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SoxF factors and Notch regulate *nr2f2* gene expression during venous differentiation in zebrafish

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Abstract

Initial embryonic determination of artery or vein identity is regulated by genetic factors that work in concert to specify endothelial cell (EC) fate, giving rise to two structurally unique components of the circulatory loop. The Shh/VEGF/Notch pathway is critical for arterial specification, while the orphan receptor *nr2f2* (COUP-TFII) has been implicated in venous specification. Studies in mice have shown that *nr2f2* is expressed in venous but not arterial ECs, and that it preferentially induces markers of venous cell fate. We have examined the role of *nr2f2* during early arterial-venous development in the zebrafish trunk. We show that expression of a subset of markers of venous endothelial identity requires *nr2f2*, while the expression of *nr2f2* itself requires *sox7* and *sox18* gene function. However, while *sox7* and *sox18* are expressed in both the cardinal vein and the dorsal aorta during early trunk development, *nr2f2* is expressed only in the cardinal vein. We show that Notch signaling activity present in the dorsal aorta suppresses expression of *nr2f2*, restricting *nr2f2*-dependent promotion of venous differentiation to the cardinal vein.

Keywords

Nr2f2; COUP-TFII; zebrafish; cardinal vein; dorsal aorta

Introduction

The proper functioning of the circulatory system as a closed loop continually re-circulating blood to and from peripheral tissues depends on the fundamental division of the circulatory system into two distinct and separate yet completely intertwined and interconnected networks of blood vessels – veins and arteries – that direct blood to and from the heart, respectively. Although the existence of these two distinct types of blood vessels has been

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appreciated for centuries, we have only begun to understand the molecular pathways leading to their differential specification and identity. In the developing zebrafish trunk, Hedgehog signaling regulates arterial differentiation via downstream activation of vascular endothelial growth factor (VEGF) and Notch pathways. Sonic hedgehog (SHH) secreted from the midline axial mesoderm promotes up-regulation and secretion of VEGF in adjacent somitic tissue, which in turn promotes Notch pathway activation and arterial differentiation in the juxtaposed assembling trunk dorsal aorta (Lawson et al., 2001; Lawson et al., 2002). VEGF and Notch signaling have also been shown to promote arterial differentiation in developing mice and other models (reviewed in (Swift and Weinstein, 2009)). While arterial specification is driven by the preferential activation of the Shh/VEGF/Notch pathways, it had been believed that venous differentiation was a default differentiation pathway for endothelial cells (Thurston and Yancopoulos, 2001).

Recently, however, this view was challenged by the identification of genetic factors required for proper venous differentiation. Chicken ovalbumin upstream promoter-transcription factor II (“COUP-TFII;” recently designated “nuclear receptor subfamily 2, group F, member 2,” or “NR2F2” in zebrafish) has been shown to act as a positive mediator of venous specification in mice. The murine *COUP-TFII* gene is specifically expressed in venous but not arterial ECs, and is required for proper vascular morphogenesis. *COUP-TFII* knockout mice die at approximately E10.5 following severe hemorrhage and edema due to enlarged blood vessels, improper development of the atria and sinus venosus, and malformed cardinal veins (Pereira et al., 1999). Endothelial-specific targeted disruption of *COUP-TFII* results in ectopic expression of arterial markers in the cardinal vein, while ectopic pan-endothelial over-expression of *COUP-TFII* results in fusion of arteries and veins, similar to phenotypes observed in *Neuropilin1* -/- or *Notch1* -/- mice (You et al., 2005). These and other results from mice and cultured cells have led to the proposal that *COUP-TFII* promotes venous identity by down-regulating pro-arterial Notch signaling, thereby releasing expression of venous factors from Notch-mediated repression and preventing Notch-mediated activation of arterial gene expression (Chen et al., 2012). However, while knockdown of *nr2f2* in zebrafish does result in reduced venous *ephb4* and *flt4* expression, it does not result in ectopic venous expression of arterial markers *grl* and *efnb2a* (Aranguren et al., 2011), suggesting additional investigation is needed to fully elucidate the role of *nr2f2* in vascular development and differentiation.

In this manuscript, we further examine the role of *nr2f2* during early arterial-venous development in the zebrafish trunk axial vasculature. We show that expression of *nr2f2* requires endothelial *sox7* and *sox18* gene function, while expression of some but not all markers of venous identity requires *nr2f2*. Importantly, our results also suggest that in addition to promoting arterial gene expression Notch signaling in the dorsal aorta suppresses expression of nr2f2, restricting nr2f2-dependent venous gene expression to the cardinal vein.

Materials and Methods

Zebrafish

Zebrafish lines used and reported elsewhere were wild-type EK, *cloche* mutant (Stainier et al., 1995), *Tg(fli1a:EGFP)^{y1}* (Lawson and Weinstein, 2002), *Tg(Tp1bglob:hmgb1-*

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mCherry)^{h11} (Parsons et al., 2009), and *Tg(UAS:N3ICDmyc)* (Scheer and Campos-Ortega, 1999). The *Tg(efnb2a:eGFP)* line was generated using a transgene construct in which cytoplasmic EGFP is driven by 10.2 kb fragment of DNA upstream from the translation start site of *efnb2a* (efnb The *Tg(fli1a:GV-EcRF')* line was generated using a transgene construct in which a drug inducible Gal4-VP16 element, GV-EcRF (Esengil et al., 2007), is driven by a minimal *fli1a* promoter/enhancer element. Zebrafish embryos and strains were maintained as described (Kimmel et al., 1995).

In Situ Hybridization and Immunohistochemistry

Whole-mount RNA *in situ* hybridization was carried out as previously described (Pham et al., 2007). Dual-color whole mount RNA fluorescent *in situ* hybridization was carried as previously described (Hauptmann and Gerster, 1994). Antisense probes for *kdrl, efnb2a, grl, tbx-20, dab2, ephB4a, fli1a*, and *cdh5* were prepared as described (Fouquet et al., 1997; Lawson et al., 2002; Siekmann and Lawson, 2007; Thompson et al., 1998; Yaniv et al., 2006). Antisense probes for *nr2f2* (full length and short form), *sox7*, and *sox18* were prepared from cDNA using primers listed in supplement table 1. Amplicons were cloned in pENTR-D/TOPO (Invitrogen) vectors. Antisense probes for *lyve1 and stab1* were obtained from commercially available clones (Open Biosystems, 7998534 and 8998853). DIG-labeled antisense riboprobes were synthesized using the DIG Labeling Kit (Roche). Immunostaining was performed as described (Yaniv et al., 2006).

Cloning and Transgene Construction

Fulllength *nr2f2, sox7*, and *sox18* were amplified from EK cDNA. The GVEcRF' cassette was amplified from pCS2+GVEcRF' (Esengil et al., 2007). *Xenopus laevis* Su(H)DBM was amplified from pCS-XSu(H)DBM (Wettstein et al., 1997). Plasmids containing *nr2f2* chimeras were made after amplifying a truncated sequence of *nr2f2* (aka 'Ct') from full length *nr2f2*. The truncated *nr2f2* was subsequently cloned into either pcGlobin2-VP16 (Ro et al., 2004) or ENG-N backbone (Kessler, 1997) to create an activating (referred to as "VCt" in this text) or a repressing ("ECt") chimera for *nr2f2*. VCt, ECt, full length *nr2f2* ("CF"), *sox7, sox18*, GV-EcRF', and XSu(H)DBM were cloned into Gateway compatible pME plasmids.

Endothelial specific *pI-SceI(kdrl-CeruleanFP-2A-VCt), pI-SceI(kdrl-CeruleanFP-2A-ECt), pI-SceI(kdrl-CeruleanFP-2A-CF), Tol2(fli1a:GVEcRF')*, and *pI-SceI(kdrl-Su(H)DBM-2A-mCherry)* constructs were assembled using Gateway technology (Kwan et al., 2007; Provost et al., 2007; Villefranc et al., 2007).

A list of primers used for cloning can be found in Supplemental Table 1.

Morpholino and Transgene Microinjections

Morpholino (Gene Tools) injections were performed at the described doses into 1- to 2-cell stage embryos. Morpholinos used in this study are as follows: *nr2f2* translation blocking MO (translation start site underlined), 5′- AGCCTCTCCACACTACCATTGCCAT-3′; *nr2f2* exon1 splice donor MO, 5′-AACAAAAATCCGAATACCTTCCCGT-3′; *nr2f2* translation block mismatch control, 5′-AGgCTgTCCACAgTACCATaGCgAT-3′; *sox7*

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translation blocking MO (Cermenati et al., 2008), 5′-ACGCACTTATCAGAGCCGCCATGTG-3′; *sox18* translation blocking MO (Cermenati et al., 2008), 5′-TATTCATTCCAGCAAGACCAACACG-3′. Tol2 and pI-SceI plasmid DNA injections were performed as previously described (Grabher et al., 2004; Kawakami et al., 2000). 100pg of Tol2 based constructs and 45pg of pI-SceI plasmids were injected for each experiment.

Microscopy

RNA *in situ* hybridization images were captured with a ProgRes C14 camera mounted on a Leica MZ12 stereo microscope, or with LAV camera/software on a LM205 stereo microscope. Confocal microscopy of transgenic and immunostained embryos was performed using an Olympus FluoView 1000 microscope.

qRT-PCR Analysis

Total cellular RNA from experimental embryos was isolated using Trizol reagent and treated with DNAse I. For all gene analysis, only trunks excised at the level of the first somite were collected, except for *prox1a* in which whole embryos were used. Briefly, heads and yolks were removed from 8-10 embryos by scalpel prior to addition of Trizol reagent to the excised trunks (within a given experiment the same number of embryos were used for preparation of cDNA for each sample). Total cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad) and qPCR analysis was carried out using iQ SYBR Green Supermix on a Bio-Rad CFX96 with *ef1a* used as a reference gene. Bio-Rad CFX Manager software was used to quantify gene expression levels and all analysis conforms with MIQE guidelines (Bustin et al., 2009).

A list of primers used for qPCR can be found in Supplemental Table 2.

Drug Treatment

Dechorionated *Tg(Tp1bglob:hmgb1-mCherry)^{h11}* embryos were treated with either 100 uM DAPT (Sigma) in 0.2 % DMSO or in 0.2 % DMSO alone as a control beginning at 6 to 8 hpf. *Tg(UAS:N3ICDmyc);Tg(fli1a:GV-EcRF')* double transgenic fish were treated with either 100 nM TBF (Sigma) in 0.05% DMSO or in 0.05% DMSO alone as a control beginning at 6 to 8 hpf.

Measuring Notch Reporter Output

For each experimental condition, ImageJ software was used to highlight and count the number of individual mCherry-positive fluorescent cells from separate, standard-sized boxed regions placed over the neural tube and the dorsal aorta. The ratio of measured mCherry-positive cells in the dorsal aorta box versus mCherry-positive cells in the neural tube box was calculated to give the relative vascular notch reporter activity in each animal. Identically sized boxes placed in equivalent positions were used for measuring reporter fluorescence in the neural tube and dorsal aorta in each animal measured. The values reported in the inset numbers in Figure 3G-K are each normalized to the control value (Figure 3G) to provide a final measurement of total notch activation. Five embryos were

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examined for each condition, with quantitation performed on one representative animal per condition (displayed in the image panels in Figure 3G-K).

Results

nr2f2 is expressed in the posterior cardinal vein during early zebrafish trunk vascular development

We used whole mount ISH to analyze the spatial and temporal expression profile of zebrafish *nr2f2*. We performed analysis with both a full open reading frame zebrafish *nr2f2* probe and with a shorter, highly specific zebrafish *nr2f2* probe against only nucleotides 1-240 of the open reading frame. Similar results were obtained with both antisense probes and images presented are from analysis with the full length probe. *nr2f2* expression is not detected prior to the 3 somite stage. Expression is detected in the hindbrain rhombomeres and anterior somites can be detected at 3 somites (Supp. Fig. 1). By the 15 and 20 somite stages, expression within the central nervous system is clearly observed in the telencephalon, ventral anterior midbrain, hindbrain rhombomeres as well as in the otic vesicles, pectoral fin buds, and developing spinal cord of the trunk (Supp. Fig. 1). While cranial and spinal cord expression remains robust throughout zebrafish development, weak expression of *nr2f2* is first observed in the posterior cardinal vein, but not in the dorsal aorta, at 24 hpf (Figure 1A,B). This expression persists through 48 hpf (Figure 1D-G), in agreement with a previously recorded expression profile of *nr2f2* (Aranguren et al., 2011). To confirm posterior cardinal vein-specific expression of *nr2f2*, we performed two color fluorescent ISH to compare expression profiles of *nr2f2* with the pan-endothelial expression of *kdrl*. While *kdrl* was observed in both the posterior cardinal vein and dorsal aorta (Figure 1E, G, red), *nr2f2* was restricted to the posterior cardinal vein (Figure 1F, G; red and green colocalize in the PCV). Endothelial expression was also confirmed by showing loss of expression in the presumptive ECs of *cloche* mutant embryos (Stainier et al., 1995) that lack most endothelium (Figure 4D and data not shown).

Reduction in *nr2f2* results in loss of venous markers without corresponding increase in arterial markers

To analyze the effect of reduction of *nr2f2* expression on vascular development, we used antisense morpholino oligonucleotides (MO) that targeted either the ATG start site of *nr2f2* translation (ATG MO, MO1), or the exon 1 splice donor site (splice MO, MO2), both of which were effective at reducing normal nr2f2 transcript levels (Supp Fig. 2). Injections of either MO at 5 or 10 ng doses resulted in embryonic lethality after 24 hpf with severe morphological defects and edema, making analysis of vascular development impossible (data not shown). At 2.5 ng most embryos developed with reasonably normal overall morphology and vascular morphology, and displayed only slight developmental delay compared to control fish injected with a control mismatch morpholino (Figure 2A-D). Using this 2.5 ng dose of *nr2f2* MO, we performed whole mount *in situ* hybridization and quantitative RT-PCR to analyze the expression of known arterial, venous, or pan-endothelial markers in the trunk vasculature of *nr2f2* deficient 24 hpf animals compared to their control siblings (Figure 2E-M). We observed significant decreases in expression of *prox1* and early vein-restricted marker *lvye1* in morphant embryos (Figure 2G,M) and modest decrease in

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vein-restricted marker *stab1* (Figure 2F,M), but no change in expression of vein-restricted markers *ephb4* and *dab2* by qRT-PCR (Figure 2M). We also saw no change in expression of arterial-restricted markers *efnb2a, grl*, and *tbx20* (Figure 2I,J,K,M). The expression of the *sox7* and *sox18* genes, previously implicated in arterial-venous fate decisions in the zebrafish (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008), was also unchanged in *nr2f2* deficient animals (Figure 2H,L,M). Other pan-endothelial markers including *fli1a, kdrl*, and *cdh5* were also unchanged (data not shown).

Since higher doses of *nr2f2* morpholinos were associated with significant developmental abnormalities and lethality, likely due to earlier essential functions of *nr2f2* in the nervous system and/or other tissues, we used transgenesis to specifically target endothelial cells for *nr2f2* activation or silencing using "activating" or "repressing" *nr2f2* chimeras, respectively. Previous reports have described methods for fusing a truncated version of *nr2f2* to either the engrailed repressor domain or the VP16 activating domain (Naka et al., 2008). We placed full-length nr2f2 or these chimeric proteins together with cerulean fluorescent protein (to mark expressing cells) under the control of the vascular-specific zebrafish *kdrl* promoter to generate constructs for endothelial-restricted expression of either wild type full-length *nr2f2* (kdrl-CF), "activating" protein containing VP16 fused to an nr2f2 polypeptide with its endogenous transactivation domain removed (kdrl-VCt). or "repressing" protein containing Engrailed fused to an nr2f2 polypeptide with its endogenous transactivation domain removed (kdrl-ECt) (Supp. Fig. 3A-D). When these constructs were injected into zebrafish embryos we found that endothelial expression of "repressing" kdrl-ECt resulted in suppression of venous-specific *lyve1* expression similar to that observed in *nr2f2* MO-injected animals (Figure 3C,E,Q). In contrast, endothelial expression of either wild type *nr2f2* or "activating" kdrl-VCt did not significantly alter *lyve1* expression (Figure 3D,F,Q).

nr2f2 does not regulate Notch in the zebrafish vasculature

Previous studies have reported that *nr2f2* modulates venous identity in mice by regulating Notch expression in the developing vascular system (Chen et al., 2012; You et al., 2005). To examine the role of *nr2f2* in regulating Notch in zebrafish, we injected *nr2f2* MO or our vascular *nr2f2* chimera-expressing constructs into either a *Tg(Tp1bglob:hmgb1-mCherry)*^{h11} ("Tp1:mCherry") Notch reporter transgenic line (Parsons et al., 2009) or into a *Tg(efnb2a:eGFP)* Notch responsive transgenic line. We found no change in the level of fluorescent protein expressed in the vasculature in either of these transgenic lines upon injection of nr2f2 MO, or kdrl-CF, kdrl-VCt, or kdrl-ECt constructs (Figure 3G-K and Supp. Fig. 4). Treatment with the Notch inhibitor DAPT did cause strong reduction in *Tg(Tp1bglob:hmgb1-mCherry)*^{h11} and *Tg(efnb2a:eGFP)* transgenic reporter expression, however, confirming that these lines are indeed responsive to reduced Notch signaling (Supp. Fig. 5). We also found no change in expression levels of the Notch–responsive gene *efnb2a* in *nr2f2* morpholino or chimera-injected animals (Figure 3L-Q). Our results indicating that Notch signaling is not regulated by *nr2f2* in zebrafish are consistent with previously published results showing that expression of *efnb2a* and *grl* are not affected in *nr2f2* morphants (Aranguren et al., 2011).

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nr2f2 is regulated by sox7 and sox18 in zebrafish

A number of previous publications have reported that the *Sry-related HMG box (Sox)* genes *sox7* and *sox18* are involved in regulating arterial-venous specification in zebrafish, although the precise nature of this regulation remains unclear (Cermenati et al., 2008; Herpers et al., 2008;Pendeville et al., 2008). We used the same *sox7* and *sox18* morpholinos used in Cermenati et al., at the same doses, to determine whether *sox7* and/or *sox18* function are required for expression of *nr2f2*. Injection of *sox7* or *sox18* morpholinos alone did not cause significant change in *nr2f2* expression (data not shown), but dual morpholino (DMO) knockdown of both *sox7* and *sox18* together resulted in strong reduction in *nr2f2* expression in the PCV, although neural tube expression was not detectably altered (Figure 4A,B). Quantitative RT-PCR of either whole embryo or excised trunk sections of pooled control and Sox DMO embryos showed that trunk levels of *nr2f2* were reduced by approximately 50%, while levels of *lyve1* were more strongly reduced (Figure 4C). The 50% reduction in *nr2f2* likely reflects reasonably complete loss of the vascular expression of *nr2f2*, since measurement of excised trunk expression levels by qRT-PCR showed that the reduction in *nr2f2* in *sox7/sox18* DMO embryos was comparable to that observed in *cloche* mutant (Stainier et al., 1995) animals that lack virtually all endothelium (but that also retain the neural tube expression of *nr2f2*; data not shown), and was not further reduced by knocking down *sox7* and *sox18* in the *cloche* mutant background (Figure 4D).

nr2f2 is regulated by Notch in zebrafish

Our results and those of others (Aranguren et al., 2011) suggest that in the zebrafish *nr2f2* is not regulating Notch, unlike *nr2f2/COUP-TFII* in mice. Furthermore, some recent *in vitro* data suggests that *nr2f2* can be a target of Notch signaling in at least some contexts. Ectopic expression of “activated” Notch intracellular domain (ICD) or Notch target transcription factors Hey1 and Hey2 represses *nr2f2* expression in cultured lymphatic endothelial cells (Kang et al., 2010). To explore whether Notch acts upstream of *nr2f2* in the zebrafish vasculature, we began by examining whether endothelial-specific activation of Notch signaling represses *nr2f2* expression, using binary transgenic inducible expression of Notch3 intracellular domain (N3ICD) in endothelium *in vivo*. We generated fish carrying a vascular-specific, tebufenozide-inducible GAL4 fusion protein *(Tg[fli1a:GVEcRF]))* and crossed these to fish we have employed previously to inducibly activate Notch signaling (Lawson et al., 2001) that carry a myc-tagged UAS-driven N3ICD transgene *(Tg[UAS:N3ICDmyc])* (Supp. Fig. 3E). Tebufenozide-induced activation of N3ICD prior to the onset of vascular development resulted in reduction in *nr2f2* expression in the PCV but not in the neural tube in *Tg(UAS:N3ICDmyc);Tg(fli1a:GV-EcRF')* double transgenic fish (Figure 5A,B). Since ectopic endothelial activation of Notch signaling resulted in reduced *nr2f2* expression, we also examined whether suppression of Notch signaling by endothelial-specific expression of a Notch signaling dominant-negative *suppressor of hairless* DNA binding domain mutant *(Su[H]DBM)* might cause increased *nr2f2* expression. To do this, we generated a construct to drive expression of *Su[H]DBM* under the control of the *kdrl* promoter *(kdrl:Su[H]DBM-2A-mCherry)* (Supp. Fig. 3F) and injected this construct into zebrafish embryos. mCherry-positive endothelial cells co-expressing Su[H]DBM also mis-expressed *nr2f2* in the dorsal aorta, although no change was noted in neural tube expression (Figure

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5C,D). Binary transgenic inducible expression of N3ICD did not alter expression of *sox7* or *sox18*, indicating that Notch does not regulate *nr2f2* expression by modulating expression of these SoxF family genes (Figure 5E-H). Taken together, these findings suggest that Notch works downstream from or in parallel to *sox7* and *sox18* to repress *nr2f2* expression in the dorsal aorta.

Discussion

Our results suggest a model for upstream regulation of, and downstream regulation by, *nr2f2* during early arterial-venous development in the zebrafish trunk axial vasculature (Figure 6). We show that endothelial expression *of nr2f2* requires endothelial *sox7* and *sox18* gene function, while expression of some but not all markers of venous identity requires *nr2f2*. Importantly, our results also indicate that in addition to promoting arterial gene expression, Notch signaling in the dorsal aorta suppresses expression of *nr2f2*, restricting *nr2f2*-dependent trunk vascular gene expression to the cardinal vein.

The function of zebrafish *nr2f2* is required for proper venous differentiation of the cardinal vein (Figure 2E-G,M). Early expression of *prox1* and *lyve1* is strongly reduced in nr2f2-deficient animals. However, other venous markers are either modestly reduced *(stab1)* or unaffected *(ephb4a, dab2)*. These results are similar to those in previously published studies in zebrafish (Aranguren et al., 2011) and mice (You et al., 2005) showing only partial effects on venous gene expression. It should be noted that the translation blocking morpholino used in our study is identical to that used in a previously published study (Aranguren et al., 2011), and the exon 1 splice donor site morpholino nearly identical (shifted 3′ by one nucleotide). Levels of endothelial Ephb4 are only slightly reduced in the cardinal veins of Nr2f2/COUP-TFII knockout mice (You et al., 2005), while only partial reduction is noted in venous expression of *flt4* and *ephb4a* in *nr2f2* knockdown zebrafish (Aranguren et al., 2011). These results suggest that factors in addition to nr2f2 may possibly be required for full establishment of venous identity in both mice and zebrafish. Various reports have suggested that nr2f2/COUP-TFII can act as either a repressor or an activator of downstream gene expression (reviewed in (Park et al., 2003)). We examined this in zebrafish using endothelial-specific expression of either wild type nr2f2, or nr2f2-engrailed (repressing) or nr2f2-VP16 (activating) fusion proteins, demonstrating that nr2f2 acts as a cell-autonomous activator required for venous gene expression in the zebrafish, since endothelial expression of an nr2f2-engrailed fusion protein (kdrl-ECt) results in reduced expression of *lyve1* in a manner similar to nr2f2 morpholino knockdown, while wild type nr2f2 or an nr2f2-VP16 fusion protein has no significant effect (Figure 3B-F,Q). These results suggest that, at least in this context, nr2f2 is acting primarily as a positive factor for venous gene expression.

Proper arterial-venous differentiation requires restricted expression of not only venous-specific genes but also arterial-specific genes. A variety of previously published studies from our laboratory and others have shown that Notch pathway activation promotes arterial differentiation downstream from hedgehog and VEGF signaling ((Lawson et al., 2001; Lawson et al., 2002), reviewed in (Swift and Weinstein, 2009)). In mice, COUP-TFII has been shown to act as a negative regulator of Notch signaling to suppress arterial gene expression in the cardinal vein (Chen et al., 2012; You et al., 2005). Endothelial-cell specific

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ablation of *COUP-TFII* results in the formation of vein-like structures that improperly express arterial markers neuropilin 1 (Np1), Jag1, *Hey1*, Notch1, and *Efnb2*, along with the partial reduction in expression of venous marker EphB4 noted above. Conversely, mis-expression of *COUP-TFII* is developing arteries leads to diminished expression of Np1 and Jag1 coincident with an increase in EphB4 (You et al., 2005). More recently, microarray analysis in HUVEC cells found the expression of several Notch target genes was regulated by COUP-TFII and that COUP-TFII regulates *foxc1*, *Np-1*, and *Hey2* at the transcriptional level (Chen et al., 2012).

In contrast to these results, we find no change in arterial-specific gene expression in *nr2f2*-deficient zebrafish. Nr2f2 morpholino-injected animals show neither ectopic expansion of arterial markers into the cardinal vein by *in situ* hybridization, nor increased trunk expression of these genes as measured by quantitative RT-PCR (Figure 2I-K,M). Again, these results are consistent with a previously published report showing lack of ectopic expression of arterial markers in veins in *nr2f2* knockdown zebrafish (Aranguren et al., 2011). In addition to the morpholino knockdown findings, we fail to observe either ectopic venous expression or increased overall expression of arterial marker *efnb2a* in zebrafish expressing the “repressing” kdrl-ECt fusion protein specifically in the endothelium (Figure 3O,Q), despite strongly reduced expression of the venous marker *lyve1* in these same animals (Figure 3E,Q). We used two different Notch-responsive transgenic zebrafish reporter lines to look more directly at the effects of either nr2f2 morpholino knockdown or endothelial-specific expression of nr2f2 fusion proteins on Notch activation. Expression of fluorescent reporters was not affected by functional manipulation of nr2f2 in *Tg(Tp1bglob:hmgb1-mCherry)^{h11}* or *Tg(efnb2a:eGFP)* transgenic animals (Figure 3G-K and Supp. Fig. 4), despite the fact that arterial expression of both reporters is clearly Notch-responsive as indicated by reduced expression upon treatment with DAPT (Supp. Fig. 5). Taken together, these results suggest that nr2f2 is not required to modulate Notch signaling activity or suppress arterial-specific gene expression in the zebrafish cardinal vein.

We examined other potential factors that might be responsible for promoting venous identity or suppressing arterial identity in the cardinal vein. A number of recent studies have reported that the soxF family members *sox7* and *sox18* modulate arterial-venous differentiation in the zebrafish, although the precise nature of their role remains somewhat unclear, with effects on expression of either arterial genes, venous genes, or both noted in different reports (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008). A recent publication suggests that Sox and Notch are required together for full expression of at least on arterial gene, Dll4 (Sacilotto et al., 2013). We find that *sox7/18* function promotes expression of at least some of the same genes dependent on *nr2f2* (Figure 4). As noted in one of those previous publications (Cermenati et al., 2008;), *sox7* and *sox18* appear to function redundantly in the regulating vein identity, since double morphants show loss of venous gene expression while single morphants do not. Interestingly, the other two studies (Herpers et al., 2008; Pendeville et al., 2008) reported ectopic expression of the venous marker *flt4* in the DA, however these studies did not examine expression beyond a single time point to see if this altered expression persists. We find that *sox7/18* function is required for expression of

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nr2f2 in the cardinal vein, suggesting that *sox7/18* function is required upstream of *nr2f2* to regulate proper venous differentiation.

While *sox7* and *sox18* are required for nr2f2 expression and for proper arterial-venous differentiation, it seems unlikely that these genes are acting as “selective factors” for promoting distinct endothelial cell fates in the dorsal aorta and cardina w in. Nr2f2 is expressed only in the cardinal vein, not in the dorsal aorta, while the two sox genes are each expressed in both the cardinal vein and the dorsal aorta during early trunk axial vessel arterial-venous differentiation. The arterial expression of *sox7* and *sox18* (but not *nr2f2*) suggests that some other factor(s) in addition to *sox7* and *sox18* is either necessary to suppress *nr2f2* expression in the dorsal aorta or required for *nr2f2* expression in the posterior cardinal vein. Surprisingly, we have now found that Notch signaling appears to be acting as an arterial-specific suppressor of *nr2f2* expression and venous identity in zebrafish. Using both whole organism and EC-specific gain-of-function experiments to mis-express Notch ICD, we find a consistent and significant loss of *nr2f2* expression in the PCV (Figure 5A,B). Mis-expression of “activated” Notch ICD does not result in altered expression of either *sox7* or *sox18*, confirming that Notch does not regulate *nr2f2* expression by modulating levels of *sox7* or *sox18* (Figure 5E-H). Conversely, endothelial-specific suppression of Notch signaling using a dominant negative suppressor of Hairless (Su(H)) DNA binding mutant driven by the *kdrl* promoter results in ectopic expression of *nr2f2* in the dorsal aorta (Figure 5C,D).

Conclusions

Together, these results suggest that Notch serves as an arterial-specific repressor of *nr2f2* expression in the zebrafish endothelium (Figure 6). Our findings lead us to hypothesize that Notch signaling functions as a “switch” regulating both arterial and venous gene expression and identity. In the dorsal aorta, the presence of active Notch signaling serves a dual function to simultaneously promote arterial gene expression and suppress *nr2f2*-driven venous gene expression. In the cardinal vein, absence of Notch signaling prevents arterial gene expression but permits expression of *nr2f2* and *nr2f2*-dependent venous gene expression. Although at least one previous study suggests that Notch may directly regulate activity of the NR2F2 promoter, our data do not establish whether suppression of nr2f2 in the zebrafish dorsal aorta is direct or due to the activity of less proximal downstream genes up-regulated in the dorsal aorta by Notch.

This model is satisfying in terms of explaining how *sox7* and *sox18*, which are both expressed in a pan-endothelial fashion, can be required upstream of *nr2f2*, which is expressed only in venous but not in arterial venous vessels. However, it is clearly an oversimplification of what is likely to be a more complex regulatory network. *nr2f2* and *sox7/18* function appear to be required for the expression of only a subset of venous-specific genes, and they also appear to also affect some arterial-specific gene expression (particularly for *sox7/18;* Herpers et al., 2008; Pendeville et al., 2008). Likewise, the effects of Notch signaling are also not absolute with regard to arterial identity, as previously published results from our laboratory and others have shown that some arterial-specific markers remain unresponsive to manipulation of Notch function (Lawson et al., 2001; Lawson et al., 2002).

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Nevertheless, our results generally support the role of Notch signaling as a key nexus for the arterial-venous fate decision in the zebrafish, not only through promotion of arterial differentiation but also through suppression of *nr2f2*-dependent venous differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

nr2f2 (aka COUP-TFII) is expressed in the cardinal vein, but not in the dorsal aorta

nr2f2 promotes expression of a subset of venous genes.

sox7/18 gene function is required for nr2f2 expression in the vasculature

Notch signaling suppresses nr2f2 expression in the dorsal aorta

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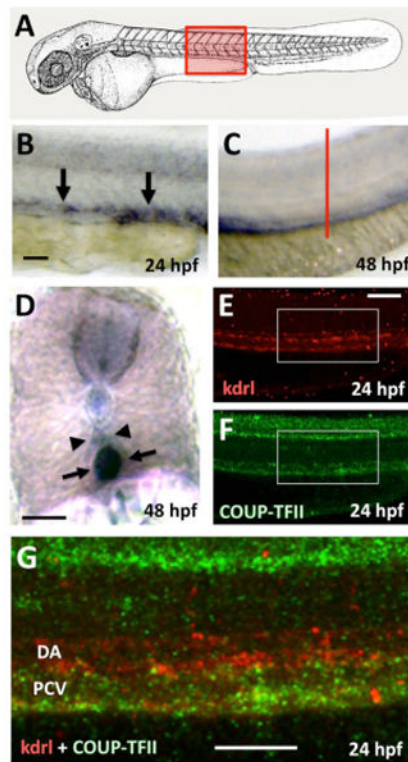

Figure 1. Expression of nr2f2 in developing veins in the zebrafish

(A) Schematic diagram of a 1 dpf zebrafish (modified from (Kimmel et al., 1995)) with a red box showing the approximate position of images in B-G. **(B-D)** Whole mount *in situ* hybridization of the trunks of 24 hpf (B) and 48 hpf (C,D) zebrafish embryos probed for *nr2f2*. **(E-G)** Two-color fluorescent in situ hybridization of the trunk of a 24 hpf animal probed for *kdrl* (in red; panels E,G) and *nr2f2* (green; panels F,G). Images are all lateral views of the trunks of whole mount stained animals (anterior to the left) except for panel D, which shows a transverse thick section through the trunk (the red line in panel C indicates the plane of section shown in panel D). Arrows in panels B, D, and G point to posterior cardinal vein expression of *nr2f2*, while arrowheads in panels D and G note the position of the dorsal aorta. Rostral is to the left and dorsal is up in all image panels. Scale bars, (B,C,E,F,G) 100 μm, (D) 50 μm.

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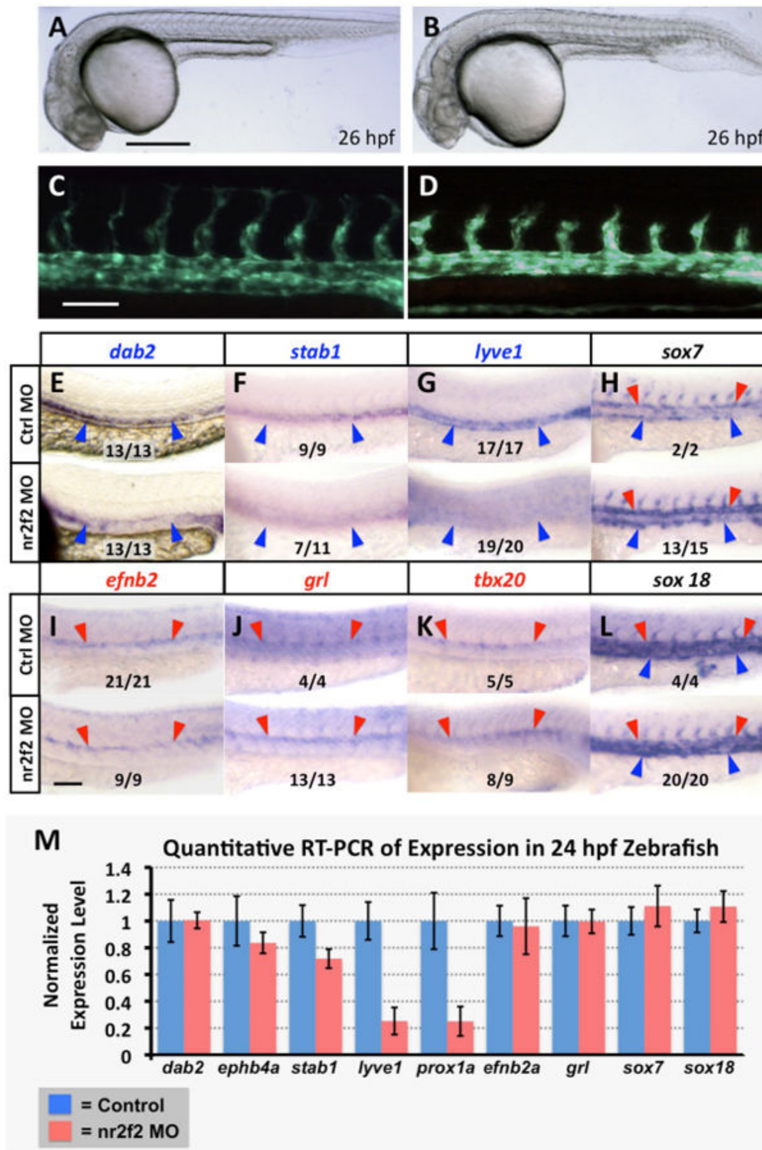

Figure 2. nr2f2 function is required for proper venous gene expression in the zebrafish vasculature

(A-D) Transmitted light (A,B) and green fluorescent light (C,D) images of control (A,C) and *nr2f2* morpholino injected (B,D) *Tg(fli1a-EGFP)*^{y1} transgenic animals. **(E-L)** Whole mount *in situ* hybridization of 24 hpf control (top) and *nr2f2* morpholino injected (bottom) zebrafish embryo trunks probed for *ephb4a* (E), *stab1* (F), *lyve1* (G), *sox7* (H), *efnb2a* (I), *grl* (J), *tbx20* (K), and *sox18* (L). Blue arrowheads note posterior cardinal vein gene expression, while red arrows note dorsal aorta gene expression. The inset numbers in panels F-L show the number of *in situ*-stained *nr2f2* MO-injected embryos exhibiting the phenotype shown in the image panel over the total number of embryos examined. **(M)** Quantitative RT-PCR measurement of gene expression in excised trunks (see Materials and Methods) of 24 hpf control and *nr2f2* morpholino injected animals. Values are all normalized to control gene expression levels, which are set equal to 1. Rostral is to the left and dorsal is up in all image panels. Scale bars, (A,B) 500 μm, (C-L) 100 μm.

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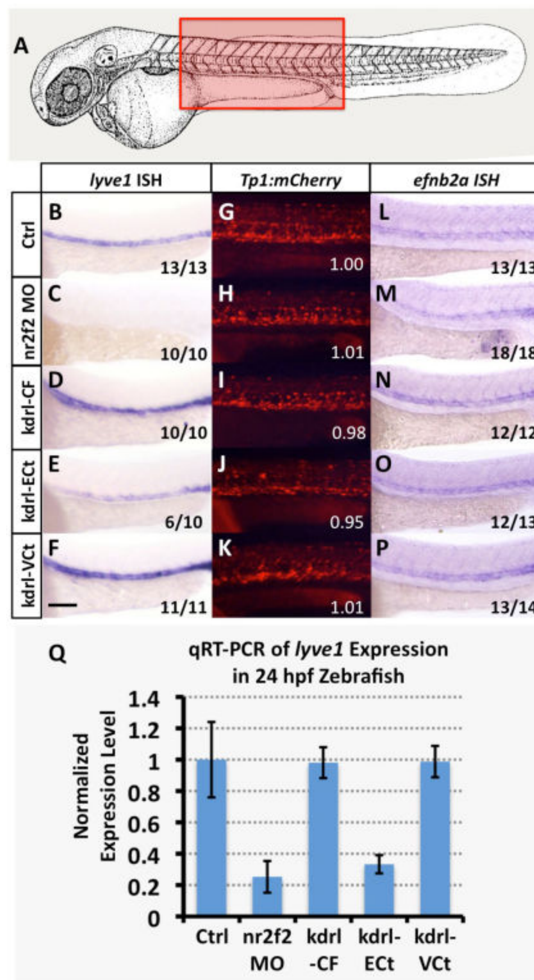

Figure 3. nr2f2 does not regulate Notch in the zebrafish vasculature

(A) Schematic diagram of a 1 dpf zebrafish (modified from (Kimmel et al., 1995)) with a red box showing the approximate position of images in B-V. **(B-F)** Whole mount *in situ* hybridization of 24 hpf zebrafish probed for *lyve1*. **(G-K)** Red fluorescent confocal micrographs of 24 hpf *Tg(Tp1bglob:hmgb1-mCherry)*^{h11} ("Tp1:mCherry") transgenic zebrafish. Inset numbers show quantitative measurements of trunk vascular mCherry fluorescence in a single representative injected fish normalized to trunk neural mCherry fluorescence and to values in untreated controls (G). See Materials and Methods for additional information on measurements. **(L-P)** Whole mount *in situ* hybridization of 24 hpf zebrafish probed for *efnb2a*. Animals in image panels B-P were either untreated (B,G,L), injected with *nr2f2* MO (C,H,M), injected with kdrl-CF (Full length nr2f2 under the control of the kdrl promoter; D,I,N), injected with kdrl-ECt (chimeric protein with nr2f2 lacking the transactivation domain fused to engrailed repressor domain; E,J,O), or injected with kdrl-VCt (chimeric protein with nr2f2 lacking the transactivation domain fused to VP16; F,K,P). The inset numbers in panels C-F and M-P show the number of *in situ*-stained embryos exhibiting the phenotype shown in the image panel over the total number of embryos examined. **(Q)** Quantitative RT-PCR measurement of gene expression in excised trunks of 24 hpf animals (see Materials and Methods). Values are all normalized to control gene

expression levels, which are set equal to 1. Rostral is to the left and dorsal is up in all image panels. Scale bar, 100 μm.

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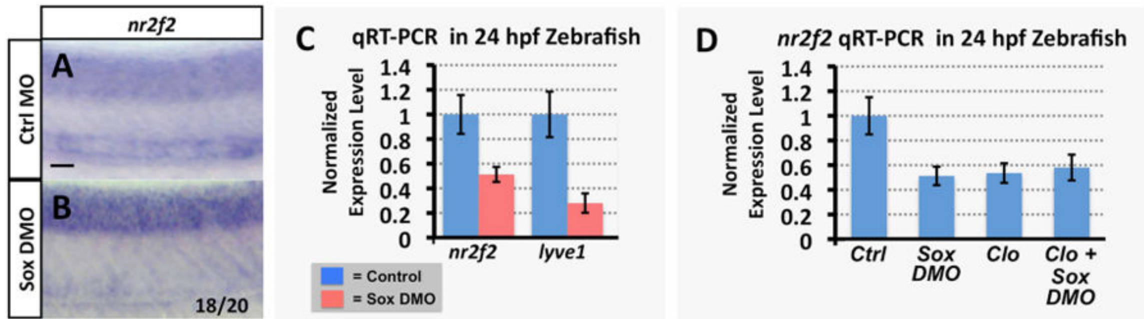

Figure 4. Sox gene function is required for nr2f2 expression in the zebrafish vasculature

(A,B) Whole mount *in situ* hybridization of 24 hpf control (A) and *sox7/sox18* double morpholino (DMO) injected (B) zebrafish embryo trunks probed for *nr2f2*. The inset numbers in panels B show the number of *in situ*-stained embryos exhibiting strongly reduced or absent vascular expression over the total number of embryos examined. **(C)** Quantitative RT-PCR measurement of *nr2f2* and *lyve1* gene expression in excised trunks (see Materials and Methods) of 24 hpf control and *sox7/sox18* DMO animals. **(D)** Quantitative RT-PCR measurement of *nr2f2* gene expression in excised trunks (see Materials and Methods) of 24 hpf control MO injected, *sox7/sox18* DMO injected, *cloche* mutant, and *sox7/sox18* DMO injected *cloche* mutant animals. All quantitative RT-PCR values are shown normalized to control gene expression levels, which are set equal to 1. Rostral is to the left and dorsal is up in all image panels. Scale bars, 50 µm.

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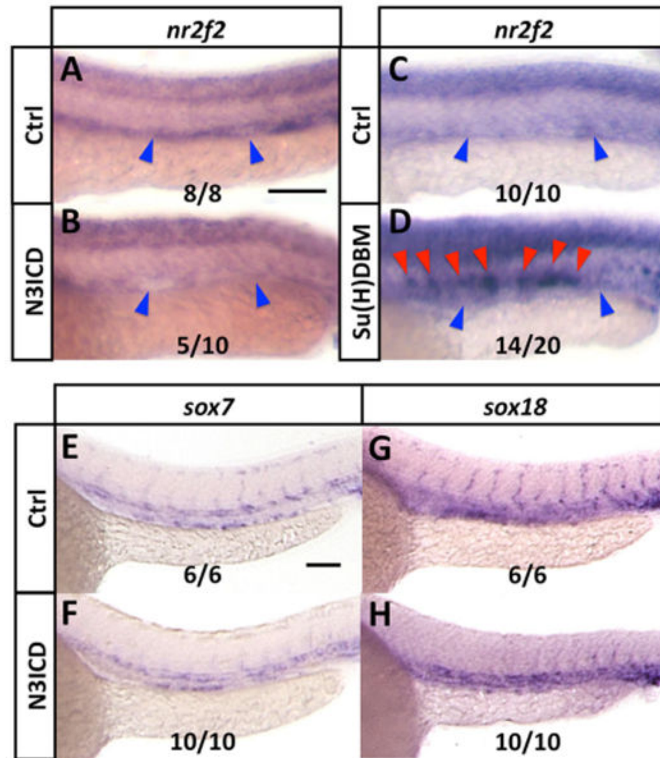

Figure 5. Notch negatively regulates *nr2f2* in the zebrafish vasculature, but not via Sox

(A,B) Whole mount *in situ* hybridization of 24 hpf control (A) and *Tg[fli1a:GV-EcRF];Tg[UAS:N3ICDmyc]* double transgenic (B) animals treated with tebufenozide from 8 hpf to 28 hpf, probed for *nr2f2*. Blue arrowheads show the position of the posterior cardinal vein. The inset numbers in panel B show the number of *in situ*-stained embryos exhibiting strongly reduced vascular expression over the total number of embryos examined. **(C,D)** Whole mount *in situ* hybridization of 24 hpf control (C) and *kdrl:Su[H]DBM-2A-mCherry* injected (D) animals, probed for *nr2f2*. Blue arrowheads show position of the posterior cardinal vein, red arrowheads show ectopic *nr2f2* expression in the dorsal aorta. The inset numbers in panel D show the number of *in situ*-stained embryos exhibiting patches of strong ectopic *nr2f2* expression in the dorsal aorta over the total number of embryos examined. **(E-H)** Whole mount *in situ* hybridization of 24 hpf control (E,G) and *Tg[fli1a:GV-EcRF];Tg[UAS:N3ICDmyc]* double transgenic (F,H) animals treated with tebufenozide from 8 hpf to 24 hpf, probed for *sox7* (E,F) or *sox18* (G,H). The inset numbers in panels F and H show the number of N3ICDmyc- expressing *in situ*-stained embryos exhibiting *sox7* or *sox18* expression comparable to that in controls, over the total number of embryos examined. Rostral is to the left and dorsal is up in all image panels. Scale bars, 100 μm.

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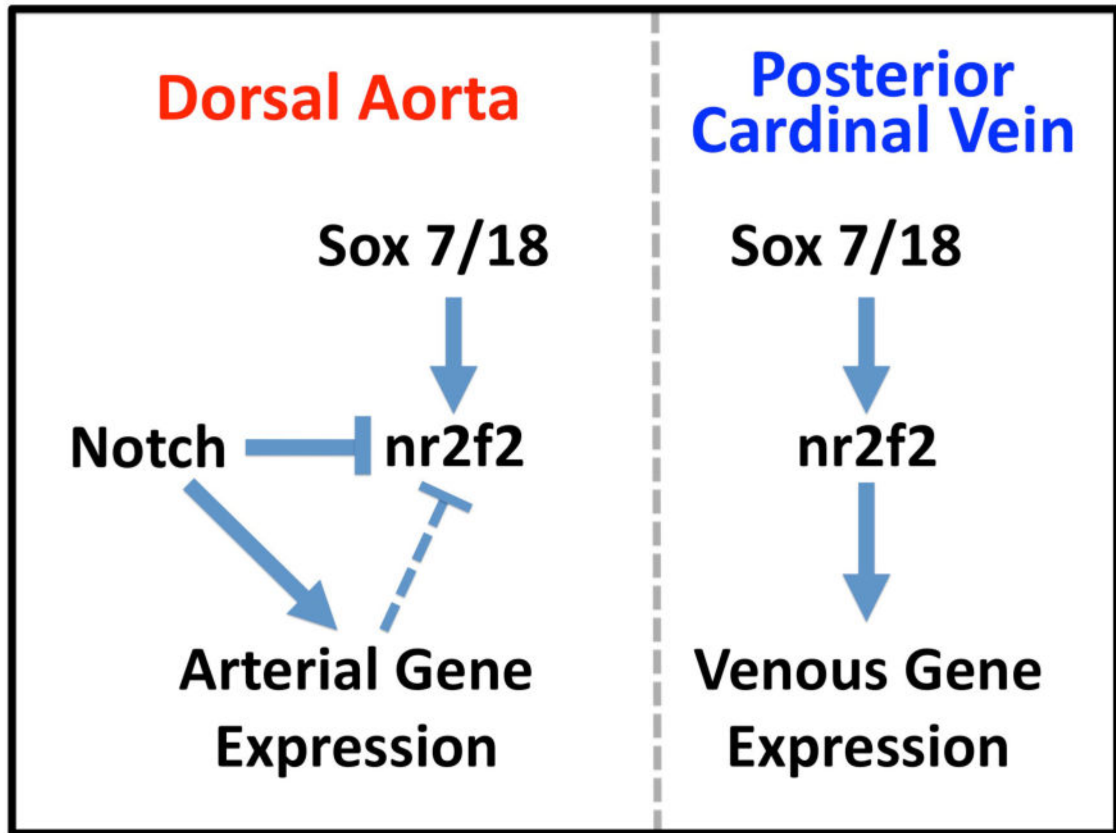

Figure 6. A proposed model for the regulation and functional role of nr2f2 in the early zebrafish trunk vasculature

The function of nr2f2 is required for some, but not all, venous gene expression in the posterior cardinal vein (PCV), most likely as a positive-acting or permissive factor. Nr2f2 expression itself is positively regulated by the combined activity of sox7 and sox18, each of which are expressed in both the venous and arterial vasculature. Although Notch does not appear to be regulated downstream from nr2f2 during early zebrafish vascular development, Notch signaling activity suppresses expression of nr2f2 in the dorsal aorta, either directly or indirectly, resulting in restriction of nr2f2 expression to the posterior cardinal vein.