EARLY DEVELOPMENTAL PATHOLOGY DUE TO CYTOCHROME C OXIDASE DEFICIENCY IS REVEALED BY A NEW ZEBRAFISH MODEL
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Running head: COX deficiency in a new zebrafish model

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Deficiency of cytochrome c oxidase (COX) is associated with significant pathology in humans. However, the consequences for organogenesis and early development are not well understood. We have investigated these issues using a zebrafish model. COX deficiency was induced using morpholinos to reduce expression of CoxVa, a structural subunit, and Surf1, an assembly factor, both of which impaired COX assembly. Reduction of COX activity to 50% resulted in developmental defects in endodermal tissue, cardiac function, and swimming behavior. Cellular investigations revealed different underlying mechanisms. Apoptosis was dramatically increased in the hindbrain and neural tube and secondary motor neurons were absent or abnormal, explaining the motility defect. In contrast, the heart lacked apoptotic cells but showed increasingly poor performance over time, consistent with energy deficiency. The zebrafish model has revealed tissue-specific responses to COX deficiency and holds promise for discovery of new therapies to treat mitochondrial diseases in humans.

Cytochrome c oxidase (COX)1 is the terminal enzyme in the mitochondrial respiratory chain. Embedded in the inner mitochondrial membrane, COX couples the vectoral movement of protons across the inner membrane to the transfer of electrons from reduced cytochrome c to molecular oxygen. Mammalian COX consists of thirteen subunits, ten of which (COXIV-COXVIII) are encoded by nuclear DNA (nDNA). The other three subunits (COXI, COXII, and COXIII) are encoded by the mitochondrial DNA (mtDNA) and constitute the hydrophobic/catalytic core of the enzyme. Assembly of COX is a complicated process involving multiple assembly factors and chaperones including COX10-11, COX15-20, 23, SCO1, SCO2, and SURF1 (1). Deficiency in COX activity is associated with significant pathology usually affecting highly metabolic tissues including brain, muscle, and eyes (2,3). For example, COX deficiency is commonly associated with Leigh syndrome, an early-onset disorder resulting in progressive central nervous system degeneration and early lethality (4,5). The majority of cases of COX-deficient Leigh syndrome are a result of mutations in SURF1 (6,7). Mutations in SURF1 impair the incorporation of subunit II, resulting in a buildup of an early COX assembly intermediate, decrease in other COX subunits, and a decrease in COX activity (8-11). COX deficiency may also be complicated by cardiac pathology including hypertrophic and dilated cardiomyopathies (12,13) and conduction defects (14).

COX deficiency is detrimental during the prenatal period as well. The incidence of this disorder has prompted the application of COX activity assays to prenatal samples obtained by chorionic villus biopsy and amniocentesis in specific cases (15,16). Prenatal diagnostics of this type have preparatory value for parents and physicians and may also be used to explain previous pregnancy losses (17). However, our incomplete understanding of the metabolic requirements of different tissues and organs during development diminishes the predictive value of these assays.

Initial studies using specific inhibitors of COX suggest that there is a large reserve of COX activity. The biochemical threshold of COX

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1 The following abbreviations are used: COX, cytochrome c oxidase; MO, morpholino; hpf, hours postfertilization; dpf, days postfertilization; qrtPCR, quantitative reverse transcriptase polymerase chain reaction.
inhibition that can be tolerated before mitochondrial respiration is impaired was observed to be 60% to 86% in isolated rat mitochondria (18-22). Studies in human cell lines using a specific COX inhibitor and mtDNA lesions revealed a range of thresholds from 21% to 82% depending upon cell type (23,24). Clinically, patient samples with COX deficiencies between 50% and 85% are associated with severe pathologies (25-29), implying that the biochemical threshold of COX impairment is exceeded. However, questions as to how these levels of COX deficiency affect a rapidly developing organism, tissue and organ sensitivity, and cellular responses remain unanswered. A vertebrate animal model of COX deficiency is therefore desirable.

Recently mouse has been used as a model system for COX deficiency. Agostino et al. generated a mouse null in Surf1 (30). The Surf1 knockout mouse exhibited defective COX activity in tissue samples, muscle weakness, motor deficits, and early-post natal lethality. However, there was approximately 90% post-implantation embryonic lethality and the 10% of mice that survived embryogenesis did not display any obvious neurological symptoms. The relative inaccessibility of in utero mouse embryonic development precludes simultaneous biochemical analysis and thorough investigation of organ development and function during the prenatal period, when the vast majority of the Surf1 knockout mice died. Furthermore, the cost and technical requirements of generating transgenic mouse mutants impede the creation of allelic series of mutants with different levels of reduction of COX components, which are needed to pinpoint the amount that best recapitulates human disorders. Therefore, we chose to dissect the mechanisms of COX deficiency associated with early developmental pathology and lethality in a zebrafish model.

Zebrafish develop ex-utero protected from the outside environment by a transparent chorion. Embryonic and larval zebrafish tissue is also transparent, a trait that has made zebrafish a powerful tool for studying development and physiology in real-time (31,32). Furthermore, compared to mouse, zebrafish is readily amenable to the analysis of dose dependent loss of function phenotypes using antisense morpholino (MO) oligonucleotides. MOs are gene-specific and block expression by binding RNA and sterically inhibiting either translation (33) or splicing (34). They can be titrated to inhibit protein expression to various degrees, resulting in a range of phenotypes from wild type to complete loss of function. The full spectrum of genetic mutations that precipitate COX deficiency is unknown. It is likely that certain mutations, or subsequent protein deficits, have not been identified because they are necessarily lethal prenatally. This may explain why no patients have been described to date with a mutation in a nDNA encoded structural component of COX (35). Tibrited MO knockdown is therefore a promising means by which the requirement for certain gene products could be identified.

In the current work we demonstrate that MOs are an effective means of inducing COX deficiency in zebrafish. We show that MOs designed to reduce expression of Surf1, an assembly factor, and CoxVa, a structural component of COX, effectively impair COX activity in developing zebrafish. We describe detrimental effects of COX deficiency on cell biology, organ development, and organism physiology. These include increased activation of programmed cell death, defective secondary motor neuron development, impaired endodermal organ development, and cardiovascular dysfunction. The progressive developmental pathologies culminate in cardiac failure and early lethality. These data provide insights into the mechanism of COX deficiency associated with embryonic loss and developmental pathology in humans. The utility of this zebrafish model is enhanced by its potential application in forward genetic screens and high throughput small molecule screens to discover modifier genes of COX activity and potential treatments of COX deficiency respectively.

**EXPERIMENTAL PROCEDURES**

*Zebrafish Maintenance and Care.* Zebrafish (*Danio rerio*) embryos were collected from natural crosses of genotype ABC or Tg(*gata2:GFP*) adults at the University of Oregon Zebrafish Facility. Embryos were reared at 28.5°C on a 14 hr. light/10 hr. dark cycle according to standard protocols (36). Embryos were staged by hours postfertilization (hpf) and days postfertilization (dpf) according to Kimmel et al. (32). All
zebrafish experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee.

**Morpholino and RNA injections.** Translation and splice-blocking MOs were designed by Gene Tools, LLC. MOs were solubilized in sterile water then diluted to working concentrations and injected as in Nasevicius et al. (33). A 1% phenol red solution was added to working concentrations for visualization of injection volume. A translation-blocking MO, which binds at the ATG start, was designed against cox5aa (5’ ACAGTCGAAGGGCGGCTCGGAACAT 3’). The cox5ab-MO (5’ AGCTGGAGACACAGCGAACACACA 3’), which binds at the fourth exon splice donor site, was designed to block appropriate splicing of target mRNA. These MOs had a 3’ fluorescein modification and fluorescence was used to select fish that had homogeneously incorporated the MOs. A splice-blocking galT-MO (5’ AAATCATTATGCACTCACCTGATGG 3’), which binds the second exon splice donor site of 1,3 galctosyl transferase, a non-mitochondrial enzyme, was used to control for non-specific effects of injection. p53-MO (5’ GCGCCATTGCTTTGCAAGAATTG 3’), a translation blocker, was used to inhibit p53-dependent apoptosis (37). emx3-MO (5’ GACGTGCTCGCTTACCTCGTGTC 3’), which binds the second exon splice acceptor site of the empty spiracles homeobox 3 gene, was used as a positive control for the effects of p53-MO.

**mRNA synthesis.** cox5aa mRNA with silent mutations at the MO binding site and surf1 mRNA were transcribed out of pcDNA3 using the T7 mMessage mMachine kit (Ambion). RNA was reverse transcribed to cDNA using the Superscript III First Strand Synthesis Kit (Invitrogen) with oligo(dT). The following primers were used for amplification of a surf1 fragment spanning the MO binding site: forward 5’ GTCAACAATCACTTCCACTGTCG 3’, reverse 5’ TTTGCTCATTTCACACTCGTGG 3’. rtPCR was carried out in an MJ Research PTC-225 Peltier Thermal Cycler. The cycling parameters were 95°C for 5 minutes then 35 cycles of 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, and finally 72°C for 10 minutes then held at 10°C.

**qrtPCR.** cDNA was synthesized from extracted RNA as described above. Genomic DNA was extracted from samples of 15 zebrafish at 3 dpf using a DNeasy Tissue Kit (Qiagen). The following primers were used in quantitative real-time rtPCR reactions: cox1 forward 5’ ACTTAGCCAACCAGGAGCAC 3’, cox1 reverse 5’ GGGTGAAGAAGTCAGAAGC 3’ (38), β-actin forward 5’ TTCTGGTCGTACTCTGGATTG 3’, and β-actin reverse 5’ ATCTTCATCAGGTAGCTCGTGCAG 3’ (38). Reactions were carried out in 25 µL volumes with 1X SYBER Green PCR Master Mix (Molecular Probes), 7.5 pmol of both forward and reverse primers, and 200 ng of DNA. All samples were run in triplicate. qrtPCR was performed in 96 well plate format in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Cycling parameters were 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The run finished with 95°C for 15 seconds followed by a 60°C for 15 seconds dissociation step.

**Microscopy.** Images of live zebrafish were obtained with a digital camera on a Zeiss Axioplan. Fluorescent images of live stained and fixed fish were captured on a Nikon Eclipse TE2000-U using epifluorescence or confocal lasers. Adobe Photoshop was used to adjust image brightness and contrast.

**Immunohistochemistry.** Zebrafish embryos were manually dechorionated and euthanized by an overdose of tricaine methane sulfonate (36). Embryos were fixed in 4% paraformaldehyde BT fix (36). 24 hpf and 31 hpf fixed embryos were
permeabilized in 0.5% triton in phosphate buffered saline (PBS). 48 hpf fixed embryos were permeabilized in 100% acetone at -20°C for 25 minutes. Permeabilized embryos were washed with 0.2% Tween-20, 1% BSA, and 1% DMSO in PBS and blocked in wash solution with 2% normal goat serum. Embryos were incubated with primary antibodies overnight at 4°C. For primary motor neuron detection 24 hpf embryos were incubated with zn1 monoclonal antibody (1:200) (39) and znpl monoclonal antibody (1:1000) (39), which were detected with goat anti-mouse Alexa 488 IgG1 and IgG2a (Molecular Probes) respectively. Secondary motor neurons were detected in Tg(gata2:GFP) transgenic zebrafish by incubation with rabbit polyclonal anti-GFP (1:200) (Molecular Probes) followed by goat anti-rabbit Alexa 488 (Molecular Probes). Initiation of apoptosis was detected by incubation with rabbit monoclonal anti-active caspase-3 (1:200) (BD Pharmingen) followed by goat anti-rabbit Alexa 594 (Molecular Probes). DAPI nuclear stain (1:1000) was added to the second to last wash.

Acridine Orange Staining. The vital dye acridine orange was used to identify apoptotic cells (41). 24 hpf, 31 hpf, 48 hpf, and 3 dpf live zebrafish were soaked in 0.5 µg/ml acridine orange for 15 minutes and washed five times in embryo medium (36) at room temperature in the dark.

Western Blotting. 20 zebrafish embryos at 3 dpf were homogenized in 100 µl 50mM Tris buffer pH 7.5 with a protease inhibitor cocktail (Sigma-Aldrich) using a Kontes Pellet Pestle. Samples were further disrupted by brief sonication with a Branson Sonifier 150. Sodium dodecyl sulfate was added to a final concentration of 0.5%. Insoluble material was removed by centrifugation at 25,000 g, then the protein concentration was determined by BCA assay according to manufacturer instructions (Pierce). Samples were electrophoresed on a 10-20% acrylamide gel using the Laemmli buffer system (42). Proteins were transferred from 1-D gels to PVDF membranes (0.45 µm pore size) as described (43). Membranes were blocked in a 5% milk/PBS solution overnight. Blots were probed with monoclonal antibodies against COXI and COXVa (Mitosciences Inc.) and a porin Ab-2 antibody (Calbiochem), all at a final concentration of 1 µg/mL in 1% milk/PBS solution. After washing three times for 5 minutes with 0.05% Tween-20 in PBS, goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson Immunolabs) was applied. Blots were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare).

RESULTS

Zebrafish have two copies of cox5a and one copy of surf1 genes. The sequence identified as NM_001024403 was recovered from an initial BLAST of the zebrafish genome, having 74% amino acid sequence identity to human COXVa (Fig. S1A). Knockdown of zebrafish cox5a with a MO designed against this sequence successfully impaired COX activity and development as described below. However, residual CoxVa protein was detected by Western blot. Therefore, another BLAST search of the zebrafish genome
was performed to look for a second cox5a gene. Due to an ancestral genome duplication, zebrafish have duplicate co-orthologs for a minimum of 20% of human genes (44). We identified a second sequence, XM_695484, as being 63% identical to human CoxVa and 76% identical to the other zebrafish gene at the amino acid level (Fig. S1A). NM_001024403 was named cox5aa and a translation blocking MO, cox5aa-MO, was designed against the ATG start site of this sequence. XM_695484 was named cox5ab and a splice-blocking MO, cox5ab-MO, was designed against the second exon splice acceptor site. cox5aa and cox5ab should encode functionally equivalent proteins. Here we refer to both proteins as CoxVa, as they are not distinguished by the antibody to human COXVa.

In a BLAST search of the zebrafish genome for surf1 one sequence, XM_678665, was identified with significant similarity to human SURF1. In a protein alignment, zebrafish Surf1 has 60% amino acid sequence identity to the homologous human sequence (Fig. S1B). A search for a duplicate gene recovered no other sequences with significant similarity. A splice-blocking MO, surf1-MO, was designed against the fourth exon splice donor site of this gene.

**Reduction of either CoxVa or Surf1 impairs COX activity.** Assembly of the COX holoenzyme requires the proper structural subunits and necessary assembly factors. Therefore, MO reduction of either CoxVa or Surf1 was expected to alter the amount of normally assembled and functional COX. To determine the effects of cox5aa-MO, cox5ab-MO, and surf1-MO on total zebrafish COX activity we measured the initial rate of cytochrome c oxidation by extracted zebrafish proteins from uninjected and MO injected samples of 20 to 30 fish at 3 dpf. Zebrafish embryos were injected with 20 ng of cox5aa-MO, cox5ab-MO, or surf1-MO. In titration experiments this dose resulted in a reproducible phenotype (described below) that was not lethal at 5 dpf in the majority of injected zebrafish. Another control group received 20 ng of galT-MO, which blocks splicing of α1,3 galactosyl transferase, a non-mitochondrial gene.2 The COX activity in each MO injected population was measured relative to an uninjected control in at least two separate experiments. For equivalent amounts of protein, COX activity in 3 dpf cox5aa-MO, cox5ab-MO, and surf1-MO samples was approximately half that of un injected and galT-MO controls (as shown in a representative experiment, Fig. 1A,B).

**Morpholino reduction of zebrafish cox5aa, cox5ab, and surf1 results in reduced COX subunits.** Surf1 deficient human patient cells exhibit decreased levels of COX protein subunits (9,10), presumably due to impaired COX assembly and subsequent proteolysis of subunits. We proposed that deficient production of the structural subunit CoxVa would similarly impair COX assembly in zebrafish. Using monoclonal antibodies to the human COX proteins that we showed cross-reacted with the zebrafish proteins of the predicted size (data not shown), we assessed the levels of CoxVa and CoxI in cox5aa-MO, cox5ab-MO, surf1-MO and control samples in Western blots. The level of CoxVa and CoxI were normalized to porin, an outer mitochondrial membrane protein, the level of which would not be expected to be altered. Proteins were extracted from samples of approximately 20 zebrafish at 3 dpf. As expected, the MO target protein, CoxVa, was decreased in cox5aa-MO and cox5ab-MO fish relative to porin compared to uninjected and galT-MO controls (Fig. 1C). A concomitant decrease in CoxI, relative to porin, was also noted.

Because no antibody was available to Surf1 in zebrafish to confirm MO inhibition of protein expression, we designed surf1-MO to inhibit splicing of the transcript. cDNA was prepared from RNA extracted from 3 dpf uninjected and surf1-MO injected embryos and used in rtPCR with primers to the third and fifth exons, which flank the MO binding site. A larger fragment was PCR amplified in the surf1-MO sample indicating failure to splice out the fourth intron (Fig. 1D). Western blot of uninjected and surf1-MO zebrafish revealed a decrease in CoxI relative to porin in the surf1-MO fish (Fig. 1E). The decrease in mtDNA encoded CoxI in cox5aa-MO, cox5ab-MO, and surf1-MO zebrafish indicates that COX assembly is impaired by reduced production of either a structural subunit or an assembly factor and that altered COX assembly results in secondary loss of other mtDNA encoded subunits in zebrafish.

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2 J. Bates and K. Guillemin, unpublished data.
mtDNA transcript levels are elevated in surf1-MO but not cox5aa-MO or cox5ab-MO zebrafish. The decrease in CoxI detected in cox5aa-MO, cox5ab-MO, and surf1-MO zebrafish could be due to proteolysis of unincorporated subunits and/or decreased mitochondrial biogenesis. To determine whether cox1 transcript abundance was altered by MO reduction of CoxVa or Surf1, quantitative rtPCR (qrtPCR) was performed comparing mtDNA encoded cox1 to nDNA encoded β-actin on cDNA synthesized from RNA extracted from 10 to 15 MO injected and control zebrafish at 3 dpf. The ratio of cox1 to β-actin transcripts was set to 1 in the uninjected samples. Compared to the uninjected control, the ratio of cox1:β-actin ranged from 0.9 to 1.2 in galT-MO controls over five experiments, 0.8 to 1.1 in cox5aa-MO fish over four experiments, and 0.9 to 1.1 in cox5ab-MO fish over five experiments (as shown in a representative experiment, Fig. 1F). These data show that transcript levels from mtDNA in fish injected with either cox5aa-MO or cox5ab-MO were unaffected relative to control samples. In contrast, the cox1:β-actin transcript ratio in surf1-MO injected fish ranged from 1.1 to 1.8 times control levels over six experiments (as shown in a representative experiment, Fig. 1F), implying activation of compensatory mechanisms when Surf1, but not CoxVa, is depleted. A similar upregulation of cox1 transcripts has been observed in COX deficient patient fibroblasts with SURF1 mutations (9,11).

The difference in transcript levels described above could reflect changes in the number of functional mitochondria. To quantify this, the cox1:β-actin ratio was measured by performing qrtPCR on extracted genomic DNA from control and MO injected 3 dpf zebrafish. While much variability was observed between genomic extractions, no significant difference was detected by one-way ANOVA between uninjected and MO samples (data not shown). Therefore regulation of subunit levels occurs at the protein level in all COX deficient samples and in addition, compensatory transcript regulation is observed in surf1-MO zebrafish.

COX deficiency results in abnormal organ development and physiology. The phenotypic effects of COX deficiency on development was assessed at 5 dpf, by which time wild type zebrafish have a functional heart (45,46), nervous system (47,48), and gut (49). The cox5aa-MO, cox5ab-MO, and surf1-MO fish all developed the same abnormal phenotypic traits. These included a shortened rostral-caudal body axis, no or uninflated swim bladder, no or delayed gut development, edematous and unabsorbed yolk sac at 5 dpf, microphthalmia (small eyes), no or diminished jaw tissue, and abnormal head shape (Fig. 2A,B). Larvae from all three COX deficient populations exhibited no or reduced swimming spontaneously and in response to touch (Movie S1A,B). Cardiovascular pathology consisted of significant pericardial edema (Fig. 2C,D), bradycardia (reduced heart rate), ventricular asystole (failure to contract), and failure to develop into a loop (Movie S2A,B). At 3 and 4 dpf the ventricle was observed to contract in series with the atrium. The bradycardia and pericardial edema was progressive, eventually leading to ventricular asystole at 5 dpf and death around 7 dpf. The cardiac pathology is consistent with the effects of other non-respiratory chain mitochondrial perturbations in zebrafish (50,51). Embryos injected with either buffer solution containing phenol red or with 20 ng of galT-MO developed equivalently to uninjected embryos.

Morpholino resistant RNA can rescue the COX deficient phenotype. To establish whether the described phenotype was specific to COX deficiency, MOs were co-injected with RNA designed to be resistant to the activity of the MO to test if they would restore expression of the target gene product. For the translation blocking MO, cox5aa-MO, a cox5aa construct was PCR amplified with silent mutations in the MO binding site, from which mRNA could be translated and used in rescue experiments. For the splice-blocking MO, surf1-MO, in vitro transcribed target mRNA was co-injected to rescue the described developmental phenotype. MOs were co-injected with a titration of the target mRNA for each experiment. Rescue experiments for each MO were performed a minimum of three times.

Phenotypes were scored at 5 dpf and qualitatively assessed as being mild (pericardial edema alone and with mild head malformation and uninflated swim bladder), moderate (having the complete developmental and physiological COX deficient phenotype described in the previous section), and severe (exaggeration of the moderate...
phenotype with severe rostral-caudal body axis truncation) (Fig. 3A). In a representative experiment, co-injection of 10 ng cox5aa-MO with 4 ng cox5aa mRNA doubled the percentage of normally developed and mild phenotype fish at 5 dpf compared to injection of 10 ng cox5aa-MO alone (Fig. 3B). Similarly, in another representative experiment, co-injection of 20 ng surf1-MO with 0.5 ng surf1 mRNA increased the percentage of 5 dpf fish with a normal phenotype by four-fold compared to injection of 20 ng surf1-MO alone (Fig. 3C).

Efforts to rescue the cox5ab-MO phenotype with in vitro transcribed cox5ab mRNA were unsuccessful (data not shown). This failure may have been due to an inability to isolate a clean sample of cox5ab mRNA, compounded with the presence of another in-frame start codon upstream of the presumed cox5ab transcriptional start. Although PCR amplification of cox5ab out of zebrafish cDNA from this upstream ATG was not successful, we could not rule out this being a functional start codon.

cox5aa-MO and surf1-MO interact synergistically to cause the COX deficient phenotype. If the abnormal phenotype observed in cox5aa-MO and surf1-MO fish were due to COX deficiency then there should be low doses of cox5aa-MO and surf1-MO that alone have little to no effect but together impair COX activity to a degree that can be read out in the phenotype. This hypothesis was tested in two separate experiments. In a representative experiment, nearly all fish injected with either 1 ng cox5aa-MO or 3.75 ng surf1-MO developed normally to 5 dpf. However, when 1 ng cox5aa-MO was co-injected with 3.75 ng surf1-MO, 34% of fish exhibited the COX deficient phenotype (Fig. 4). If the phenotype described previously were the result of two different metabolic or cellular impairments then a reduction in one pathway would have no effect on the endpoint of the other pathway. Instead a combination of cox5aa-MO and surf1-MO, each below the minimum level needed for an individual effect, had a synergistic effect on phenotype. These data indicate that while cox5aa-MO and surf1-MO have different gene-specific actions, they target the same pathway.

COX deficient zebrafish exhibit increased apoptosis. Mitochondria play a pivotal role in progression through apoptotic pathways (52,53). Therefore, COX deficient mitochondrial pathology might alter the homeostatic levels of apoptosis that normally occur during development. We utilized fluorescent stains of apoptotic cells to visualize these effects. Live 24 hpf, 31 hpf, 48 hpf, and 3 dpf zebrafish were stained with the vital dye acridine orange, a marker of apoptotic cells (41). More apoptotic cells were observed in cox5aa-MO, cox5ab-MO, and surf1-MO fish compared to uninjected and galT-MO controls at 24 hpf and most dramatically at 31 hpf, subsiding at subsequent time points (Fig. 5A-E and data not shown). Apoptotic cells were stained throughout the head region, neural tube, and trunk of developing COX deficient fish but were not detected in the heart.

Because the acridine orange staining was most intense at 31 hpf, immunohistochemical staining of active caspase-3 was performed at this time point. The caspase-3 protease is activated during early apoptosis and plays an essential role in the proteolytic cleavage of a number of proteins involved in apoptosis (54,55). An increase in the amount of the active form of caspase-3 was observed in cox5aa-MO, cox5ab-MO, and surf1-MO injected zebrafish compared to uninjected and galT-MO controls, indicating progression through apoptotic pathways in COX deficient zebrafish (Fig. 5F-J). Active caspase-3 staining was especially clustered in the head and hindbrain of COX deficient fish.

A p53-MO has been shown to block p53-dependent cell death in zebrafish (37). Recently, Robu et al. have proposed that an increase in p53-dependent cell death is an off-target non-specific effect of some MOs and they showed that it can be rescued by co-injection of a p53-MO of the same sequence (56). Using a MO to empty spiracles homeobox (emx3-MO), which targets a transcription factor important for brain development (57) and has been previously shown to induce such non-specific cell death, we saw similar levels of acridine orange staining as in surf1-MO injected fish. As previously observed by our colleagues, co-injection of the same p53-MO with emx3-MO ameliorated the apoptosis detectable with acridine orange staining in a dose dependent manner. In contrast, when p53-MO

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3 G. Aspock and M. Westerfield, unpublished data.
was injected with either 10 ng or 20 ng of surf1-MO, which induced high levels of apoptosis, little to no amelioration of the apoptosis detected by acridine orange staining was observed. These data argue that the increased apoptosis observed in the COX deficient zebrafish is not an off-target effect of MO injection.

Motor neuron development is impaired in COX deficient zebrafish. At 5 dpf cox5aa-MO, cox5ab-MO, and surf1-MO injected fish had severely impaired motility, with no or diminished swimming spontaneously and in response to touch (Movie S1A,B). These motility defects, along with the staining of apoptotic cells in the neural tube of COX deficient fish at 31 hpf (Fig. 5C), prompted us to examine the effects of COX deficiency on motor neuron development. The axial muscles of zebrafish are innervated by primary and secondary motor neurons, which are distinguishable by location, size, and axonal pathways (58). Primary motor neurons develop earlier and are responsible for the spontaneous contractions that can first be observed at 17 hpf (59). At 26 hpf cox5aa-MO, cox5ab-MO, and surf1-MO zebrafish displayed spontaneous contractions that were comparable to those seen in uninjected and galT-MO controls (Movie S1C,D). Furthermore, immunohistochemical assays using primary antibodies to zn-1 and znp-1, primary neuronal markers at 24 hpf (39), confirmed normal development of primary motor neuron structure in all three COX deficient zebrafish populations (Fig. 6A,B).

Axonal outgrowth of secondary motor neurons, which are most similar to mammalian and avian motor neurons (60), occurs later than primary motor neurons (61). They are also more numerous and some innervate more than one hemisegment (58,62). To assess the effects of COX deficiency on secondary motor neuron development, cox5aa-MO, cox5ab-MO, and surf1-MO were injected into embryos of Tg(gata2:GFP), a transgenic line of zebrafish that express GFP in a subset of secondary motor neurons and occasionally in descending interneurons and Rohon-Beard cells (63). To enhance the fluorescent signal from secondary motor neurons, 48 hpf Tg(gata2:GFP) embryos were fixed and immunohistochemically stained with a primary antibody to GFP. Development of secondary motor neurons in the majority of cox5aa-MO, cox5ab-MO, and surf1-MO zebrafish was severely impaired (Fig. 6). Most axons in the rostral two-thirds of the COX deficient fish were absent (Fig. 6D). Other secondary motor neuron axons developed but were stunted or irregularly spaced compared to uninjected and galT-MO controls (Fig. 6E). Axonal disruptions were often accompanied by a decrease in the number of secondary motor neuron cell bodies in the neural tube (Fig. 6F). The caudal one-third of the COX deficient zebrafish generally displayed more secondary motor neuron axons and these were more likely to have normal morphology than in the rostral portions. These data may indicate an increased vulnerability of rostral secondary motor neurons. Alternatively, as motor neurons develop from rostral to caudal (64,65), it may be that disruption of secondary motor neurons is a consequence of temporal biochemical derangements, possibly reflecting dilution and decreased efficacy of the MOs.

DISCUSSION

The effects of COX deficiency in humans are usually manifest in tissues with high metabolic activity. However, the clinical presentation of COX deficiency is variable in terms of the combination of tissues affected and severity of pathology. Onset of pathology is generally early and has been detected in the prenatal period. To date, an incomplete understanding of the genetic causes of COX deficiency (66) and the variability in presentation (35) have hindered our comprehension of pathophysiologic mechanisms. Previous studies in mouse have yielded important information, although the relative inaccessibility of in utero development prevents a thorough dissection of the effects of COX deficiency on early development. Additionally, the all-or-none phenotypes that result from deleting a gene preclude investigation of gene dosages required for development. Zebrafish offer an attractive alternative disease model because they develop ex-utero, are transparent in embryonic and larval stages, and rely on many of the same major organs and systems as humans (67). By titrating the injection of MOs targeting a COX structural subunit, CoxVa, and an assembly factor, Surf1, we have been able to reduce the level of COX activity to a degree that impairs normal development but is
not immediately lethal. Capitalizing on the unique characteristics of zebrafish, we have investigated the effects of COX deficiency on organ development and function, revealing zebrafish as an informative and powerful model of COX deficient mitochondrial disease.

MOs designed against *cox5aa*, *cox5ab*, and *surf1* reduced COX activity in developing zebrafish to approximately 50% of wild type levels (Fig. 1A,B). The accompanying decrease in CoxI protein (Fig. 1C,E) likely indicates altered enzyme assembly and subsequent proteolysis of unused subunits, as has been suggested by work in SURF1 deficient human cell lines (10,11). Levels of mtDNA encoded *cox1* transcripts in *cox5aa-MO* and *cox5ab-MO* fish were approximately equal to control samples, indicating little or no secondary effects of CoxVa reduction on mitochondrial biogenesis (Fig. 1D). However, the decrease in CoxI induced by MO reduction of Surf1 was accompanied by an upregulation of *cox1* transcripts (Fig. 1E), which implies stimulation of a compensatory feedback mechanism in response to the reduction of Surf1 but not CoxVa. Inadequate Surf1 may itself instigate upregulation of mtDNA transcripts. Alternatively, the loss of Surf1 likely stalls assembly of COX at a different point than loss of CoxVa and the transcript level compensation may be secondary to the assembly effects. Mammalian COX assembly proceeds through a series of intermediates (68). The first is COXI alone followed by the addition of COXIV. The next phase involves incorporation of COXII, COXIII, and all nuclear encoded factors except subunits VIa and VIIa or VIIb, which are added in the last phase before dimerization. Work by Stiburek et al. and Williams et al. identified an assembly intermediate composed of subunits I, IV, and Va, implying that the SURF1 mediated incorporation of subunit II occurs after Va is added (69,70). According to this model, MO reduction of zebrafish Surf1 would disrupt assembly at a later stage than loss of CoxVa, providing a possible temporal explanation for the differing effects on mtDNA transcripts.

MO reduction of either the CoxVa structural subunit or the Surf1 assembly factor resulted in decreased COX activity to approximately 50% of that in control fish (Fig. 1A,B). Pooled samples of live zebrafish with greater than 57% COX impairment were not identified at 3 dpf. In contrast, studies performed on tissue samples and cell culture showed that COX activity could be impaired by as much as 82% in a human cell line (23) and 88% in isolated rat kidney mitochondria (22) without affecting cellular respiration. With these types of samples, however, threshold represents an arbitrary parameter of assaying mitochondrial throughput rather than a phenotypic determinant. An understanding of the effects of cellular COX deficiency on tissue development, organ function, and organism survival requires a more complex model system. In our zebrafish model 50% impairment of COX activity was adequate for initial stages of zebrafish development but it was insufficient for maintenance of cellular homeostasis, cardiac function, development of the peripheral nervous system, and was lethal by 7 to 9 dpf due to cardiac failure.

Several controls were employed to demonstrate that these effects were specific to COX deficiency and not secondary to injection trauma or MO toxicity. First, three different MOs targeting COX factors, *cox5aa-MO*, *cox5ab-MO*, and *surf1-MO*, precipitated the same phenotype. Second, control fish injected with buffer containing phenol red or 20 ng of *galT-MO*, which targets a non-mitochondrial protein, developed the same as uninjected fish. Third, co-injections of MO with in vitro transcribed target mRNA rescued the COX deficient phenotype, significantly increasing the percentage of normally developed fish at 5 dpf (Fig. 3). Fourth, the doses of *cox5aa-MO* and *surf1-MO* could be titrated down to levels that individually have no phenotypic effect but when injected together synergistically induce the COX deficient phenotype (Fig. 4). Finally, co-injection of a target MO with *p53-MO* has been reported to alleviate apoptosis associated with general MO off-target effects (56). We observed no significant reduction in the COX deficient apoptosis in zebrafish co-injected with *surf1-MO* and *p53-MO* relative to *surf1-MO* alone (Fig. 5K) arguing against the phenotype being due to MO off-target effects.

Our investigations into the progression of pathology that culminates in the COX deficient phenotype revealed a dramatic increase in apoptosis, peaking at 31 hpf, in *cox5aa-MO*,
cox5ab-MO, and surf1-MO samples (Fig. 5A-E). Immunohistochemical staining of active caspase-3 showed that some, if not all, of this programmed cell death occurs through a caspase-dependent pathway (Fig. 5F-J). Furthermore, co-injection of p53-MO with surf1-MO failed to ameliorate the increase in apoptosis (Fig. 5K). Knockdown of p53 by MO technology may be insufficient to block all p53-dependent cell death in a COX deficient background. Alternatively, the observed increase in apoptosis may be p53-independent.

Interestingly, apoptotic cells were not identified in the heart of COX deficient zebrafish, suggesting that the cardiac pathology and eventual failure is a functional pathology due to energy failure, not an anatomic pathology secondary to cell loss. The fact that apoptotic cells were not identified in the heart is also indicative of the different cellular responses to COX deficiency, which may be a tissue dependent phenomenon reflecting different thresholds of tolerable COX impairment. Similarly, tissues mature at different rates and stages of organism development. The cellular energy requirements likely fluctuate depending upon stage of maturation or differentiation and may contribute to whether cells with the same level of COX deficiency undergo apoptosis or survive but are functionally impaired. Also, cells in different tissues probably have different sensitivities to stress and hence different propensities to undergo apoptosis in response to stress. Qualitatively, the COX deficient zebrafish hearts did not appear hypertrophic or dilated. However, it may be that the timeline over which heart failure occurred was not long enough to result in sufficient physiological feedback to precipitate identifiable changes in cardiac wall or chamber size. Electrophysiological studies would be required before attributing the observed ventricular asystole to heart block.

In contrast to the heart, extensive apoptosis was observed in the neural tube (Fig. 5C). This finding, along with the decreased motility phenotype at 5 dpf (Movie S1A,B), prompted us to investigate the development of motor neurons in the COX deficient background. Primary motor neuron development was unaltered by COX deficiency, as indicated by immunohistochemical staining of primary motor neurons at 24 hpf (Fig. 6A,B) and observations of normal spontaneous contractions at 26 hpf in cox5aa-MO, cox5ab-MO, and surf1-MO zebrafish (Movie S1C,D). Interestingly, development of secondary motor neurons, which more closely resemble mammalian motor neurons (60), was severely impaired in the COX deficient zebrafish. Secondary motor neuron axons extend later than primary motor neurons (61) and some innervate more than one hemisegment (58,62). In most cases the majority of secondary motor neuron axons in the rostral 2/3 of the COX deficient fish were absent (Fig. 6D). In some cases shortened and irregularly spaced axons were present (Fig. 6E). Secondary motor neuron cell bodies were also reduced in number (Fig. 6F). The temporal disparity in development of primary versus secondary motor neurons may account for the different effects of COX deficiency on these two populations of neurons. The maternal contribution of mitochondrial proteins and transcripts may be sufficient to allow normal development of primary motor neurons but insufficient to compensate for the MO reduction of COX activity during later secondary motor neuron development. Grunwald et al. have shown that a ned-1 mutation in zebrafish that results in central nervous system cell death and prevents formation of secondary motor neurons, but has no effect on primary motor neuron development, is sufficient to prevent coordinated swimming activity (71). Additionally, Cheesman et al. demonstrated that MO reduction of Nkx6.1, which also results in loss of secondary motor neurons and spares primary motor neurons but does not increase cell death, prevents swimming in response to touch (72). The lack of secondary motor neurons and increased apoptosis in the COX deficient zebrafish could therefore account for the impaired motility at 5 dpf.

Peripheral neuropathies have been described in a number of human mitochondrial diseases with COX deficiency and or sequenced COX mutations (25,73-76) including mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) (28), myoclonus epilepsy with ragged red fibers (MERRF) (76), and Leigh Syndrome (26,27,77). In a study of 43 patients Stickler et al. concluded that peripheral neuropathy is a common component of genetic mitochondrial disorders and is likely the result of dysfunctional cellular energy production (76). They propose that it is often unrecognized on clinical examination because of

Cox deficiency in a new zebrafish model
confounding neuromuscular degeneration. In humans, the peripheral nervous system pathologies are generally primary demyelination and axonal changes (26,27,29,76,78). Based on our findings using a zebrafish model, we postulate that COX deficiency during early development is particularly detrimental to the peripheral nervous system, preventing even the development of motor neurons. COX deficiency is also detrimental to cardiac function at this stage, which results in early lethality. A similarly severe phenotype has been documented in a human infant with a mutation in SCO2, another COX assembly factor (79). This patient died of heart failure at 2 months of age and autopsy revealed moderate-to-severe motor neuron loss from the ventral horns of the spinal cord (77). Together, these data indicate that we should anticipate cardiac and peripheral nerve pathology following diagnosis of COX deficiency in prenatal and early post-natal patients. Similarly, mitochondrial respiratory chain diseases should be included on a list of differential diagnoses for infants displaying signs of peripheral neuropathy and cardiac pathology.

Finally, zebrafish have been used in developmental screens to identify small molecules that modify development (80). Here we have identified a COX deficient developmental phenotype that includes cellular, organ, and organism pathology. To date, there are only a limited number of palliative and supportive treatments of COX deficient disease (81). The identification of a specific and titratable COX deficient phenotype in zebrafish has promising future application in screens for small molecule that ameliorate the biochemical and developmental pathologies.

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FIGURE LEGENDS

Fig. 1. Morpholino knockdown of *cox5aa*, *cox5ab*, and *surf1* impairs COX activity and results in reduced COX subunits. (A and B) The oxidation of ferrocytochrome c by extracted zebrafish protein from samples of 20 uninjected, *galT*-MO, *cox5aa*-MO, *cox5ab*-MO, and *surf1*-MO zebrafish at 3 dpf. COX activity was normalized to the uninjected control. (C and E) Zebrafish lysates were electrophoresed on 10-20% acrylamide gels under denaturing conditions. Membranes were blotted with monoclonal antibodies to CoxI, CoxVa, and porin. (D) cDNA from 3 dpf uninjectected and *surf1*-MO embryos was PCR amplified using primers to the third and fifth exons of *surf1*. The larger amplicon in the *surf1*-MO sample reflects failure to splice out the fourth intron. (F) Real-time quantitative rPCR amplification of *cox1* to *β-actin* transcripts in cDNA synthesized from RNA extracted from 3 dpf uninjected, *galT*-MO, *cox5aa*-MO, *cox5ab*-MO, and *surf1*-MO zebrafish. The ratio of *cox1* to *β-actin* transcripts in the uninjected sample was set to one and the other samples are described relative to the uninjected.

Fig. 2. COX deficiency results in abnormal organ development and physiology. (A) Normally developed 5 dpf uninjected zebrafish. (B) 5 dpf COX deficient zebrafish characterized by shortened rostral-caudal body axis, no or uninflated swim bladder, no or delayed gut development, microphthalmia, no or diminished jaw tissue, abnormal head shape, and yolk sac and pericardial edema. (C) 5 dpf uninjected zebrafish heart. The ventricle is looped behind the atrium. (D) 5 dpf COX deficient zebrafish heart. The heart is not looped and is surrounded by pericardial edema. sb=swim bladder, a=atrium, v=ventricle.

Fig. 3. MO resistant RNA can rescue the COX deficient phenotype. *cox5aa*-MO and *surf1*-MO were co-injected with in vitro transcribed target RNA resistant to the respective MO. Phenotypes were scored by severity and compared to those resulting from MO injection alone. (A) Scale of COX deficient phenotype severity. The mild phenotype includes pericardial edema alone and with uninflated swim bladder. The moderate phenotype includes all abnormalities described in Figure 2. The severe phenotype is an exaggeration of the moderate phenotype with severe rostral-caudal body axis truncation. (B) Pie charts of phenotypes resulting from 10 ng of *cox5aa*-MO alone and 10 ng of *cox5aa*-MO with 4 ng of *cox5aa* mRNA with silent mutations in the MO binding site. (C) Pie charts of phenotypes resulting from 20 ng of *surf1*-MO alone and 20 ng of *surf1*-MO with 0.5 ng of *surf1* mRNA.

Fig. 4. *cox5aa*-MO and *surf1*-MO interact synergistically to cause the COX deficient phenotype. The phenotypes of uninjected (n=66) and fish injected with 20 ng *galT*-MO (n=50), 20 ng *cox5aa*-MO (n=24), 20 ng *surf1*-MO (n=93), 1 ng *cox5aa*-MO (n=58), 3.75 ng *surf1*-MO (n=63), and 1 ng *cox5aa*-MO + 3.75 ng *surf1*-MO (n=70) were compared at 5 dpf. Percentages of normally developed fish are listed above each sample bar. 1 ng *cox5aa*-MO and 3.75 ng *surf1*-MO separately had little effect but together resulted in the COX deficient phenotype.

Fig. 5. COX deficient zebrafish exhibit increased apoptosis. Live 31 hpf zebrafish were stained with acridine orange, anesthetized with tricaine, and imaged with epifluorescence; (A) uninjected (B) *galT*-MO (C) *cox5aa*-MO (D) *cox5ab*-MO (E) *surf1*-MO. 31 hpf fixed zebrafish were stained with an anti-active caspase-3 antibody followed by goat-anti-rabbit Alex 594 (red) and DAPI (blue); (F) uninjected (G) *galT*-MO (H) *cox5aa*-MO (I) *cox5ab*-MO (J) *surf1*-MO. Scale bar: 300 µm for A,B,C,D,E,J; 240 µm for H,I. (K) Live 31 hpf zebrafish embryos were stained with acridine orange and visualized by epifluorescence. Staining was scored from + to +++, where + represents minimal scattered stained cells and +++ indicates staining of apoptotic cells throughout the fish too numerous to count. Control fish were not injected with MO. One dot represents one fish and the line signifies the group average.
Fig. 6. Secondary motor neuron development is impaired in COX deficient zebrafish, whereas primary motor neurons are spared. Immunohistochemical staining of 24 hpf zebrafish with zn1 and znpi antibodies followed by goat-anti-mouse Alexa 488 IgG1 and IgG2a revealed normal primary motor neuron axons extending from the ventral spinal cord in (A) uninjected and (B) COX deficient zebrafish. 48 hpf Tg(gata2::GFP) zebrafish expressing gfp in a subset of secondary motor neurons were fixed, immunohistochemically stained with anti-GFP followed by goat-anti-rabbit Alexa 488, and visualized on a confocal microscope. (C) Uninjected zebrafish with normally developed secondary motor neurons. COX deficient zebrafish secondary motor neuron phenotypes included (D) lack of axons, (E) stunted and irregularly spaced axons with decreased lateral branches, and (F) decreased cell bodies. Scale bar: 140 µm for A,B; 100 µm for C,D,E,F.
Figure 1

A

% COX activity

uninjected  galT-MO  cox5aa-MO  cox5ab-MO

B

% COX activity

uninjected  surfl-MO

C

uninjected  galT-MO  cox5aa-MO  cox5ab-MO

D

CoxI
porin

500bp
300bp

E

uninjected  surfl-MO

F

ccox1/β-actin transcription

uninjected  galT-MO  cox5aa-MO  cox5ab-MO  surfl-MO
Figure 2

A

2 mm

sb

B

2 mm

eye

v/a

C

D

v

a
Figure 3

A

B

cox5aa-MO
(n=92)
cox5aa-MO + cox5aa mRNA
(n=53)

C

surfl-MO
(n=93)
surfl-MO + surfl mRNA
(n=91)

1% 20% 12%
67%

4% 39%
34%

23%

22% 4% 20%
54%

22% 16% 13%
49%
Figure 4

A

![Bar chart showing the percentage of sample population with different phenotypes across different conditions.]

- **Phenotypes**: normal and abnormal
- **Conditions**: un.injected, 200 ng gat/MO, 200 ng coxaa/MO, 200 ng surfl/MO, 100 ng coxaa/MO, 3.75 ng surfl/MO, 1 ng coxaa/MO + 3.75 ng surfl/MO
- **Data points**:
  - un.injected: 97% normal, 3% abnormal
  - 200 ng gat/MO: 92% normal, 8% abnormal
  - 200 ng coxaa/MO: 0% normal, 100% abnormal
  - 200 ng surfl/MO: 4% normal, 96% abnormal
  - 100 ng coxaa/MO: 93% normal, 7% abnormal
  - 3.75 ng surfl/MO: 95% normal, 5% abnormal
  - 1 ng coxaa/MO + 3.75 ng surfl/MO: 66% normal, 34% abnormal
Figure 5

|                    | uninjected | galT-MO | cox5aa-MO | cox5ab-MO | surf1-MO |
|--------------------|------------|---------|-----------|-----------|----------|
| Acridine orange    | A          | B       | C         | D         | E        |
| Active caspase-3   | F          | G       | H         | I         | J        |

K

| p53-MO | 0 | 9 | 18 | 24 | 0 | 9 | 18 | 0 | 9 | 18 | 0 | 24 |
|--------|---|---|----|----|---|---|----|---|---|----|---|----|
| emx3-MO| 0 | 0 | 0  | 0  | 2.5| 2.5| 2.5| 0 | 0 | 0  | 0 | 0  |
| surf1-MO| 0 | 0 | 0  | 0  | 0  | 0  | 0  | 10| 10| 10 | 10| 20 |
| # of fish| 52| 19| 14 | 20 | 15| 19| 16 | 27| 20| 18 | 20| 19 |
Figure 6

A B
primary mns
C D
secondary motor neurons
E F

Cox deficiency in a new zebrafish model
Early developmental pathology due to cytochrome c oxidase deficiency is revealed by a new zebrafish model

Katrina N. Baden, James Murray, Roderick A. Capaldi and Karen Guillemin

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