; Rhodes et al., 2005), in *gata1* morphants *pu.1* was dramatically increased at the 20-somite stage in the ICM region (Fig. 6A,B). In contrast, *mpx* expression was absent at the 20-somite stage in *gata1* morphants, as compared to uninjected control embryos (Fig. 6C,D). *pu.1* expression was also increased in the ICM region at the 24-somite, 26 hpf and 32 hpf stages, when it is not observed in control uninjected embryos (Fig. 6E,F,I,J,M,N). *mpx* expression was also strongly decreased in *gata1* morphants at the 24-somite stage (Fig. 6G,H). Other myeloid markers such as neutrophil-specific *lyz* and macrophage-specific *interferon regulatory factor 8* (*irf8*) were not expressed at the 24-somite stage in control uninjected embyros (Suppl. Fig. S1A,C) but were expressed in a small number of cells at the 24-somite stage in *gata1* morphants (Suppl. Fig. S1B,D). At the 26 hpf and 32 hpf stages, *mpx* expression was increased in the *gata1* morphants as compared to control uninjected embryos (Fig. 6K,L,O,P), which is in accordance with the previous reports (Galloway et al., 2005; Rhodes et al., 2005). Consistent with this result, *lyz* and *irf8* expression was increased in *gata1* morphants at 32 hpf (Suppl. Fig. S1E-H).

To confirm that the increased *mpx* expression at 32 hpf represents posteriorly-derived cells only, we used the *troponin T2a (tnnt2a)* morpholino to stop blood circulation (Sehnert et al., 2002). Posterior *mpx* expression was normal in embryos injected with *tnnt2a* MO only (Suppl. Fig. S2A, B). The average number of anterior and posterior *mpx*-expressing cells in control uninjected (75 ± 22.7 in anterior, 14 ± 6.7 in posterior, n=19) and in *tnnt2a* morphants (65 ± 20.4 in anterior, 17 ± 11.7 in posterior, n=15) was not significantly different ($p>0.1$). Injection of *gata1* MO and *tnnt2a* in combination caused a similar increase in posterior *mpx* expression as injection of *gata1* MO alone, indicating that the additional

posterior cells are derived from the initial posterior ICM expression domain (Suppl. Fig. S2C,D). These results argue that *gata1* has a specific requirement in initiating *mpx* expression in the ICM, while at later stages its role switches to the repression of myelopoiesis.

Discussion

In this study, we demonstrate that *etv2* has a critical cell autonomous requirement during neutropoiesis in the anterior lateral plate mesoderm. We further show that *scl* functions downstream of *etv2* in ALPM-derived neutropoiesis. In contrast, posterior *mpx* expression largely does not depend on *etv2* or *pu.1* function, but is regulated by *scl* and, surprisingly, *gata1* expression. Similar to its zebrafish homolog, mouse *etv2* function has been previously implicated in both vasculogenesis and hematopoiesis (Ferdous et al., 2009; Kataoka et al., 2011; Lee et al., 2008). Interestingly, HSC-specific knockout of mouse *er71/etv2* displays defects in myelopoiesis, similar to the zebrafish knockdown phenotype (Lee et al., 2011). This argues for a high degree of functional conservation between mammalian and zebrafish Etv2 function.

Our results show that *mpx* expression in the zebrafish ALPM and PLPM are regulated differently. *mpx* expression in the ALPM depends on *etv2* function while its expression in the PLPM is largely *etv2*-independent. Because *etv2* expression is not observed in ALPM-derived neutrophil progenitors, while *etv2*:GFP is, this supports the hypothesis that neutrophil progenitors in the ALPM are derived from *etv2*-expressing cells that downregulate *etv2* expression upon myeloid differentiation. Although not all ALPM-derived *mpx* cells displayed *etv2*:GFP expression, it is quite possible that some cells downregulated *etv2*:GFP beyond the detectable level. Cell transplantation data provide further support for a cell autonomous function of *etv2* during myelopoiesis. However, our experimental data do not exclude the possibility that *etv2* also has a non-autonomous function during neutropoiesis in the ALPM.

As evident from this and previous studies, *mpx* expression is initiated in the ALPM and PLPM regions at distinct times and under different genetic control. Two independent studies have demonstrated using fate mapping that ALPM region gives rise to neutrophil progenitors (Jin et al., 2012; Le Guyader et al., 2008). On the other hand, the fate of posterior *mpx*-expressing cells is currently less clear. Our data show that all *mpx*-positive cells in the ICM are also positive for the erythroid marker *gata1* and embryonic globin *hbae3* expression. This argues for two possibilities. First, $gata1^{+}mpx^{+}$ cells in the ICM represent erythro-myeloid progenitors and may contribute to both erythroid and myeloid lineages. A second possibility is that all $gata1^{+}mpx^{+}$ cells in the ICM express *mpx* only transiently, and lose *mpx* expression as they undergo erythroid differentiation. This scenario supports the model where all primitive myeloid lineages (macrophages and neutrophils) originate within the ALPM while all PLPM gives rise only to erythroid progenitors (as well as endothelial cells). The coexpression of *mpx* and *hbae3* in the ICM supports this model. However, hematopoietic progenitors in the ICM do have myeloid potential and can differentiate into neutrophils and macrophages if *gata1* function is inhibited.

In a previous study, single cell labeling in the ICM region at 24-26 hpf produced neutrophils and erythrocytes among its progeny (Warga et al., 2009). This result would support bipotent fates of *gata1*$^{+}$*mpx*$^{+}$ progenitors. However, it is possible that some of the ICM derived cells contribute to the erythro-myeloid progenitors (EMPs) that have been previously observed in the posterior blood island (PBI) at slightly later stages of 30-42 hpf (Bertrand et al., 2007). In fact, cell labeling has shown that these definitive EMPs originate in the ICM region (Bertrand et al., 2007). It remains to be determined what percentage of *gata1*$^{+}$*mpx*$^{+}$ cells in the ICM contribute to myeloid and erythroid lineages.

It is intriguing that *mpx* expression is regulated differently in the ALPM and PLPM. Our results argue that the majority of ALPM-derived neutrophil precursors originate from *etv2*-expressing cells, while the majority of *mpx*-positive cells in the PLPM are not derived from *etv2*-expressing cells. *scl* is required for *mpx* expression in both anterior and posterior regions, while only the anterior *scl* domain is regulated by Etv2. We have previously demonstrated that *scl* expression in the PLPM is induced independently of Etv2 (Sumanas et al., 2008). It is likely that there are other yet unidentified ETS factors that regulate posterior *scl* and *mpx* expression.

In situ hybridization analysis revealed that *mpx* expression overlaps with both *scl* and *gata1* in the ICM of the early embryo. Previous studies have also demonstrated overlap between *mpx* and *pu.1*-expressing cells (Bennett et al., 2001). Our results show that *scl* is required for the initiation of *mpx* expression in the ICM, consistent with the previous studies that analyzed *mpx* expression at later stages. Surprisingly, we and others (Galloway et al., 2005) have revealed an early requirement for the erythroid transcription factor *gata1* to initiate *mpx* expression in the ICM region. This is a transient loss in *mpx* expression since *mpx* becomes upregulated at 26 hpf in *gata1* morphants. As expected, when *gata1* is knocked down, expression of *pu.1* increases dramatically. In the previous studies an opposite effect of increased *mpx* expression was observed, as *pu.1* expression has been shown to drive *mpx* expression and *pu.1* and *gata1* have been demonstrated to have an antagonistic relationship (Bennett et al., 2001; Rhodes et al., 2005). However, this analysis was performed after 24 hpf, and our results indicate an early role for *gata1* but not *pu.1* in initiating *mpx* expression in the ICM region. Both *pu.1* and *gata1* are known to function in a transcriptional complex with multiple other co-factors. Presence or absence of one of these *gata1* co-factors may account for the switch in permissive and inhibitory roles of *gata1*.

Our results describe the transcriptional regulatory interactions during the earliest steps of primitive neutropoiesis, which have been poorly understood. Because the factors that regulate hematopoiesis are highly conserved between different vertebrates, our findings will promote further understanding of the molecular mechanisms that control hematopoiesis in mammalian embryos and may ultimately contribute to the development of novel therapeutic strategies to treat hematopoietic disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Zebrafish Etv2 regulates anterior but not posterior myeloperoxidase *mpx* expression.
- Anterior neutrophils originate from Etv2-expressing cells.
- Scl functions downstream of Etv2 during anterior neutropoiesis.
- Gata1 is required to initiate posterior mpx expression.

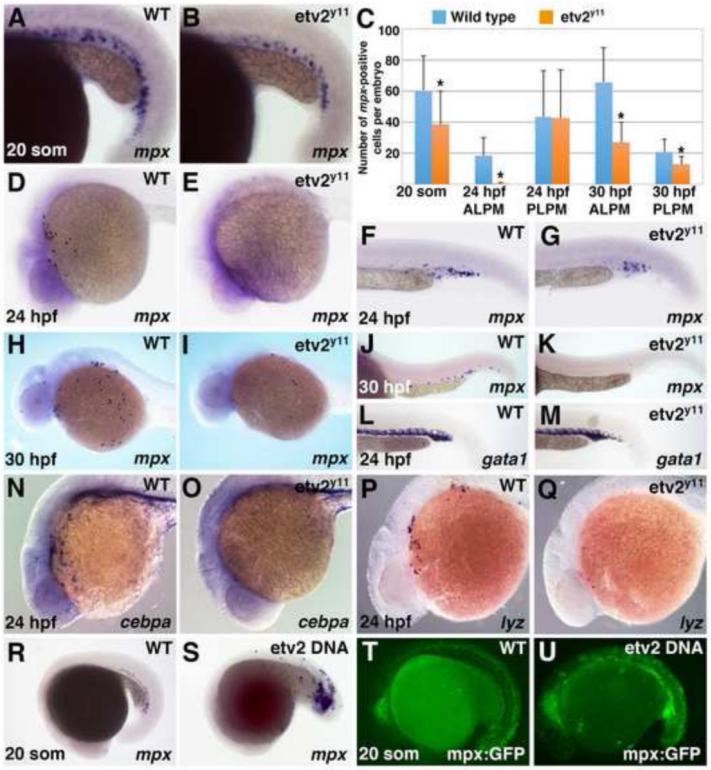

Figure 1. Etv2 function is necessary for neutropoiesis in the ALPM and is sufficient to initiate *mpx* expression

(A-K) Anterior but not posterior *mpx* expression is greatly reduced or absent in *etv2*^{y11}mutants, as analyzed by in situ hybridization. (A,B) *mpx* expression is slightly reduced in the ICM region of *etv2*^{y11} mutants (B) as compared to their wild-type siblings (A) at the 20 somite stage. (C) Number of *mpx*-expressing cells at various stages in the anterior and posterior lateral plate mesoderm of wild type and *etv2*^{y11} mutant embryos. The cells were counted in 30 wt and 23 *etv2*^{y11} mutant embryos at the 20-somite stage, 16 wt and 16 mutant embryos at 24 hpf and 10 wt and 9 mutant embryos at 30 hpf. Error bars correspond to ± standard deviation. Asterisks denote significant differences ($p<0.05$) calculated by Student's T-Test. (D, E) *mpx* expression is strongly reduced at 24 hpf in the ALPM of *etv2*^{y11} mutants (E) compared to wild type siblings (D). (F, G) *mpx* expression has only a minor reduction in the ICM region of *etv2*^{y11} mutants (G) compared to their wild-type siblings (F). (H-K) *mpx* expression is reduced at 30 hpf in the anterior (I) and posterior (K) of *etv2*^{y11} mutants compared to wild type siblings (H, J). (L, M) *gata1* expression is not significantly affected in *etv2*^{y11} mutants (M) as compared to wild-type siblings at 24 hpf (L). (N, O) *cebpa* expression at 24 hpf is strongly reduced in 100% of *etv2*^{y11} mutants (O, n=11) while it is strongly expressed in 100% of wild-type siblings (N, n=11). (P, Q) *lyz* expression is strongly reduced in 100% of *etv2*^{y11} mutants (Q n=14) compared to 100% of wild-type siblings with normal *lyz* expression (P, n=15). (R-U) Etv2 DNA overexpression results in ectopic *mpx* expression as analyzed by in situ hybridization (R,S) and *mpx*:GFP live fluorescence (T,U) at the 20-somite stage (65% of *etv2*-injected embryos with increased or ectopic *mpx*:GFP, n=115 as opposed to 0% controls, n=39). Note that *mpx*:GFP exhibits

non-specific GFP fluorescence in the neural tube. All embryos are positioned with the anterior to the left and dorsal side up.

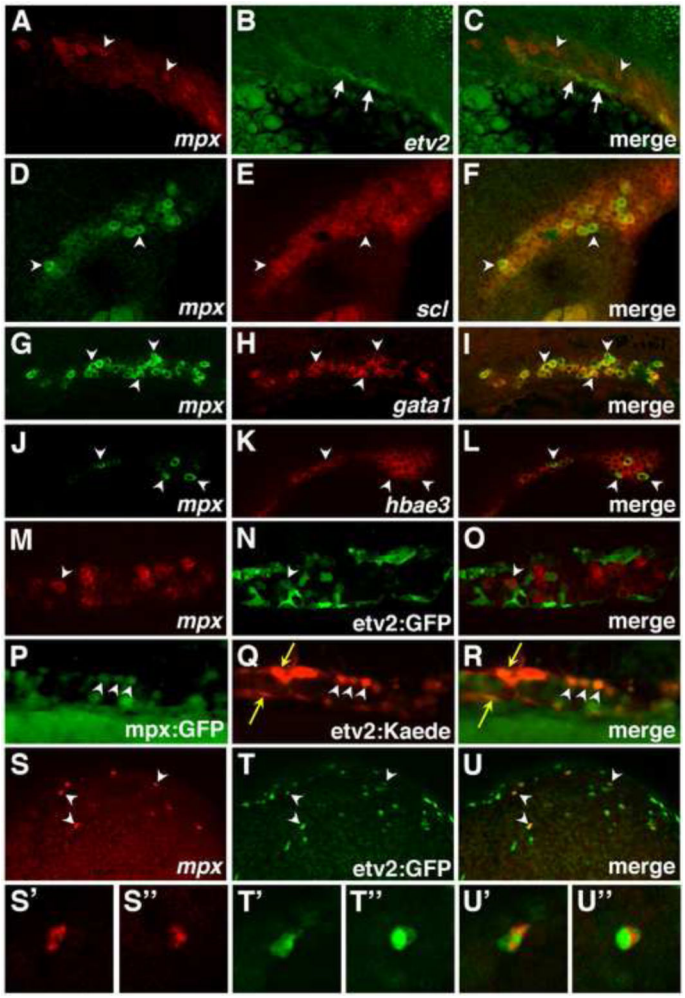

Figure 2. Comparison of *mpx, etv2, scl, gata1, hbae3, etv2*, GFP and *etv2*:Kaede expression

(A-L) *mpx* expression overlaps with *scl*, *gata1* and *hbae3,* but not *etv2,* in the ICM of wild-type embryos at the 22-somite stage, as analyzed by two color in situ hybridization. (A-C) *etv2* (arrows), and *mpx* (arrowheads) expression domains largely do not overlap. Maximum intensity projections of confocal images are shown . Autofluorescent yolk granules are apparent in the lower left corner in (B,C). (D-F) *mpx* expression partially overlaps with *scl* expression (selected overlapping cells, arrowheads). Single confocal slices are shown. (G-I) *mpx* and *gata1* expression largely overlap (arrowheads, selected cells). Single confocal slices are shown. (J-L) *mpx* and *hbae3* expression largely overlap (arrowheads, selected cells). Note that not all *hbae3* cells are positive for *mpx* expression. Single confocal slices are shown. (M-O) Only a fraction (10.8%) of *mpx*-expressing cells in the ICM overlap with *etv2*:GFP expression at the 24 somite stage, as observed by combined ISH for *mpx* expression and immunohistochemistry for GFP. (P-R) *etv2:*Kaede and *mpx:*GFP expression overlaps in neutrophils (white arrowheads) in the trunk ICM region of embryos at 24hpf. Yellow arrows denote vascular endothelial cells. (S-U) Multiple (64%) ALPM-derived *mpx*-expressing cells, positioned over the yolk, overlap with *etv2*:GFP expression at 24 hpf (white arrowheads) as observed by combined ISH for *mpx* expression (red) and

immunohistochemistry for GFP (green). (S'-U'') Magnified views of individual cells from S-U. Images show anterior to the left and dorsal up in all panels.

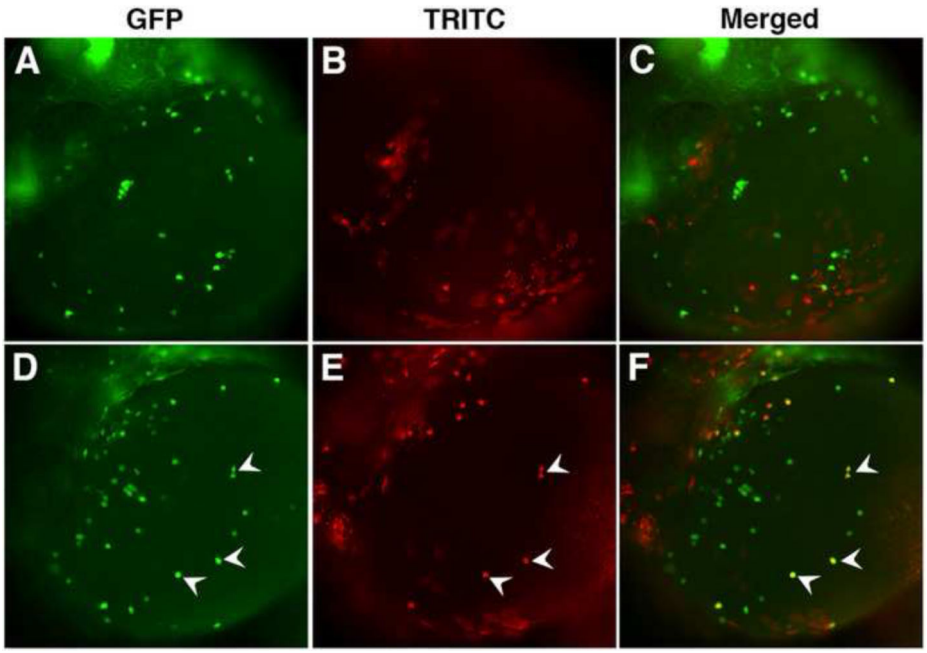

Figure 3. *etv2* functions cell autonomously in neutropoiesis

Donor *mpx*:GFP embryos were injected with either TRITC-dextran tracer or TRITC-dextran and *etv2* DNA, and the cells were transplanted at the sphere stage (4 hpf) into *mpx*:GFP recipient embryos. (A-C) Wild type cells transplanted into a wild type host do not induce *mpx* expression. (A) *mpx*:GFP, (B) TRITC and (C) merged fluorescence in the recipient embryos at 26 hpf stage. (D-F) A subset of *etv2*-overexpressing cells transplanted into a wild type host induce *mpx* expression autonomously. (D) *mpx*:GFP, (E) TRITC and (F) merged fluorescence in the recipient embryos at 26 hpf stage. White arrowheads point to overlapping cells, indicating that they are derived from the donor *etv2*-overexpressing embryos.

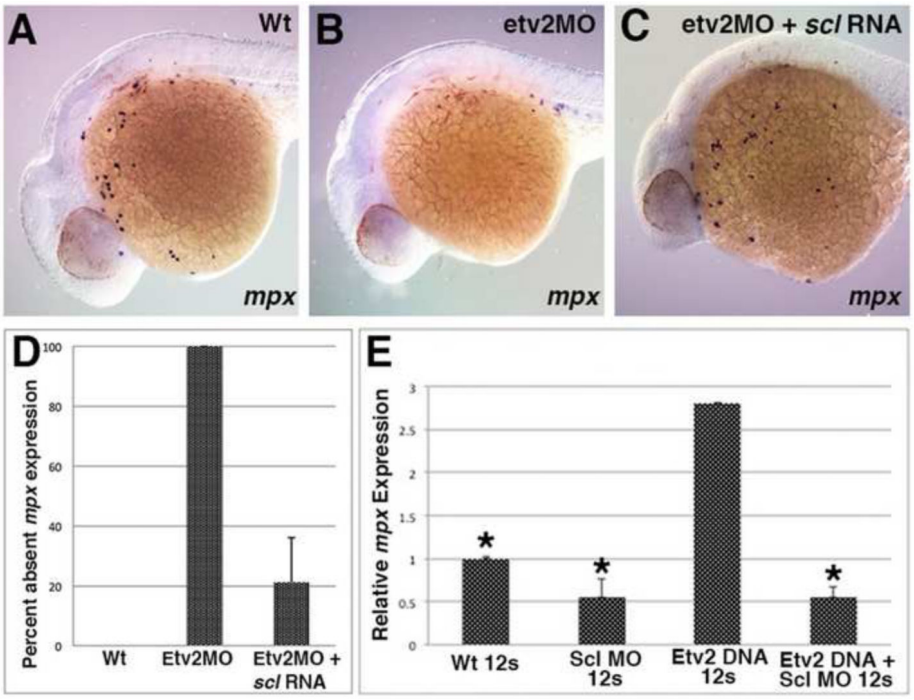

Figure 4. *scl* functions downstream of *etv2* during neutropoiesis in the ALPM

(A-D) *scl* RNA can rescue anterior *mpx* expression in *etv2* knockdown embryos, as analyzed at 24 hpf. *mpx* expression in *etv2* morphants (B, n=19/19) is decreased as compared to the wild type siblings (A, n=26/26). Injection of *scl* RNA rescues *mpx* expression in *etv2* morphants (C, n=23/29). All embryos are positioned with anterior to the left and dorsal side up. (E) Morpholino knockdown of *scl* reduces the induction of *mpx* by *etv2* DNA, as analyzed by real-time RT-PCR at the 12-somite stage. *mpx* expression is increased in *etv2* DNA injected embryos, relative to uninjected controls but is not changed significantly when *etv2* DNA is co-injected with *scl* MO. Relative expression levels have been normalized to EF1α expression. Asterisk denotes statistical significance vs. etv2 DNA over-expression ($p<0.01$) as analyzed by one-way ANOVA test with pair-wise comparison. Error bars correspond to ± standard deviation.. (n=3).

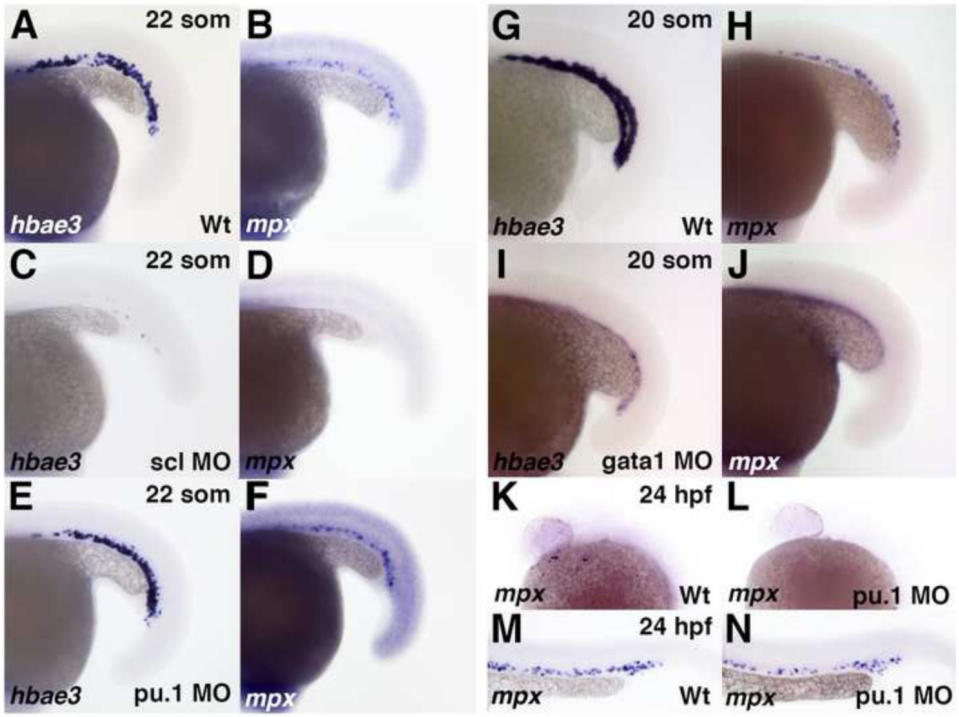

Figure 5. Knockdown of *scl* and *gata1*, but not *pu.1*, results in the loss of PLPM-specific *mpx* expression at the 22- and 20-somite stage

(A, B) Wild type expression of embryonic globin *hbae3* (A), and *mpx* (B) is normal in 100% (n=23) and 61% (n=28) of control uninjected embryos, respectively, in the intermediate cell mass region at the 22-somite stage. (C, D) *scl* morpholino knockdown results in the loss of *hbae3* (C) and *mpx* (D) expression in 100% (n=24) and 97% (n=32) of embryos, respectively. (E, F) Knockdown of *pu.1* does not affect *hbae3* expression (E, 100% of embryos, n=54), or *mpx* expression (F, 70% of embryos, n=37). (G, H) Wild type expression of embryonic globin *hbae3* (G), and *mpx* (H) in the intermediate cell mass region at the 20-somite stage is normal in 100% (n=20) and 78% (n=60) of control uninjected embryos, respectively. (I, J) Knockdown of *gata1* results in the loss of expression of both *hbae3* (I) and *mpx* in 100% of embryos, n=28 and n=100, respectively (J). (K, L) *mpx* expression is strongly reduced or absent in the anterior of *mpx* morpholino knockdown embryos (L, 92% of embryos, n=25) compared to uninjected controls (K, 13% of embryos, n=8). (M, N) *mpx* expression is normal in the posterior of *mpx* morpholino knockdown embryos (N, 88% of embryos, n=25) compared to uninjected controls (M, 75% of embryos, n=8).

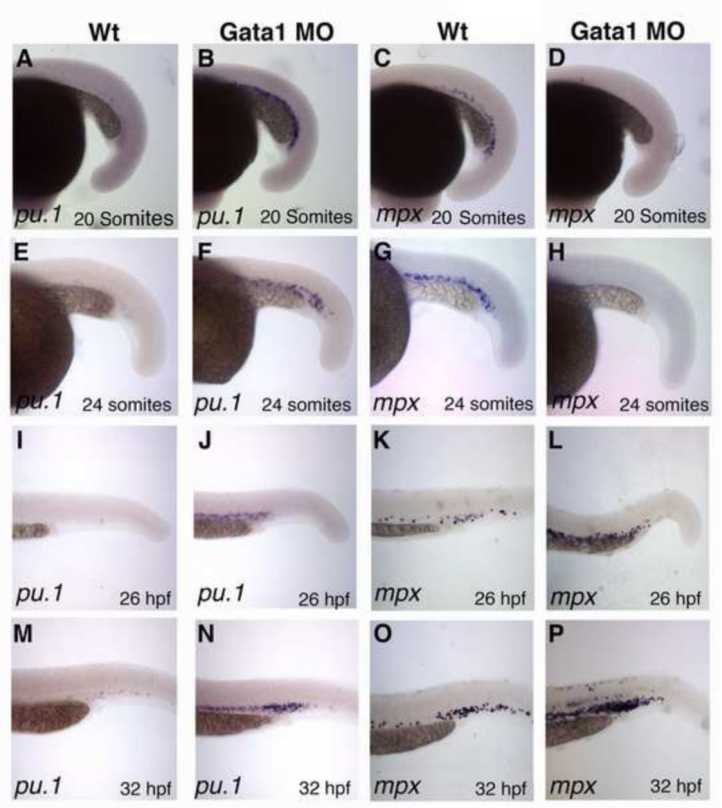

Figure 6. *gata1* is required to initiate *mpx* expression in the ICM region, but later represses it

(A-H) *pu.1* expression is greatly increased (n=16/16 and 31/31) while *mpx* expression is greatly reduced (n=66/66 and 26/26) as analyzed by ISH in *gata1* MO knockdown embryos at the 20-somite (A-D) and 24-somite (E-H) stages, respectively. (I-P) Both *pu.1* and *mpx* expression are greatly increased in *gata1* morphant embryos at 26 hpf and 32 hpf (n=35/35 and 30/33 for *pu.1* expression; n=27/31 and 21/24 for *mpx* expression at 26 hpf and 32 hpf, respectively). Dorsal is up and anterior is to the left in all panels.