Pax2a regulates angiogenesis to facilitate fusion of the optic fissure

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ABSTRACT:

Formation of the vertebrate retina involves timely and precise fusion of the optic fissure, failure of which results in the congenital blinding disorder coloboma. The optic fissure forms in consequence of optic cup morphogenesis and has a functional role as a conduit for migration of endothelial vasculature during hyaloid vasculogenesis. Recent studies have linked hyaloid vasculature precursor cells to be potential triggers, initiating optic fissure fusion. In order to examine this link, we analyzed the molecular events associated with optic fissure fusion and hyaloid vasculogenesis in the zebrafish pax2a mutant coloboma model. By means of wholemount immunohistochemistry, we determined that optic fissure fusion initiation, as indicated by laminin remodeling, is preceded by a spike in F-actin signaling. In contrast, the spike in F-actin activity and the disassembly of the basement membrane (BM) were misregulated in pax2a-/- embryos.

To examine how the fusion process is disrupted in the absence of pax2a function, we compared transcriptomic profiles between pax2a-/- and wildtype (WT) embryos. This analysis uncovered a novel connection between regulation of angiogenesis and fusion. Loss of pax2a function resulted in significant reduction of talin1 expression, a known regulator of endothelial vasculature migration associated with fissure fusion. In addition, loss of pax2a also resulted in increased expression of an anti-angiogenic protease, ADAMTS1. 3D confocal and live imaging of retinal hyaloid vascularization in Tg[kdrl:mCherry] embryos indicated a significant deficit in pax2a-/- embryos while pharmacological inhibition of VEGF using DMH4 or ADAMTS1 mRNA overexpression phenocopied the pax2a mutant phenotype. Taken together, we propose that Pax2a positively regulates taln1 while negatively regulating ADAMTS1 expression. This enables timely hyaloid vascularization of the retina which in turn directly signals to initiate fissure fusion via cytoskeletal rearrangements and subsequent BM remodelling.
Formation of a fully functioning human eye requires precise and coordinated cellular mechanisms during very early development. One such mechanism is responsible for the formation of the eyeball structure from a flat sheet of cells to a hemispherical organ. In order to facilitate the biophysical properties of this shape change, an opening on the ventral side of the eye forms and must be closed in order to properly form the eyeball. Failure of this event leads to pediatric blindness in the form of a disease termed Coloboma. While it has been studied by researchers for almost a century, we still lack a significant understanding of how and why some children are born with this disease. In this study, we aimed to better understand how the closure of this opening is triggered by the developing tissues. What we found is, that non-eye cells which are found within this opening and are making vasculature networks to nourish the eye, are likely triggers for the fusion event. We found that regulating their migration and proliferation, also known as angiogenesis, is required for closure of this opening and therefore proper development of the eye.
INTRODUCTION:

Ocular development is a highly conserved process amongst vertebrate species. Assembly of the hemispherical retinal structure from an initially flat sheet of cells requires many complex morphogenetic movements. One such morphogenetic movement involves the invagination of the optic vesicle which results in a fissure forming at the ventral region of the developing retina. This fissure, known as the choroid or optic fissure (OF), enables hyaloid vasculature endothelial cell migration into the developing retina and subsequent establishment of the hyaloid vasculature. Hyaloid vasculature is a temporary circulatory system required for ocular development, and in most cases will degenerate once mature blood vessels begin to grow (1-4). As soon as the hyaloid vasculature has been established, the two opposing retinal epithelial sheets of the optic fissure will undergo fusion. Thereby, they encase the ganglion cell axons localized in the optic stalk and complete retinal morphogenesis. Failure of OF fusion leads to a congenital blinding disorder known as coloboma (5-7). Coloboma is a prevalent cause of pediatric blindness, accounting for approximately 10% of cases worldwide (6, 8). This makes it one of the leading causes of pediatric blindness. Coloboma is a spectrum disorder presenting unilaterally or bilaterally and ranging in severity from minor visual impairment, to complete blindness in the affected eye (9). This spectrum of severity is associated with the location and degree to which the choroid fissure was able to fuse and the severity of subsequent loss of ganglion cell axons (7). The process of epithelial tissue fusion is not unique to the eye and occurs throughout development, encompassing neural tube closure, palatal shelf formation and eyelid development to name a few. Similar to the failure of optic fissure fusion, congenital disorders have been associated with various organs where epithelial fusion fails (10). Epithelial fusion has been studied for over a century, and is known to involve transcriptional regulation, cell signaling pathways, and morphogen gradients (11, 12).

However, in regard to the optic fissure we are still lacking a basic understanding of how these molecular mechanisms coordinate and directly facilitate fusion. Recent work has begun to implicate some of the cellular processes involved in optic fissure fusion. For example, the actin cytoskeleton is known to be a crucial component of the machinery driving fusion of the optic fissure as well as other tissues (13). The importance of the
The actin cytoskeleton during epithelial fusion is thought to involve lamellipodia and filopodia projections between the two opposing epithelia. These help to “zipper” the cells together to form a single continuous sheet. When lamellipodial and filopodial projections are precluded, fusion often fails (14). Interestingly, these projections were observed during OF fusion almost 3 decades ago (15-17). However, while it has been observed that actin protrusions likely play a functional role during OF fusion, it still remains to be determined what signals rearrangement of the actin cytoskeleton. The second cellular mechanism known to be directly involved in epithelial fusion is the remodeling of the basement membrane (BM). During epithelial fusion, the BM acts as a physical barrier restricting the establishment of cell to cell contacts, which must be removed in order for fusion to complete. Recent work, including our own, across several species has characterized progressive removal of the BM during choroid fissure fusion (18, 19). However, the molecular mechanisms facilitating this process, in particular BM remodeling, remain largely unknown. It was recently shown that migrating hyaloid vasculature precursors found in the OF could potentially signal BM remodeling in anticipation of fusion (19). James et al. 2016, showed that mutations in zebrafish talin1, an actin cytoskeleton scaffolding protein known to be required for endothelial cell migration (20), result in choroid fissure fusion defects. Theirs, and previous studies also indicated that cloche mutants, which lack all early hyaloid vasculature, have delayed basement membrane breakdown in the region of the choroid fissure (19, 21). Hyaloid vasculature has long been hypothesized to be an initiating factor of optic fissure fusion. It’s role during retinal development is to establish a vasculature supply to the developing retina and lens while connecting to the choroid vasculature for proper blood flow (3). Hyaloid vasculogenesis takes advantage of an open optic fissure so that vasculature cells can migrate into the developing optic cup. Once optic fissure fusion is completed, hyaloid vasculature is fully established. It has therefore been proposed that because hyaloid vasculature requires an open fissure to complete establishment of its network, migrating hyaloid vasculature cells could directly signal to the opposing retinal lobes and regulate the timing of fissure fusion. This mechanism could potentially involve vasculature-mediated activation of the fusion machinery within the retinal rim cells, or direct supply of molecular factors, such as matrix proteases (22). In additional support
of this hypothesis, recent optic cup transplantation experiments in zebrafish embryos confirm that in the absence of hyaloid vasculature, ectopic retinal OFs fail to initiate fusion (23). There is therefore a clear link between OF fusion initiation and hyaloid vasculature migration through the fissure.

Coloboma has been studied for many decades in many different species. This has led to a general outline of the signaling and morphogenetic pathways required for proper OF formation and fusion (recently reviewed in (24)). In particular, opposing action of bone morphogenetic protein (BMP) and sonic hedgehog (Shh) signaling establishes the dorso-ventral pattern of the optic vesicle and ensures proper expression of optic stalk and OF regulators pax2, vax1 and vax2 (25, 26). However, as outlined above, the actual molecular mechanisms driving the fusion process remain largely unknown. Recent work from our lab has begun to decipher the composition and timing of remodeling of the OF BM (27), however, the field still lacks a detailed understanding of the cellular processes during and leading up to OF fusion. Hence, we have undertaken a detailed analysis of zebrafish OF fusion comparing WT and the pax2a<sup>−/−</sup> coloboma model (28). In particular we have characterized the timing of BM remodeling, F-actin dynamics, morphological apposition and hyaloid vascularization. Furthermore, using transcriptomic analysis to compare WT and pax2a<sup>−/−</sup> eyes we discovered misregulation in angiogenic signaling, resulting in decreased vasculogenesis of the early retina and OF fusion failure. In particular we found that pax2a<sup>−/−</sup> embryos exhibit a decrease in talin1 expression coupled with an increase in A disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1) expression, an anti-angiogenic protease (29, 30). Modulation of vascularization via pharmacological inhibition of VEGF signaling or overexpression of ADAMTS1 both phenocopied the pax2a<sup>−/−</sup> hyaloid vasculature and coloboma phenotypes. This has led us to propose a novel pathway for the regulation of optic fissure fusion where pax2a mediates proper regulation of anti-angiogenic factors in order to facilitate hyaloid vasculature migration through the OF. Timely migration of hyaloid vasculature cells in turn signals to the retina rim cells, via F-actin, and initiates the fissure fusion machinery, in particular remodeling of the BM.
**RESULTS:**

Optic Fissure basement membrane remodeling is preceded by F-actin signaling.

Several recent studies have undertaken a detailed time course to map out the exact timing of optic fissure fusion in numerous species, including zebrafish (18, 19). Overall, in zebrafish, the data point to ~32-36hpf as the time of OF fusion initiation, observed by BM remodeling. To better understand the molecular mechanisms regulating OF fusion, we also performed a detailed time course analysis of OF BM remodeling, using laminin immunohistochemistry (IHC) as a read out of this process. We performed whole mount IHC for laminin deposition in embryos starting at 24hpf and sampling every 4 hours up to 48hpf and then at 56 and 72hpf (Fig 1A). To analyze the progression of the fusion process, we quantified the laminin signal within the OF from 3D z-stacks of confocal scans. Regions of the BM outside the OF and juxtaposed to the developing lens were used to normalize the laminin signal (Fig S1A). In particular we quantified laminin signal in the central proximal region of the fissure (Fig S1B). This region has been shown to represent the site of fusion initiation. In agreement with recent studies in zebrafish, we found that OF fusion initiates at ~32-36hpf and does so in a central region of the fissure, subsequently proceeding proximally and distally (Fig 1B). By 48hpf, we observed most of the laminin signal is removed from the OF, and by 72hpf, little to no laminin persists in the region (Fig 1A, B). In addition to examining the timing of OF BM remodeling, we also determined whether changes in F-actin dynamics correlate to the timing of fissure fusion. James et al, 2016 had recently suggested that infiltrating vasculature endothelial cells migrating through the fissure could be a source of signal for fissure fusion. In fact, they showed that vasculature cell interaction with the fissure results in an increase of cytoskeletal, F-actin, rearrangements (19). To track F-actin signaling during fusion we stained the laminin labeled embryos using phalloidin and quantified the signal within the fissure. To normalize staining intensity we used an interior region of the retina (Fig S1A). Our analysis revealed a spike in OF associated F-actin signal between 24-32hpf, preceding the time that BM remodeling is initiated (Fig 1A,B). We also noticed a second spike of F-actin activity around 44hpf, a time of active
fusion (Fig 1C). Interestingly, the observed spikes in F-actin activity correlate with the
timing of hyaloid vasculature migration (24-32hpf) through the fissure and the expected
timing of actin protrusions facilitating epithelial fusion (36-48hpf). When measuring
distance between the fusing lobes of the retina we observed apposition to complete
around 36-40hpf (Fig S2). Taken together, we concluded that both apposition and BM
remodeling are preceded by a spike in F-actin within the OF driving initiation of BM
remodeling and subsequently fusion.

Optic fissure fusion mechanics are altered and disrupted upon loss of Pax2a
function.

In order to test our theory that an F-actin signaling wave precedes and drives
subsequent OF fusion we compared our findings to an established model of coloboma,
the pax2a\textsuperscript{noi} line (28). Pax2 is a critical regulator of OF fusion in several model systems
and has been documented in human coloboma cases (31-33). The noi mutation is
predicted to result in a loss of Pax2a function due to a premature stop codon at position
198 (34). Originally characterized for their no-isthmus phenotype, pax2a\textsuperscript{noi/noi} embryos
(referred to as pax2a\textsuperscript{-/-} from this point on) elicit a fully penetrant unfused OF in addition
to severe heart defects. Unfortunately, homozygotes are not viable and do not enable
the study of coloboma at juvenile or adult stages. We hypothesized that examining the
molecular events leading to OF fusion in the pax2a\textsuperscript{noi} system would inform us whether
these events are functionally important. Similar to our WT study, we examined lobe
distance, in addition to laminin and F-actin levels throughout the time OF fusion (Fig 2A,
Fig S1C). In stark contrast to WT, pax2a\textsuperscript{-/-} embryos did not exhibit a significant decrease
in laminin signal between 32-48hpf, in fact, laminin appears to be largely retained in the
OF of pax2a\textsuperscript{-/-} embryos (Fig 2B). While there is moderate decrease in laminin signal in
pax2a\textsuperscript{-/-} embryos from 32-36hpf, this is well above the levels observed in WT. IHC
images clearly show that laminin persists in the fissure up to and including 72hpf (Fig
2A). A time when WT OF fusion is already completed and clearly absent of laminin
signal (Fig 1A). When examining F-actin levels in pax2a\textsuperscript{-/-} embryos we did not detect a
spike in activity between 24 and 32hpf. Also, F-actin levels fluctuate between 36 and
44hpf and finally drop to WT levels by 48hpf. As such, it appears the absence of F-actin
signaling at early timepoints, 24-32hpf, correlates with failure to initiate fusion. Lastly, when examining retinal lobe distance in pax2a<sup>−/−</sup> embryos we do not observe any significant defects in apposition (Fig S2). Taken together, we conclude that in the absence of pax2a the molecular mechanism regulating OF fusion, likely involving F-actin signaling, fails to initiate. This is apparent by the persistence of OF BMs. While neither F-actin signaling or BM remodeling is not initiated in pax2a<sup>−/−</sup> embryos, apposition of the lobes appears largely unaffected indicating that pax2a does not regulate morphogenetic movements of the retinal lobes. Based on these data we propose that the F-actin signaling spike we observe just prior to initiation of OF BM remodeling, in conjunction with pax2a activity, is critical for the completion of OF fusion.

**Pax2a retinal transcriptomic profile during optic fissure fusion.** Interestingly, although pax2a has been the subject of intensive research, there are currently no obvious transcriptional targets of pax2a that would directly point to mechanistic regulation of OF fusion. In an effort to understand the absence of OF BM remodeling and misregulation of F-actin activity observed in pax2a<sup>−/−</sup> embryos we compared retinal transcriptomic profiles between WT and pax2a<sup>−/−</sup> embryos. Whole retinas from 48hpf WT and pax2a<sup>−/−</sup> embryos were isolated and total RNA was subsequently purified and sequenced using Illumina sequencing. 48hpf was chosen for our experimental time point as pax2a<sup>−/−</sup> embryos are easily phenotyped at this age due to severe heart malformation. When comparing three replicates for WT and pax2a<sup>−/−</sup> embryos using RSEM software, we detected 1215 transcripts significantly upregulated (>95% confidence interval) and 1202 transcripts significantly downregulated (Fig 3A and 3D). Gene ontology analysis indicated a wide spread of biological function being affected, including cytoskeletal signaling, adhesion and developmental processes for both down and upregulated genes (Fig 3B, E). In fact, the distribution of biological function between up and downregulated genes was highly similar (Fig 3B, D). A list of top 20 up and down regulated genes is outlined in Figure 3C and F. Results of all the statistically significant up and downregulated genes identified in our assay are presented as supplementary data (Table 1).
From our analysis of downregulated targets, one, talin1 (tln1), stood out in particular. As outlined in the introduction, tln1 was recently shown to be involved in the migration of vasculature endothelial cells and subsequently fusion of the OF (19). As such, we next sought to investigate the relationship between pax2a, tln1, vasculogenesis and optic fissure fusion mechanics.

**Loss of pax2a function leads to a reduction of hyaloid vasculature migration into the optic fissure.**

Tln1 has recently been shown to play a role in optic fissure fusion and our discovery of reduced tln1 expression in pax2a<sup>−/−</sup> retinas engaged us to evaluate this connection further. To validate our RNAseq data, we performed wholemount *in situ* hybridization (WISH) for tln1 comparing WT siblings to pax2a<sup>−/−</sup> mutant embryos. WT expression of tln1 was observed in the optic fissure between 28 and 48hpf coinciding with pax2a expression (Fig 4A, Fig S3). In pax2a<sup>−/−</sup> embryos, OF tln1 expression appears significantly reduced compared to WT while retaining similar expression in periocular regions (Fig 4A). Tln1 expression is also reduced in the mid brain-hind brain boundary, another region of strong pax2a expression (Fig 4A, S3A). To determine whether the reduction of tln1 expression in pax2a<sup>−/−</sup> embryos had functional consequences on optic fissure fusion and hyaloid vasculogenesis we examined pax2a<sup>−/−</sup> embryos expressing mCherry in vasculature cell, Tg[kdrl:mCherry]. Using 3D *in vivo* time-lapse confocal microscopy we recorded migration of mCherry expressing cells through the OF from 24 to 30hpf (Fig 4B, Movie 1). Both WT and pax2a<sup>−/−</sup> embryos contain vasculature endothelial cells within the fissure at 24hpf, however, over the next 6 hours of imaging it becomes clear that a higher number of mCherry expressing cells migrate through the OF in WT than in pax2a<sup>−/−</sup> embryos (Fig 4B, Movie 1). To quantify this effect, we processed WT and pax2a<sup>−/−</sup> Tg[kdrl:mCherry] embryos at 24, 32, 36 and 48hpf, collected 3D confocal stacks and counted the number of mCherry positive cells found within the fissure (Fig 4C). The data clearly indicate that there is a significant reduction in the number of kdrl:mCherry cells found within the pax2a<sup>−/−</sup> optic fissure at both 32 and 36hpf (Fig 4D). Furthermore, using 3D rendering rotation, we observed that in 48hpf pax2a<sup>−/−</sup> embryos the hyaloid vasculature established in the back of the lens is
reduced in size and lacks the proper connections to the newly forming choroidal and superficial vasculature systems (Movie 2-3). Overall, our data indicate that loss of pax2a function leads to a decreased and impaired migration of hyaloid vasculature endothelial cells into the optic fissure. Ultimately leading to a disorganized and reduced hyaloid vasculature.

**Inhibition of VEGF signaling impair optic fissure fusion.**

Based on our discovery of reduced hyaloid vasculature in pax2a\(-/-\) embryos, we next sought to examine whether this phenomenon is associated with failure of optic fissure fusion. To do so, we turned our attention to vascular endothelial growth factor (VEGF) signaling. VEGF, the ligand for vascular endothelial growth factor receptor (VEGFR), is an angiogenic factor and the prime candidate for signaling the migration and proliferation of hyaloid vasculature endothelial cells in the optic fissure. To inhibit VEGF signaling, we took advantage of the dorsomorphin derivative DMH4, which has been shown to selectively inhibit VEGF signaling independent from BMP (35). Based on previously published working concentrations, we conducted a dose response to examine DHM4 effects on hyaloid vasculature formation using Tg[kdrl:mCherry] as a readout. Treatment of embryos from 12-24hpf, ranging from 1-100µM, resulted in a dose response of reduction of mCherry signal in the developing retina (Fig S4). We decided to use the 100µM concentration for subsequent experiments as this concentration was able to completely inhibit vascularization of the retina up to 72hpf (Fig 5A). Embryos were treated starting at 12hpf and examined for fissure fusion status via whole mount laminin IHC at 24, 32, 36, 48, 56 and 72hpf (Fig 5B). 3D confocal imaging revealed a persistence of laminin signal within the fissure up and including 72hpf (Fig 5B) upon DMH4 treatment. Measurement of the laminin signal over our time course generated very similar results to what we observed in pax2a\(-/-\) embryos, with laminin persisting within the OF at timepoints where WT embryos have completed remodeling (Fig 5C). In addition to laminin, we also quantified F-actin activity during the treatment (Fig 5B). Again, similar to pax2a\(-/-\) embryos we did not observe a spike in F-actin activity between 24-32hpf as observed in WT embryos (Fig 5D). Similar to pax2a\(-/-\) embryos, DMH4 treatment also did not affect the size of the retina nor the apposition of
retinal lobes (Fig S2). Based on these findings, we conclude that inhibition of VEGF signaling results in failure of basement membrane breakdown, presumably due to the absence of vasculature endothelial cell migration through the optic fissure. This is further supported by a lack of increased F-actin signaling during the time of vasculature migration through the OF. As such, our results give direct evidence for the necessity of vasculature endothelial cell migration through the OF in order to initiate BM remodeling and ultimately achieve fusion of the fissure.

**Pax2a negatively regulates ADAMTS1 expression to inhibit anti-angiogenesis.**

Having established a clear connection between pax2a function and proper recruitment of hyaloid vasculature endothelial cells into the optic fissure, we sought to examine the mechanisms behind this phenotype. Our transcriptomic comparison of WT and pax2a−/− eyes, with a focus on angiogenesis regulation, revealed misexpression of a disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1), a secreted protease known to be involved in regulating VEGF signaling (36). Being a member of the ADAMTS family, ADAMTS1 encodes three thrombospondin motifs enabling it to directly bind and sequester VEGF (30). Furthermore, the protease activity of ADAMTS1 has been shown to target thrombospondin (thsb) 1 and 2 for cleavage, ultimately liberating their active forms of which bind to and block VEGF from binding the VEGF receptor (29). In our transcriptomic comparison we found that ADAMTS1 expression was upregulated almost two-fold in pax2a−/− retinas. This would suggest that the expression of ADAMTS1, and therefore its anti-angiogenic activity, is normally somehow kept in check by pax2a. Pax2a has not been reported to harbor transcriptionally repressive function, so this may involve an indirect mechanism. To confirm our RNAseq results we performed WISH, examining ADAMTS1 expression at 28 and 36 hpf. In agreement with our transcriptomic analysis, we observed an upregulation in ADAMTS1 in eyes of pax2a−/− embryos, specifically in the region of the optic fissure (Fig 6A). To test whether upregulation of ADAMTS1 expression would have direct effects on hyaloid vasculature migration and optic fissure fusion we injected Tg[kdrl:mCherry] expressing embryos with 100pg ADAMTS1 mRNA. We subsequently examined hyaloid vasculature using 3D confocal imaging between 24-48hpf. Injection of
ADAMTS1 mRNA resulted in a significant decrease in the number of kdrl:mCherry cells found within the optic fissure (Fig 6B, Movie 5). Quantification of mCherry positive cells within the optic fissure confirmed this observation (Fig 6C). The reduction in the numbers of mCherry positive cells within the fissure was similar to what we observed in pax2a^-/- embryos (Fig 4, Movies 2, 5). This result indicated that the anti-angiogenic function of ADAMTS1 applies to regulating hyaloid vasculogenesis. In contrast to ADAMTS1 mRNA overexpression, we also examined the consequences of ADAMTS1 loss of function using morpholino-mediated transient knockdown of ADAMTS1. Tg[kdrl:mCherry] expressing embryos injected with ADAMTS1 morpholinos had a contrasting phenotype to that of pax2a^-/- or ADAMTS1 mRNA injected embryos. Morphant ocular vasculature appeared expanded and significantly disorganized, in particular within the dorsal regions (Fig 6D, Movie 6). Morphants did not display any distinct phenotypes in hyaloid vasculature of the optic fissure at 48hpf, however, we did note improper connections being formed as well as a disorganization of the dorsal vasculature network as compared to WT (Fig 6D and Movies 4,6). These findings suggest that while ADAMTS1 activity needs to be suppressed in the optic fissure, other regions of the retinal vasculature may rely on proper levels of ADAMTS1 to enact proper connections within the hyaloid/choroidal network.

Misregulation of ADAMTS1 expression leads to optic fissure fusion failure

Having mimicked the vasculature phenotype of pax2a mutants, we next examined whether overexpression of ADAMTS1 would also inhibit optic fissure fusion. To do so, we performed whole mount laminin and F-actin IHC in ADAMTS1 mRNA injected embryos at 24, 32, 36, 48 and 72hpf (Fig 7A). Confocal imaging of the fissure indicated that like the vasculature phenotype, ADAMTS1 mRNA injection also phenocopied pax2a^-/- associated persistence of laminin and therefore failure of optic fissure fusion (Fig 7B). Levels of laminin persisting at 48hpf was similar to pax2a^-/- embryos. As was observed with pax2a^-/-, timing for apposition of the lobes was not affected in ADAMTS1 overexpressing embryos. Furthermore, as documented for pax2a^-/- and DMH4 treated embryos, we again noted a lack of the F-actin signaling spike between 24-32hpf, and no significant difference to WT levels by 48hpf (Fig 7C).
Maintaining proper levels of ADAMTS1 expression is therefore necessary to ensure timely fusion of the fissure. Taken together, we envision that pax2a functions to restrict expression of ADAMTS1 within the optic fissure to ensure proper recruitment and migration of hyaloid vasculature endothelial cells. One established, the hyaloid vasculature in turn signals to the retinal rim cells to initiate BM remodeling and leads to subsequent OF fusion.

**DISCUSSION**

Pax2 is the best recognized regulator of optic fissure fusion with a direct connection to pediatric cases of coloboma. Interestingly, while the association of pax2 and coloboma is undoubted, little molecular detail exists of how pax2 actually confers this important function. At the same time, studies of optic fissure fusion dating back several decades have been suggesting a direct connection between the fusion process and migrating vasculature endothelial, or periocular mesenchyme, cells found within the fissure prior to and during fusion. A notion strongly supported by recent findings where reduction of hyaloid vasculature in the fissure, or removal of optic vesicles from sources of vasculature inhibits or significantly delays fusion (19, 23). In our study, we outline a novel connection between pax2a function and hyaloid vasculature-mediated initiation of optic fissure fusion, further strengthening the hypothesis that vasculature directly signals initiation of fissure fusion.

Several recent reports, including this one, have comprehensively characterized optic fissure fusion timing. Work from zebrafish, mice and chick all point to an orderly progression involving: **1)** retinal growth and cellular rearrangement leading to nasal and temporal retinal lobe apposition, **2)** invasion of the fissure by endothelial and neural crest cells forming the hyaloid vasculature system, **3)** cellular signaling, either between retinal rim cells or between rim cells and the migrating vasculature cells, likely in the form of cytoskeletal activation (f-actin), **4)** remodeling or removal of the basement membrane to enable physical connection of the rim cells and subsequent formation of a continuous retinal epithelial sheet via re-polarization and cell-cell adhesion. Step 1 has been nicely characterized in a few recent publications outlining the flow of retinal cells
and morphological formation of the fissure (37-39). For step 2, several reports have carefully characterized the formation of the hyaloid vasculature system, including migration of hyaloid vasculature precursor cells into the fissure as soon as it forms (1-3). Importantly, perturbation of this process, or removal of the developing eye from its source, has been shown to lead to fissure fusion failure (19, 23). To date, steps 3 and 4 are the least well understood. Several molecular components associated with cell-cell adhesion and epithelial sheet fusion have been associated with the fissure, including α-catenin, n-cadherin, and netrin (19, 40-43). However, the timing or the molecular mechanism organizing and regulating these components remain uncharacterized.

Furthermore, very little information exists pertaining to the identity of protease enzymes responsible for BM remodeling. Recent work from our lab has characterized the composition of core BM components within the fissure, but to date, only ADAM16 has been functionally examined in context of fissure fusion, while MMP2 was detected during fusion in mice and MMP23bb implicated from transcriptomic comparison of pre and post fusion fissure expression (42, 44, 45). Identifying proteases active in OF fusion continues to be an area of needed attention. In our current study, we attempted to address the questions of step 3 by concurrently analyzing f-actin and BM remodeling. In doing so we discovered that BM remodeling is preceded by an uptick in F-actin signaling. The timing of this coincides with the active migration of hyaloid vasculature precursors through the fissure. An event shown previously to involve in F-actin signaling within in the fissure (19). Furthermore, we show both genetically and pharmacologically that hyaloid vasculature precursors are necessary for OF fusion, and ultimately outline a mechanism where suppression of anti-angiogenic activity within the fissure is critical for OF fusion. These findings not only confirm a long-held hypothesis of hyaloid vasculature involvement in OF fusion, but also uncover previously unexplored mechanisms of angiogenesis playing a role in the OF fusion process.

Based on our IHC time course analysis of optic fissure fusion dynamics, in particular F-actin and laminin status, we propose that BM remodeling is preceded by an increase of F-actin activity. This likely stemming from the interaction of retinal and vasculature cells within the fissure (Fig 1). Interestingly, when examining this process in
the pax2a\textsuperscript{mut} mutant line we did not detect an increase of F-actin signaling at times preceding fusion. This coincided with a decrease in the number of vasculature cells found within the fissure and ultimately lead to failure of optic fissure basement membrane remodeling and lack of fusion. In an attempt to understand the molecular consequence driving these differences we uncovered misregulation of tln1 expression in the absence of pax2a function. Tln1 is a key regulator of endothelial cell migration recently shown to be directly involved in OF fusion (19). In fact, loss of tln1 function leads to a decrease in fissure associated vasculature and persistence of the OF BM. Our finding that pax2a regulates expression of tln1 has several compelling implications. Talin1 is known to be a direct link between the actin cytoskeleton and the BM via integrin (46, 47). Furthermore, tln1 has been associated with the formation of adhesion junctions and other cell-cell interaction processes (48, 49). One could therefore hypothesize several different models for its role in optic fissure fusion. Our, and previous analyses of tln1 expression do not directly indicate which cells within the fissure are expressing tln1. It could be the retinal lobe rim cells, the migrating vasculature cells or both. As such, we can envision that tln1 could function through differing mechanisms. If expressed solely in the retinal cells, then tln1 may function to act as a sensor for the apposition of retinal lobes, or for the presence, or absence, of vasculature endothelial cells. James et al. 2016 showed that vasculature cells within the fissure trigger F-actin accumulation and presumably cytoskeletal signaling, potentially facilitated by tln1. A feature that is missing in pax2a mutant fissures. Furthermore, tln1 may also act as a direct mechanism required for the formation of adhesions between the fusing lobes. However, as observed in both tln1 and pax2a mutants, the BM persists at times fusion should be occurring, suggesting that tln1 first plays a functional role in the initiation of BM remodeling, and it remains unclear whether its directly involved in cell-cell adhesion. Future work will focus on answering these questions. 

Our study clearly supports the notion that vasculature plays an integral part in OF fusion. As such, regulation of angiogenesis may also be part of the mechanism. Using a transcriptomic comparison of WT and pax2a mutants we did in fact discover a connection between angiogenesis and pax2a. In the absence of pax2a function we
observe an upregulation of ADAMTS1. Encoding 3 thrombospondin (thsb) motifs able to directly bind and sequester VEGF, in addition to targeting thsbn1 and 2 for cleavage into their VEGF inhibitory active forms, ADAMTS1 has been extensively studied anti-angiogenic factor in cancer, aortic and renal biology (50). In fact, we showed that upregulating ADAMTS1 expression partially inhibits hyaloid vasculature migration into the optic fissure, an outcome similarly observed in pax2anoi embryos. Up regulation of ADAMTS1 also prevented the F-actin spike and OF BM remodeling while having no effect on eye size or retinal lobe apposition. Along those same lines we also showed that optic fissure fusion and preceding F-actin signaling are abolished when pharmacologically inhibiting VEGF using DHM4. Interestingly, knockdown of ADAMTS1 using morpholino resulted in hyaloid vasculature disorganization, suggesting anti-angiogenesis may function to guide and/or shape the developing vasculature network during retinal development. Taken together, our study confirms an undeniable link between hyaloid vasculogenesis and optic fissure fusion. It also uncovers a molecular mechanism involving angiogenic regulation to ensure proper and timely establishment of hyaloid vasculature and subsequent OF fusion.
MATERIALS AND METHODS

Zebrafish and embryo maintenance

Zebrafish were maintained using husbandry procedures approved by University of Kentucky IACUC committee. Embryos were kept at 28.5°C in E3 embryo media. AB and TL strains were used as wild-type, Tg[kdrl:mCherry] transgenic line was used to visualize retinal vascularization (51).

Pax2<sup>noi</sup> embryos were a gift from Dr. Gregory-Evans. Genotyping analysis was conducted by amplifying the region of gDNA with the noi mutation using the forward primer: 5’- CTCGCTCTGCCTCCATGATTG3’- and the reverse: 5’-GGCACTGAAAGAGCACAGG -3’. The resultant 460bp amplicon was digested with TaqI (NEB) which would recognize and digest the WT allele sequence but not the mutant allele.

Immunohistochemistry (IHC)

Dechorionated embryos were fixed with 4% PFA in PBS at room temperature for 3h and washed with PBST 4 times for 5 minutes. Embryos were then permeabilized with Proteinase K, 30µg/mL 10 minutes for 24-28 hpf, 50µg/mL 15-20 minutes for 32-48 hpf and 75µg/mL 20 minutes for 56-72 hpf, washed 2 times in PBST for 5 minutes and blocked overnight at 4°C with 10% sheep serum, 0.8% Triton X-100 and 1% BSA in PBS. Primary mouse anti-laminin antibody (ThermoFisher – 1:100) in blocking buffer (1% sheep serum, 1% BSA and 0.8% Triton X-100 in PBS) were incubated overnight at 4°C and washed 5 times in PBST for 15 minutes. Secondary antibody, goat anti-rabbit (Alexa Fluor® 488 – Abcam – 1:1000), DAPI 1:1000, and phalloidin (Alexa Fluor® 555 - 1:50) were incubated overnight at 4°C in the dark. Tg[kdrl:mCherry] embryos were treated with proteinase K as described above and stained with DAPI 1:1000 overnight. The embryos were washed 2 times in PBST for 15 minutes and visualized using a Nikon C2+ confocal microscope equipped with a 40X (1.15NA) water immersion objective. Embryos were embedded in 1.2% low melting point agarose on glass bottom 35mm dishes (Fluorodish, World Precision Instruments). Images were captured in steps of 3.5 microns for a total of 31.5 microns using Nikon Elements software. Image
adjustment, such as cropping and brightness/contrast was performed using Adobe Photoshop.

Analysis of Fluorescence signal

Fiji software (https://fiji.sc) was used to measure the fluorescence intensity of laminin and phalloidin signal from raw image data. In order to account for variability in staining, normalization values were measured for laminin and actin pixel intensity where an area directly outside of the choroid fissure was measured and a ratio was generated between the two values (Fig S1B). For Tg[krdl:mCherry], 3D reconstructions of the optic fissure were generated and individual cells were counted (from the opening of the OF through the back of the lens).

Stats

Student’s t-test was used to compare individual time points. One-way ANOVA was used to analyze across treatments. Graphs are displayed as mean +/- standard deviation. Analysis was performed using Prism8 graphing software (GraphPad).

Total RNA Sequencing

WT and Pax2⁻/⁻ embryos were dissected and collected at 48 hpf and RNA was extracted from dissected eyes using trizol. Pax2a⁻/⁻ embryos were phenotyped by distinct heart defects only observed in pax2a⁻/⁻ embryos, as verified by previous genotyping experiments. Dissected eyes were suspended in 1 mL of trizol and sheared with a 22-gauge needle. Samples were incubated at room temperature for 5 minutes, then 200 μL of chloroform was added and vortexed for 1 minute. Samples were then centrifuged at 12,000 g for 15 minutes at 4°C. Aqueous phase was removed and put into fresh RNAse free tube. 3 μL of Glycobule was added to samples and vortexed for 5 seconds. 500 μL of 100% Isopropanol was then added and vortexed for an additional 10 seconds. Samples then were incubated at room temperature for 10 minutes. Samples were then centrifuged at 12,000 g for 10 minutes at 4°C. Supernatant was removed and 1 mL of 75% EtOH was added and vortexed for 10 seconds. Samples were then centrifuged at 7,500 g for 5 minutes at 4°C. Supernatant was removed and samples then
underwent a pulse centrifugation and any remaining supernatant was removed. Samples were then air-dried for 7 minutes at room temperature under a fume hood. 20 μL of RNAse free ddH₂O was added to the samples and mixed until pellet dissolved. Samples were finally incubated at 60°C for 12 minutes and then stored at -80°C.

The RNA then underwent a DNase treatment using the DNA-free Kit. 2 μL of 10X DNasel Buffer, and 1 μL of rDNasel was added to the entire 20 μL sample. Samples were incubated at 37°C for 20 minutes, and then 2 μL of DNase Inactivation Reagent was added. Samples were then incubated at room temperature for 2 minutes, mixing samples 3 times. Samples were centrifuged at 10,000 g for 1.5 minutes. Supernatant was then removed and placed into fresh RNase free tube and stored at -80°C.

Purified total RNA was sent to Applied Biological Materials Inc. for Illumina sequencing. Bioinformatic analyses were completed using RSEM. Those transcripts which had a greater than 0.95 posterior probability of being differentially expressed were considered significant. Panther (http://pantherdb.org) was used for gene ontology and pie chart generation.

Whole-mount in situ hybridization (WISH)

Whole-mount in situ hybridization was performed as previously described (52). RNA probes were generated using PCR with T7 promoter sequence and subsequently transcribed [DIG labeled] using T7 polymerase (Roche). Primer sequences were 5'-GAGGGCAAGAGAATTCAGTAC-3' and 5'-TAATACGACTCTAGGGCAGATTGCACAAAGGGTCAGAAG-3' for ADAMTS1, 5'-ATGGATATTCACTGCAAAGCAG-3' and 5'-TAATACGACTCTAGGGCTAGTGGGCTGCCGATAGGCAGTG-3' for pax2a, 5'-GCCACAGCTAAAGCAGTGGCTG-3' and 5'-TAATACGACTCTAGGGCTAGTGGGCTGCCGATAGGCAGTG-3' for talin 1. Images were captured using a Nikon Digital sight DS-Fi2 camera mounted on a Nikon SZM800 stereo scope using Elements software. Dissected eyes from 24, to 72hpf embryos were mounted in 70% glycerol and imaged under DIC using a Nikon TiE compound microscope equipped with a 20X (0.7NA) objective and Elements software. Image
adjustment, such as cropping and brightness/contrast was performed using Adobe Photoshop.

Morpholino Injections
Translation blocking Morpholino was obtained from Gene Tools, LLC and used to knock down the expression of ADAMTS1. The morpholino used in this study was:

Control MO: 5’-CCTCTTACCTCAGTTACAATTTATA-3’, Adamts1: 5’-AGACATTATATTCCTGTCAAGTTCT-3’. 6ng of of ADAMTS1 or control morpholino was injected into 1-2 cell stage embryos.

Live imaging analysis
Live imaging of Tg[kdrl:mCherry] embryos was conducted using a Nikon C2+ confocal microscope equipped with at 20X (0.95NA) water immersion objective. 22 hpf embryos were imbedded in 1.1% low gelling agarose in 1-inch glass bottomed Flourodish cell culture dishes (World Precision Instruments) and covered in embryo media, 3-amino benzoic acid ethylester (tricaine) to anaesthetize the embryos and 1-phenyl 2-thiourea (PTU) to inhibit pigmentation. Z-stacks 75μm thick with a step size of 2.5μm were captured over the course of 6 hours at 10 minute intervals. The time lapse data were reconstructed in 3D using Elements software. Image adjustment, such as cropping and brightness/contrast was performed using Adobe Photoshop. After imaging, embryos were removed and genotyped.

DMH-4 treatment
Embryos were incubated in embryo media with 5, 25, 50, or 100 μM of DMH-4 (Sigma Life Science) in DMSO starting at 12hpf. Fresh DHM4 containing media was added every 12hpf for timepoints past 24hpf.

Cloning and mRNA synthesis.
Full coding domain sequences for ADAMTS1 (NCBI reference sequence XM_021475923.1), Pax2a (Ensembl transcript ID: ENSDARG00000028148) were amplified from 24hpf zebrafish cDNA using primers 5’-
CATGGATCCATGGATATTCACTGCAAAGCA-3' and 5'
ATGCTCGAGCTAGTGCGTCATAGGCAGTG-3' for pax2a, 5'
GCGATCGCATGTCTTTTTGCGTGTGGT-3' and 5'
CTCGAGTCAACAGGGAGTCAGATTG-3' for ADAMTS1, cloned into pGEMT
(Promega), digested with AsiSI/XhoI, BamHI/XhoI respectively and subsequently cloned
into pCS2+. All constructs were verified by sanger sequencing (Eurofinsgenomics).
mRNA was synthesized from linearized pCS2 constructs using SP6 mMessage
mMachine kit (Ambion) and purified using YM-50 Microcon columns (Amicon, Millipore).

**Ethics Statement**
The use of zebrafish in this study was approved by the University of Kentucky IACUC
committee, Institutional PHS Assurance #D16-00217 (A3336-01) with a protocol
number: 2015-1370.

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**FIGURE LEGENDS**

**Figure 1:** An increase in F-actin dynamics precedes laminin remodeling during optic fissure fusion.

A) Whole mount Immunohistochemistry was used to simultaneously visualize F-actin (red) and laminin (green) during optic fissure fusion, 24-72hpf. Central-proximal sections obtained using confocal imaging were collected and quantified. Scale bar = 50um. B) Quantification of laminin signal intensity within the optic fissure, normalized to regions of laminin staining juxtaposed to the lens. Relative pixel intensities are displayed. A green dotted line depicts the trend in laminin intensity over time. ANOVA p<0.0001. C) Quantification of F-actin signal intensity (phalloidin staining) within the optic fissure, normalized to regions of F-actin signal within the lobe of the retina. Relative pixel intensities are displayed. A red dotted line represents the trend in F-actin intensity over time. ANOVA p<0.0001.

**Figure 2:** Pax2 mutant embryos lack F-actin activity increase and laminin remodeling within the optic fissure.

A) Whole mount Immunohistochemistry was used to simultaneously visualize F-actin (red) and laminin (green) during optic fissure fusion, 24-72hpf, in pax2^-/- embryos. Central-proximal sections obtained using confocal imaging were collected and quantified. Scale bar = 50um B) Quantification of laminin signal intensity within the optic fissure, normalized to regions of laminin staining juxtaposed to the lens. Relative pixel intensities are displayed. A green dotted line depicts the trend in laminin intensity over time. Blue dotted line represents the trend observed in wildtype embryos. ANOVA p<0.0001. C) Quantification of F-actin signal intensity (phalloidin staining) within the optic fissure, normalized to regions of F-actin signal within the lobe of the retina. Relative pixel intensities are displayed. A red dotted line represents the trend in F-actin intensity over time. Blue dotted line represents the trend observed in wildtype embryos. ANOVA p<0.0001.
Figure 3: Retinal transcriptomic comparison between WT and pax2a<sup>−/−</sup>.  
A) Heat map representing transcripts found to be significantly down regulated in pax2a<sup>−/−</sup> embryos.  
B) Gene ontology for pax2a<sup>−/−</sup> downregulated transcripts.  
C) Top 20 downregulated transcripts are outlined in order of fold change.  
D) Heat map representing transcripts found to be significantly upregulated in pax2a<sup>−/−</sup> embryos.  
E) Gene ontology for pax2a<sup>−/−</sup> upregulated transcripts.  
F) Top 20 upregulated transcripts are outlined in order of fold change.

Figure 4: Pax2a is necessary for tln1 expression and recruitment of vasculature into the optic fissure.  
A) Wholemount in situ hybridization comparing tln1 expression between WT and pax2a<sup>−/−</sup> embryos at 28 and 32hpf. Tln1 signal within the optic fissure (yellow arrowhead) is clearly reduced in Pax2a<sup>−/−</sup> embryos (red *).  
B) In vivo 4D confocal imaging of Tg[kdrl:mCherry] WT or pax2a<sup>−/−</sup> embryos. Time lapse series depicting the region of the optic fissure (dotted white lines) and mCherry positive vasculature endothelial cells migrating through the fissure. Compared to WT, pax2a<sup>−/−</sup> embryos display a reduction in the number of vasculature cells migrating in the fissure from 24-29hpf. Scale bar = 10um.  
C) Comparison of WT and pax2a<sup>−/−</sup> vascularization during optic fissure fusion, 24-48hpf. 3D reconstructions of whole mount Tg[kdrl:mCherry] (red) WT or pax2a<sup>−/−</sup> embryos with DNA stained by DAPI (blue). Scale bar = 50um.  
D) Quantification of mCherry positive cells from 3D confocal stacks within the region of the optic fissure. Individual embryo results are depicted. ANOVA p<0.0001.

Figure 5: Inhibiting angiogenesis prevents optic fissure fusion.  
A) 3D confocal imaging of Tg[kdrl:mCherry] (red) embryos treated with DMH4 at 24-72hpf. DNA was stained with DAPI (blue). Scale bar = 50um. DMH4 treatment restricts the migration of vasculature endothelial cells into the fissure and retina.  
B) Whole mount Immunohistochemistry was used to simultaneously visualize F-actin (red) and laminin (green) in DMH4 treated embryos during the time of optic fissure fusion, 24-72hpf. Central-proximal sections obtained using confocal imaging were collected and quantified. Treatment with DMH4 inhibits laminin removal and results in optic fissure
fusion failure. Scale bar = 50um. C) Quantification of laminin signal intensity within the optic fissure, normalized to regions of laminin staining juxtaposed to the lens. Relative pixel intensities are displayed. A green dotted line depicts the trend in laminin intensity over time, a blue dotted line represents the WT trend. ANOVA p<0.0001. D) Quantification of F-actin signal intensity (phalloidin staining) within the optic fissure, normalized to regions of F-actin signal within the lobe of the retina. Relative pixel intensities are displayed. A red dotted line represents the trend in F-actin intensity over time, a blue dotted line represents the WT trend. ANOVA p<0.0001.

**Figure 6: ADAMTS1 inhibits retinal vascularization.**
A) Wholemount in situ hybridization comparing ADAMTS1 expression between WT and *pax2a*-/- embryos at 28 and 32hpf. ADAMTS1 signal within the optic fissure (yellow arrowhead) is clearly increased in Pax2a/-/- embryos (red *). B) Comparison of WT and ADAMTS1 mRNA injected embryo vascularization during optic fissure fusion, 24-48hpf. 3D reconstructions of whole mount Tg[kdrl:mCherry] (red) WT or ADAMTS1 mRNA injected embryos with DNA stained by DAPI (blue). Scale bar = 50um. C) Quantification of mCherry positive cells from 3D confocal stacks within the region of the optic fissure. Individual embryo results are depicted. * p<0.001, ANOVA p<0.0001. D) 3D reconstruction of Tg[kdrl:mCherry] (red) WT or ADAMTS1 MO injected embryos at 48hpf. DNA is stained with DAPI (blue). Inhibiting ADAMTS1 function leads to significant disorganization of the dorsal vasculature (yellow *) as well as misconnections within the vascular network (yellow arrowhead). Scale bar = 50um.

**Figure 7: ADAMTS1 misregulation leads to failure of optic fissure fusion.**
A) Whole mount Immunohistochemistry was used to simultaneously visualize F-actin (red) and laminin (green) during optic fissure fusion, 24-72hpf in ADAMTS1 mRNA injected embryos. Central-proximal sections obtained using confocal imaging were collected and quantified. Scale bar = 50um. B) Quantification of laminin signal intensity within the optic fissure, normalized to regions of laminin staining juxtaposed to the lens. Relative pixel intensities are displayed. A green dotted line depicts the trend in laminin intensity over time, blue line depicts WT trends. ANOVA p<0.0001. C) Quantification of
F-actin signal intensity (phalloidin staining) within the optic fissure, normalized to regions of F-actin signal within the lobe of the retina. Relative pixel intensities are displayed. A red dotted line represents the trend in F-actin intensity over time, blue line represents WT trends. ANOVA p<0.0001.
Supplemental Material

Figure S1: Optic Fissure quantification strategy
A) Graphical representation of the region of OF analyzed using confocal microscopy. B) Sample image depicting regions of the OF (1), laminin normalization (2) and f-actin normalization (3) used for signal intensity quantification.

Figure S2: Optic Fissure apposition measurements.
Measurements of the distance between retinal lobes (apposition) in WT, pax2a^-/-, DMH4 treated or ADAMTS1 mRNA injected embryos at 48hpf. Measurements were made using laminin staining as reference for edges of retinal lobes. Distance was measured as pixels.

Figure S3: pax2a and tln1 expression during development
A) Whole mount in situ hybridization of pax2a probe at 24, 48, 54 and 72hpf. Lateral (top) and ventral (bottom) images depicting optic fissure expression are shown. Pax2a expression persists in the fissure up to 54hpf. B) Whole mount in situ hybridization of tln1 probe at 24, 48, 54 and 72hpf. Lateral (top) and ventral (bottom) images depicting optic fissure expression are shown. Tln1 expression is detected in the optic fissure from 24-48hpf (yellow arrowheads).

Figure S4: DMH4 dose response
3D reconstructions of 48hpf whole mount Tg[kdrl:mCherry] (red) embryos treated with 1, 5, 50 or 100uM DMH4. DNA stained with DAPI (blue). Increasing concentration of DMH4 eliminates mCherry expressing cells from the optic fissure and retina. Scale bar = 50um.

Movie 1: WT Tg[kdrl:mCherry] migration within the optic fissure, 24-30hpf

Movie 2: pax2a^-/- Tg[kdrl:mCherry] migration within the optic fissure, 24-30hpf
Movie 3: 3D rotation of WT Tg[kdrl:mCherry] embryo at 48hpf

Movie 4: 3D rotation of pax2a^-/- Tg[kdrl:mCherry] embryo at 48hpf

Movie 5: 3D rotation of 48hpf Tg[kdrl:mCherry] embryo injected with ADAMTS1 mRNA

Movie 6: 3D rotation of 48hpf Tg[kdrl:mCherry] embryo injected with ADAMTS1 morpholino

Table 1: pax2^-/- 48hpf retinal expression changes.
Figure 2

A

| Time  | laminin | F-actin | merge |
|-------|---------|---------|-------|
| 32 hpf | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| 36 hpf | ![Image](image4) | ![Image](image5) | ![Image](image6) |
| 44 hpf | ![Image](image7) | ![Image](image8) | ![Image](image9) |
| 48 hpf | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| 72 hpf | ![Image](image13) | ![Image](image14) | ![Image](image15) |

pax2a−/−  WT

B

**Pax2a−/− Optic Fissure Laminin Intensity**

![Graph](image16)

C

**Pax2a−/− Optic Fissure F-actin Intensity**

![Graph](image17)
Figure 7

A

ADAMTS1 mRNA:

laminin

F-actin

merge

Control

24 hpf  32 hpf  36 hpf  48 hpf

B

ADAMTS1 overexpression
Laminin Intensity

C

ADAMTS1 overexpression
F-actin Intensity
Supplementary Figure 1

A

distal

nasal ←→ temporal

proximal

central

B

1: Optic Fissure Signal

2: Laminin signal normalization

3: Phalloidin (F-actin) signal normalization
Supplementary figure 2

Optic Fissure Apposition

Distance between lobes

WT  pax2^−/−  DMH4  ADAMTS1 mRNA

ns  ns  ns
Supplementary Figure 3

A

24 hpf  48 hpf  54 hpf  72 hpf

pax2a

B

24 hpf  28 hpf  32 hpf  48 hpf  54 hpf  72 hpf

tl1n1
Supplementary Figure 4

|           | DMH-4 |
|-----------|-------|
| kdr1:mCherry |       |
| 1 uM      | ![Image](image1) |
| 5 uM      | ![Image](image2) |
| 50 uM     | ![Image](image3) |
| 100 uM    | ![Image](image4) |

| kdr1:mCherry | DAPI |
|--------------|------|
| ![Image](image5) | ![Image](image6) |
| ![Image](image7) | ![Image](image8) |
| ![Image](image9) | ![Image](image10) |

48hpf