Ubiad1 is an antioxidant enzyme that regulates eNOS activity by CoQ10 synthesis

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SUMMARY

Protection against oxidative damage caused by excessive reactive oxygen species (ROS) by an antioxidant network is essential for the health of tissues, especially in the cardiovascular system. Here we identified a new gene with important antioxidant features by analyzing a null allele of zebrafish ubiad1, called barolo (bar). bar mutants show specific cardiovascular failure due to oxidative stress and ROS-mediated cellular damage. Human UBIAD1 is a non-mitochondrial prenyltransferase that synthesizes CoQ10 in the Golgi membrane compartment. Loss of UBIAD1 reduces the cytosolic pool of the antioxidant CoQ10 and leads to ROS-mediated lipid peroxidation in vascular cells. Surprisingly, inhibition of eNOS prevents Ubiad1-dependent cardiovascular oxidative damage suggesting a crucial role for this enzyme and non-mitochondrial CoQ10 in NO signaling. These findings identify UBIAD1 as a new non-mitochondrial CoQ10-forming enzyme with specific cardiovascular protective function via the modulation of eNOS activity.

INTRODUCTION

Reactive oxygen species (ROS) play an important role in signal transduction and physiological regulation of the cardiovascular system by facilitating various biological responses such as gene expression, cell proliferation and migration, apoptosis, and senescence in heart and endothelial cells (ECs) (Lander et al., 1996; Finkel et al., 2000, 2003; Hare et al., 2005; D'Autréaux and Toledano, 2007). In cardiovascular tissues ROS are normally produced in response to RTK and GPC receptors signaling, as well as during mechanical (shear) stress. Such oxidative species contribute to the regulation of cardiovascular homeostasis (Colavitti et al., 2002; Werner, 2004; Ushio-Fukai, 2006). An antioxidant network is required to balance these ROS. This will prevent excessive oxidative damage which may contribute to different cardiovascular pathologies, including endothelial apoptosis and dysfunction, atherosclerosis, and also heart failure (Finkel, 2003; Giordano, 2005; Kuster et al., 2010). While several enzymes responsible for ROS production in ECs have been identified, many of the molecules involved in the endogenous antioxidant network are still unknown.

A promising molecule known to play an important antioxidant role in the cardiovascular system is CoQ10 (also known as Coenzyme Q10 or ubiquinone) (Pepe et al., 2007; Kumar et al., 2009). CoQ10 is the only endogenously synthesized lipid-soluble antioxidant (Crane, 2007; Bentinger et al., 2007). It is available in the membranes of the Golgi where it is present at an even higher concentration than in mitochondria, as well as in plasma membranes (Kalen et al., 1987; Crane, 2001; Bentinger et al., 2008). While the biosynthesis of CoQ10 in mitochondria has been studied in detail only a limited amount of data is available in regards to the synthesis of cellular membrane CoQ10. Interestingly, several in vivo experiments suggest that

CoQ10 synthesis may also occur in the Golgi and endoplasmic reticulum membranes. This synthesis provides the cellular membrane CoQ10 pool (Kalén et al., 1990). However, although hypothesized, a "non-mitochondrial" CoQ10 biosynthetic enzyme has not been identified as yet (Bentinger, et al., 2010). Identification of such an enzyme could be extremely useful to devise strategies to counteract the excess of ROS responsible for cellular oxidative damage (such as lipid peroxidation, protein nitrosylation and DNA oxidation) and therefore balance redox signaling especially in cardiovascular tissues.

CoQ10 is a mobile lipophilic electron carrier which is critical for electron transfer both in the mitochondrial membrane for respiratory chain activity as well as in Golgi and plasma membranes for NAD(P)H-oxidoreductase-dependent reactions such as in NO synthesis (Navas et al., 2007). Endothelial nitric oxide synthase (eNOS) is a critical regulator of cardiovascular functions by generating nitric oxide (NO), which is an important mediator of cardiovascular homeostasis (Alp and Channon, 2004; Fostermann and Sessa, 2011). As in the case with non-mitochondrial CoQ10, eNOS is specifically localized in the Golgi and plasma membrane of heart and endothelial cells and it can be differentially regulated in these two cellular compartments (Fulton et al., 2002). Interestingly, different reports over the years have suggested that CoQ10 may improve endothelial dysfunction by 'recoupling' eNOS and modulating NO-related signaling (Chew and Watts, 2004; Tsai et al., 2011).

Here we identify UBIAD1 as an enzyme for CoQ10 synthesis at the level of Golgi membranes. By using a zebrafish null mutant for Ubiad1 and primary human cells silenced for UBIAD1, we show that UBIAD1 is a critical enzyme for oxidative stress protection. In particular UBIAD1 plays a strategic role by protecting cardiovascular tissues from eNOS-dependent oxidative stress. We propose a possible functional link

between UBIAD1, CoQ10 and NO signaling during cardiovascular development and homeostasis.

RESULTS

Oxidative stress and cardiovascular failure characterize *barolo* mutants

During two independent forward genetic screens for cardiovascular mutants (Tuebingen Screen, 2005; Jin et al., 2007), we identified mutations in the barolo (*bar*) gene (bar^{t31131} and bar^{s847}). These mutations are recessive and show complete penetrance and expressivity. *barolo* mutants show distinct cranial vascular hemorrhages and pericardial edema in the context of wild-type body morphology (Figure 1A). To examine whether this phenotype is associated with cardiovascular defects, we crossed the mutations into *Tg(kdrl:GFP)*^{s843} (Figure 1B). Fluorescence microscopy analyses show that the hemorrhagic phenotype in *bar* mutants is accompanied by specific endothelial regression and fragmentation in the cranial and trunk vascular compartments (Figure 1C and 1D). Time-lapse analyses of trunk vasculature indicate that the *bar* phenotype starts around 36 hours post-fertilization (hpf)(Supplementary Movies 1-4) and that it is not due to general loss of blood flow. The cardiac edema of *bar* mutants is caused by a gradual breakdown of endocardial and myocardial cells in the heart leading to the collapse of the heart between 60 and 72hpf (data not shown). All these features lead to the complete cardiac and vascular organ failure by 72hpf in *bar* mutant embryos.

It has been demonstrated that cardiovascular failure is related to altered redox signaling and an increased production of reactive oxidative species (ROS)(Hare and Stamler, 2005). Intriguingly, *bar* mutants compared to wild-type siblings show altered redox balance and oxidative stress features evaluated as an increase of the NADP/NADPH ratio and as an overexpression of anti-oxidant genes, respectively (Figure 1E and 1F). Increase of ROS production occurs specifically in cardiovascular tissues as demonstrated by measuring oxidizing species in ECs of *bar* mutants using

the selective and stable ROS detector CellROX (Figure 1G). To better establish whether *bar* mutants are characterized by redox imbalance and oxidative stress we measured S-nitrosylation levels on heart and blood vessels sections of *bar* mutants. *bar* mutants show higher levels of S-nitrosylated proteins in heart and blood vessel cells as well as in pronephros as measured by an antibody against S-Nitroso-Cysteine (SNO-Cys) (Figure 1H). A high level of S-nitrosylated proteins is normally associated with altered redox signaling and cellular protection against oxidative stress (Stamler et al., 2001; Hess et al., 2005; Sun and Murphy, 2010).

A particularly destructive aspect of oxidative stress is the production of ROS that, if not overcome by antioxidant mechanisms or compounds, cause extensive cellular DNA damage. We found that cardiovascular tissues from *bar* mutants are positive for 8-hydroxy-2'-deoxyguanosine (8-OHdG), one of the predominant free radical-induced oxidative lesions in DNA (Figure 1I). Analyses of heart and blood vessel sections show that both myocardial and endothelial tissues of bar mutants exhibit ROS-mediated DNA damage compared to controls.

Since an unbalanced increase of ROS might lead to apoptosis and organ failure we evaluated whether oxidative stress in *bar* mutants ends up in causing cell death at later stages. Indeed, endothelial and endocardial cells in bar mutants were positive for TUNEL staining, and show typical morphological features of apoptotic cells at this stage (Figure 1L). In summary, both mutant embryos carrying the *barolo* mutation show increased oxidative stress that cause DNA damage and then cell death specifically in blood vessels and the heart.

By positional cloning, we determined that bar encodes Ubiad1 (UbiA-domain containing protein 1)(Figure 1M). Sequence analyses show that the bar^{t31131} mutant

allele contains a nonsense mutation (123 T>A) in ubiad1 introducing a premature stop codon at amino acid position 41, likely generating a null allele. The second allele of *bar* (bar^{s847}) bears a base change (185 T>A) leading to the substitution of a conserved leucine residue to glutamine at amino acid position 62 ($Ubiad1^{L62Q}$)(Figure 1N). bar^{s847} shows the same phenotypical features as bar^{t31131} except for the fact that the phenotype is delayed by 12 hours (Figure S1A-E and S1T). Both bar allele mutants do not express Ubiad1 (Figure 1O) and are fully rescued by Ubiad1 mRNA expression. On the other hand, $Ubiad1^{L62Q}$ mRNA does not rescue the bar^{t31131} mutant indicating that this amino acid mutation is sufficient to knock-out Ubiad1 activity (Figure 1P).

To further test whether Ubiad1 regulates cardiovascular protection and survival, we knocked down Ubiad1 expression using an antisense morpholino oligonucleotide. Ubiad1 morphants show the same hemorrhagic phenotype and cardiovascular defects as bar mutants (Figure S1F-I).

Analyses of ubiad1 mRNA expression in developing zebrafish embryos by RT-PCR and in situ hybridization reveal a ubiquitous expression at 24hpf in addition to a distinct expression in the heart at 48hpf (Figure S1J-L), in agreement with mammalian expression data (Nakagawa et al., 2010). In addition, cell transplantation and tissue-specific rescue experiments showed that Ubiad1 functions autonomously within endothelial and endocardial cells (Figure S1M-P and Supplementary Table).

These data indicate that loss of *ubiad1* triggers a progressive cardiovascular failure by increasing oxidative stress and apoptosis in heart and blood vessels suggesting a role for Ubiad1 as a controller of redox state and ROS levels in vertebrate cardiovascular tissues (Figure S1Q). Thereby, *bar* represents a unique model to study oxidative stress conditions and redox signaling during zebrafish development.

UBIAD1 is a new CoQ10 biosynthetic enzyme

Bioinformatic analyses show that Ubiad1 is conserved amongst species and contains an UbiA prenyltransferase domain, present also in the vertebrate protein, Coq2. So far, the mitochondrial enzyme Coq2 is the only vertebrate enzyme known to synthesize CoQ10 through the prenylation of 4-hydroxybenzoic acid by oligoprenyl diphosphates (Forsgren et al., 2004). The CoQ10 molecule is composed of a redox active benzoquinone ring, which is connected to a polyisoprenoid side chain of variable length (typically 9/10-units in vertebrates)(Crane, 2001). As for Coq2, we speculate that Ubiad1 might catalyze the biosynthesis of CoQ10. To test our hypothesis we have measured CoQ10 levels in bar mutants and ubiad1 morphants by HPLC-MS technique (Figure 2A and 2B). The absence of Ubiad1 in zebrafish larvae significantly reduces CoQ10 levels (Figure 2C), while cholesterol levels are not significantly affected (Figure S2A). By time-course of synthesis analyses we found that CoQ10 increases during zebrafish embryonic development and is almost entirely produced by the zygote without any contribution from the yolk sac (Figure S2B). On the other hand, a significant amount of embryonic cholesterol is provided by the yolk sac throughout early development (Figure S2C). These data suggest that de novo zygotic synthesis of CoQ10 by Ubiad1 (and/or Coq2) is required for zebrafish development.

To test whether it is the absence of CoQ10 synthesis in zebrafish embryos that causes the bar phenotype we injected CoQ10 in one-single cell stage *bar* mutants (Figure 2D). Three different CoQ10 formulations significantly rescue bar mutants suggesting that the intake of CoQ10 in Ubiad1 deficient embryos may restore normal

cardiovascular development. A recent paper reports that UBIAD1 is required for Vitamin K2 production (Nakagawa et al., 2010). However, exogenous Vitamin K2 injections did not rescue bar mutants (Figure 2D) and Vitamin K2 was undetectable in our experimental conditions (Figure S2D-K). Although it is possible that UBIAD1 may catalyze several enzymatic activities, our data indicate that the lack of Vitamin K2 is not responsible for the cardiovascular phenotype in bar mutants.

To provide biochemical evidence that Ubiad1 is able to synthesize CoQ10 we performed metabolic studies in zebrafish embryos by analyzing the ability of *bar* mutants to convert hydroxy-4-benzoic acid to CoQ10 (Figure 2E). We first confirmed that administration of $^{13}C_6$-labelled hydroxy-4-benzoic acid (hydroxy-4-benzoic acid-$^{13}C_6$) is metabolized to CoQ10-$^{13}C_6$ in zebrafish embryos by injecting this C^{13}-labeled precursor in zebrafish embryos at one-cell stage. After 72hpf of incubation, lipids were extracted and analyzed by HPLC-MS to quantify the amount of CoQ10-$^{13}C_6$ formed in this time frame (Figure 2E). As a matter of fact, *bar* mutants show a reduced CoQ10-$^{13}C_6$ production compared to wild-type siblings. Interestingly, we also detected a strong impairment of CoQ9-$^{13}C_6$ production (Figure 2F) indicating that zebrafish larvae can synthesize both CoQ9 and CoQ10 as can human cells (Turunen et al., 2004). In summary, Ubiad1 is a 4-OH-benzoic acid-prenyl-tranferase enzyme required for CoQ10 and CoQ9 biosynthesis *in vivo*.

Ubiad1 and HMG-CoA reductase inhibitors act in the mevalonate pathway for CoQ10-dependent cardiovascular oxidative stress protection.

The rate-limiting reaction in the biosynthesis of CoQ10 is the transfer of the polyprenyl-pyrophosphate (polyprenyl-PP) chain, derived from mevalonate, on to 4-

OH-benzoic acid, derived from tyrosine metabolism (Bentinger et al., 2008)(Figure 2A). We reduced the pool of polyprenyl-PP available for CoQ10 synthesis by treating zebrafish embryos with statins, inhibitors of its precursor (e.g. mevalonate)(Figure S3A). By blocking HMG-CoA reductase statins impair the mevalonate and FPP synthesis which is mandatory to synthetize the polyprenyl-PP chain (Figure 2A). Wild-type embryos (*ubiad1*$^{+/+}$) treated with 100 or 500 nM of statin developed a bar-like phenotype characterized by cranial hemorrhages, vascular regression and collapsed heart with high penetrance (Figure 3A-C). Statin treatments efficiently reduced CoQ10 synthesis without interfering with cholesterol levels probably due to the fact that statin treatment blocks de novo cholesterol biosynthesis, but does not interfere with the maternally-provided cholesterol (Figure 3D and 3E). Wild-type zebrafish embryos treated at the same developmental stages with the squalene synthase inhibitor (SQI) or with farnesyl or geranyl-geranyl tranferase inhibitors (FTI or GGTI) did not exhibit a *bar*-like phenotype (Figure S3B) indicating that the absence of CoQ10 is the cause of the statin-induced cardiovascular oxidative phenotype. Furthermore, while treatment with concentration of 2.5 or 5 nM (low-dose) of statin did not produce any phenotype in wild-type (*ubiad1*$^{+/+}$) embryos, it caused hemorrhages, endothelial dysfunction, and altered heart morphology (e.g. *bar*-like phenotype) in embryos heterozygous for *ubiad1* (*ubiad1*$^{+/-}$) (Figure 3F-I). The *bar*-like phenotype caused by statin treatments was efficiently rescued by exogenous administration of liposomal CoQ10 (Figure 3J and 3K and S3C). To address the role of the mevalonate pathway in protection from oxidative stress in vivo, we measured ROS levels in zebrafish embryos treated with HMG-CoA reductase inhibitors. A significant increase in ROS level is detected in statin-treated zebrafish embryos, and this oxidative stress was fully rescued by CoQ10 treatment (Figure 3L). These data

suggest that HMG-CoA reductase and Ubiad1 are on the same metabolic pathway to produce CoQ10 as a potent anti-oxidant molecule which protects cardiovascular tissues during development. Altogether, these findings lead us to propose UBIAD1 as an important target for therapeutic strategies involved in limiting side effects of statin treatments.

UBIAD1 synthesizes CoQ10 in the Golgi compartment

To verify whether Ubiad1 could be a new enzyme responsible for cellular CoQ10 production, we examined its subcellular localization. Based on its amino-acid structure, Ubiad1 is well conserved among species and is predicted to be a transmembrane protein (Nickerson et al., 2010). Using human primary ECs we performed localization studies for endogenous UBIAD1. UBIAD1 localizes in an asymmetric perinuclear region that resembles the Golgi compartment (Figure S4A). Co-localization studies using the Golgi specific marker TGN46 demonstrate that endogenous UBIAD1 distributes in the proximity of the Golgi compartment (Figure 4A). Also a GFP-tagged zebrafish Ubiad1 fusion protein when expressed in human ECs localizes in the same perinuclear region compartment and co-localize with the Golgi markers, GM-130 and γ-adaptin (Figure 4B and4C). To confirm that Ubiad1 is a Golgi protein, we treated Ubiad1-GFP-expressing cells with brefeldin A (BFA), an antibiotic that causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum (Fujiwara et al., 1988). Under these conditions, we observe a rapid redistribution of Ubiad1-GFP proteins in cells treated with BFA thus supporting its Golgi localization (Figure 4D).

We then hypothesized that since UBIAD1 is localized in the Golgi compartment, this enzyme might be a non-mitochondrial CoQ10 prenyltransferase. To test this assumption we performed subcellular fractionation experiments on human ECs. Golgi membranes or mitochondria were isolated and analyzed for UBIAD1 expression. Corroborating our previous immunofluorescence analyses, we detected UBIAD1 only in Golgi compartments and not in mitochondria (Figure 4E). We then analyzed *de novo* CoQ10 synthesis in these subcellular fractions by incubating ECs with the hydroxy-4-benzoic acid-$^{13}C_6$ precursor. These experiments show a significant decrease in CoQ10-$^{13}C_6$ production in Golgi compartments compared to mitochondrial fractions when UBIAD1 expression is reduced (Figure 4F). These data strongly indicate that UBIAD1 is a new CoQ10 biosynthetic enzyme located in the Golgi membrane compartment where large amounts of cellular CoQ10 are normally synthesized (Kalén et al., 1990; Swiezewska et al., 1993).

SCCD mutations protect cardiovascular tissues from oxidative stress by CoQ10 production

Mutations in the *UBIAD1* gene are linked to the autosomal dominant eye disease Schnyder Crystalline Corneal Syndrome (SCCD; OMIM 121800)(Weiss et al., 2007; Orr et al., 2007; Yellore et al., 2007; Weiss et al., 2008). By genetic analyses, two of the most common *UBIAD1* mutations found in SCCD patients lead to the N102S and D112G substitutions. A major unresolved issue is whether these mutations affect the activity of the UBIAD1 protein. We generated human UBIAD1 constructs carrying these mutations (UBIAD1-N102S and UBIAD1-D112G) and performed rescue experiments in the *bar* mutants to test their effect on UBIAD1 function (Figure 5A).

The SCCD variants were able to rescue the loss of Ubiad1 during zebrafish development indicating that these variants are biologically active. We then tested these variants for levels of expression and CoQ10 synthesis in human EC. Compared to wild-type UBIAD1 protein, SCCD variants were expressed at higher levels (Figure 5B). Furthermore, human ECs expressing the UBIAD-SCCD mutant proteins produced higher CoQ10 levels compared to wild-type protein (Figure 6C and D). Intriguingly, SCCD mutations did not affect cholesterol synthesis in human ECs (Figure 5E). These findings suggest that the SCCD variants are effective in CoQ10 production in tranfected cells. Altough it remains unclear why SCCD mutation would results in the SCCD phenotype these results do suggest a reassessment of the pathological features of SCCD patients.

Ubiad1 and Coq2 play different roles in CoQ10-mediated cardiovascular oxidative protection.

CoQ10 is a mobile lipophilic electron carrier critical for electron transfer by the mitochondrial inner membrane respiratory chain (Mitchell, 1961; Turunen et al., 2004; Duncan et al., 2009). Primary deficiency of CoQ10 (OMIM 607426) is characterized in humans by five major clinical phenotypes: 1) an encephalomyopathic form with seizures and ataxia; 2) a multisystem infantile form with encephalopathy, cardiomyopathy and renal failure; 3) cerebellar ataxia with marked cerebellar atrophy; 4) Leigh syndrome with growth retardation and 5) pure myopathy. Primary CoQ10 deficiencies have been associated with mutations in mitochondrial CoQ10 biosynthetic genes, such as *COQ2* (Salviati et al., 2005; Quinzii et al., 2006).

In order to investigate whether the Golgi-localized Ubiad1 and the mitochondrial-localized Coq2 can have different functions in living organisms, we knocked-down Coq2 expression during zebrafish development by morpholino (MO) injections. Similar to *Drosophila sbo*/*coq2* mutants (Liu et al., 2011), *coq2* morphants are characterized by a general developmental delay, small body and severe hindbrain edema (Figure S5A-C). Interestingly, *coq2* morphants display a short but normal swimming behavior, indicating that muscles are unlikely to be directly affected but that the energy is possibly inadequate for normal swimming (data not shown). These phenotypes do not occur in *bar* mutants or *ubiad1* morphants, and, although a strong increase in oxidative stress occurs in *coq2* morphants (Figure 6B), they did not show blood vessel regression (Figure 6A). These data indicate that loss of Coq2 leads to oxidative stress but it does not significantly affect vascular integrity and survival. We measured CoQ9 and CoQ10 levels, and as expected, they dropped in *coq2* and *ubiad1* morphants compared to controls and even more in double morphants (Figure 6C and S5D). Loss of Ubiad1 and Coq2 in vivo unequivocally causes a decrease in CoQ10 levels which possibly increases oxidative damage in different subcellular compartments leading to different embryonic phenotypes.

To test whether Coq2 might compensate for loss of Ubiad1 we performed rescue experiments by injecting mRNA encoding for Coq2 in *bar* mutants (Figure 6D). While *coq2* mRNA injection could rescue *coq2* morphants (data not shown) it did not rescue *bar* mutants suggesting that Coq2 does not protect cardiovascular tissues from oxidative stress during zebrafish development. MitoQ is a mitochondria-targeted antioxidant (Murphy and Smith, 2007). Due to its selective accumulation within mitochondria, MitoQ only protects against the mitochondrial oxidative damage that is caused in embryos and cells by blocking mitochondrial CoQ10 synthesis through

knocking down Coq2 (Figure S5E). However, MitoQ (which will not interact with the Golgi apparatus) did not rescue *bar* mutants (Figure 6D) indicating that the two different subcellular pools of CoQ10 contribute in quite distinct ways to the phenotype of living organisms.

To demonstrate the existence of a functional difference between the subcellular pools of CoQ10, we used human primary ECs in which we could silence to the same extent UBIAD1 and COQ2 genes. The silencing of these two genes causes an increase in oxidative stress and accumulation of oxidative damage (Figure 6E). We then compared the effects of these two genes on CoQ10 production, mitochondrial viability and oxidative degradation of cellular lipids. Initially we analyzed the efficiency of UBIAD1 and COQ2 in total CoQ10 biosynthesis by performing labeled precursor experiments where 4-hydroxybenzoic acid-$^{13}C_6$ was added to human ECs previously transfected with siRNA for UBIAD1 (siUBIAD1) or for COQ2 (siCOQ2) or both. Lipids were extracted and analyzed by HPLC-MS to quantify the amount of CoQ10-$^{13}C_6$ formed (Figure S5F). Silencing of UBIAD1 or COQ2 individually or together significantly reduced CoQ10-$^{13}C_6$ production. We also confirmed the efficiency of UBIAD1 and COQ2 siRNAs in decreasing endogenous total CoQ10 levels in ECs (Figure 6F). Interestingly the silencing of the UBIAD1 gene did not significantly interfere with the transcriptional regulation of COQ2 and vice versa, indicating that the effect on CoQ10 levels from the silencing of one gene could not be altered by upregulation of the other (Figure 6G).

To further evaluate the function of UBIAD1, we analyzed mitochondrial morphology and viability in ECs that have been silenced for UBIAD1 or COQ2. The absence of COQ2 significantly altered mitochondrial morphology (Figure S5G and S5H) and mitochondrial membrane potential (Figure 6H) which can indeed be rescued by

MitoQ (Figure S5I). Mitochondrial damage could not be detected in UBIAD1-silenced cells indicating that the lack of the mitochondrial pool of CoQ10 in COQ2-silenced cells exclusively impaired mitochondrial function.

High levels of CoQ10 have been found in cellular membranes, including the Golgi and plasma membranes (Kalen et al., 1987; Turunen et al., 2004). The presence of CoQ10 in non-mitochondrial membranes have been associated with its role in protecting membrane lipids from peroxidation as well as from an extracellular-induced ceramide-dependent apoptotic pathway (Navas et al., 2007). Since UBIAD1 is specifically localized in Golgi membrane and Ubiad1 null mutants die by apoptosis we hypothesize a specific role for this enzyme in membrane protection from oxidative damage. When we first evaluated oxidative degradation of lipids by measuring lipid peroxidation in siUBIAD1-treated cells (Figure 6I). ECs without a cellular membrane pool of CoQ10 showed twice the level of oxidized fatty acid lipids indicating a functional role for UBIAD1 in protection from oxidative species specifically localized in cellular but not mitochondrial membrane. We conclude that UBIAD1-derived CoQ10 prevents lipid peroxidation either directly or through the regeneration of other antioxidants such as Vitamin E.

In summary, we have demonstrated that in endothelial cells (and possibly all cardiovascular tissues) both UBIAD1 and COQ2 are CoQ10 biosynthetic enzymes. Their different subcellular localization drives distinctive functions inside the cells: the UBIAD1-synthetized CoQ10 works mainly as antioxidant for cellular membranes, while the COQ2-synthesized CoQ10 controls mitochondrial function.

UBIAD1 prevents a shear stress/Klf2a/eNOS-mediated oxidative stress pathway

A hemodynamic environment, such as shear stress, is known to lead to ROS generation which is responsible for vascular remodeling and angiogenesis (Ungvari et al., 2006; Matlung et al., 2009). Several studies have shown that Kruppel-like factor 2 (Klf2) is an immediate mediator of hemodynamic forces created by blood flow. Klf2 regulates flow-dependent blood vessel integrity both in zebrafish and mice (Lee et al., 2006; Nicoli et al., 2010). We tested if a shear stress-Klf2a pathway can modulate the *bar* phenotype and oxidative stress signaling by injecting cardiac troponin T2 (tnnt2) morpholino to block blood circulation (Sehnert et al., 2002) and *klf2a* morpholino (Nicoli et al., 2010) in bar mutants. In both cases we detected a significant delay of the bar phenotype as assessed by intersegmental vessels (Se) disintegration (Figure S6A and S6B). *klf2a* morpholino injection in *barolo* mutants lead to a partial recovery of oxidative stress in ECs (Figure S6C). These data indicate that a Klf2a-dependent shear stress pathway is required for oxidative stress conditions in *bar* mutants and that loss of Ubiad1 *in vivo* can be protected by impairment of this pathway.

In human endothelial cells KLF2 has been found to be directly responsible for endothelail nitric oxide synthase (eNOS) expression and signaling (Searles, 2006). Recently, a blood flow-dependent klf2a-NO signaling cascade has been identified in developing zebrafish embryos to be required for stabilization of the hematopoietic stem cell (HSC) program (Wang et al., 2011). We have investigated the role of eNOS in *bar* mutants. Endothelial *nos* isoforms have been well characterized in zebrafish development (Fritsche et al., 2000; Pelster et al., 2005; North et al., 2009; Wang et al., 2011). We performed knock-down experiments of *nos1* (the zebrafish orthologue of mammalian endothelial *Nos*) in *bar* mutants. Inhibition of *nos1* expression significantly rescued the *bar* mutant phenotype as well as endothelial regression (Figure 7A and 7B). Because abrogation of *nos1* expression may interfere with HSC

development and blood flow we treated *bar* mutants at the onset of oxidative stress (32hpf) with the selective inhibitor of eNOS activity, N-nitro-L-arginine methyl ester (L-NAME)(North et al., 2009). L-NAME treatment promoted a full morphological and functional cardiovascular recovery of a statistically significant number of *bar* mutants (Figure 7C and 7D and S6E). This recovery was carefully evaluated for ROS level and altered redox signaling (SNO-Cys staining) in ECs. Blocking of eNOS activity by L-NAME treatment significantly reduced oxidative stress in endothelial cells of *bar* embryos (Figure 7E) and restored normal redox signaling (Figure 7F and S6E). Interestingly, pronephric duct cells affected by oxidative stress in *bar* mutants did not improve after L-NAME treatments (Figure S6F). To test whether the source of ROS in the absence of UBIAD1 was caused by eNOS, we silenced eNOS in primary human endothelial cells. ROS levels could be fully normalized by reducing eNOS expression in siUBIAD1-treated cells (Figure 7G).

To further define the role of NO signaling in causing the *bar* mutant phenotype, we evaluated the effect of the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) on *bar* mutants. SNAP treatments did aggravate the *bar* phenotype by increasing oxidative species in these mutant embryos (Figure S6D-F).

Altogether these data indicate that eNOS may be responsible for ROS accumulation in *bar* mutants. It has been demonstrated that when eNOS is in a "coupled" conformation it produces NO, but when essential cofactors are limited, superoxide ($O_2^{\cdot}$) is produced by eNOS instead of NO (a so-called "uncoupled" conformation)(Fostermann and Sessa, 2011): as a consequence eNOS no longer produces NO but instead generates superoxide and causes oxidative stress in cardiovascular tissues. To verify whether the NO-forming activity of eNOS was compromised due to the lack of Ubiad1 and Golgi-CoQ10, we measured NOS activity

in *bar* mutants and human ECs silenced for UBIAD1. The lack of Ubiad1 drastically reduced the formation of NO in zebrafish embryos (Figure S6H) as well as in human ECs (Figure 7H).

We measured tissue specific NO levels in *bar* mutants by imaging DAF-2DA staining in live embryos. NO accumulation was reduced in the notochord (NC) and was completely absent in the bulbus arteriosus (BuA) of *bar* mutants compared with controls (Figure S6I). All together we show a specific role for Ubiad1 on regulation of eNOS-mediated NO formation in endothelial and cardiovascular cells in vivo.

These data indicate that the primary source of ROS causing cardiovascular oxidative stress in *bar* mutant as well as in ECs lacking UBIAD1 is dependent on eNOS activity. Therefore, we propose an unexpected functional link in the Golgi compartments between UBIAD1 and COQ10 in regulating eNOS activity and eventually NO signaling during cardiovascular development and homeostasis.

DISCUSSION

Ubiad1 is a prenyltranferase enzyme required for CoQ10 biosynthesis in Golgi membranes.

CoQ10 is an important cellular and mitochondrial redox component and the only endogenously produced lipid-soluble antioxidant (Bentinger et al., 2010). Although the genes encoding for the CoQ10 biosynthetic enzymes have been identified in bacteria and yeast, there is still only limited information about these synthetic enzymes in vertebrates (Turunen et al., 2004). The rate-limiting enzyme for the biosynthesis of CoQ10 is the enzyme that catalyzes the condensation of the polyisoprenoid chain with the benzoquinone ring. So far, the mitochondrial COQ2 enzyme has been considered the only prenyltransferase able to catalyze this reaction (Trevisson et al., 2011). Here we identified UBIAD1 as a novel vertebrate CoQ10 prenyltransferase. UBIAD1 contains an UbiA prenyltransferase domain also present in vertebrate COQ2. While COQ2 encodes a mitochondrial prenyltransferase, we found that UBIAD1 resides in the Golgi compartment where it produces CoQ10. While the presence of CoQ10 in non-mitochondrial membranes was previously explained by the existence of specific mechanisms for its redistribution within the cell (Crane and Morre, 1977; Jonassen and Clarke, 2000), our data now formally demonstrate that CoQ10 are synthetized in the Golgi compartment.

In favour of our hypothesis of a Golgi-synthetized CoQ10 it has been recently reported that COQ6, COQ7, and COQ9 which are critical enzymes for CoQ10 maturation are also localized in the Golgi compartment (Heeringa et al., 2011). Similarly, these authors suggested that the Golgi-localized pool of CoQ10 may

function in specific cells as an essential antioxidant for plasma membrane lipids that are normally derived from the Golgi compartment.

In this work we demonstrated that the existence of two distinctive ubiquinone prenyltransferases act to resolve different molecular functions inside the cells and in living organisms. We found that COQ2-mediated CoQ10 production is mainly for mitochondrial respiratory chain function and energy production, while UBIAD1-mediated CoQ10 production is important for membrane redox signaling and protection from lipid peroxidation. These results are in agreement with the different subcellular compartmentalization of these two enzymes.

Ubiad1 and CoQ10: an anti-oxidant system controlling redox cellular (plasma) membrane in cardiovascular tissues.

Oxidative damage is caused by an imbalance between the production of ROS and the antioxidant network, in favor of the former. Although ROS are predominantly implicated in causing cell damage and premature aging via oxidation of DNA, lipids and proteins, they also play a major physiological role in several aspects of intracellular signaling and regulation especially in cardiovascular tissues (Hare and Stamler, 2005; Kuster et al., 2010). Therefore, heart, endothelial and vascular smooth muscle cells need an efficient anti-oxidant network to balance ROS levels. We have shown a set of genetic and cellular data that reveal an unexpected role for UBIAD1 as an essential antioxidant gene with important functions in the protection of heart and endothelial cells from oxidative stress at the level of cellular membranes by producing CoQ10 in the Golgi for distribution to non-mitochondrial membranes throughout the cell. In addition to its crucial role in oxidative phosphorylation, CoQ10 plays another

vital role in cellular function as an antioxidant molecule. The antioxidant nature of CoQ10 is derived from its function as an electron carrier: in this role, CoQ10 continually shuttles between oxidised and reduced forms. As it accepts electrons, it becomes reduced. As it gives up electrons, it becomes oxidized. In its reduced ubiquinol form, the CoQ10 molecule will quite easily give up one electron and, thus, act as an antioxidant. In such a way, CoQ10 inhibits lipid peroxidation by acting as a chain breaking antioxidant. Moreover, $CoQH_2$ reduces the initial lipid peroxyl radical, with concomitant formation of ubisemiquinone and an alkyl peroxide. This quenching of the initiating peroxyl radicals thereby prevents the propagation of lipid peroxidation and protects not only lipids, but also proteins from oxidation. In addition, the reduced form of CoQ10 might also contribute to the stabilization of the plasma membrane, regenerating antioxidants such as α-tocopherol. A crucial role in all these processes is played by NAD(P)H-dependent reductase(s) acting at the plasma membrane to regenerate the reduced ubiquinol form of CoQ10, contributing to the maintainance of its antioxidant properties (Navas et al., 2007). This finding also opens a new and interesting link among cellular redox-state and metabolic pathways such as the mevalonate pathway.

An essential function for Ubiad1/CoQ10 in regulation of NO signaling

Here we have demonstrated that UBIAD1 protects cardiovascular tissue from ROS-mediated oxidative stress by producing CoQ10 located in Golgi and plasma membranes. Major enzymatic pathways responsible for the generation of ROS in cardiovascular tissues are mainly NADPH oxidases and eNOS (Browning et al., 2012). By using drug inhibition and gene inactivation approaches we identify eNOS

dysfunction as the primary cause of ROS increase in *bar* mutant and UBIAD1-silenced human ECs. The NO synthetized by eNOS is an essential factor for cardiovascular development and homeostasis in vertebrates (Alp and Channon, 2004; Pelster et al., 2005; North et al., 2009; Wang et al., 2011). It has been suggested that CoQ10 might have a positive role in modulating NO-related pathways by recoupling eNOS in endothelial cells (Stuehr et al., 2001; Chew and Watts, 2004; Tsai et al., 2011). eNOS is a "L-arginine, NADPH:oxygen oxidoreductases, NO forming enzyme" (EC 1.14.13.39) that coupled reduction of molecular oxygen to L-arginine oxidation and generation of L-citrulline and NO. eNOS controls the flow of electrons donated by NADPH to flavins FAD and FMN in the reductase domain of one monomer through BH4 to the ferrous–dioxygen complex (Fe) in the oxygenase domain. When NADPH and BH4 cofactors are limiting, electron transfer becomes uncoupled from L-arginine oxidation, the ferrous–dioxygen complex dissociates, and superoxide ($O_2^{-\bullet}$) is generated from the oxygenase domain (Schmidt and Alp, 2007).

Our data indicate that *barolo* mutants lacking the Golgi pool of CoQ10 die due to the accumulation of oxidative damage in cardiovascular tissues caused by ROS produced by eNOS. Here, we suggest a speculative mechanism whereby UBIAD1 is required in the Golgi compartment to produce CoQ10 as an important cofactor for eNOS-mediated NO production (Figure 7I). Such a model could fit with the oxido-reductive properties of CoQ10 and eNOS: the electron flux inside eNOS that is mandatory to produce NO from L-arginine might require, together with NADPH and BH4, also CoQ10. Such function would not be very different from what CoQ10 does in the mitochondrial electron transport chain (ETC) coupling electron transfer between an electron donor (such as NADH) and an electron acceptor (such as O_2) with the transfer of H^+ ions across the mitochondrial inner membrane. In the Golgi membranes

the flow of electrons within eNOS transfers electrons from NADPH to the flavins FAD and FMN, which have the capacity to reduce molecular oxygen. If the ETC is disturbed, such as in the absence of UBIAD1/CoQ10, the ferrous-dioxygen complex dissociates, and O^{-}_{2} is generated from the FMN oxygenase domain (Liu et al., 2002; Rigoulet et al., 2011). In cardiovascular tissues the CoQ10 produced by UBIAD1 in the Golgi compartment may be an important cofactor to maintain eNOS in a coupled conformation (e.g. required to produce physiological NO) and, eventually, quench leaking-uncoupled electrons. We speculate that when UBIAD1/CoQ10 in the Golgi compartment is absent or decreased (*bar* mutants and siUBIAD1-treated cells) eNOS switches to an "uncoupled" conformation producing oxidative species instead of NO and causing cardiovascular oxidative damage (Figure 7I). Further experiments need to be done to assess this hypothesis as well as the possibility that other NOS (e.g. nNOS) could be affected by the lack of Ubiad1 in vivo.

In summary, our study places UBIAD1 in a new pathway with important therapeutic implications for cardiovascular failure, such as the opportunity to decrease oxidative damage and counteract some of the side effects of statins. In addition, pharmacological or genetic stimulation of UBIAD1, as a new CoQ10 biosynthetic enzyme, represents a new promising therapeutic approach for anti-oxidant-related diseases such as aging and cancer.

EXPERIMENTAL PROCEDURES

Zebrafish strains, mapping, genotyping

Embryos and adult fishes were raised and maintained under standard laboratory conditions. *bar*^{s847} and *bar*^{t31131} mutants were generated by ENU mutagenesis as previously described (Jin et al., 2007).

Oxidative stress analyses in zebrafish embryos and cells

Oxidative damage was detected by immunofluorescence on agarose sections of zebrafish embryos with following markers: anti-8-hydroxy-2'-deoxyguanosine (1:500, Abcam) and anti-S-Nitroso-cysteine (1:250, Sigma).

ROS levels were quantitated in isolated human (HUAEC) and zebrafish (*Tg(Kdrl:GFP)*^{s843}) endothelial cells with CellROX Deep Red Reagent (Invitrogen; 2,5µM) and analyzed by FACS.

Western blot analyses

Western blot analyses were performed with following primary antibodies: mouse anti-UBIAD1 (9D4; 1:500), goat anti TERE1 (UBIAD1)-N16 (1:250; SantaCruz), mouse anti actin (1:5000; Sigma), mouse anti Rab11 (1:1000; BD Biosciences), rabbit anti HADHA (Hydroxyacyl-CoA Dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA Hydratase (trifunctional protein), Alpha subunit)(1:5000; Abcam).

Generation of human SCCD mutation in UBIAD1

Constructs containing the SCCD mutation N102S and D112G were introduced in pCS2+ plasmids and used for expression in zebrafish embryos as well as human ECs.

NO levels and NOS activity in zebrafish embryos and human ECs

NOS activity was assayed using the NOS Activity Assay Kit (Cayman Chemicals). NO levels were detected using the DAF-2 DA (Calbiochem).

Cell culture experiments

Human primary EC were purchased from Lonza and cultured according to the manufacturer's protocols (Lonza). Cells were electroporated with siRNA (Dharmacon). Detailed protocols are available on Supplemental Experimental Procedures.

HPLC analyses

Samples for HPLC-MS analysis were zebrafish embryos (n=25) or human HUAEC (2×10^6 cells). CoQ10 and CoQ9 levels were detected by HPLC-MS or HPLC-UV (as indicated). Total cholesterol from zebrafish embryos was analyzed by HPLC-UV. Vitamin K2 from zebrafish embryos and cell lysates was analyzed by HPLC-MS/MS. Metabolomics experiments were carried on ECs and zebrafish embryos from bar^{t31131} and bar^{s847} heterozygote intercrosses. 4-Hydroxy-benzoic acid-$^{13}C_6$ (Euriso-Top) was stocked in DMSO and dissolved in cell culture medium (1mM) or microinjected in zebrafish embryo at one cell stage (2mM). ECs or embryos were extracted after 48 or 72 hours, respectively, after labeled precursor administration. Extracts were quantified by HPLC-MS for following molecules: CoQ10, CoQ9, and CoQ9-$^{13}C_6$, CoQ10-$^{13}C_6$. For detailed procedure of sample extraction and analysis refer to Supplemental Experimental Procedures.

Exogenous CoQ10 and Vitamin K administration

All chemicals and solvents for liposomal CoQ10 and Vitamin K formulation were

purchased from Sigma Aldrich and Avanti Polar Lipids. Liposomal SINAMIT® was a gift from Dr. Franz Enzmann (MSE Pharmazeutika GmbHT), liposomal LiQsorb® was from Tishcon Corp. MitoQ was synthetized as previously reported (Murphy and Smith, 2007). All CoQ10 and vitamin K formulations were tested for toxicity and microinjected at specific concentrations into zebrafish embryos at one cell stage.

See Supplemental Data for more details and all other experimental procedures.

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FIGURE LEGENDS

Figure 1: Loss of Ubiad1 induces cardiovascular failure in zebrafish embryos by increasing oxidative stress.

(A-B) Siblings (sib) and *barolo* ^{t31131} (bar) mutant embryos at 65 hpf. (A) Vascular hemorrhages (asterisks) and heart failure (arrowhead) in *bar*. (B) *Tg(kdrl:GFP)*^{s843} bar show vascular integrity defects (arrows) and collapsed endocardium (arrowhead). Scale bar, 300μm. (C) *Tg(Fli1a:GFP)*^{y1} bar show severe loss of cranial network vasculature and fragmented endothelium (arrow) at 65hpf. (D) Confocal images of trunk vasculature of *Tg(kdrl:GFP)*^{s843} bar report loss of ECs at the level of Se, DA (arrows) and PCV. Scale bar, 50μm. (E) $NADP^+$/NADPH ratio at 60hpf. Increased ratio is indicative of oxidative stress state. (F) Real-time PCR analysis show up regulation of the antioxidant genes *glutathione peroxidase1a* (*gpx1a*) and *heme-oxigenase1* (*hmox1*) in *bar* at 56hpf. Data are means ± SD. (G) Quantification of oxidative stress level in ECs derived from *Tg(kdrl:GFP)*^{s843} embryos at 56hpf. (H) Immunofluorescence analyses for S-Nitroso-Cysteine (SNO-Cys, red) and DNA(blue) in *Tg(kdrl:GFP)*^{s843} bar and sib embryos at 60hpf. Upper left: heart transverse sections; upper right: quantification of SNO-Cys positive cells. Lower left: trunk vasculature transverse sections; lower right: quantification of SNO-Cys positive cells. Scale bar, 75 μm. Specific SNO-Cys signals were also detectable in pronephros and myocardium of *bar* (arrowhead). (I) Immunofluorescence analyses for 8-hydroxy-2'deoxyguanosine (8OHdG, red) and DNA (blue) in *Tg(kdrl:GFP)*^{s843}*bar* and sib at 56 hpf. Upper left: heart transverse sections; upper right: quantification of 8OHdG positive cells. Scale bars, 50 μm. Lower left: trunk vasculature transverse sections; lower right; quantification of 8OHdG positive. Scale bar, 20μm. *bar* show specific

8OHdG staining in endocardial and endothelial cells (arrows) and myocardium (arrowhead). (L) TUNEL-positive (red) cells show apoptosis in endocardium and endothelium (arrows). Upper left: heart transverse sections of *Tg(kdrl:GFP)*^{s843} bar ^{s847} and sib ; upper right: quantification of TUNEL-positive cells. Lower left: transverse sections of trunk vasculature; lower right: quantification of positive cells in DA and PCV. Scale bars, 20 μm. (M) Genetic map of the zebrafish barolo locus. The SSLP markers (z22307) was used to identify the "zero recombinant" region by analysis of 987 diploid mutants. (N) Schematic representation of zebrafish Ubiad1. UbiA: UbiA domain. (O) Western blot analysis of protein extracts from *bar*^{s847} and *bar*^{t31131} and respective siblings at 72hpf. (P) Embryos from *bar*^{s847} and *bar*^{t31131} heterozygote intercrosses were injected with mRNA encoding for wild-type *ubiad1* mRNA, *ubiad1*^{L62Q} or fluorescent protein *cherry* (ctrl). *bar*^{s847} as well as *bar*^{t31131} were not rescued by microinjection of *ubiad1*^{L62Q} mRNA.

A, atrium; V, ventricle; DA, dorsal aorta; PCV, posterior cardinal vein; BuA bulbus arteriosus; PD, pronephric ducts; E, endocardium; M myocardium. All data are means ± SEM **p<0.01, ***p<0.001.

See also Figure S1.

Figure 2: Ubiad1 is a new prenyltranferase required for CoQ10 biosynthesis in vertebrates.

(A) Schematic representation of the ^{13}C-based metabolomic approach used to demonstrate that Ubiad1 is a 4-OH-benzoic acid-prenyl tranferase enzyme. Coenzyme Q10 (CoQ10) biosynthesis starts with formation of a hydroxy-benzoic acid head group (4-OH-benzoic acid) and a lipophilic polyisoprenoid tail (polyprenyl-PP). The aromatic precursor of the benzoquinone ring (4-OH-benzoic acid) derives from tyrosine, an essential amino acid in vertebrates. Synthesis of the polyisoprenoid tail takes place in the cytoplasm starting from acetyl-CoA through the mevalonate pathway (which is shared in common with the cholesterol and dolichol biosynthetic pathways). The polyisoprenoid tail is assembled by polyprenyl diphosphate synthase, which is responsible for determining the number of isoprene units (designated as n). Next critical step is the condensation of the polyisoprenoid chain with the benzoquinone ring to form the 4-hydroxy-3-polyprenyl benzoic acid intermediate (PPHB) by a 4-OH-benzoic-polyprenyl-tranferase (UbiA-containing enzyme). The subsequent reactions (O-methylations and C-methylations, hydroxylations and decarboxylations) modify the structure of the aromatic ring, but some of the enzymes involved are still unknown. Exogenous 4-OH-benzoic acid-$^{13}C_6$ containing heavy carbon element (^{13}C) is administered to embryos and cells so that de novo synthesis of PPHB and therefore CoQ10 can be monitored by HPLC-MS analyses. Loss of UbiA-containing enzymes (red) blocks the condensation of the polyisoprenoid chain with the benzoquinone ring and then formation of CoQ-$^{13}C_6$. $^{13}C_6$-labeled compounds used or detected in this work are shown in green. (B) High resolution MS spectrum of lipid extracts from wild-type zebrafish embryos. The MS spectrum of the HPLC fraction containing CoQ10 reveals the analyte as [CoQ10-Na^+] at *m/z* = 885.6751.

(C) HPLC-MS analyses show that *bar* mutants are characterized by a reduced level of endogenous CoQ10 in comparison to their siblings. (D) Exogenous supplement of CoQ10, but not of VitaminK2, can efficiently rescue bar mutants. $CoQ10^{F1}$; liposomal CoQ10 preparation 0,6 mM; $CoQ10^{F2}$, LiQsorb® Liposomal CoQ10 Gel 0,6 mM; $COQ10^{F3}$, SANOMIT® nano-particles CoQ10 liquid, 6,9 mM; VitK ; Liposomal Vitamin K2 0,3 mM. (E-F) Levels of $^{13}C_6$-labeled CoQ10 (CoQ10-$^{13}C_6$) and $^{13}C_6$-labeled CoQ9 (CoQ9-$^{13}C_6$) are detected by HPLC-MS in *bar* mutants and siblings at 72hpf. Loss of Ubiad1 expression protein significantly reduces *de novo* CoQ10-$^{13}C_6$ synthesis by 78% (E) and *de novo* CoQ9-$^{13}C_6$ synthesis by 66,5% (F). All data are means ± SEM **p<0.01,***p<0.001.

See also Figure S2.

Figure 3: Block of mevalonate pathway causes cardiovascular failure in zebrafish embryos by reducing CoQ10 synthesis.

(A) wild-type (wt) embryos at 72hpf treated from 54hpf with statin (mevastatin) or DMSO. Statin treatments induce a bar-like phenotype which is characterized by hemorrhages (asterisk) and heart failure (arrowhead). Scale bar, 300 μm. (B) 3D projections of trunk vessels at 72hpf of DMSO and statin-treated zebrafish embryos. Statin treatments induce specific endothelial vessels regression and fragmentation in DA and Se (arrows). Scale bar, 100 μm. (C) Quantification of bar-like phenotype after statin treatments. MEV, mevastatin 500nM; SIM; simvastatin 500nM; MEN, mevinolin 500nM. Levels of CoQ10 (D) and cholesterol (E) detected by HPLC-UV analyses in statin-treated embryos. Bright-field (F) and fluorescent (G) images of *Tg(kdrl:GFP)*^{s843} *ubiad1*$^{+/+}$ and *ubiad1*$^{+/-}$ embryos at 65hpf treated with mevastatin (2.5nM and 5nM) from 32 to 50hpf. Treatment with low dose of statin caused hemorrhages (asterisks) and altered heart morphology (arrowheads) in *ubiad1*$^{+/-}$ but not in *ubiad1*$^{+/+}$ embryos. Although treatment with low dose of statin did not induce specific alterations of the main vasculature, *ubiad1*$^{+/-}$ embryos showed head vasculature, Se and DA regression; scale bar: 300μm. (H) Images of *Tg(kdrl:GFP)*^{s843} *ubiad1*$^{+/+}$ and *ubiad1*$^{+/-}$ embryos trunk vasculature show thinner and collapsed Se and DA morphology in statin-treated *ubiad1*$^{+/-}$ but not in *ubiad1*$^{+/+}$ embryos. Scale bar: 75μm. (I) Penetrance of *bar*-like phenotype at 65hpf , after treatment with mevastatin. *ubiad1*$^{+/-}$ embryos are significantly more sensitive to develop a *bar*-like phenotype than normal embryos (*ubiad1*$^{+/+}$). (J) CoQ10 supplemented embryos show less susceptibility to statin treatments in terms of developing a *bar*-like phenotype as indicated by histograms showing the percentage of statin-treated embryos having *bar*-like phenotype after exogenous CoQ10 delivery. (K) CoQ10 supplementation before

statin treatment prevents cardiac edema (arrowhead) and brain hemorrhages (asterisk) in *barolo*. Images of vehicle- and CoQ10-injected embryos after statin treatment. Scale bar, 300μm. (L) Histograms show oxidative stress in embryos after statin treatment with or without CoQ10.

All data are means ± SEM ***p<0,001.

See also Figure S3.

Figure 4: UBIAD1 is a prenyltransferase responsible for CoQ10 synthesis in the Golgi compartment.

(A-B) Confocal images of human ECs showing Ubiad1 colocalization with Golgi markers. (A) DNA (blue), human Ubiad1(red), and TGN46 (green). (B) DNA (blue), zebrafish Ubiad1-GFP (green), GM130 (red). Scale bar, 20μm. (C) High magnification confocal images of Golgi compartment showing colocalization between Ubiad1-GFP (green) and the Golgi marker γ-adaptin (red). Scale bar, 10μm. (D) Fluorescence images of human ECs transfected with Ubiad1-GFP and treated or not with brefeldin A (+BFA). After BFA treatment, Ubiad1 localization becomes diffuse throughout the cell. Scale bar, 10μm. (E) Golgi compartment (Golgi, ER and endosome fractions) and mitochondrial fractions from human ECs were analyzed by western blot with the following antibodies: γ–Adaptin and Rab11, as marker for Golgi/ER/endosome fractions, HADHA (Hydroxyacyl-CoA Dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA Hydratase (trifunctional protein), Alpha subunit) as marker for mitochondrial fractions. (F) Levels of $^{13}C_6$-isotope labeled CoQ10 (CoQ10-$^{13}C_6$) detected by HPLC-MS. Compared to control (siCTRL) the level of CoQ10-$^{13}C_6$ present in cells silenced for UBIAD1 (siUBIAD1) is significantly reduced in Golgi compartment but not in mitochondria. Data are means ± SD **$p<0.01$.

See also Figure S4.

Figure 5: SCCD **UBIAD1 variants rescue *barolo* mutants and positively regulate CoQ10 synthesis**.

A) wild-type UBIAD1 (WT) or SCCD UBIAD1 isoforms (N102S; D112G) rescue *bar* mutants at a significant extent. (B) Western blot analysis of protein extracts from HUAEC transfected with plasmids encoding for wild-type UBIAD1 (WT) or SCCD UBIAD1 isoforms (N102S; D112G). (C-E) HUAEC were transfected with plasmids encoding for wild-type UBIAD1 (WT) or SCCD UBIAD1 isoforms (N102S; D112G) and total lipid extracts were quantitated for CoQ10-$^{13}C_6$ (C) and CoQ10 by HPLC-MS (D) and for cholesterol by HPLC-UV (E). Expression of all UBIAD1 proteins rises levels of CoQ10 as well as de novo synthetized CoQ10-^{13}C but does not change cholesterol levels in human ECs.

All data are means ± SEM **p<0.01, ***p<0.001.

Figure 6: Ubiad1 and Coq2 play different functions during development.
(A) Se integrity is affected in *Tg(kdrl:GFP)*^{s843} embryos injected with *ubiad1* morpholino (*ubiad1* MO) but not in *coq2* morphants (*coq2* MO). Scale bar, 100 μm. (B) Oxidative stress levels in entire *ubiad1* morphants (*ubiad1* MO), *coq2* morphants (*coq2* MO), and relative control (ctrl MO). (C) CoQ10 levels detected by HPLC-MS are significantly lower in *ubiad1* morphants (*ubiad1* MO), *coq2* morphants (*coq2* MO), *ubiad1* and *coq2* double morphants (*ubiad1+ coq2* MO) than control morphants (ctrl MO). (D). Embryos from *bar* heterozygote intercrosses were injected with *coq2* mRNA and mitochondria-targeted CoQ10 analog called MitoQ (10μM) or *cherry* mRNA and Decyl-TPP (10μM) as respective controls. Phenotypes were scored at 72hpf. *bar* phenotype is not caused by lack of mitochondria CoQ10. (E) Silecing of both CoQ10 biosynthetic enzymes (siUBIAD1 and siCOQ2) cause increase of total cellular ROS in ECs. (F) Levels of CoQ10 detected by HPLC-MS in total lipid extracts from HUAEC silenced for UBIAD1 (siUBIAD1), COQ2 (siCOQ2) or both (siUBIAD1+siCOQ2). UBIAD1 and COQ2 were silenced with 50pmoles (black bars) or with 100pmoles (gray bars). Levels of CoQ10 are significantly reduced in cells silenced for UBIAD1 or COQ2 expression. (G) Quantitative PCR analyses of UBIAD1 and COQ2 mRNA levels in HUAEC cells. (H) Quantification of loss of mitochondrial membrane potential ($\Delta\psi_m$) in HUAEC silenced for UBIAD1 (siUBIAD1) or COQ2 (siCOQ2). (I) Quantification of lipid peroxidation by MDA adducts levels in HUAEC silenced for UBIAD1 (siUBIAD1) or COQ2(siCOQ2).

All data are means ± SD **p<0,01, ***p<0,001.

See also Figure S5.

Figure 7: NOS activity in cardiovascular tissues is regulated by UBIAD1 and CoQ10. (A) Knock-down of *endothelial nitric oxide synthase 1 by* morpholino (*nos1* MO) reduces the penetrance of *bar* phenotype at 72 hpf. (B) Knock down of *nos1* rescue endothelial integrity defects of *bar* mutants. Images of Se of *bar* at 72hpf injected with *nos1* morpholino (*bar* + *nos1*MO) or control morpholino (*bar* + *ctrl* MO) Scale bar, 50μm. (C) Bright-field images (left) and fluorescent micrographs showing the trunk vasculature (right) of *Tg(kdrl:GFP)*^{s843} bar treated from 48hpf with the specific eNOS inhibitor L-NAME (500μM). Heart failure (arrowhead) and endothelial regression (arrows) were fully rescued by L-NAME treatment. Scale bar, 100μm. (D) Penetrance of the *bar* phenotype at 65hpf is significantly decreased by inhibition of eNOS activity with L-NAME treatment from 32hpf. (E) Oxidative stress level in ECs derived from *Tg(kdrl:GFP)*^{s843} bar mutant embryos is decreased by L-NAME treatment. (F) eNOS inhibition by L-NAME treatment significantly reduces ECs positive for S-Nitroso-cysteine (SNO-Cys) in DA and PCV of bar embryos. (G) Silencing of eNOS (sieNOS) rescues oxidative stress induced by the lack of UBIAD1 (siUBIAD1). Silencing eNOS alone did not decrease ROS level in ECs. (H) Silencing of UBIAD1 gene (siUBIAD1) causes a significant decrease of eNOS activity in ECs evaluated as [^{3}H]-L-citrulline. (I) Schematic representation of Ubiad1 molecular function in CoQ10 production and maintenance of nitrix oxide (NO) signalling. In wild-type cells, Ubiad1 localizes in the Golgi compartment and produces CoenzymeQ10 (CoQ10), an antioxidant molecule, important to counteract oxidative damage in particular in cellular membranes (cytosol and plasma membrane). In the Golgi compartment CoQ10 as an electron carrier might also play a fundamental role as a cofactor for eNOS activity by maintaining its "coupled" conformation and allowing normal NO production. On the other hand, lack of UBIAD1 and lowering of

CoQ10, as occurs in the cardiovascular tissues of *bar* mutants, might "uncouple" eNOS causing loss of NO production and consequently reactive oxygen species overload leading to cellular oxidative damage (e.g. lipid peroxidation). Thereby, the "*barolo"* phenotypes can be rescued by impairment of eNOS activity or expression.

Data are means ± SEM *p<0.05**p<0,01***p<0,001.

See also Figure S6.