1 Hemato-vascular specification requires *arnt1* and *arnt2* genes in zebrafish embryos

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8 ABSTRACT

9 During embryonic development, a subset of cells in the mesoderm germ layer are specified as
10 hemato-vascular progenitor cells, which then differentiate into endothelial cells and
11 hematopoietic stem and progenitor cells. In zebrafish, the transcription factor *npas4l*, also
12 known as *cloche*, is required for the specification of hemato-vascular progenitor cells.
13 However, it is unclear if *npas4l* is the sole factor at the top of the hemato-vascular specification
14 cascade. Here we show that *arnt1* and *arnt2* genes are required for hemato-vascular
15 specification. We found that *arnt1;arnt2* double homozygous mutant zebrafish embryos (herein
16 called *arnt1/2* mutants), but not *arnt1* or *arnt2* single mutants, lack blood cells and most
17 vascular endothelial cells. *arnt1/2* mutants have reduced or absent expression of *etv2* and *tal1*,
18 the earliest known endothelial and hematopoietic transcription factor genes. *npas4l* and *arnt*
19 genes are PAS domain-containing bHLH transcription factors that function as dimers. We
20 found that Npas4l binds both Arnt1 and Arnt2 proteins *in vitro*, consistent with the idea that
21 PAS domain-containing bHLH transcription factors act in a multimeric complex to regulate
22 gene expression. Our results demonstrate that *npas4l*, *arnt1* and *arnt2* act together as master
23 regulators of endothelial and hematopoietic cell fate. Our results also demonstrate that *arnt1*

and *arnt2* act redundantly in a transcriptional complex containing *npas4l*, but do not act redundantly when interacting with another PAS domain-containing bHLH transcription factor, the aryl hydrocarbon receptor. Altogether, our data enhance our understanding of hemato-vascular specification and the function of PAS domain-containing bHLH transcription factors.

INTRODUCTION

Endothelial and hematopoietic cells are derived from the mesoderm during vertebrate embryogenesis. These two cell types are closely linked and arise from a common progenitor during development (Huber et al., 2004; Kataoka et al., 2011; Liao et al., 2000). The differentiation and specification of endothelial and hematopoietic cells occurs in two waves—primitive and definitive hematopoiesis (Costa et al., 2012; Paik and Zon, 2010; Palis et al., 1999). During primitive hematopoiesis the first hemato-vascular progenitors are observed in the lateral plate mesoderm of zebrafish and in the extraembryonic yolk sac mesoderm of mice (Lee et al., 1994; Palis et al., 1999; Stainier et al., 1993). These progenitors give rise to blood islands which contain primitive erythroid cells (Palis et al., 1999). During definitive hematopoiesis in mice and zebrafish, fully multipotent hematopoietic stem cells (HSCs) are derived from specialized epithelial cells, hemogenic endothelium (Hirschi, 2012; Kobayashi et al., 2014).

In humans, the genes that drive the specification of hemato-vascular progenitors from the multipotent mesoderm are not completely understood. We have a more complete understanding of this process in other vertebrates. In zebrafish and chicken, Neuronal PAS 4-like (*npas4l*) is essential for the specification of hemato-vascular progenitors from multipotent mesoderm (Reischauer et al., 2016; Weng et al., 2020). Mammals do not appear to have a

47 *npas4l* homolog in their genomes and the mammalian functional equivalent to zebrafish *npas4l*
 48 is unknown. Many genes involved in embryonic hematopoiesis are conserved between
 49 zebrafish, mice, and humans (Aplan et al., 1992; Brownlie et al., 1998; Casie Chetty et al.,
 50 2017; Dooley et al., 2005; Ferrara et al., 1996; Gong et al., 2004; Kalev-Zylinska et al., 2002b;
 51 Kataoka et al., 2011; Okuda et al., 1996; Wang et al., 1996), but *npas4l* appears to be an
 52 exception. Understanding the specific mechanisms of hemato-vascular specification in
 53 zebrafish may help identify functional homologs in humans and other mammals.
 54 Understanding the specific mechanisms of hemato-vascular specification may prove useful in
 55 regenerative therapies for blood and vascular diseases. Recipients of embryonic stem cell-
 56 based regenerative therapies have an increased risk of tumor formation. A promising
 57 alternative, which ameliorates the risk of tumor formation, is to produce specific multipotent
 58 progenitors from embryonic stem cells prior to transplantation (Doss et al., 2012; Gunaseeli et
 59 al., 2010).

60 Zebrafish embryos and larvae are an ideal organism in which to study hemato-vascular
 61 development *in vivo*. Zebrafish oocytes are fertilized externally and embryos are optically
 62 transparent. Zebrafish embryos can survive up to 7 days post fertilization without circulating
 63 red blood cells because oxygen can freely diffuse through the skin (Hughes et al., 2019;
 64 Pelster and Burggren, 1996). This trait allows for characterization of cardiovascular
 65 phenotypes that would be lethal in embryos from other organisms.

66 In zebrafish, endothelial cells and HSCs can be derived from a single hemato-vascular
 67 progenitor cell population called the hemangioblast, which is hypothesized to exist in mammals
 68 but has not been definitively confirmed (Huber et al., 2004; Moore et al., 2018; Shu et al.,
 69 2015; Zhang and Featherstone, 2020). It has been previously reported that the basic helix-

loop-helix Per-Arnt-Sim (bHLH-PAS) transcription factor, *npas4l*, is the master regulator of hemato-vascular specification in zebrafish (Reischauer et al., 2016) and in chicken (Weng et al., 2020).

bHLH-PAS transcription factors are classified as Class I or Class II, which must dimerize to regulate transcription (Erbel et al., 2003; Pongratz et al., 1998)(Figure 1A). *Npas4l* is a Class I bHLH-PAS protein, but its binding partner during hemato-vascular progenitor specification is not known. We hypothesized that *Arnt1* or *Arnt2* is the dimerization partner of *Npas4l* in zebrafish because there are only 4 bHLH-PAS Class II proteins encoded in the zebrafish genome and *arnt1* and *arnt2* genes are expressed at the the same stages of embryo development as *npas4l* (Prasch et al., 2006; Reischauer et al., 2016; Tanguay et al., 2000).

To test this hypothesis, we examined hemato-vascular development in zebrafish with loss-of-function mutations in *arnt1* and *arnt2* genes. We find that *Arnt1* and *Arnt2* are essential for hemato-vascular specification. Double homozygous *arnt1;arnt2* zebrafish embryos phenocopy *npas4l* mutants (also called *cloche*), showing lack of circulating blood, absence of hematopoietic stem and progenitor cells, and dramatic reduction in the number of vascular endothelial cells. Additionally, we show that zebrafish *Npas4l* protein interacts with both zebrafish *Arnt1* and zebrafish *Arnt2* proteins *in vitro*. Together, our results suggest that *Arnt1* and *Arnt2* act redundantly and form a transcriptional complex with *Npas4l* that sits atop the hemato-vascular specification cascade.

MATERIALS AND METHODS

Zebrafish

Adult zebrafish were raised at 28.5°C on a 14-h light, 10-h dark cycle in the UAB Zebrafish Research Facility or the BCM Zebrafish Research Facility in an Aquaneering recirculating water system (Aquaneering, Inc., San Diego, CA) and a Tecniplast recirculating water system (Tecniplast USA, Inc., West Chester, PA). All zebrafish used for experiments were wild-type AB strain (Westerfield, 2000) and all mutant lines were generated on the AB strain. All procedures were performed in accordance with Institutional Animal Care and Use Committees (IACUC) and BCM guidelines.

Embryo collection

Adult zebrafish were allowed to spawn naturally in pairs or in groups. Embryos were collected in intervals of 20 minutes to ensure precise developmental timing or staged following collection, placed in 60 cm² Petri dishes at a density of no more than 100 per dish in E3B media (60X E3B: 17.2g NaCl, 0.76g KCl, 2.9g CaCl₂-2H₂O, 2.39g MgSO₄ dissolved in 1L Milli-Q water; diluted to 1X in 9L Milli-Q water plus 100 µL 0.02% methylene blue), and then stored in an incubator at 28.5°C on a 14-h light, 10-h dark cycle.

Genotyping adult zebrafish

Genomic DNA was isolated from tail biopsies from individual adult zebrafish by incubation in 50 µL ELB (10 mM Tris pH 8.3, 50 mM KCl, 0.3% Tween 20) with 0.5 µL proteinase K (800 U/ml, NEB) in 96 well plates, one sample per well, at 55°C for 8 hours (tail biopsies). Proteinase K was inactivated by incubation at 98°C for 10 minutes and DNA was stored at -20°C. Genotyping was performed by PCR and high-resolution melting curve analysis as described (Parant et al., 2009; Romano et al., 2017). All melting curves were generated with a

Bio-Rad CFX96 Real-Time System over a 70-95°C range and analyzed with the Bio-Rad CFX Manager 3.1 or the Bio-Rad CFX Maestro 4.1 software. All mutations were confirmed by TA cloning and sequencing.

CRISPR-Cas9 Mutant Generation

Cas9 mRNA and gRNAs for *arnt1*, and *arnt2* mutants were generated as previously described (Romano et al., 2017; Souder and Gorelick, 2019). Cas9 mRNA was transcribed from a linearized pT3TS-nCas9n plasmid, Addgene #46757 (Jao et al., 2013), and purified. The target sequences were identified using CHOPCHOP (Labun et al., 2016; Labun et al., 2019). Oligonucleotides were annealed to each other and cloned into a pT7-gRNA plasmid, Addgene #46759 (Jao et al., 2013). Oligonucleotides utilized are shown in Table 1. gRNAs were synthesized from plasmids using a MEGAshortscript T7 Transcription Kit (Invitrogen AM1354) and purified.

One-cell-stage embryos were injected using glass needles pulled on a Sutter Instruments Fleming/Brown Micropipette Puller, model P-97 and a regulated air-pressure micro-injector (Harvard Apparatus, NY, PL1–90). Each embryo was injected with a 1nL solution containing one gRNA for *arnt1* and one gRNA for *arnt2* (30 ng/μL per target), Cas9 mRNA (150 ng/μL), and 0.1% phenol red. Mixtures were injected into the yolk of each embryo. Approximately, 100 injected embryos per gRNA pair were raised to adulthood and crossed to AB zebrafish to generate F1 embryos. F1 offspring with heritable mutations were sequenced to identify mutations predicted to cause loss of function.

Live Imaging

Embryos were imaged with a Nikon SMZ25 microscope equipped with a Hamamatsu ORCA-Flash4.0 digital CMOS camera. Images were equally adjusted for brightness and contrast in Adobe Photoshop CC 2020. Embryos and larvae were anesthetized with 0.04% tricaine and imaged in Petri dish containing E3B. All embryos were genotyped after imaging.

Whole mount *in situ* hybridization

etv2, *tal1*, *runx1*, and *flk1* antisense RNA probes were used as described (Moore et al., 2013; Thisse and Thisse, 2008). All DNA clones were verified by sequencing. Digoxigenin-labeled antisense RNA probes were synthesized using T7 for *runx1* and *flk1*, or T3 for *etv2*. Colorimetric *in situ* hybridization (ISH) was performed on fixed zebrafish embryos and larvae as previously described, with 5% dextran in the hybridization buffer (Lauter et al., 2011; Thisse and Thisse, 2008). Following ISH, embryos were washed several times in 1x PBST (phosphate buffered saline, 0.01% Tween), cleared with glycerol or mounted in 3% methylcellulose on a glass coverslip, and imaged on either a Nikon SMZ18 stereoscopic microscope or an inverted Nikon ECLIPSE Ti2-E microscope. Embryos were then genotyped. Genomic DNA was extracted using the HotSHOT protocol (Dobrzycki et al., 2018). Briefly, embryos were suspended in 20 μ L of lysis buffer (25 mM NaOH, 0.2 mM EDTA) and incubated at 95°C for 30 minutes, cooled to 4°C, then an equal volume of neutralization buffer (40 mM Tris-HCl) was added. DNA regions of interest were amplified with JumpStart REDTaq ReadyMix (Sigma-Milipore, P0982) PCR according to manufacturer's protocols.

Cell Culture and Transient Transfection

HEK293T cells (gift of Dr. Charles Foulds), were thawed and maintained at 37°C in humidified 5% CO₂ atmosphere incubator. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, 2062564), and 1% penicillin-streptomycin (Gibco, 15140-122). After 24 hours passaging in a 100mm plate at a density of 2.2 x 10⁶ cells per dish and reaching at least 70 confluency, the media was changed to DMEM supplemented with 1% dialyzed FBS. Cells were transiently transfected with 12 µg of total DNA and Lipofectamine 2000 according to manufacturer's instructions (Invitrogen, 2400439) with *npas4l-v5* together with either *arnt1-myc* or *arnt2-HA* plasmid DNA.

Cloning of plasmids for expression in cultured cells was performed as follows. For *npas4l-V5*, we took pCS2+*npas4l*ORF (gift of Dr. Leonard Zon) that contains the open reading frame (ORF) of zebrafish *npas4l* downstream of the CMV promoter. This plasmid was modified to include a C-terminal V5 tag using Q5 Site-Directed Mutagenesis Kit (New England Biolabs, #E0554S). For *arnt1-myc*, we synthesized a plasmid containing CMV promoter driving a codon-optimized zebrafish *arnt1* ORF (ENS DART00000081852.5) with a C-terminal myc tag (Twist Bioscience, San Francisco, CA). For *arnt2-myc*, we synthesized a gene block of the zebrafish *arnt2* ORF (ENS DART00000158162.2, Integrated DNA Technologies). The gene block was integrated into a plasmid containing a CMV promoter using pcDNA3.3-TOPO TA cloning kit (Invitrogen, K8300-01) in accordance with manufacturer's instructions. A C-terminal HA tag was added using Q5 Site-Directed Mutagenesis Kit (New England Biolabs, #E0554S).

Co-immunoprecipitation and Western blot

Transiently transfected cells were washed with ice cold PBS and lysed in 500 µL of 1x RIPA buffer containing Pierce Protease (ThermoScientific, A32955). Cells were sonicated in 6 x 30

184 second intervals using a Bioruptor 300 Machine (Diagenode), then centrifuged for 10 minutes
185 at 10,000g at 4°C. The lysate (supernatant) was removed from the pellet and placed in fresh
186 1.5mL Eppendorf tube. Cell lysate was precleared with Protein A beads (Invitrogen #159 18-
187 014) for 30 minutes 4°C, incubated with either 5 µg of V5 antibody (ThermoFisher Scientific
188 R960-25) or myc antibody (SantaCruz, SC-40) diluted 1:200 for 1 hour, then
189 immunoprecipitated with 20 µL of Protein A beads for 1 hour at 4°C. The beads were washed
190 three times with RIPA buffer and once with PBS and finally suspended in 30µL of PBS. Equal
191 volume of 2x Laemmli Sample Buffer (BioRad, cat# 1610737) was added to each sample and
192 boiled for 5 minutes at 95°C then placed on ice. Samples were run on 4-20% Mini-PROTEAN
193 TGX gels (BioRad, cat# 4561093) and transferred onto PVDF membrane using Trans-Blot
194 Turbo Transfer System (BioRad) using the pre-defined Mixed MW program. The membranes
195 were washed several times with 1x Tris-Buffered Saline, 0.1% Tween 20 (TBST)
196 (GBiosciences, R043) and incubated for 1 hour with agitation in 5% milk in 1x TBST.
197 Membranes were probed with 1:1000 V5-HRP (ThermoFisher Scientific, R960-25), 1:1000
198 myc-HRP (SantaCruz, SC-40), or 1:200 HA (Cell Signaling, 3724) antibodies rocking overnight
199 at 4°C. Membranes were washed 5 times with 1xTBST on orbital rocker for 15 minutes at room
200 temperature. Membranes probed with the anti-HA antibody were then probed with 1:10,000
201 IRDye 800CW donkey anti-rabbit (Licor, 926-32213) in 2.5%-5% milk in 1x TBST for 1 hour
202 and then washed multiple times with 1xTBST on orbital rocker for 5 minutes prior to imaging.
203 Membranes probed with HRP conjugated antibodies were developed using Clarity™ Western
204 ECL Substrate (BioRad, 170-5061). Membranes were imaged using BioRad ChemiDoc™ MP
205 Imaging System.
206

207 **TCDD exposure**

208 At 1-day post fertilization (dpf) embryos were exposed to 10 ng/mL 2,3,7,8-
 209 tetrachlorodibenzodioxin (TCDD, AccuStandard Inc, D404N) or vehicle (0.1% DMSO). All
 210 treatments were performed on a single clutch of at least 20 embryos; embryos treated with
 211 vehicle were from the same clutches as embryos treated with TCDD. Chemical exposures
 212 were performed on at least 2 clutches per genotype. Embryos were raised at 28.5°C on a 14-h
 213 light, 10-h dark cycle until 3 dpf, when they were imaged and then genotyped.

214

215

RESULTS

arnt1/2 homozygous mutants lack circulating blood

To determine if *arnt1* or *arnt2* is required for hemato-vascular development, we generated heritable mutant alleles for *arnt1* (*arnt1^{bcm1}* and *arnt1^{bcm2}*) and *arnt2* (*arnt2^{bcm3}*) using CRISPR/Cas9. For *arnt1^{bcm1}*, we generated zebrafish with a 5 bp insertion in exon 5, which results in a frameshift at amino acid (AA) 126 and a premature stop codon in the first PAS domain at AA139 (Fig. 1B). For *arnt1^{bcm2}*, we generated zebrafish with an 11 bp deletion in exon 10, which results in a frameshift at AA230 and a premature stop codon between the two PAS domains at AA250 (Fig. 1B). We did not observe any phenotypic differences between homozygous *arnt1^{bcm1/bcm1}* and *arnt1^{bcm2/bcm2}* mutant embryos. These mutants are morphologically indistinct from wildtype during development and homozygous adults are viable and fertile, which is consistent with previously published *arnt1* mutants (Marchi et al., 2020). We also generated maternal zygotic *arnt1* mutants (derived from homozygous *bcm1* or *bcm2* parents) and these were morphologically indistinct from wildtype. For *arnt2^{bcm3}*, we generated zebrafish with a 3 bp deletion and 1 bp insertion in exon 9, which resulted in a missense mutation at AA286 and a premature stop codon between the two PAS domains at AA287 (Fig. 1C). Homozygous *arnt2* mutants (*arnt2^{bcm3/bcm3}*) were viable at 5 days post fertilization (dpf) and grossly morphologically normal, however they did not survive to adulthood. Our observations are consistent with independently generated *arnt2* mutant zebrafish lines (Hill et al., 2009; Lohr et al., 2009).

We crossed *arnt1^{bcm2/+}*; *arnt2^{bcm3/+}* adult fish to each other and analyzed the phenotype and genotype of the resulting embryos. We observed all genotypes at the expected Mendelian ratios (Table 1). The single mutant larvae, *arnt1^{bcm2/bcm2}*, herein called *arnt1* mutants (Fig. 1D and D'), and *arnt2^{bcm3/bcm3}*, herein called *arnt2* mutants (Fig. 1E and E'), are phenotypically indistinguishable from their wildtype siblings by gross morphology (Fig. 1C and C'). However, *arnt1^{bcm2/bcm2}*; *arnt2^{bcm3/bcm3}* larvae, herein called *arnt1/2* mutants, showed a clear phenotype (Fig. 1F). These larvae lack all circulating blood (Fig. 1H, M3 and M4). All other intermediate *arnt1*; *arnt2* genotypes, e.g.,

arnt1^{bcm2/+};arnt2^{bcm3/bcm3}, appeared phenotypically wildtype (Fig. 1G, and data not shown). These results suggest that *arnt1* and *arnt2* function redundantly, because only the *arnt1/2* double homozygous mutants show a phenotype. Additionally, the *arnt1/2* mutant phenotype appears identical to the previously described *npas4l* mutant phenotype (also known as *cloche*) (Reischauer et al., 2016; Stainier et al., 1995a). Like *npas4l* mutants, *arnt1/2* mutants exhibit cardiac edema with enlarged heart atrium, likely a secondary effect of the lack of blood and endocardial cells (Fig 1G). Taken together, these data suggest that *arnt1* and *arnt2* have a similar function as *npas4l*.

***arnt1/2* mutants have reduced expression of hematopoietic and endothelial cell markers at 2 dpf**

We hypothesized that the Arnt proteins are dimerization partners with Npas4l and that *arnt1/2* mutants would exhibit the same phenotypes as *npas4l* mutants. Therefore, we examined *arnt1/2* mutant embryos for additional phenotypes found in *npas4l* mutants. Previous studies determined that *npas4l* mutants lack almost all endothelial cells, except some cells located in the lower trunk and caudal tail of the larvae (Liao et al., 1997; Stainier et al., 1995a). To determine if our *arnt1/2* mutants showed a similar reduction in endothelial cells, we performed whole-mount *in situ* hybridization (WISH) on 2 dpf embryos for *flk-1* (also known as *kdr*). *flk-1*, a widely used marker of endothelial cells in blood vessels, is essential for the development of blood vessels in mice and zebrafish (Dumont et al., 1995; Liao et al., 1997; Shalaby et al., 1995). We found that *maternal zygotically arnt1* mutants (*MZarnt1*) and *arnt2* mutants showed clear expression of *flk-1* in the vasculature, similar to wild type (**Fig 2B-C**). In contrast, *arnt1/2* mutants had a reduced number of *flk-1*-positive cells (**Fig. 2D and D'**). The *flk-1*-positive cells present in the *arnt1/2* mutant were in the caudal tail and likely comprised a portion of the caudal vein (**Fig. 2D'**), similar to the *flk-1* expression pattern seen in *npas4l* mutants (Liao et al., 1997). This suggests that *npas4l* and *arnt1* or *arnt2* are required for the development of most blood vessels.

In addition to a significant reduction of endothelial cells, *npas4l* mutants show a reduction in the number of hematopoietic stem cells (HSC) (Stainier et al., 1995a; Thompson et al., 1998). To

determine if *arnt1/2* mutants contain HSCs, we examined *runx1* expression by WISH. *runx1* is a marker of HSCs and is required for definitive hematopoiesis in zebrafish (Kalev-Zylinska et al., 2002a). By 48 hpf, *runx1*-positive HSCs are localized to the area between the dorsal aorta and the posterior caudal vein (Lam et al., 2009). At 2 dpf, wildtype, *arnt1*, and *arnt2* single mutants all contain *runx1*-positive cells in the blood island region (**Fig 2E-G**), indicating the presence of HSCs. However, *arnt1/2* mutants lack *runx1*-positive cells in this region (**Fig. 2H**). This suggests that *arnt1* or *arnt2* is necessary for hematopoietic cell development during embryogenesis, similar to *npas4l* (Liao et al., 1998; Stainier et al., 1995a; Thompson et al., 1998).

Hematopoietic and endothelial progenitor populations are reduced in *arnt1/2* mutants

Hematopoietic cells and endothelial cells arise from a common progenitor population in zebrafish (Vogeli et al., 2006). To determine if *arnt1/2* mutants have a reduction of hemato-vascular progenitor cells, we performed WISH on embryos at the 4-6 somite stage and examined expression of the earliest known regulators of endothelial and hematopoietic cell differentiation: *etv2* and *tal1*, both of which are expressed in the posterior lateral plate mesoderm (PLPM) during early somitogenesis stages (Qian et al., 2005; Reischauer et al., 2016; Stainier et al., 1995a; Thompson et al., 1998). We bred *arnt1^{bcm2/+}*; *arnt2^{bcm3/+}* adults to each other to generate *arnt1/2* mutant, *arnt1* mutant, *arnt2* mutant, and wildtype embryos. We then performed WISH to examine the expression of *etv2* (**Fig. 3A and B**, n=516 embryos from 5 clutches) or *tal1* (**Fig. 3C and D**, n=665 embryos from 3 clutches). 8% and 6% of embryos showed reduced expression of *etv2* and *tal1*, respectively, neither of which is statistically significantly different from the expected Mendelian ratio of 6.25% double homozygous mutant embryos (binomial test, *etv2* n=516 $p>0.05$; *tal1* n=665 $p>0.05$). To confirm that the observed reduction of *etv2* and *tal1* in these embryos correlates with the *arnt1/2* double homozygous mutant genotype, following WISH we genotyped 24 of the embryos with absent or reduced expression of *etv2* and 17 with absent or reduced expression of *tal1*. 100% of the low/no expressing *etv2* embryos were *arnt1/2* double

homozygous mutants, while 83% of the low/no expressing *tal1* embryos were *arnt1/2* double homozygous mutants, demonstrating a strong correlation between the reduced expression of *etv2* and *tal1* and the *arnt1/2* double homozygous genotype. Additionally, we genotyped 26 of the embryos with normal expression of *etv2* and 7 with normal expression of *tal1*. 7.7% of the normal expressing *etv2* embryos were *arnt1/2* mutants (genotypes of the 26 embryos: 2 *arnt1/2* mutants, 1 wildtype, 3 *arnt1^{bcm2/bcm2}*, 4 *arnt1^{bcm2/bcm2};arnt2^{bcm3/+}*, 4 *arnt1^{bcm2/+}*, 5 *arnt1^{bcm2/+};arnt2^{bcm3/+}*, 4 *arnt1^{bcm2/+};arnt2^{bcm3/bcm3}*, 1 *arnt2^{bcm3/bcm3}*, and 3 *arnt2^{bcm3/+}*), while 0% of the normal expressing *tal1* embryos were *arnt1/2* mutants (genotypes of the 7 embryos: 1 *arnt1^{bcm2/+};arnt2^{bcm3/+}*, 1 *arnt1^{bcm2/+};arnt2^{bcm3/bcm3}*, 1 *arnt1^{bcm2/+}*, 1 *arnt2^{bcm3/+}*, and 3 *arnt1^{bcm2/bcm2};arnt2^{bcm3/+}*). We conclude that *arnt1* and *arnt2* are required for hemato-vascular progenitor specification. Previous findings demonstrated that *npas4l* is a master regulator of hemato-vascular progenitor specification (Reischauer et al., 2016). Together, these results demonstrate that *npas4l*, *arnt1* and *arnt2* are co-master regulators of hemato-vascular specification.

Npas4l interacts with both Arnt1 and Arnt2

Npas4l is a Class I bHLH-PAS domain transcription factor (Reischauer et al., 2016), and as such, must heterodimerize with Class II bHLH-PAS transcription factors, like *Arnt1* and *Arnt2*, to initiate gene transcription. To determine if *Npas4l* forms a complex with either *Arnt1* or *Arnt2*, we performed a coimmunoprecipitation experiment. Due to the absence of commercially available antibodies that recognize zebrafish *Npas4l*, *Arnt1* or *Arnt2* proteins, we co-expressed tagged versions of zebrafish *npas4l* (*Npas4l*-V5), *arnt1* (*Arnt1*-myc), and *arnt2* (*Arnt2*-HA) in HEK293T cells. We performed co-immunoprecipitation studies and found that *Arnt1*-myc interacts with *Npas4l*-V5 and that *Arnt2*-HA interacts with *Npas4l*-V5 (**Fig. 4A, B**). These data suggest that *Npas4l* uses either *Arnt1* or *Arnt2* or both as a binding partner. Furthermore, these data indicate that *Arnt1* and *Arnt2* may be functionally interchangeable and redundant, at least in the case of *Npas4l*.

Arnt1 and Arnt2 cannot functionally compensate for one another in the case of Ahr2

Our results demonstrate that Arnt1 and Arnt2 are functionally interchangeable and redundant when it comes to interacting with Npas4l in the context of hemato-vascular development. It is unclear if Arnt1 and Arnt2 are functionally interchangeable with all Class I bHLH-PAS domain transcription factors or just with Npas4l. To determine if *arnt1/arnt2* redundancy is unique to Npas4l, we examined if *arnt1* and *arnt2* are interchangeable in the case of a different class I bHLH-PAS transcription factor, the aryl hydrocarbon receptor 2 (Ahr2). Previous studies demonstrate that Ahr2 can bind DNA in the presence of either Arnt1 or Arnt2 *in vitro* (Andreasen et al., 2002; Hirose et al., 1996; Tanguay et al., 2000), but whether Arnt1 and Arnt2 bind Ahr2 interchangeably *in vivo* is not known. Ahr2 is a ligand-dependent transcription factor. A known ligand of Ahr2 is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which we have previously shown induces cardiotoxicity in zebrafish larvae and requires wild-type *ahr2* (Souder and Gorelick, 2019). We asked whether TCDD causes cardiotoxicity in *arnt1* or *arnt2* single mutants, reasoning that if Ahr2 interacts with Arnt1 or Arnt2 interchangeably, then only *arnt1/2* double mutants, but not single mutants, would be resistant to TCDD exposure. We treated wildtype, *arnt1* mutants, and *arnt2* mutants with either vehicle (0.1% DMSO) (**Fig. 5A, B, and C**) or with 10 ng/mL TCDD (**Fig. 5D, E, and F**) from 1 dpf to 3 dpf. Wildtype and *arnt2* mutants treated with TCDD exhibit cardiac edema and the heart fails to loop properly (**Fig. 5D, and F**), unlike vehicle treated siblings (**Fig. 5A and C**). In contrast, *arnt1* mutants treated with TCDD showed normal heart looping and lack cardiac edema (**Fig. 5E**), like their vehicle treated siblings (**Fig. 5B**). This suggests that *arnt1*, but not *arnt2*, is required for Ahr2-dependent TCDD toxicity. This also shows that *arnt1* and *arnt2* are not always redundant *in vivo* and that the Class I dimerization partner may influence whether the Class II dimerization partners are redundant.

DISCUSSION

Class I bHLH-PAS proteins must dimerize with Class II bHLH-PAS proteins to transcribe their target genes (Ema et al., 1996; Erbel et al., 2003; Jiang et al., 1996; Lees and Whitelaw, 1999; Michaud et al., 2000; Wu and Rastinejad, 2017). It has previously been established that *Npas4l*, a Class I bHLH-PAS transcription factor, is required for hemato-vascular development in zebrafish (Reischauer et al., 2016). To determine the Class II bHLH-PAS binding partner of *Npas4l*, we generated *arnt1* mutant and *arnt2* mutant zebrafish and expected one of these mutants to mimic the *npas4l* mutant phenotype. However, both *arnt1* mutant and *arnt2* mutant larvae are viable to 5dpf and are indistinguishable from wildtype embryos by eye. Our results demonstrate that double homozygous *arnt1/2* mutant embryos have a similar phenotype to *npas4l* mutants (Reischauer et al., 2016; Stainier et al., 1995b; Thompson et al., 1998) and that *Npas4l* can function with either *Arnt1* or *Arnt2*. Thus, *Arnt1* and *Arnt2* act redundantly to specify hemato-vascular progenitor cells.

Endothelial cell development and hematopoietic cell development are closely related during embryogenesis. Both cell types primarily arise from the lateral plate mesoderm (LPM) and share a common progenitor (Qian et al., 2005; Reischauer et al., 2016; Stainier et al., 1995a; Thompson et al., 1998). Additionally, during definitive hematopoiesis, some HSCs are derived from specialized endothelial cells, called hemogenic endothelium (Boisset et al., 2010; Kissa and Herbomel, 2010). Both *npas4l* and *arnt1/2* mutants lack all blood cells but do have a small population of endothelial cells, which is restricted to the most posterior region of the animal (Figure 2 and (Reischauer et al., 2016)). In zebrafish, there is a population of endothelial cells in the caudal region of the embryo that are not derived from the LPM and differentiate in the absence of *npas4l* expression (Pak et al., 2020). We hypothesize that *arnt1* and *arnt2* are

required for the differentiation of endothelial cells from the LPM but are not required for the differentiation of endothelial cells, such as the caudal population, from outside the LPM. The transcriptomic signature of the caudal population of endothelial cells was distinct from endothelial cells that require *npas4l* for differentiation, with genes for somitogenesis and neurogenesis highly enriched compared to LPM-derived endothelial cells (Pak et al., 2020). Based on this transcriptome analysis, we speculate that the caudal population of endothelial cells fails to express genes required for hemogenic endothelium and that no blood cells are derived from the caudal population of endothelial cells. This is consistent with the observation that the caudal population of endothelial cells, but not blood cells, are present in *npas4l* or *arnt1/2* mutants.

Our results show that either Arnt1 or Arnt2, Class II bHLH-PAS proteins, can act as a required co-factor for Npas4l-dependent mesodermal specification to hematopoietic and endothelial progenitors. Likewise, Arnt1 is essential for adult hematopoietic stem cells in mice and is required for survival of hematopoietic progenitor cells in the fetal liver (Krock et al., 2015). Homozygous Arnt1 mutant mice are not viable beyond E10.5 and show severe defects in vascularization, suggesting that Arnt1 is essential for normal vascular development (Kozak et al., 1997; Maltepe et al., 1997). The role of Arnt1 during mammalian embryonic hematopoiesis remains unclear.

Based on these data we speculate that the mammalian equivalent to zebrafish Npas4l is likely to be a Class I bHLH-PAS protein that heterodimerizes with ARNT1. Zebrafish Npas4l shows the most sequence homology to mouse Npas4 (Reischauer et al., 2016). Although Npas4 is known to dimerize with Arnt1 and Arnt2 *in vivo* (Brigidi et al., 2019), mammalian Npas4 is unlikely to be the functional equivalent to zebrafish Npas4l because homozygous

Npas4 mutant mice are viable to adulthood and do not show any overt vascular or hematopoietic defects (Bloodgood et al., 2013).

Similarly, we speculate that Npas1, 2, and 3 are unlikely to be the mammalian equivalent to Npas4l, as mice mutant for these genes are viable to adulthood and no endothelial or hematopoietic phenotype has been reported (Dudley et al., 2003; Erbel-Sieler et al., 2004). Additionally, we think that the Sim (Sim1/2) and Circadian Locomotor Output Cycles Kaput (CLOCK) genes are unlikely to be the master regulators of hemato-vascular specification in mice or humans because mutant mice appear to have normal blood cells and vasculature (DeBruyne et al., 2007; Epstein et al., 2000; Holder et al., 2004). We cannot exclude the possibility that Npas1, 2, 3 and 4 proteins act redundantly to specify hemato-vascular progenitors in mammalian embryos because mice with mutations in multiple Npas genes have not been reported.

Three other Class I bHLH-PAS proteins, Ahr, Hif1 α , and Hif2 α , are known to be involved in mammalian hematopoiesis and vascularization. Lack of Ahr *in vitro* and *in vivo* promotes adult hematopoietic progenitor expansion and resulted in aberrant ratios and functionality of some blood cell populations, suggesting that Ahr could be functionally relevant during definitive hematopoiesis (Singh et al., 2011; Smith et al., 2013). However, there is no evidence that Ahr is expressed during primitive hematopoiesis in mice and Ahr mutant mice are viable to adulthood and do not show any vascular defects (Singh et al., 2011).

Hif1 α and Hif2 α are involved in mouse vascular development and hematopoiesis (Iyer et al., 1998; Krock et al., 2015; Peng et al., 2000; Scortegagna et al., 2003; Takubo et al., 2010). However, the role of Hif1 α and Hif2 α during early mouse embryonic hematopoiesis and endothelial development is not well characterized. Hif1 α is required for cell cycle quiescence of

adult hematopoietic stem cells (Takubo et al., 2010). Hif1 α mRNA is detected during mouse primitive hematopoiesis and Hif1 α homozygous mutant mice die *in utero* before E11.5 (Iyer et al., 1998; Kotch et al., 1999). The Hif1 α mutant mice are reported to have defects in blood vessels, increased death of vascular-supporting mesenchymal cells, and cardiovascular defects (Iyer et al., 1998; Kotch et al., 1999). In mice, Hif2 α mRNA is expressed during primary hematopoiesis specifically within the yolk sac, where primary hematopoiesis originates (Flamme et al., 1997). Global knockout of *Hif2 α* is reported to be embryonic or postnatal lethal depending upon the mutation and strain of mouse used (Compernelle et al., 2002; Peng et al., 2000; Tian et al., 1998). Embryonic lethal Hif2 α mutants showed severe vascular disorganization and increased frequency of hemorrhaging *in utero* (Compernelle et al., 2002; Peng et al., 2000). Both *Hif1 α* and *Hif2 α* mutants have endothelial and blood cells, thus neither mutation mimics the zebrafish *npas4l* phenotype. But both *Hif1 α* and *Hif2 α* mutant mice do have a vascular phenotype (Compernelle et al., 2002; Compernelle et al., 2003; Iyer et al., 1998; Peng et al., 2000; Ryan et al., 1998). Although neither Hif1 α nor Hif2 α single mutant mice recapitulate the phenotype observed in zebrafish *cloche* or *arnt1;arnt2* mutants, the HIFs are strong candidates to be the functional equivalent of Npas4l.

One reason we may not see an Npas4l-like phenotype in Hif1 α or Hif2 α single mutant mice could be because of genetic compensation. Genetic compensation occurs in both zebrafish and mice (Bunton-Stasyshyn et al., 2019; Rossi et al., 2015). There is evidence in zebrafish and mice of *Hif1 α* and *Hif2 α* functionally compensating for one another. Zebrafish with reduced expression of all 4 Hif1 and Hif2 transcripts (*hif1aa*, *hif1ab*, *hif2aa*, *hif2ab*) lack hemogenic endothelium, suggesting that at least one HIF gene is required for the specification of hemogenic endothelium (Gerri et al., 2018). These results also indicate that *hif1 α* and *hif2 α*

are functionally redundant for the generation of hemogenic endothelium (Gerri et al., 2018). Global knockout of mouse *Hif1α* is embryonic lethal (Compornolle et al., 2003; Iyer et al., 1998; Ryan et al., 1998) and global knockout of mouse *Hif2α* is embryonic or postnatal lethal depending upon the strain of mouse used (Compornolle et al., 2002; Peng et al., 2000; Tian et al., 1998).

To our knowledge, no one has generated a mouse in which both *Hif1α* and *Hif2α* are globally knocked-out. Future studies could examine if global or mesodermal-specific knockout of both *Hif1α* and *Hif2α* genes in mouse results in a phenotype similar to knockout of both *arnt1* and *arnt2* in zebrafish. Our results demonstrate that under some conditions, Arnt1 and Arnt2 act redundantly. In other contexts, Arnt1 is the preferred binding protein for a class I PAS transcription factor. The extent to which this occurs during different developmental stages *in vivo*, or in different cell types, warrants further study.

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Gene	Allele	CRISPR target	Oligo 1	Oligo 2
<i>arnt1</i>	<i>bcm1</i>	GGATGGTTTATAGGTGCCAT <u>CGG</u>	TAGGATGGTTTATAGGTGCCAT	AAACATGGCACCTATAAACCAT
<i>arnt1</i>	<i>bcm2</i>	GGCGACTGTGAGGATGAGTATGG	TAGGCGACTGTGAGGATGAGTA	AAACTACTCATCCTCACAGTCG
<i>arnt2</i>	<i>bcm3</i>	GGTCTCGGTCCGTCTAAGGAG <u>GGG</u>	TAGGTCTCGGTCCGTCTAAGGA	AAACTCCTTAGACGGACCGAGA

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698 Table 1. CRISPR target sequence and oligos used to generate *arnt1* and *arnt2* mutants. PAM sequence in

699 CRISPR target is underlined

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701

702 Figure 1

Figure 1. *arnt1/2* double mutant embryos, but not *arnt1* or *arnt2* single mutants, lack circulating blood, have cardiac edema and atrial enlargement. (A) bHLH-PAS transcription factors act as heterodimers, where a class I and indicated class II protein interact and bind DNA. Asterisks indicate proteins encoded by genes present in zebrafish but absent in humans. (B,C) Generation of zebrafish with mutations in *arnt1* and *arnt2* genes. The amino acid sequences for the zebrafish Arnt1 (B), and Arnt2 proteins (C) are represented by the black line. Basic helix-loop-helix (bHLH), Per-Arnt-Sim (PAS) domains, and the motif C-terminally located to the PAS (PAC) are represented by the labeled bubbles. The numbers below each domain indicate the starting and ending amino acid (AA) for each motif. The *arnt1*^{bcm1} mutation causes a premature stop codon to occur at AA139 and loss of the PAS-B, PAC, and majority of the PAS-A. The *arnt1*^{bcm2} mutation causes a premature stop codon to occur at AA250 and loss of the PAS-B and PAC domains (B). The *arnt2*^{bcm3} mutation causes a premature stop codon to occur at AA287 and loss of the PAS-B and PAC domains (C). (D-I) Live embryos from *arnt1*^{bcm2/+};*arnt2*^{bcm3/+} heterozygous parents were imaged at 2 days post fertilization (dpf) prior to genotyping. All double homozygous *arnt1*^{bcm2/bcm2};*arnt2*^{bcm3/bcm3} (*arnt1/2* mutant) embryos showed cardiac edema and atrial enlargement (G and G'). The wildtype (D and D'), *arnt1*^{bcm2/bcm2} (*arnt1* mutant) (E and E'), and *arnt2*^{bcm3/bcm3} (*arnt2* mutant) (F and F') siblings of the *arnt1/2* mutants showed normal heart development. Black arrows in panels D'-G' indicate the atrium and ventricle of the heart. Additionally, all siblings of *arnt1/2* mutants had circulating blood (H and M1) while *arnt1/2* mutants lacked visible circulating blood cells (I and M2). The white arrows in panel H indicate blood cells in the dorsal aorta. Images are representative of 7 different clutches, each clutch contained between 20-300 embryos. For all images of zebrafish embryos (D-I) anterior is towards the right and dorsal is towards the top of the image. The scale bar in Panel D applies to Panels D-G and is 1000 μ m in length. The scale bar in Panel D' applies to Panels D'-G' and is 100 μ m in length.

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705 Figure 2

Figure 2. *arnt1/2* double homozygous mutants show reduced levels of *flk-1*-positive endothelial cells and *runx-1*-positive cells in blood island (A-H). Embryos were probed for *flk-1* (A-D) or *runx-1* (E-H) using whole-mount in situ hybridization at 2 days post fertilization (dpf). Wild-type embryos were derived from an in-cross of laboratory strain AB parents (A and E). *arnt1* mutant embryos were derived from *arnt1;arnt2* heterozygous parents or are maternal zygotic derived from *arnt1* homozygous parents (MZarnt1). *arnt2* mutant embryos were derived from heterozygous parents. *arnt1/2* double homozygous mutant embryos (D and H) were derived from *arnt1;arnt2* double heterozygous parents and identified by cardiovascular phenotype prior to fixation. Panels A'-D' are enlarged images of each embryo's trunk near the end of the yolk extension (area indicated by red box in panels A-D). (A'-D') Black arrows indicate *flk-1* staining in the dorsal longitudinal anastomotic vessels (DLAV), white arrows indicate the dorsal aorta and caudal vein (DA/CV), arrowheads indicate inter-segmental vessels. (E-H) *runx-1* labeling near the end of the yolk extension, arrows indicate blood island region. n=2-5 clutches for each genotype, 10-100 embryos per clutch. All embryos are oriented with anterior to the right and dorsal towards the top. The scale bar in D applies to A-D, the scale bar in D' applies to A'-D', and the scale bar in H applies to E-H. All scale bars are 10 μ m.

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708 Figure 3

Figure 3. *arnt1/2* mutants show decreased *etv2* and *tal1* expression in the posterior lateral plate mesoderm (PLPM) at 4-6 somite stages. Whole-mount in situ hybridization for *etv2* and *tal1* expression was performed on 4-6 somite stage embryos which were offspring of *arnt1^{bcm2/+}*; *arnt2^{bcm3/+}* adult zebrafish. (A) Expression of *etv2* was observed in the PLPM in 92% of embryos (n=477 of 516 total embryos). (B) Limited or no expression of *etv2* was observed in 8% of embryos (n=39 of 516), consistent with the expected Mendelian ratios for *arnt1/2* mutants (6.25%). 24 of the 39 presumptive *arnt1/2* mutants were genotyped and 24/24 embryos were confirmed *arnt1/2* mutants. (C) Expression of *tal1* was observed in 94% of embryos (n=624 of 665 total embryos). (D) Limited or no expression of *tal1* was observed in 6% of embryos (n=41 of 665), consistent with the expected Mendelian ratios for *arnt1/2* mutants (6.25%). 17 of the 41 presumptive *arnt1/2* mutants were genotyped and 14/17 embryos were confirmed *arnt1/2* mutants. All embryos are oriented with dorsal towards the top of the image and posterior is closest to the reader. Scale bar is 10 μ m and applies to all images.

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711 Figure 4

Figure 4. Arnt1 and Arnt2 co-immunoprecipitate with Npas4l. (A) HEK293T cells were transfected with zebrafish Npas4l-V5 and Arnt1-myc. Cells were homogenized (input) and immunoprecipitated (IP) with antibodies against V5 or an immunoglobulin G control (IgG). Samples were run on an immunoblot and probed using myc antibodies (anti-myc) to detect the presence of Arnt1-myc (top) or samples were probed using V5 antibodies (anti-V5) to verify successful pulldown of Npas4l-V5 (bottom). (B) HEK293T cells were transfected with zebrafish Npas4l-V5 and Arnt2-HA. Cells were homogenized and immunoprecipitated with antibodies against HA or an IgG control. Samples were run on an immunoblot and probed using V5 antibodies (anti-V5) to detect the presence of Npas4l-V5 (top) or samples were probed using HA antibodies (anti-HA) to verify successful pulldown of Arnt2-HA (bottom).

715 Figure 5

Figure 5. Mutation in *arnt1*, but not *arnt2*, prevents AHR2-dependent TCDD toxicity. Wild type or mutant larvae were exposed to 0.1% DMSO (A, B, C), or 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (D, E, F) from 1-3 dpf and imaged at 3 dpf. Wildtype embryos exposed to TCDD (D) lack an S-shaped cardiac loop and exhibit cardiac edema, unlike their vehicle treated siblings (A). *arnt1* homozygous mutant larvae (*arnt1^{bcm2/bcm2}*) treated with vehicle and TCDD (B,E) show normal heart looping and do not have cardiac edema. *arnt2* homozygous mutant larvae (*arnt1^{bcm3/bcm3}*) treated with vehicle (C) show expected heart looping similar to wild type siblings, but *arnt2* homozygous mutant larvae treated with TCDD (F) lack an S-shaped cardiac loop similar to wild type and exhibit cardiac edema similar to TCDD treated wild-type embryos. Black arrows indicate location of the heart. Embryos were genotyped following imaging and representative images are shown. TCDD treated wildtype siblings of *arnt1* mutant embryos, n= 9 embryos across 3 clutches; TCDD treated *arnt1* mutant larvae, n=21 larvae across 3 clutches. TCDD treated wildtype siblings of *arnt2* larvae, n=19 embryos across 3 clutches; TCDD treated *arnt2* mutant larvae, n=22 embryos across 4 clutches. All embryos are oriented with anterior to the right and dorsal towards the top of the image. Scale bar is 100 μ m.

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