Title
Identification of ubiad1 as a gene involved in cardiovascular homeostasis and development

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Publication Date
2011

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Identification of *ubiad1* as a gene involved in cardiovascular homeostasis and development

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Jeffrey Miles Hegarty

Committee in charge:

Professor Neil Chi, Chair
Professor Deborah Yelon, Co-Chair
Professor David Traver

2011

The Thesis of Jeffrey Miles Hegarty is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

_____
Chair

University of California, San Diego

2011

I dedicate this thesis to my Mom and Dad, and Colleen, for their motivation and support.

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ACKNOWLEDGEMENTS

I would first like to thank Dr. Neil Chi for allowing me to work in his lab and also for his hard work mentoring me. Neil's passion for science and drive to succeed has made me a better scientist.

Next I would like to thank everyone from the Chi Lab for your help and advice. In particular, I want to thank Dr. Hongbo Yang, Dr. Ruilin Zhang, Dr. Kim Ly, Diana Lopez, and Neil Tedeschi for contributing to my success while I worked towards my Master degree. Also, I would also like to thank Michael Dickover for giving me zebrafish embryonic heart cDNA and Jonathan Grinstein for contributing human fetal heart and HUVEC cDNA.

In addition, I would like to thank Dr. David Traver and Dr. Deborah Yelon for participating in my thesis defense, and also for allowing me to use their confocal microscope.

Lastly, I would like to thank Colleen Shannon for pushing me to join the BS/MS graduate program, without your love and encouragement this project would not be possible.

ABSTRACT OF THE THESIS

Identification of *ubiad1* as a gene involved in cardiovascular homeostasis and development

by

Jeffrey Miles Hegarty

Master of Science in Biology

University of California, San Diego, 2011

Professor Neil Chi, Chair

Professor Deborah Yelon, Co-Chair

Cardiovascular disease is a major cause of morbidity and mortality throughout the world. However, many of the genetic factors rendering humans susceptible to these diseases are still largely unknown. In order to identify novel genes related to vertebrate cardiovascular development and disease, many forward genetic screens have been carried out utilizing zebrafish as a model organism. In 2007, a screen for

vascular integrity defects identified a cerebrovascular hemorrhage mutant named *reddish*^{s587}(Jin *et al*., 2007). Using confocal microscopy we determined that the *reddish* mutant's hemorrhaging was likely caused by cerebrovascular dysgenesis and regression. Furthermore, we discovered that *reddish* mutants exhibit a previously unreported cardiac phenotype, characterized by ventricular failure by 72 hours post fertilization. Using positional cloning, we identified the *reddish* defect as a T-to-A missense mutation in *ubiad1*. Although heterozygous mutations in *UBIAD1* have been discovered, this is the first report showing the implications of homozygous mutations. Moreover, the cardiovascular phenotype in the *reddish* mutants provides the first evidence of *ubiad1*s role in the heart and vascular system. Lastly, the expression of *UBIAD1* in both embryonic zebrafish hearts and human fetal heart, could suggest that UBIAD1 has a conserved cardiac function amongst vertebrates. Although the molecular role for UBIAD1 in the cardiovascular system has not been established, this study demonstrates that UBIAD1 is necessary for its proper functioning.

I.

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in many nations, and contributes to over 700,000 deaths annually in the United States alone (Xu J. *et al., 2007)*. Nevertheless, the exact mechanism of many of these diseases is largely unknown. One interesting class of molecules that has been implicated in cardiovascular disease is quinone derivatives. These molecules have diverse functions, for example ubiquinone is known to participate in cardiac bioenergetics (Kumar *et al., 2009*), and both Menaquinone-4(vitamin K2) and phylloquinone (vitamin K1) are required for endothelial homeostasis (Burstyn-Cohen *et al.,* 2009). Consequently, defects in ubiquinone and vitamin K biosynthesis results in cardiomyopathy and vascular hemorrhage, respectively. Nevertheless, it is currently unknown whether the enzymes that synthesize these quinone derivatives have additional functions involved in the cardiovascular system. In this thesis we will discuss the known functions of UBIAD1, the enzyme responsible for Menaquinone-4 biosynthesis, and its role in cardiovascular homeostasis. In addition, we will address the potential role of UBIAD1 in the heart.

UBIAD1

The main findings of this thesis are that homozygous mutations in the N-terminus of the zebrafish *ubiA prenyltransferase domain containing 1* gene (*ubiad1)* lead to vascular hemorrhage and cardiac failure. Interestingly*, UBIAD1* has recently been implicated in several human diseases such as Schnyder Corneal Dystrophy (SCD), and transitional cell carcinoma (TCC) of the bladder. SCD is characterized by an abnormal accumulation of unesterified cholesterol on the cornea that typically leads

to blindness (Rodrigues *et al.,* 1987), whereas TCC is a cancer arising from the transitional epithilium that lines organs such as the bladder (McGarvey *et al*., 2001). Although no mutations have been identified in TCC patients, UBIAD1 protein levels are significantly reduced in TCC (McGarvey *et al*., 2001). Alternatively, mutations in *UBIAD1* are known to cause SCD (Weiss *et al.*, 2007). However, the mechanism by which UBIAD1 is down-regulated in TCC as well as how *UBIAD1* mutations contribute to SCD are both still unknown. Nevertheless, a recent study presenting UBIAD1 as the human enzyme responsible for Menaquinone-4 (MK-4) biosynthesis could suggest SCD is caused by corneal deficiency of MK-4 (Nakagawa *et al.*, 2010). Conversely, data showing differing distributions of *Ubiad1* and MK-4 in certain mouse tissues (Nakagawa *et al.*, 2010) could also propose an unidentified function of UBIAD1 that is altered in SCD patients. Since all of the patients discovered with SCD are heterozygous for missense mutations in *UBIAD1*, the complete loss of UBIAD1 function on the cornea or other tissues has not yet been elucidated. Determining the role of UBIAD1 in non-pathological conditions is required to fully understand the etiology of SCD and would allow the identification of other diseases associated with UBIAD1 defects. Furthermore, the implications of mutations in *UBIAD1* on the vitamin K cycle, and its relevance to human disease, still needs to be examined.

The vitamin K cycle

Vitamin K is an important co-factor for γ-glutamyl carboxylase (GGCX), an enzyme that catalyzes the posttranslational carboxylation of specific glutamine

residues on vitamin K-dependent proteins. Additionally, vitamin K has recently been reported to possess biological functions outside of its canonical co-factor role, such as prevention of oxidative cell death in oligodendrocytes (Li *et al.,* 2009) and involvement in the synthesis of sphingolipids as well (Sundaram *et al.,* 1988). In humans, two types of vitamin K are utilized: menaquinones (MKs), which are synthesized by a wide variety of organisms, including humans, and the plant form known as phylloquinone (PK). Both MKs and PKs are 2-methyl-1, 4-naphthoquinone derivatives and differ in their isoprenoid side chains at position 3. MK nomenclature is based on the number of prenyl units attached to its isoprenoid side chains, and in vertebrates, such as humans, MK is synthesized with 4 prenyl units, and thus named MK-4. In humans, vitamin K is primarily acquired as PK from dietary sources such as leafy green vegetables, and then converted to MK-4 in specific tissues by the enzyme UBIAD1 (Nakagawa *et al.*, 2010). Although PK can be converted to MK-4 in most tissues, studies have shown that MK-4 is abundant in all organs with the exception of the liver and heart (Thijssen *et al.,* 1995). Conversely, PK levels are highest in the hepatic and cardiac tissues, and relatively low in other organs (Thijssen *et al.,* 1995). Although PK accumulates in the heart, the absence of PK-epoxide shows that the heart does not use PK as a cofactor for GGCX (Thijssen *et al.,* 1996). Nevertheless, the differential tissue distribution of PK and MK-4 could indicate that organs such as the liver preferentially use PK as a co-factor and other organs, MK-4. This notion is supported by studies demonstrating that only the liver accumulates PK-epoxide and other organs such as the pancreas and submaxillary gland have increased MK-4-

epoxide levels (Thijssen *et al.,* 1995). Further studies are needed to identify the role of MK-4 in GGCX carboxylation and the non-hepatic proteins requiring this posttranslational modification.

The role of vitamin K has been most extensively studied in the context of coagulation. Many vitamin K-dependent proteins are involved in blood coagulation, such as prothrombin, factor VII, factor IX, factor X, protein C, protein S, and Protein Z (Stafford 2005), all of which help maintain haemostasis as well as prevent aberrant bleeding. In order for GGCX to carboxylate its targets, it requires a reduced form of vitamin K known as vitamin K hydroquinone (KH_2). During the carboxylation reaction, KH_2 is converted to vitamin K epoxide (KO), which is no longer able to serve as a cofactor for GGCX. Following GGCX mediated carboxylation, many organisms have developed a system that allows KO to be rapidly converted back to KH_2, which can once again function as a cofactor for GGCX. The enzyme that reduces KO back to vitamin K is known as vitamin K epoxide reductase (VKOR). Inhibition of VKOR by Warfarin, a commonly prescribed anticoagulant, is recognized to cause hemorrhaging in many organisms including humans and zebrafish (Hanumanthaiah *et al*., 2001), likely due to the defects in vitamin K-dependent coagulation protein function. Although Warfarin is known to affect the carboxylation of vitamin K-dependent proteins in the liver, the consequence of VKOR inhibition in non-hepatic tissues has not been significantly researched; thus, non-hepatic vitamin K-dependent proteins might also contribute to the bleeding phenotype seen during Warfarin treatment. Following KO reduction to vitamin K, VKOR has also been shown to

reduce vitamin K to KH_2 (Jin *et al.,* 2007); however, studies show that complete VKOR inhibition by Warfarin does not fully inhibit the reduction of vitamin K to KH_2 suggesting that another Warfarin-resistant enzyme must be the primary contributor to this reaction (Tie *et al.*, 2011). This cycle in which vitamin K is reduced, oxidized by GGCX, and then reduced back to vitamin K by VKOR is generally referred to as the vitamin K cycle.

Although the role of vitamin K in the liver has been well established, the function of vitamin K in the heart is largely unknown. Interestingly, research has shown that the heart contains very low levels of both MK-4 epoxide (Nakagawa *et al.*, 2010) and PK-epoxide (Thijssen *et al.,* 1996), indicating that the heart does not use vitamin K as a cofactor for gamma carboxylation. Nevertheless, cardiac PK levels are nearly as high as the liver, suggesting an alternative role for vitamin K in the heart (Thijssen *et al.,* 1995). Intriguingly, UBIAD1, the enzyme responsible for MK-4 biosynthesis, and PK, which is converted to MK-4 by UBIAD1, both have the highest levels in the heart; thus, it would be expected that the heart would have the highest concentration of MK-4. However, numerous studies have established that the heart has one of the lowest concentrations of MK-4 (Thijssen *et al.,* 1996; Nakagawa *et al.*, 2010), suggesting that UBIAD1 has an alternative role in the heart not related to MK-4 biosynthesis. Since UBIAD1 appears to have a role in the heart and is linked to MK-4 production in many other tissues, it is possible that defects in this gene might contribute to both heart disease and conditions related to vitamin K-deficiency, such as

spontaneous hemorrhaging. Thus, further studies are required to elucidate the impact of UBIAD1 defects and their relation to human disease.

Ubiquinone

Ubiquinone is an essential component of the electron transport chain and is required for aerobic respiration. Deficiencies in ubiquinone production have been shown to cause cardiomyopathy, retinitis pigmentosa, and other conditions in human patients (Rötig *et al.*, 2000). In addition, defects in genes involved in the ubiquinone biosynthesis pathway are associated with mitochondrial defects affecting bioenergetics (López-Martín *et al.*, 2007). Although the production of ubiquinone varies between prokaryotes and eukaryotes, several enzymes discovered share homology. For example, the prokaryotic ubiA is a prenyltransferase that converts 4-hydrobenzoic acid and geranyl diphosphate into 3-geranyl-4-hydroxybenzoate, a molecule required for the synthesis of ubiquinone. This same reaction is carried out by coq2, a ubiA homologue in yeast, and COQ2 in humans (Forsgren *et al.*, 2004). The functional similarity between COQ2 in humans and coq2 in yeast was determined by functional complementation. The results of this study demonstrated that human COQ2 could rescue yeast coq2 null mutants, albeit at a lower efficacy than wildtype yeast coq2 (Forsgren *et al.*, 2004). The reason for this decreased efficacy could indicate that human COQ2 does not have the same capacity to generate 3-geranyl-4-hydroxybenzoate as its yeast homologue. Thus, humans might require additional enzymes in order to adequately produce ubiquinone. Interestingly, UBIAD1 shares homology with ubiA, which could mean UBIAD1 participates in the same reaction as

COQ2 during ubiquinone biosynthesis. Nevertheless, symptoms of ubiquinone deficiency have not been linked to UBIAD1, and whether or not UBIAD1 has functions outside of MK-4 biosynthesis has not been determined.

Although studies have shown COQ2 has similar functions to yeast coq2, the expression patterns of *COQ2* and the distribution of ubiquinone indicate that another enzyme might contribute to ubiquinone production. The expression of *COQ2* has been reported to be highest in skeletal muscle with moderately high expression in other tissues (Forsgren *et al.*, 2004). In fact, the expression of *COQ2* in skeletal muscle was more than double that of all tissues examined (Forsgren *et al.*, 2004). Interestingly, amounts of ubiquinone are much higher in organs such as the liver and the heart, with cardiac ubiquinone levels reported as three times greater than that found in skeletal muscle (Åberg *et al.*, 1992). How the heart is able to produce so much more ubiquinone without an increase in *COQ2* has not been determined. One possibility is that in these organs with higher energy requirements, another enzyme may contribute to the same reaction as COQ2. Additionally, organs such as the heart might absorb dietary ubiquinone more efficiently than other organs. Nonetheless, the existence of another enzyme, carrying out similar functions as COQ2, is supported by the identification of two human siblings with homozygous missense mutations in *COQ2*, which render their COQ2 defective (López-Martín *et al.*, 2007). When COQ2 from these individuals was used to rescue yeast coq2 null mutants, growth was significantly reduced in comparison to rescue with wildtpye human COQ2 (López-Martín *et al.*, 2007). The fact that these individuals were viable beyond birth without

supplementation of ubiquinone suggests another enzyme was compensating for the COQ2 deficiency. Although it is probable that the *COQ2* missense mutation resulted in partial function of COQ2, it is unlikely that tissues such as the heart and liver, which require very large amounts of ubiquinone, were adequately supplied for survival by COQ2 alone. The possibility that UBIAD1 is the enzyme contributing to this increase in cardiac and hepatic ubiquinone is further supported by protein docking models which demonstrated 4-hydrobenzoic acid could fit into the active site of human UBIAD1 (Nickerson *et al.*, 2010). Furthermore, mouse *Ubiad1* is most highly expressed in the heart, with moderately high expression in the liver; however, MK-4 biosynthesis is very low or absent in both organs (Nakagawa *et al.*, 2010). Thus, it is likely that Ubiad1 plays an important role outside of MK-4 biosynthesis in mice. Further studies are warranted to determine the function of UBIAD1 in human heart and liver, and also whether COQ2 is the only enzyme responsible for 3-geranyl-4-hydroxybenzoate production.

Using zebrafish to study human development and disease

The use of model organisms to replicate human diseases has been widely used throughout the scientific community. Several studies have shown that mammalian cardiovascular development and haemostasis share many evolutionarily conserved mechanisms among vertebrates. One emerging model organism, the zebrafish (*Danio rerio)*, shares a similar cardiovascular development with mammals. Surprisingly, zebrafish share many conserved cardiac structures with mammals, including a ventricle, atria, heart valves, and cardiac conduction system (Stainier *et al.,*

2001; Walsh *et al.,* 2001; and Sedmera *et al.,* 2002). Additionally, conserved genetic pathways regulate cardiac size and shape in vertebrates (Auman H. J. and Yelon D. 2004). Similarly, the vascular system is patterned and maintained using similar genetic pathways as mammals (Ungos *et al.,* 2007), and during the establishment of both zebrafish and mammalian cardiovascular systems, aberrant bleeding is prevented by the process known as haemostasis (Jagadeeswaran *et al.,* 2007). Studies have confirmed that haemostasis is affected in both mammals and zebrafish by the anticoagulant Warfarin, and they require the activity of vitamin K-dependent gamma carboxylase to prevent hemorrhage (Hanumanthaiah *et al.,* 2001). The remarkable conservation in many of the processes directing cardiovascular development and haemostasis exemplify zebrafish as practical models for the study of human disease and development.

Zebrafish have many advantages compared to other vertebrate model organisms. For example, zebrafish are externally fertilized, allowing observation and manipulations to be made outside of the mother. Conversely, mouse embryos are internally fertilized and develop within the mother during embryonic development, making manipulations and observations very difficult. Furthermore, zebrafish embryos are optically clear, allowing the heart and vasculature to be easily visualized with reporter lines. Using tissue specific promoters, fluorescent proteins can be used to visualize, in real time, events such as the assembly of blood vessels or the migration of cardiac progenitors (Fujita *et al.,* 2011 and Holtzman *et al.,* 2007). Additionally, fate mapping can be carried out, to identify distinct cardiac progenitor populations,

such as those for the atrium and ventricle (Schoenebeck and Yelon 2007). Similar experiments in mice are technically difficult, and *in vivo* observations are nearly impossible. Another advantage of zebrafish is that they develop very quickly, and the majority of the zebrafish cardiovascular system is established by 72 hours post-fertilization (hpf), with a functional heart pumping blood through a vascular system as early as 24hpf (Stainier *et al.,* 1993). Because of the relatively small size of zebrafish embryos, a functional cardiovascular system is not required during the first several days following fertilization. Thus, severe cardiac and vascular defects, which would likely kill mammalian or avian model organisms, can be monitored for extended periods of time. One of the most important features of zebrafish is their amenability to high throughput forward genetic screens. For example, in 2007, a forward genetic screen was carried out using the mutagen ethylnitrosourea (ENU), which causes point mutation in premeiotic germ cells of treated zebrafish. Subsequent work isolated 30 unique vascular integrity mutants (Jin *et al*., 2007). Because of the reproductive capacity of zebrafish, identification of these mutations can be achieved in a relatively short amount of time using positional cloning. Thus, the primary goal of this thesis project is to identify the *reddish* mutant isolated from the aforementioned screen, and to study the implication of similar mutations in humans.

II.

Results

reddish^{s587} exhibit vascular hemorrhage, disorganized cranial vessels, and ventricular failure.

The _reddish_^{s587} mutant was discovered in a _flk1_ transgene-assisted ethynitrosourea forward genetic zebrafish vascular screen (Jin _et al._, 2007). The _reddish_^{s587} mutant establishes proper circulation around 24 hours post-fertilization (hpf), much like their wildtype siblings; however, circulation is progressively lost by 36-56 hpf (Figure 3B and E). As early as 48 hpf, _reddish_ mutants have visible cerebrovascular hemorrhage (Figure 1E and 2B). Nevertheless, vascular development appears unaffected in _Tg(flk1:gfp)_^{s843} _reddish_ mutants during the initial 48 hours following fertilization, and mutants develop the same vessels as wildtype siblings, including dorsal aorta, cardinal vein and intersomitic vessels (Figure 3A). Following cerebral hemorrhaging at 48 hpf, the patterning of the cranial central arteries is altered, and angiogenic sprouting of new vessels is significantly diminished (Figure 3L and N). Additionally, between 56-72 hpf, when central arteries become patent and further vascular remodeling occurs in wildtype siblings, we observe endothelial regression in the _reddish_ mutant and cessation of further vascular development (Figure 3L). Furthermore, between 56-72 hpf all _reddish_ mutants begin to show cardiac morphology defects and decreased ventricular contraction (Figure 1F). All _reddish_ mutants die around 7 days, with cerebrovascular hemorrhaging, non-contractile ventricles, and severe edema throughout whole body.

Positional cloning of _reddish_^{s587} revealed a missense mutation in _ubiad1_.

To identify the _reddish_ genetic defect, positional cloning was implemented. Using numerous Simple Sequence Length Polymorphisms makers (_SSLP_), we were

able to localize the genetic defect to chromosome 8. Using bulk segregate analysis with several SSLPs localized to chromosome 8, the critical region was determined to be between z10048 and Z14412. For fine mapping, we assembled the critical region using syntenic regions of *Takifugu rubripes* and t*etraodon nigroviridis* genomes. The predicted map allowed us to generate several *SSLP* markers that were used to chromosome walk toward the genetic defect. Using the markers zv81100.13B, zv81100.12, and snp1, we were able to narrow the critical region to a less than 60 kb interval (Figure 2A). This region contained the genes, *ubiad1, mmp23ba,* and *cdk11b.* Sequence analysis of *reddish* genomic DNA revealed a T-to-A missense mutation in *ubiad1,* resulting in an amino acid change from Leucine to Glutamine. This mutation occurred in a region highly conserved among both vertebrates and invertebrates, suggesting a biological conserved importance of the mutated residue (Figure 2C).

Loss of *ubiad1* results in vascular hemorrhage

To verify that loss of *ubiad1* resulted in the *reddish* phenotype, we generated an antisense morpholino to reduce expression of functional ubiad1. Since *ubiad1* was determined to have maternal contribution (figure 5a), we decided to use a morpholino that interfered with splicing of zygotic *ubiad1* mRNA instead of blocking translation of all *ubiad1* mRNA. Using 8ng of splice morpholino, we were able to recapitulate the *reddish* hemorrhage (Figure 4a). Furthermore, rescue experiments using 150 pg of wild type *ubiad1* mRNA were sufficient to partially rescue *reddish* mutants (Figure 4c). These findings confirm that the *reddish* phenotype is caused by loss of *ubiad1* function.

Maternal and zygotic expression of *ubiad1* in zebrafish

The *ubiad1* gene is comprised of three exons that give rise to one protein containing 333 amino acid. Early expression of *ubiad1* was observed in one-cell stage embryos using reverse transcription PCR (RT-PCR), indicating that *ubiad1* is maternally derived (Figure 5a). Since the *reddish* phenotype is not evident until 48 hpf, we hypothesize that the delayed phenotype could be due to the perdurance of maternally derived ubiad1. In order to determine the expression patterns of *ubiad1* during the *reddish* phenotype emergence, we carried out *in situ* hybridization of embryos at 36, and 48 hpf. *ubiad1* mRNA show prominent expression in the head and heart at 36 hpf,(Figure 5d) with cardiac expression disappearing by 48 hpf (Figure 5d). To verify that cardiac expression of *ubiad1* was really lost by 48 hpf, we carried out RT-PCR using cDNA from 48 hpf zebrafish hearts (Figure 5a). Oddly, the RT-PCR showed that *ubiad1* is present in the 48 hpf hearts. This discrepancy between the *in situ* data and the RT-PCR data still needs to be examined. In order to determine if the mutation seen in *reddish* could have an impact on human heart development and function, we carried out RT-PCR on fetal heart, and the various chambers of an adult heart. *UBIAD1* expression was present in both the fetal heart and all of the chambers of the adult heart (figure 5 b and c). The early expression of UBIAD1 in the heart of both zebrafish and humans could indicate that UBIAD1 has a conserved cardiac function or is involved in cardiogenesis. In regards to the vascular phenotype, we were unable to show that *ubiad1* is expressed in zebrafish endothelial cells; however, RT-PCR using RNA from HUVECs did show that *UBIAD1* is present in human endothelial cells. Thus, UBIAD1 defects in human endothelial cells could potentially produce vascular defects similar to the *reddish* defects.

Figure 1. The *reddish* mutant exhibits cranial hemorrhages and cardiac defects.

(A-F) Bright field micrographs of 48 hpf wildtype (wt (+/+)) and *reddish* mutant (*s587 (* −/−)) embryos.
(A-C) At 48hpf wild type zebrafish show no sign of hemorrhage (A-B) and never develop pericardial edema by 72hpf (C).
(D-F) At 48hpf *reddish* mutants have visible hemorrhaging in the head (D-E). In addition, by 72hpf all mutants exhibit pericardial edema (F) and the ventricle is non-contractile.

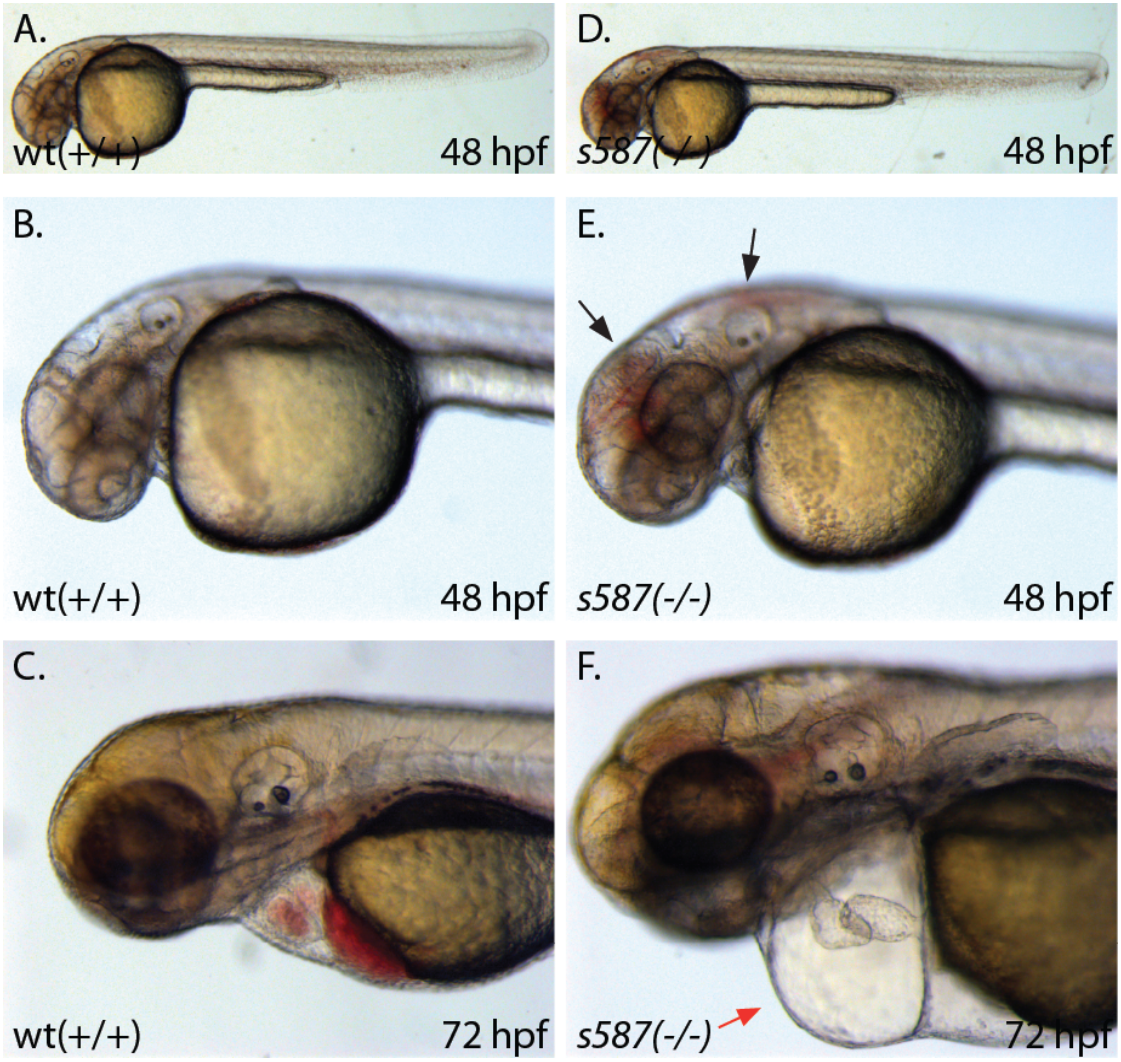
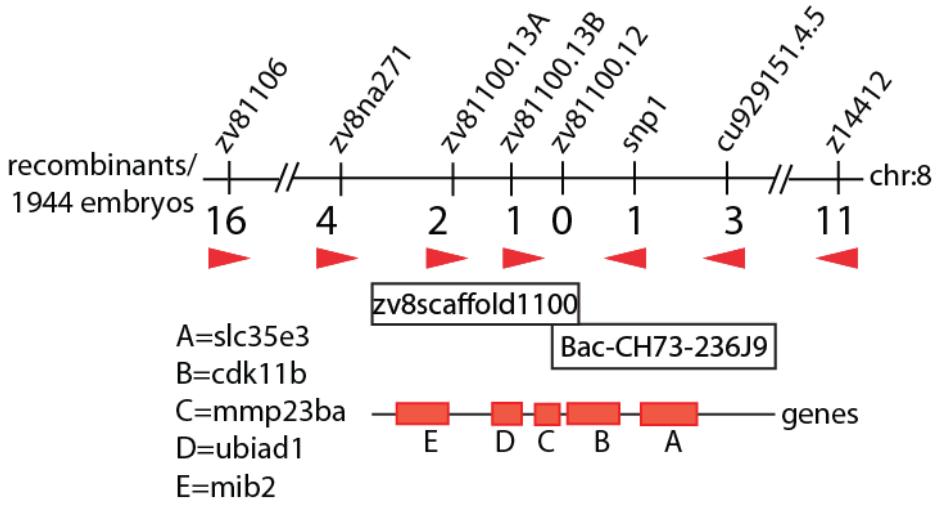

A.
wt(+/+)
48 hpf
D.
s587(-/-)
48 hpf
B.
wt(+/+)
48 hpf
E.
s587(-/-)
48 hpf
C.
wt(+/+)
72 hpf
F.
s587(-/-)
72 hpf

Figure 2. Genetic map of *reddish* critical region and causative mutation responsible for the deleterious phenotype.

(A) Genetic map of the *reddish* critical region. Numbers below SSLP markers indicate recombination events out of 1944 diploid embryos.
(B) Sequencing of *reddish* (-/-) genomic DNA revealed a T-to-A mutation within the coding sequence of *ubiad1* resulting in a Leu-to-Gln substitution at residue 65.
(C) The location of the *reddish* mutation in a highly conserved region, that is homologous with human UBIAD1.

A.


zv81106
zv8na271
zv81100.13A
zv81100.13B
zv81100.12
snp1
cu929151.4.5
z14412
recombinants/
1944 embryos
chr:8
16
4
2
1
0
1
3
11
zv8scaffold1100
Bac-CH73-236J9
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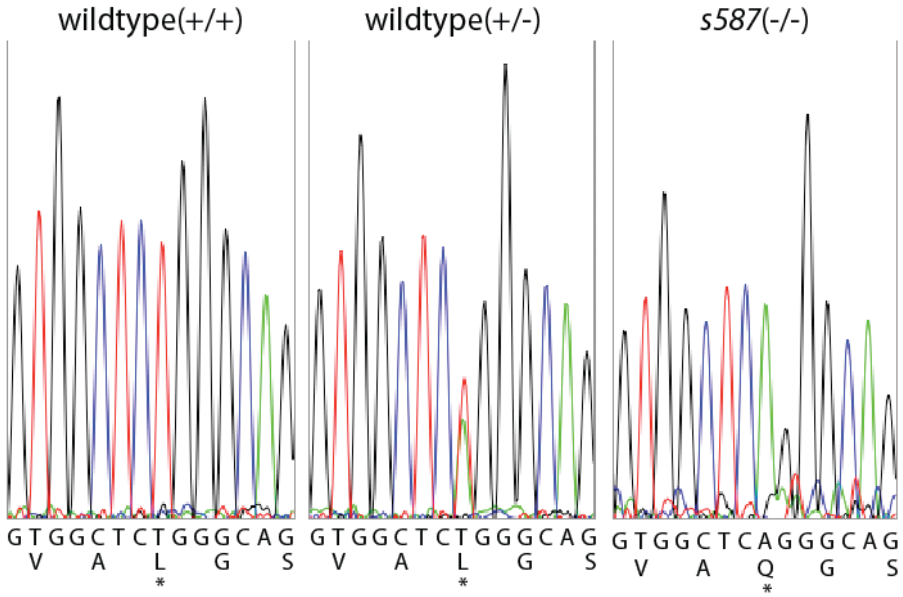
B=cdk11b
C=mmp23ba
D=ubiad1
E=mib2
genes
E
D
C
B
A

B.


wildtype(+/+)
wildtype(+/-)
s587(-/-)
G T G G C T C T G G G C A G
V A L * G S
G T G G C T C T G G G C A G
V A L * G S
G T G G C T C A G G G C A G
V A Q * G S

C.

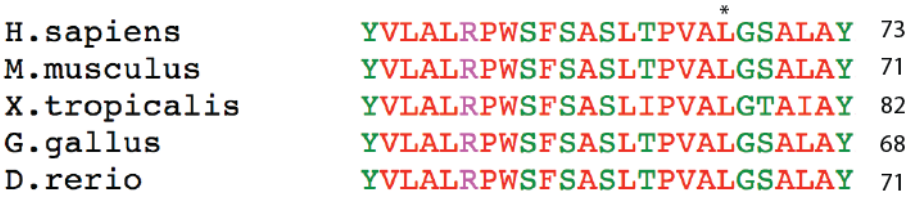

H.sapiens YVLALRPWSFSASLTPVALGSALAY 73
M.musculus YVLALRPWSFSASLTPVALGSALAY 71
X.tropicalis YVLALRPWSFSASLIPVALGTAIAY 82
G.gallus YVLALRPWSFSASLTPVALGSALAY 68
D.rerio YVLALRPWSFSASLTPVALGSALAY 71

Figure 3. Phenotypic analysis of the *reddish* mutant.

(A-F) Stereomicrographs of blood vessels visualized with *flk1:gpf* (A and D), an endothelial specific reporter line, and hemorrhaging visualized with *gata1:dsred* (B and E), a blood reporter line. Figures C and F shows a merge of *flk1:gpf* and *gata1:dsred* in reddish mutants and wildtpye siblings, respectively. All of the *reddish* mutants develop trunk vessels similar to wildtype siblings (A and E). However, unlike the siblings (red arrowhead E), *reddish* mutants do not have blood circulation by 48hpf and blood can be seen pooling in the head and trunk (red arrowheads in B).
(G-N) Brightfield micrographs (G, I, K, and M) and confocal micrographs (H, J, L, and N) of *reddish* and wildtpye siblings. Endothelial cells are visualized using *flk1:mcherry-ras* (H and L) and *flk1:gfp* (J and N). *reddish* mutants exhibit cranial hemorrhaging (K and M black arrow) and a disorganized cranial vascular system (L) compared to wildtpye that show no hemorrhage (G and I) and have a very organized cranial vasculature system (H). Furthermore, the vessels that are present in the head of *reddish* mutants never become patent and appear collapsed (white Asterisk in N) compared to wildtpye (J).

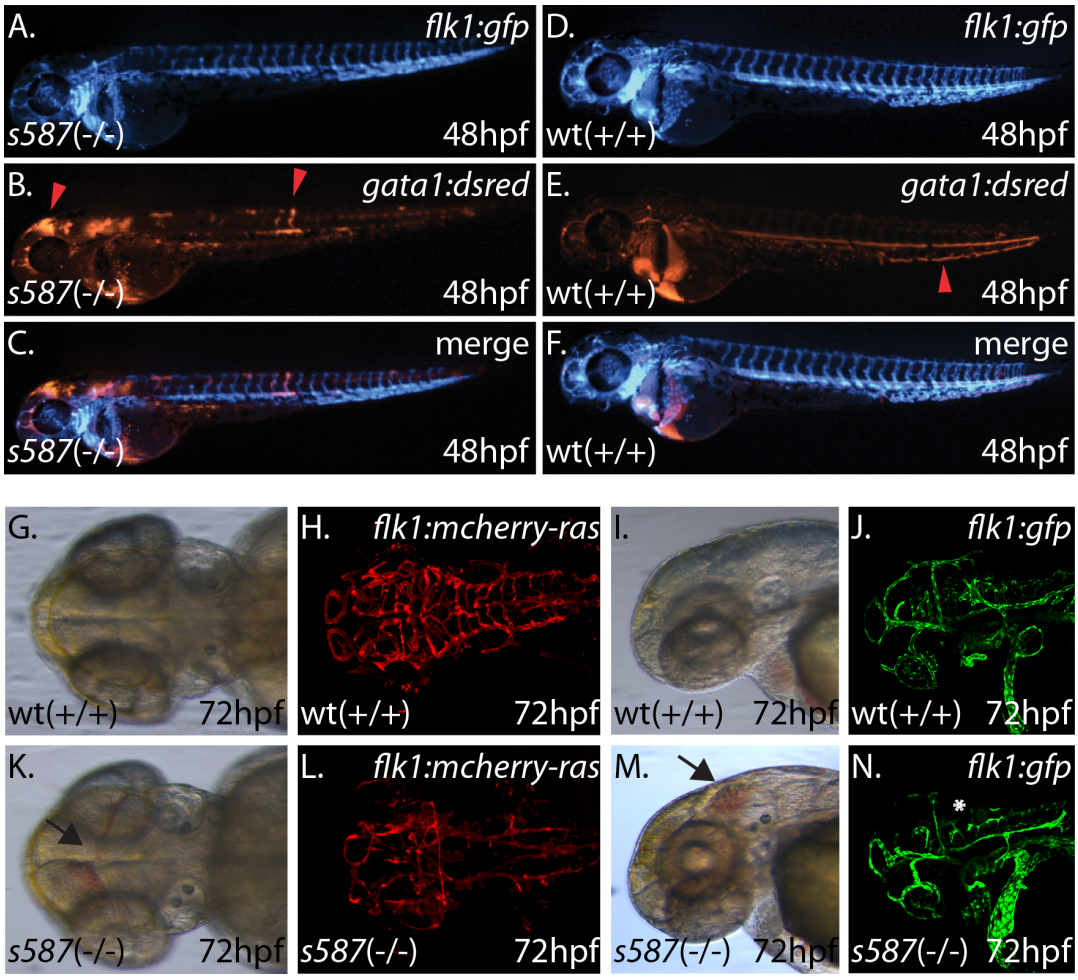
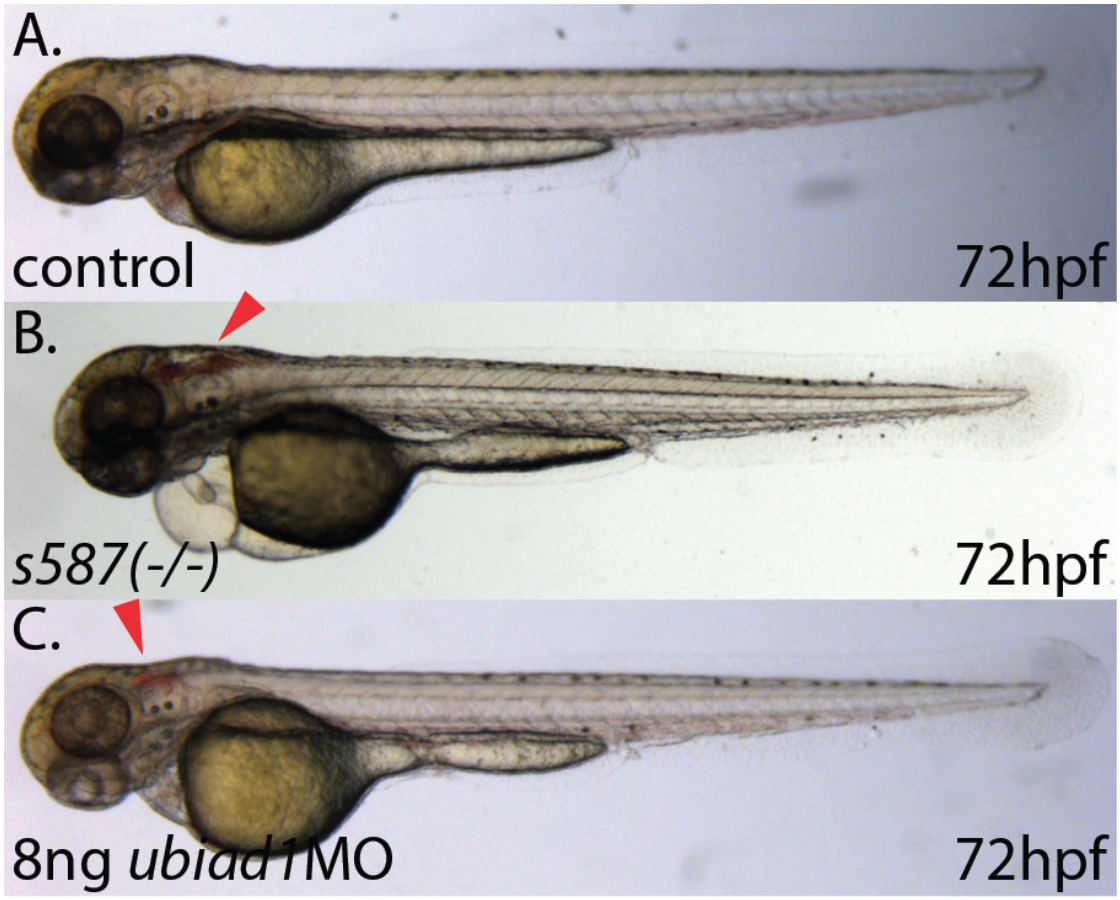
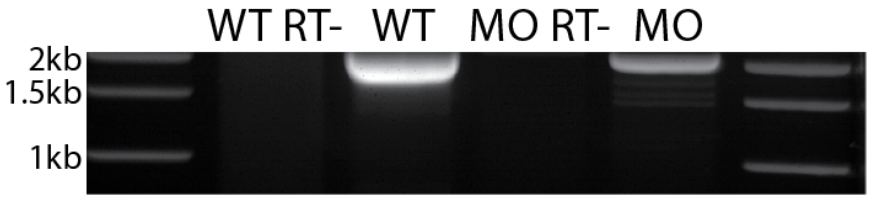

A.
flk1:gfp
s587(-/-)
48hpf
D.
flk1:gfp
wt(+/+)
48hpf
B.
gata1:dsred
s587(-/-)
48hpf
E.
gata1:dsred
wt(+/+)
48hpf
C.
merge
s587(-/-)
48hpf
F.
merge
wt(+/+)
48hpf
G.
wt(+/+)
72hpf
H.
flk1:mcherry-ras
wt(+/+)
72hpf
I.
wt(+/+)
72hpf
J.
flk1:gfp
wt(+/+)
72hpf
K.
s587(-/-)
72hpf
L.
flk1:mcherry-ras
s587(-/-)
72hpf
M.
s587(-/-)
72hpf
N.
flk1:gfp
s587(-/-)
72hpf

Figure 4. The *reddish* mutant can be recapitulated by knocking down zygotic *ubiad1* with a splice morpholino and also rescued by over expression of *ubiad1* mRNA.

(A-C) Bright field micrographs of 72 hpf control morphant (A), *reddish*^{s587} (B), and *ubiad1* morphant (C). Using 8ng of *ubiad1* splice morpholino we are able to recapitulate the *reddish* hemorrhage (red arrows head in B-C), whereas the control morpholino produced no hemorrhaging (A).
(D) RT-PCR using cDNA from morphant embryos(MO) and wild type(WT). Decreased intensity of the morphant band indicates that the splice morpholino reduces expression of full-length *ubiad1* mRNA.
(E) Injection of 8ng of splice morpholino recapitulates the *reddish* phenotype in 67% percent of the injected embryos. In addition, injection of 150pg of wt *ubiad1* mRNA rescues approximately 50% of injected *reddish* mutants. Rescue percentage was determined by taking the total number of embryos that showed hemorrhaging and dividing by the predicted number of mutants (25% of 118 embryos).



D.

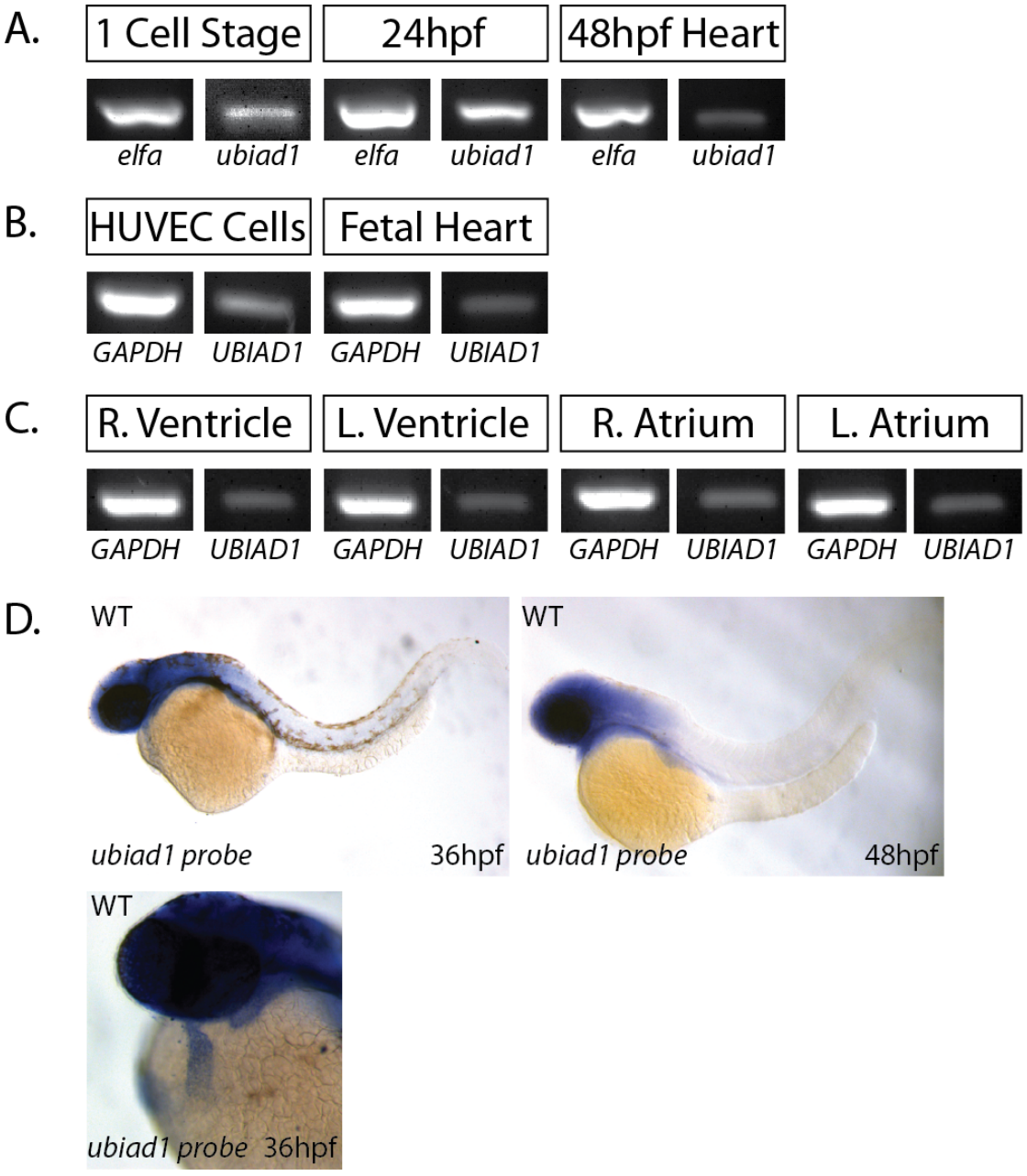


E.

	Total embryos	wildtype phenotype	*reddish* phenotype	Approximate % rescue	% *reddish* phenotype recapitulation
Non-injected *reddish clutch*	87	65	22	0%	-
reddish clutch injected with 150pg of wt *ubiad1* mRNA	118	103	15	50%	-
8ng control morpholino	174	174	0	-	0%
8ng *ubiad1* splice morpholino	207	69	138	-	67%

Figure 5. *ubiad1* expression in developing zebrafish and human tissues.

(A) Reverse transcription PCR (RT-PCR) using total RNA from 1 cell stage and 24hpf zebrafish embryos. The last column shows RT-PCR using RNA from 48hpf zebrafish hearts. We checked for the presence of *ubiad1* in each sample and used Elongation factor alpha (*elfa)* as a positive control.
(B) RT-PCR using human umbilical vein endothelial cell (HUVEC) RNA and human fetal heart RNA. We checked for the presence of *UBIAD1* in each sample and used glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* as a positive control.
(C) RT-PCR using RNA from the various chambers of an adult human heart. We checked for the presence of *UBIAD1* in each sample and used *GAPDH* as a positive control.
(D) *In situ* hybridization of 36 and 48hpf zebrafish embryos using a dig-labeled *ubiad1* probe. The bottom picture shows expression of *ubiad1* in the heart of 36 hpf embryo.


A.
1 Cell Stage
24hpf
48hpf Heart
elfa
ubiad1
elfa
ubiad1
elfa
ubiad1
B.
HUVEC Cells
Fetal Heart
GAPDH
UBIAD1
GAPDH
UBIAD1
C.
R. Ventricle
L. Ventricle
R. Atrium
L. Atrium
GAPDH
UBIAD1
GAPDH
UBIAD1
GAPDH
UBIAD1
GAPDH
UBIAD1
D.
WT
ubiad1 probe
36hpf
WT
ubiad1 probe
48hpf
WT
ubiad1 probe
36hpf

III.

Discussion

In the present thesis we discovered a recessive missense mutation in the zebrafish *ubiad1* gene that results in vascular hemorrhage and cardiac arrest. The vascular hemorrhage coincided with disrupted vascular development, and the cardiac defect is characterized by ventricular failure by 72hpf. Interestingly, we found that *UBIAD1* is expressed in both human and zebrafish hearts very early in development, suggesting that UBIAD1 could have an essential role in cardiac function during embryonic development. Furthermore, we detected *UBIAD1* in human umbilical vein endothelial cells, which could also indicate that UBIAD1 plays an important role in blood vessels. Although heterozygous missense mutations have been discovered in human *UBIAD1*, this is the first report of homozygous missense mutations in a vertebrate. The absence of homozygous mutations in humans could indicate that UBIAD1 is essential not only for cardiac and vascular function, but also viability in general. Since UBIAD1 is highly homologous in all vertebrates, it is likely that UBIAD1 has similar functions in these organisms, and thus, the genetic defects seen in the *reddish* mutant might translate to similar phenotypes in humans with loss of heterozygosity mutations similar to *reddish*.

The expression of *UBIAD1* in human umbilical vein endothelial cells could explain why *reddish* exhibits a vascular phenotype. Although we were unable to show that zebrafish endothelial cells express *ubiad1*, we still believe the vascular hemorrhage observed in *reddish* mutants is likely caused by MK-4 deficiency, because similar human deficiencies cause vascular hemorrhages in infants (Komatsu *et al.,* 2011). Although PK is primarily used as a cofactor in the liver, a study indicating that

PK is converted in many tissues to MK-4 by UBIAD1 could suggest that infant hemorrhaging is also caused by non-hepatic deficiencies of MK-4. Since MK-4 is the preferred cofactor for GGCX outside of the liver in vertebrates (Thijssen *et al.,* 1996), any protein that requires gamma carboxylation outside of the liver would likely be affected by MK-4 deficiency. For example, Protein S, a vitamin K-dependent protein that has 43 percent of its expression derived from the endothelium, has recently been linked to intracranial hemorrhaging in mice null for Protein S (Burstyn-Cohen *et al.,* 2009). These hemorrhages only occurred in protein S null mice and not in conditional knockouts of hepatic or endothelial Protein S (Burstyn-Cohen *et al.,* 2009), suggesting that non-hepatic Protein S plays a significant role in hemostasis. Thus, the hemorrhaging seen in the *reddish* mutant could be caused by reduced activity of endothelial derived coagulation factors, such as Protein S. Further work will be required to determine the role of UBIAD1 in the endothelium, and also whether MK-4 or PK is the predominant co-factor of GGCX in this tissue. One experiment that can be done to prove that MK-4 deficiency is the primary contributor to the *reddish* hemorrhage is to inject MK-4 into the circulation of developing zebrafish. Taking into consideration PK supplementation cures vitamin K-dependent hemorrhaging in humans, similar supplementation with MK-4 might be advantageous to *reddish* zebrafish as well. Furthermore, if the hemorrhaging in *reddish* is caused by endothelial specific deficiency of MK-4, then endothelial specific rescues experiments could be useful. For example, we could over-express wildtype *ubiad1*, via transient expression, using an endothelial promoter driving the

expression of wildtype *ubiad1*.Nevertheless, the findings that human and mouse hearts do not use MK-4 for gamma carboxylation could indicate that UBIAD has a novel function in the heart. If this novel function is conserved in zebrafish, then Mk-4 and endothelial specific rescue experiments might not be sufficient to full rescue *reddish* mutants alone. Further experiments will be required to elucidate the function of UBIAD1 in both zebrafish and mammalian hearts.

The localization of UBIAD1 to different sub-cellular locations in distinctive cell types suggests that UBIAD1 function varies between certain tissues. For example, UBIAD1 has been show to localize to the mitochondria in keratocytes (Nickerson *et al.*, 2010), and the Endoplasmic reticulum (ER) in osteoblast-like cell (Nakagawa *et al.*, 2010). Since GGCX has been shown to localize primarily to the endoplasmic reticulum (ER), it is predicted that in cells utilizing MK-4 as a cofactor for GGCX, UBIAD1 might also localize to the ER. Conversely, in cells where MK-4 is not utilized, UBIAD1 might localize to other sub-cellular regions and carry out novel functions. In order to elucidate the function of UBIAD1, it will be important for future experiments to determine the sub-cellular localization of UBIAD1 in various tissues.

Although the function of UBIAD1 in the heart is not currently known, the observation that *ubiad1* loss of function in zebrafish results in ventricular failure does indicate that ubiad1 plays an important role in the heart. Furthermore, a study demonstrating that *Ubiad1* has the highest expression in the mouse heart, which does not correlate with MK-4 biosynthesis, supports the concept that UBIAD1 has novel functions in the heart. One intriguing possibility is that UBIAD1 is involved in the

ubiquinone biosynthesis pathway. Protein docking models demonstrate that the UBIAD1 is able to bind the same substrate as COQ2 (Nickerson *et al.* 2010); thus, it is possible that in certain tissues UBIAD1 shares a redundant role with COQ2 in the ubiquinone pathway. In fact, *COQ2* expression varies in different chambers of the heart (right ventricle>left atrium>right atrium>left ventricle (Forsgren *et al.*, 2004)), with the left ventricle having nearly half the *COQ2* expression as the right ventricle (Forsgren *et al.*, 2004). The low expression in the left ventricle, which is likely to have the highest ATP requirement, as it needs to pump blood throughout the body, might indicate another enzyme is contributing to the same reaction as COQ2 in the left ventricle. Since the *reddish* mutant exhibited decreased ventricular contractility, we hypothesize that this phenotype might be due to decreased production of ATP by ubiquinone mediated aerobic respiration. In order to prove UBIAD1 participates in the ubiquinone pathway, we have several experiments planned. First, if UBIAD1 does participate in the ubiquinone pathway, then it would need to be localized to mitochondrial; thus, immunostaining using antibodies specific for UBIAD1, in addition to markers for mitochondria, can be used to show if UBIAD1 co-localizes to the mitochondria. We will also try to rescue the *reddish* cardiac phenotype by injecting ubiquinone into the heart of developing zebrafish embryos. However, since we predict UBIAD1 defects lead to MK-4 deficiency, ubiquinone might not be completely sufficient to rescue mutants; therefore, we also plan on trying to rescue by co-injection of both MK-4 and ubiquinone into developing *reddish* mutants.

IV.

Materials and Methods

Zebrafish strains.

Embryos and adult fish were raised and maintained under standard laboratory conditions. We used the following lines *reddish*^{s587} (Jin *et al.,* 2007), *Tg(flk1:mcherry-ras)*^{s896}*,(*Chi *et al.,* 2009). *Tg(flk1:GFP)*^{s843}*,*(Jin *et al., 2005)* and *Tg(gata1:dsred)*^{sd2}(Traver *et al,* 2003)

Mapping.

We mapped the *reddish* mutation to chromosome 8 using a set of simple sequence length polymorphism markers. For fine mapping, 1944 mutant embryos were genotyped with simple sequence length polymorphism markers within the critical region. *ubiad1* complementary DNA was isolated from *reddish*^{s587} embryos and their wildtpye siblings, sequenced, and then analyzed. A point mutation discovered in *ubiad1* was verified by sequencing of *reddish*^{s587} genomic DNA.

In situ hybridization.

Whole mount in situ hybridization was performed on 36 and 48hpf zebrafish embryos as previously described (Thisse *et al.,* 2007), using a *ubiad1* RNA probe. The *ubiad1* in situ hybridization probe was generated by PCR, using the primers F 5'-ATGCAGGAGATGAAGCCGGCTGC -3' and R 5'GTAATA CGACTCACTATAGGGCTCACAATAACGGCA G-3.

Morpholino Knockdown and mRNA rescue

To knock down expression of *ubiad1,* we used an antisense morpholino oligonucleotide targeted against the 5' splice site of exon 2, with the following sequence: 5'-GAAGCCAATCGGTATATTCACCTCC-3'. Embryos were injected at the one cell stage with 8-10ng of *ubiad1* morpholino, and analyzed at 72hpf. For control injections, 8-10ng of mmp23ba MO, which we established in previous experiment to cause no phenotype (data not shown), was also injected into one cell stage embryos. For mRNA rescue experiments, one cell stage embryos were injected with 100-150pg wildtype zebrafish *ubiad1* mRNA, and analyzed at 72hpf.

Confocal microscopy

For confocal imaging embryos were anesthetized using .5-1% tricane, and then mounted in 1% low melt agarose. Imaging was carried out using the Leica SP5 confocal microscope. Z-stacks were generated using an optical slice thickness of 1um. The 3D projections were generated using the Leica software.

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