Lack of Developmental Redundancy between Unc45 Proteins in Zebrafish Muscle Development

Sophie A. Comyn, David Pilgrim*
Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

Abstract
Since the majority of protein-coding genes in vertebrates have intra-genomic homologues, it has been difficult to eliminate the potential of functional redundancy from analyses of mutant phenotypes, whether produced by genetic lesion or transient knockdown. Further complicating these analyses, not all gene products have activities that can be assayed in vitro, where the efficiency of the various family members can be compared against constant substrates. Two vertebrate UNC-45 homologues, unc45a and unc45b, affect distinct stages of muscle differentiation when knocked down in cell culture and are functionally redundant in vitro. UNC-45 proteins are members of the UCS (UNC-45/CRO1/She4p) protein family that has been shown to regulate myosin-dependent functions from fungi to vertebrates through direct interaction with the myosin motor domain. To test whether the same functional relationship exists between these unc45 paralogs in vivo, we examined the developmental phenotypes of doubly homozygous unc45b−/−; unc45a−/− mutant zebrafish embryos. We focused specifically on the combined effects on morphology and gene expression resulting from the zygotic lack of both paralogs. We found that unc45b−/− and unc45b−/−; unc45a−/− embryos were phenotypically indistinguishable with both mutants displaying identical cardiac, skeletal muscle, and jaw defects. We also found no evidence to support a role for zygotic Unc45a function in myoblast differentiation. In contrast to previous in vitro work, this rules out a model of functional redundancy between Unc45a and Unc45b in vivo. Instead, our phylogenetic and phenotypic analyses provide evidence for the role of functional divergence in the evolution of the UCS protein family.

Introduction
Members of the UCS (UNC-45/CRO1/She4p) protein family are present in single-celled eukaryotes and metazoa [1] and participate in essential myosin-dependent functions by directly interacting with the myosin motor domain. This interaction is mediated by a conserved 400 residue carboxyl-terminal domain, a defining feature of UCS proteins [1,2]. In contrast to the fungal UCS homologues, metazoan UNC-45 proteins also contain an amino terminal tetratricopeptide repeat (TPR) domain which acts as a flexible central region then joins the TPR and UCS domains and facilitates the interaction between UNC-45 and Hsp90 [3,4]. A flexible central region then joins the TPR and UCS domains and acts in concert with the UCS to bind to, and mediate the folding of, the myosin head domain [3,4]. Through the formation of a stoichiometric complex with Hsp90, UNC-45 acts in vitro as both a direct myosin chaperone and an Hsp90 co-chaperone [4].

UNC-45 is essential for muscle assembly and function in C. elegans where it co-localizes with thick filaments [3,5,6,7]. Thick filaments in C. elegans are assembled from two different myosin heavy chains, MHC A and B, which are asymmetrically arranged. The minor myosin isoform (MHC A) is found only in the central 2 μm of the filament, while MHC B is found along the majority of the lateral arms [8]. UNC-45 shows myosin isotype specificity, interacting with muscle myosin heavy-chain B (MHC B) through the UCS domain, but not with MHC A [1,3,6,9]. If MHC B is removed (as in unc-54 null mutants), C. elegans muscle thick filaments can be induced to form using only MHC A [10]. In such animals UNC-45 no longer localizes to the thick filament, and missense mutations in unc-45 have no effect on thick filament assembly [6]. Thus, MHC A does not require UNC-45 activity despite the fact that the two myosins are 65% identical in sequence, and MHC A can functionally substitute for MHC B [11].

Vertebrates have two UNC-45 genes, unc45a and unc45b, whose expression patterns differ dramatically [12,13,14]. Unc45b expression is restricted to striated muscle, where it interacts with Hsp90 during myofibrillogenesis and is required for folding the myosin motor domain [4,13]. In humans, Unc45b is a candidate locus for cardiomyopathies and a protein determining hereditary inclusion-body myopathy (p97) regulates Unc45b stability [15,16]. Knockdown of unc45b and hsp90a in zebrafish or Xenopus produce similar phenotypes, including cardiac dysfunction, disordered sarcomeres and paralysis, and regulation of these two genes is interdependent [13,17]. Genetic mutations affecting unc45b have been identified in zebrafish (steif) and Xenopus (dicky ticker) [13,14,18]. The unc45b mutant steif has defective myofibrils containing disorganized thick and thin filaments and reduced levels of muscle myosin protein [13,14]. Consequently, embryos are paralyzed, lack circulation, and develop extensive heart and yolk edema by 5 days post-fertilization (dpf). As is seen in C. elegans...
for unc-45, overexpression of unc45b in zebrafish leads to severely disorganized myosin filaments, indicating the need for tight regulation of unc45b expression [19].

Both Unc45a and Unc45b are expressed in differentiating C2C12 myoblasts and such cells show defects in myogenesis when expression is reduced [12]. Antisense-oligonucleotides directed against Unc45a affected myoblast proliferation, while Unc45b knockdown affected sarcomere formation [12]. In vitro, both Unc45a and Unc45b have the capacity to fold the myosin motor domain in an Hsp90/ATP-dependent manner [20]. In that assay, Unc45a was shown to be the better UNC-45 chaperone for myosin folding [20]. In zebrafish and mouse, unc45a has a broad expression pattern with transcripts present in the brain, pharyngeal arches, and retina, although no significant expression is seen in the striated muscles of the trunk or heart [12,21]. Unexpectedly, a mutation in zebrafish unc45a (kurzschluss) results in aortic arch defects, which is not easily explained via a role in myosin regulation [21]. The unc45a mutants develop an arteriovenous malformation involving aortic arches 5 and 6 that causes blood to shunt from the primary head sinus back into the heart. In contrast to unc45b, myofibrillar organization and thick filament assembly appear normal in unc45a mutants. These observations have led to the hypothesis that Unc45a may either regulate myosin assembly in non-muscle cells, or contribute to muscle myosin assembly solely in muscle groups other than the trunk or heart.

In C. elegans, the single unc-45 gene is responsible for both striated muscle assembly and function through the muscle myosin UNC-54/MHC-B, as well as cytokinesis and cell polarity via the type II non-muscle myosin NMY-2 [6,22,23]. These observations, in conjunction with those from the UCS protein family member Rga3, which also interacts with a type II myosin and is required for cytokinesis [24], led to hypotheses that the vertebrate orthologues of UNC-43 may divide these functions between them. A cytokinesis defect has not been reported for either of the unc45 mutants in zebrafish [14,21]. Several models could explain both the in vivo biochemical redundancy toward myosin folding and the unc45a aortic arch phenotype. First, functional redundancy between the two vertebrate Unc45 proteins could be masking a cytokinesis phenotype in either of the unc45 zebrafish mutants. Second, Unc45a contributes to muscle myosin assembly only in the tissues in which it is expressed and only during later stages of development. A subset of specific muscles lacking both isoforms would express a phenotype more severe than that seen in the single unc45b mutant. Finally, given the striking lack of a muscle malformation involving aortic arches 5 and 6 that causes blood to shunt from the primary head sinus back into the heart. In contrast to unc45b, myofibrillar organization and thick filament assembly appear normal in unc45a mutants. These observations have led to the hypothesis that Unc45a may either regulate myosin assembly in non-muscle cells, or contribute to muscle myosin assembly solely in muscle groups other than the trunk or heart.

In C. elegans, the single unc-45 gene is responsible for both striated muscle assembly and function through the muscle myosin UNC-54/MHC-B, as well as cytokinesis and cell polarity via the type II non-muscle myosin NMY-2 [6,22,23]. These observations, in conjunction with those from the UCS protein family member Rga3, which also interacts with a type II myosin and is required for cytokinesis [24], led to hypotheses that the vertebrate orthologues of UNC-43 may divide these functions between them. A cytokinesis defect has not been reported for either of the unc45 mutants in zebrafish [14,21]. Several models could explain both the in vivo biochemical redundancy toward myosin folding and the unc45a aortic arch phenotype. First, functional redundancy between the two vertebrate Unc45 proteins could be masking a cytokinesis phenotype in either of the unc45 zebrafish mutants. Second, Unc45a contributes to muscle myosin assembly only in the tissues in which it is expressed and only during later stages of development. A subset of specific muscles lacking both isoforms would express a phenotype more severe than that seen in the single unc45b mutant. Finally, given the striking lack of a muscle malformation involving aortic arches 5 and 6 that causes blood to shunt from the primary head sinus back into the heart. In contrast to unc45b, myofibrillar organization and thick filament assembly appear normal in unc45a mutants. These observations have led to the hypothesis that Unc45a may either regulate myosin assembly in non-muscle cells, or contribute to muscle myosin assembly solely in muscle groups other than the trunk or heart.

In C. elegans, the single unc-45 gene is responsible for both striated muscle assembly and function through the muscle myosin UNC-54/MHC-B, as well as cytokinesis and cell polarity via the type II non-muscle myosin NMY-2 [6,22,23]. These observations, in conjunction with those from the UCS protein family member Rga3, which also interacts with a type II myosin and is required for cytokinesis [24], led to hypotheses that the vertebrate orthologues of UNC-43 may divide these functions between them. A cytokinesis defect has not been reported for either of the unc45 mutants in zebrafish [14,21]. Several models could explain both the in vivo biochemical redundancy toward myosin folding and the unc45a aortic arch phenotype. First, functional redundancy between the two vertebrate Unc45 proteins could be masking a cytokinesis phenotype in either of the unc45 zebrafish mutants. Second, Unc45a contributes to muscle myosin assembly only in the tissues in which it is expressed and only during later stages of development. A subset of specific muscles lacking both isoforms would express a phenotype more severe than that seen in the single unc45b mutant. Finally, given the striking lack of a muscle malformation involving aortic arches 5 and 6 that causes blood to shunt from the primary head sinus back into the heart. In contrast to unc45b, myofibrillar organization and thick filament assembly appear normal in unc45a mutants. These observations have led to the hypothesis that Unc45a may either regulate myosin assembly in non-muscle cells, or contribute to muscle myosin assembly solely in muscle groups other than the trunk or heart.

In C. elegans, the single unc-45 gene is responsible for both striated muscle assembly and function through the muscle myosin UNC-54/MHC-B, as well as cytokinesis and cell polarity via the type II non-muscle myosin NMY-2 [6,22,23]. These observations, in conjunction with those from the UCS protein family member Rga3, which also interacts with a type II myosin and is required for cytokinesis [24], led to hypotheses that the vertebrate orthologues of UNC-43 may divide these functions between them. A cytokinesis defect has not been reported for either of the unc45 mutants in zebrafish [14,21]. Several models could explain both the in vivo biochemical redundancy toward myosin folding and the unc45a aortic arch phenotype. First, functional redundancy between the two vertebrate Unc45 proteins could be masking a cytokinesis phenotype in either of the unc45 zebrafish mutants. Second, Unc45a contributes to muscle myosin assembly only in the tissues in which it is expressed and only during later stages of development. A subset of specific muscles lacking both isoforms would express a phenotype more severe than that seen in the single unc45b mutant. Finally, given the striking lack of a muscle malformation involving aortic arches 5 and 6 that causes blood to shunt from the primary head sinus back into the heart. In contrast to unc45b, myofibrillar organization and thick filament assembly appear normal in unc45a mutants. These observations have led to the hypothesis that Unc45a may either regulate myosin assembly in non-muscle cells, or contribute to muscle myosin assembly solely in muscle groups other than the trunk or heart.
Immunohistochemistry and histology

Embryos were fixed in 2% TCA and washed four times with 0.8% Triton X-100 in PBS (PBS-Tx) for 5 minutes at room temperature. Samples were blocked in 5% BSA in PBS-Tx for 1 hour at room temperature and incubated overnight at 4°C in a 1:10 dilution of monoclonal antibody MF-20 (Developmental Studies Hybridoma Bank) in blocking solution. Embryos were washed five times with PBS-Tx for 5 minutes at room temperature. Subsequent to a single 5 minute PBS wash, embryos were incubated overnight at 4°C in a 1:1,000 dilution of anti-mouse Alexa 488 (Molecular Probes) secondary antibody. Antibody was removed by three washes in PBS-Tx for 10 minutes each at room temperature prior to mounting embryos in 3% methylcellulose. Images were captured using a Nikon Eclipse 80i confocal.

Alcian Blue stains proteoglycan components of the extracellular matrix [33,34]. The following method is adapted from [35]. 5 dpf larvae were fixed 2 hr in 4% PFA and then dehydrated in 50% ethanol for 10 minutes. Once dehydrated, larvae were stained overnight in: 0.02% Alcian Blue (Sigma), 60 mM MgCl2, and 70% ethanol. Following a rinse in water, embryos were bleached for 20 minutes in equal volumes of 3% H2O2 and 2% KOH in open tubes on the bench top. To visualize cartilage more readily, tissues were cleared for 20 minutes with gentle rocking using 1 mg/mL trypsin dissolved in saturated sodium tetraborate [36]. Larvae were cleared in 20% glycerol with 0.25% KOH for 1 hour followed by 50% glycerol with 0.25% KOH for 2 hours and stored at 4°C in 100% glycerol.

Results

Phylogenetic analysis of UNC-45

Although it has been suggested that the UNC-45 gene duplication in vertebrates emerged during the chordate radiation, it is unclear whether the two UNC-45 genes present in all vertebrates share a common origin dating to early in the chordate lineage, or whether those present in teleosts are unique, having arisen from a separate whole-genome duplication event in the common ancestor to teleosts followed by selective loss [12,37]. To clarify the evolutionary relationship between UNC-45 proteins we performed a phylogenetic analysis before embarking on a detailed phenotypic analysis of the unc45b−/−; unc45a−/− mutant (Fig. 1). In particular, we tested whether sequence divergence patterns could help to clarify the phylogenetic relationship between the two vertebrate UNC-45 genes.

A neighbour-joining tree generates three major sequence groups: Unc45a, Unc45b, and UNC-45 (Figure 1). Vertebrate genomes contain two UNC-45 gene copies, whereas non-vertebrates have a single UNC-45 gene. In vertebrates, both Unc45a and Unc45b amino acid sequences share more similarity with homologs in other vertebrate species than with each other. For example, the Danio rerio Unc45a and Unc45b proteins are 77% similar to each other, but are ~85% similar to human Unc45a and Unc45b, respectively. The UNC-45 amino acid sequence from Drosophila melanogaster, and Unc45a and Unc45b from Danio rerio are all approximately 60% similar to the single Caenorhabditis elegans UNC-45 protein.

One fifth of all D. rerio genes have been retained as duplicates following the whole-genome duplication event thought to have occurred prior to the telost radiation, approximately 330 million years ago [38,39]. We found no evidence for additional copies of either Unc45a or Unc45b in our searches of T. rubripes, O. latipes, G. aculeatus, T. nigroviridis, or D. rerio genomes, suggesting that the Unc45 gene duplicates, if generated at that time, were lost early during the teleost lineage. Therefore, it appears that vertebrate Unc45a and Unc45b have diverged equally from a single ancestral UNC-45 locus and given the comparable sequence divergence from their human orthologs, it is likely that the two Unc45 proteins were generated early in the vertebrate lineage following a whole-genome duplication event.

Gross morphology of unc45 mutants

We used a genetic approach to examine the functional relationship between unc45 genes in vivo. The phenotypic assessment of unc45b−/−; unc45a−/− mutants focused on gross morphology and gene expression. Novel phenotypes not present in the single mutants, or a double mutant displaying phenotypes more severe than those present in the single mutants, would be consistent with functional redundancy between the two vertebrate unc45 genes. The unc45b−/−; unc45a−/− mutant line was created through the crossing of adult unc45b+/− and unc45a+/− fish that have been previously described [13,21]. The two mutants are predicted to be molecular nulls, since each is a nonsense mutation in the UCS domain, and in each case, morpholino-oligonucleotide knockdown of the cognate gene in wild-type embryos phenocopies the mutant phenotype [13,14,21]. Since progeny that are homozygous at either of the unc45 loci are zygotic-lethal, strains were maintained as unc45b+/−; unc45a+/− double heterozygotes. Crosses between these fish yielded embryonic progeny genotypes in the expected Mendelian ratios (data not shown). Specifically, no underrepresentation of the unc45b−/−; unc45a−/− genotype was observed, which might have suggested lethality during early developmental stages. Additionally, no evidence for a heterozygous effect was observed such that unc45a+/− and unc45b+/− genotypes appeared phenotypically identical in all combinations.
In regard to gross morphology, the unc45b<sup>−/−</sup>; unc45a<sup>−/−</sup> and unc45b<sup>−/−</sup> embryos appear identical (Fig. S1). Both mutants are paralyzed and unc45b<sup>−/−</sup>; unc45a<sup>−/−</sup> embryos have reduced somite birefringency as has been reported previously for the unc45a<sup>−/−</sup> mutants [13,14]. The absence of both circulation and cardiac contraction in the unc45b<sup>−/−</sup> and unc45a<sup>−/−</sup>; unc45a<sup>−/−</sup> mutants, results in accumulation of fluid in the pericardial space (cardiac edema) and yolk sac edema by 5 dpf. To a lesser extent, the unc45a<sup>−/−</sup>; unc45b<sup>−/−</sup> mutants also develop cardiac edema, but yolk sac edema does not advance to the degree seen in the other mutants.

**Differential distribution of unc45a and unc45b transcripts**

The expression patterns of zebrafish unc45a and unc45b transcripts have been reported, but expression was not examined in the reciprocal unc45a mutant, which might suggest compensatory regulation between the two homologues [13,14,21]. As reported in [21], wild type embryos have a diffuse pattern of unc45a expression in the brain and pharyngeal arch region (Fig. 2a). At 48 hpf, we detected a similar unc45a expression pattern in unc45b<sup>−/−</sup>, and unc45a<sup>−/−</sup>; unc45a<sup>−/−</sup> embryos (Fig. 2c,d). In contrast to wild type, however, unc45a transcripts are almost absent in the unc45a<sup>−/−</sup> mutants consistent with nonsense-mediated decay (Fig. 2b). Expression of unc45a in the unc45b<sup>−/−</sup>; unc45a<sup>−/−</sup> embryos appeared slightly more variable than in the unc45a single mutants, although none of the genotypes demonstrated an expansion of the unc45a expression domain.

We tested whether unc45b expression is affected in the unc45a mutants (Fig. 2e–h). Transcripts were detected in the extraocular, cranial, cardiac, pectoral fin, and trunk muscles of unc45a<sup>−/−</sup>; unc45a<sup>−/−</sup> and wild type embryos (Fig. 2c, f). In contrast, expression is either present at low levels or absent in the extraocular, cranial, cardiac, and pectoral fin muscles of the unc45b<sup>−/−</sup> and unc45b<sup>−/−</sup>; unc45a<sup>−/−</sup> mutants (Fig. 2g, h). Expression in the trunk musculature however, is up-regulated in the unc45b mutants as compared to wild type siblings consistent with previous reports [13]. Expression levels of unc45 genes therefore, are diminished in their respective mutants except in the cases of the unc45b<sup>−/−</sup> and unc45a<sup>−/−</sup>; unc45a<sup>−/−</sup> where unc45b expression is increased in the trunk musculature. Since no functional protein is made from this allele, and no expansion of the expression domain is seen, it is unlikely that this can have any compensatory effect on the phenotype.

unc45a<sup>−/−</sup> and unc45b<sup>−/−</sup> mutants have different hsp90 expression profiles

The hsp90a chaperone is co-expressed with unc45b in striated muscle and the proteins interact in vitro [13]. Moreover, the striking phenotypic similarity of the hsp90a<sup>1</sup> and unc45b homozygous mutants suggests that this interaction is necessary for myofibrillogenesis [13,17,40]. Both unc45b and hsp90a mRNA transcripts are upregulated in unc45b<sup>−/−</sup> mutants; however, expression of the hsp90 genes has not been examined in the unc45a<sup>−/−</sup> mutant [13]. Unc45a (GCUNC45) has been associated with Hsp90 isoforms in vivo and in vitro [41,42]. Thus, we examined the expression patterns of hsp90a<sup>1</sup>, hsp90a<sup>2</sup>, and hsp90ab<sup>1</sup> transcripts in unc45 mutants to determine if a similar relationship exists for unc45a<sup>−/−</sup> and unc45b<sup>−/−</sup>; unc45a<sup>−/−</sup> mutants which could suggest that Unc45a activity affects expression of an Hsp90 chaperone isoform in vivo. Whole-mount in situ hybridization performed at 48 hpf shows similar expression levels and patterning of the three hsp90 genes for unc45a<sup>−/−</sup> mutants and wild type siblings (Fig. 3a–c, d–f). No appreciable increase in hsp90 expression levels or spatial distribution was observed in these embryos. A notable increase in hsp90a<sup>1</sup> and hsp90a<sup>2</sup> expression was, however, observed in the unc45b<sup>−/−</sup> and unc45b<sup>−/−</sup>; unc45a<sup>−/−</sup> mutants (Fig. 3g, h, j, k). Also, the spatial distribution...
of *hsp90a* transcripts was expanded in these embryos to include the trunk musculature. In contrast to the *hsp90a* genes, *hsp90ab1* expression was similar among wild type, unc45a2/−; unc45b2/−, and unc45b2/−; unc45a2/− embryos (Fig. 3c, f, i, l). Therefore, while this analysis cannot completely rule out a role of Unc45a as an Hsp90 co-chaperone, we can conclude that there is no reciprocal regulatory relationship between *hsp90a* and unc45a as is seen with unc45b.

**unc45a** mutants do not show a delay in differentiation of trunk muscle precursors

Both Unc45a and Unc45b are required during the early stages of myogenesis in mouse C2C12 cells [12]. However, *unc45a* mutants do not display a gross muscle phenotype, nor do we see co-regulation of *unc45a* with *hsp90a*. Given that *myoD* expression is an early marker of muscle commitment, it should reflect any significant delay in myogenic commitment in vivo. At 48 hpf cranial myogenesis was normal in *unc45a* mutants as *myoD* expression levels were unchanged compared to wild type siblings in the cranial and extraocular muscle precursors to the constrictor hyoideus, intermandibularis, inferior rectus, lateral rectus, medial rectus, superior rectus, pharyngeal arches, and sternohyoideus (Fig. 4a–d). The *unc45b*2/− and *unc45b*2/−; *unc45a*2/− mutants appear identical, exhibiting a displacement of the intermandibularis, constrictor hyoideus, sternohyoideus, and pharyngeal muscle precursors to positions that are lateral and dorsal to those of wild type siblings (Fig. 4c, d). In the trunk musculature, *unc45b*2/− and *unc45b*2/−; *unc45a*2/− mutants show darker staining *myoD* transcript expression by *in situ* hybridization compared to *unc45a*2/− and wild type embryos with levels appearing to be similar between the two (Fig. 4c’, d’). However since the somite chevrons are also often compacted in the dorsal/ventral axis in whole mount *unc45b* and other mutants affecting contractility of trunk musculature [13,43], possibly due to the defect in thick filament contractility, this may not reflect an increase in MyoD expression per cell. As determined by *myoD* expression, the *unc45a*2/− mutants display no disruption in myogenesis of the trunk musculature. Moreover, given the observed *myoD* expression in the *unc45b*2/−; *unc45a*2/− mutants, the lack of *unc45a* in these embryos does not suppress the *myoD* staining seen in the *unc45b*2/− mutants, suggesting that *unc45a* does not act epistatically to *unc45b* during zebrafish myoblast differentiation.

**Craniofacial Muscle Organization**

Considering that *unc45b*2/− and *unc45b*2/−; *unc45a*2/− mutants have defective myofibril organization in the trunk musculature, we examined the cranial musculature to see if this muscle population also contained gross morphological abnormalities, a feature that has not been previously described for *unc45a* mutants. Although craniofacial myogenesis appeared normal in the *unc45a* mutants, we examined the muscle fibre arrangement in older embryos to see if any abnormalities were present (Fig. 5). No cranial muscles were lost in any of the mutants compared to wild type siblings consistent with patterns of *myoD* expression (Fig. 5). The sternohyoideus muscle is displaced laterally in *unc45b*2/− and *unc45b*2/−; *unc45a*2/− mutants and the muscle fibres appear to be bowed in shape. This modified placement of muscle groups is similar to the *myoD* expression observed at 48 hpf and is likely due to the pericardial edema that develops in the *unc45b*2/− and *unc45b*2/−; *unc45a*2/− mutants (Fig. 4).

**Pharyngeal Arch Cartilage is disrupted in unc45 Mutants**

Cranial cartilage and muscle are tissues related by development, function, and evolution. The pharyngeal arches form through the interaction of neural crest, mesoderm, and endoderm tissues, and develop in concert with their associated muscles [36,44]. Furthermore, both *unc45a* and *unc45b* are expressed in this region: *unc45a* within the arches themselves and *unc45b* in the surrounding musculature [14,21]. We therefore examined potential *unc45* redundancy in the pharyngeal arches during myoblast differentiation and organization in the *unc45b*2/−; *unc45a*2/− mutant.

The majority of zebrafish skull bones develop indirectly through cartilaginous intermediates that can be visualized using Alcian Blue dye, which stains proteoglycan components of the extracellular matrix of chondrocytes [36]. At 5 dpf, the *unc45b*2/− and *unc45b*2/−; *unc45a*2/− mutants display lower levels of Alcian Blue

---

**Figure 4. Whole-mount *in situ* hybridization of the myogenic regulatory factor *myoD* in cranial and trunk muscle precursors at 48 hpf.** Wild type siblings (a,a'); *unc45b*2/− (b,b'); *unc45b*2/−; *unc45a*2/− (c,c'); and *unc45b*2/−; *unc45a*2/− (d,d') mutants. Compared to wild type siblings (a,a'); *unc45a*2/− (b,b'); *unc45b*2/− (c,c'); and *unc45b*2/−; *unc45a*2/− (d,d') mutants have increased expression of *myoD* in the trunk precursors compared to wild type siblings (a,a'); *unc45a*2/− (b,b'); *unc45b*2/− (c,c'); and *unc45b*2/−; *unc45a*2/− (d,d') mutants have increased expression of *myoD* in the trunk precursors.
staining as compared to the unc45a<sup>−/−</sup> mutants and wild type siblings (Fig. 6). Reduced staining is seen in all seven pharyngeal arches. Compared to their wild type siblings, the unc45b<sup>−/−</sup> and unc45a<sup>−/−</sup> mutants (and to a lesser extent the unc45a<sup>−/−</sup> mutants) display improper angling of the ceratobranchial and ceratohyal cartilages and shortening of the palatoquadrates and Meckel’s cartilage (Fig. 6b, c, d). Also, the pectoral girdle is missing or reduced in the unc45b<sup>−/−</sup> and unc45a<sup>−/−</sup> mutants and does not connect with the pelvic fins (Fig. 6c, d). Further, in situ hybridization of marker gene expression patterns showed no consistent differences between the unc45b<sup>−/−</sup> and unc45a<sup>−/−</sup> mutants (Figure S2). There is no evidence for patterning defects in pharyngeal neural crest (hand2, dlx2a), pharyngeal endodermal pouches (nkx2.3), or pharyngeal mesenchyme (nkx3.2) (Figure S2a–d, e–h, i–l, m–p). Moreover, the decreased Alcian Blue staining observed in the unc45b<sup>−/−</sup> and unc45a<sup>−/−</sup> mutants cannot be associated with defects in the regulation of chondrogenesis as the levels of sox9a transcripts remain unchanged in the unc45a<sup>−/−</sup> and unc45b<sup>−/−</sup> embryos, and the unc45a<sup>−/−</sup> mutants, compared to wild type (Fig. S2q–t).

Discussion

Based on reports of at least partial molecular redundancy from several types of in vitro assay, it was assumed that the vertebrate paralogs of UNC-45 would have overlapping in vivo functions, acting as myosin-specific co-chaperones with primary action at different stages of myogenesis. To directly test this, we undertook a genetic-based examination of the in vivo function and potential redundancy between vertebrate unc45 genes using null alleles of unc45a and unc45b. Of particular interest for phenotypic examination were regions of the embryo where unc45 gene expression domains overlap, such as in myoblasts during muscle differentiation and the pharyngeal arches at later stages of development. No phenotype was observed that was novel to the double mutants, nor did the embryos display phenotypes that were more pronounced than those of either of the unc45a or unc45b mutants alone. This suggests that in contrast to experiments in myoblast cell culture, UNC45a and UNC45b do not participate in redundant functions in the embryo.

We find no evidence for compensation between unc45a and unc45b or an expansion of unc45 expression domains in the double mutant. To our knowledge only one other study has examined the effects of both vertebrate unc45 paralogs. From their work in vitro, Price et al. proposed that UNC45a and UNC45b play a role in early myogenesis and myoblast fusion [12]. Were this the case in vivo, one would expect to see a difference compared to wild type siblings in the extent or timing of expression of the myogenic regulatory factor MyoD in the unc45a mutants, which we did not detect. Instead, our findings confirm reports by others that unc45a mutants
have no discernable muscle phenotypes [21] and show that this is not due to compensation by Unc45b function. Surprisingly, myoD expression levels were increased in the trunk muscle precursors of the *unc45b* mutants compared to wild type siblings and *unc45a* mutants. This increase may reflect the similar increase in *hsp90α* levels observed in the *unc45b* mutants. A subset of cells located in the somites and pectoral fin buds express both myoD and *hsp90α*, and both genes are down regulated subsequent to the establishment of striated muscle fibres [45,46]. It was not surprising that *hsp90α* expression was similar in all genotypes tested since *hsp90αβ*1, unlike *hsp90α*, is not involved in the cellular stress response [17]. In contrast to *unc45b*, the loss of *unc45a* has no effect on the levels of *hsp90* transcripts. This indicates that although both *unc45a* and *unc45b* can directly interact with Hsp90, and have the ability to act as Hsp90 co-chaperones, they elicit distinct Hsp90 responses at the gene level. This would suggest that *unc45a* and *unc45b* are under differential regulatory control, which may be a reflection of their divergent functions.

Previously, myofibril organization had only been examined in the trunk musculature of *unc45b* mutants. Although craniofacial myogenesis was normal in the *unc45* mutants, we examined the muscle fibre arrangement in older embryos to see if any abnormalities were present. The altered placement of muscle groups in the mutants is similar to the pattern of *myoD* expression observed at 48 hpf and is likely due to the pericardial edema that develops in the *unc45b*; *unc45b−/−*; *unc45a−/−* mutants. The combination of muscle phenotypes observed in the *unc45* mutants indicates that craniofacial myogenesis is unperturbed in the *unc45a* mutants and that the *unc45b*; *unc45b−/−*; *unc45a−/−* mutants have an equal degree of myofibril disorganization. Since no changes in gene expression were observed, it may be that the cartilage defects seen in the *unc45* mutants are associated with pericardial edema. While evidence in support of edema as the origin of a set of universal abnormal pharyngeal arch phenotypes is compelling, it remains to be determined whether the interplay between muscle contraction and cartilage formation is a requirement for proper jaw organization and development. Although the *unc45b*; *unc45b−/−*; *unc45a−/−* mutants accumulate fluid to a much larger extent than the *unc45a−/−* mutants, it seems unlikely that edema alone could account for the decreased cartilage staining observed in the *unc45b* mutants. Schilling and Kimmel [36] have proposed that despite the craniofacial muscles differentiating slightly later than cartilages in the same region, the scaffold created through the interaction between the muscle and cartilage precursors might play a role in the patterning of the pharyngeal region.

While the cellular phenotypes of *C. elegans* UNC-45 and fungal UCS protein mutants vary considerably, they all have an effect on myosin assembly and/or function [1,17]. Consequently, structures that incorporate myosin molecules are also affected in these mutants. Phenotypic differences may be ascribed to the myosin classes with which fungal UCS proteins associate as well as their different domain structure. In contrast to UNC-45, fungal UCS proteins interact with both conventional and non-conventional myosin, leading to their participation in numerous cellular functions [1]. The amino terminal TPR domain is absent in fungi and the central domain, if present, shares little homology with UNC-45 or other fungal proteins [47]. The UCS domain, however, is highly conserved amongst species, with approximately 53% similarity between She4p and human UNC-45 proteins [47].

The abnormal aortic arch development and arteriovenricular malformations in the *unc45a−/−* mutants remain puzzling. The UCS proteins found in most non-vertebrates function in processes that require non-muscle myosin, such as cytokinesis [22,48], and data from cell culture studies suggested that Unc45a may have a role in cell division and proliferation, likely mediated by an interaction with non-muscle myosin. We were therefore expecting that the double mutant might exhibit a novel phenotype indicative of cytokinesis defects, but no such phenotype was observed. In an *in vitro* folding assay Liu et al. [20] demonstrated that in comparison to UNC-45b, UNC-45a has a higher affinity for the smooth muscle myosin motor domain and a greater efficiency of folding for this region. Importantly, smooth muscle myosin is closely related to non-muscle myosin II. In human cell lines, Unc45a inhibits retinoic acid signaling and its overexpression in tumors is associated with increased proliferation and metastasis [49,50]. Unc45a has been linked to cancer progression through its overexpression and ability to confer resistance to histone deacetylase inhibitors and retinoic acid. In *in vitro* overexpression of unc45a leads to increased cell proliferation and an accumulation of non-muscle myosin and Unc45a at the cleavage furrow during cytokinesis and both proteins also localize to the filopodia of motile cells [49]. In humans, there is a positive correlation between levels of Unc-45a and the stage and grade of ovarian cancer. This can be attributed, in part, to the elevated levels of Unc45a protein in ovarian carcinoma tumours compared to healthy ovarian epithelium [49]. This suggests that Unc45a is involved in cellular functions that are not manifested at the embryonic stage we are analysing. Regardless, this analysis has eliminated developmental roles for Unc45a that are dependent on a muscle-myosin-dependent chaperone function, which might have been masked by redundancy with Unc45b.

Since mammals and teleosts have two *unc45* genes, which show no evidence of functional redundancy and appear to be involved in distinct cellular processes, it is useful to consider how these different cellular functions may have arisen. The duplication/degeneration/complementation model (DDC) is based on the observation that many genes required for development have a number of independent functions based on their spatial and temporal expression patterns [51]. One way for both gene duplicates to be preserved is through subfunctionalization, whereby both gene copies accumulate degenerative mutations resulting in the partitioning of the ancestral expression domains and/or functions [52]. When duplicate gene pairs accrue loss-of-function mutations that affect separate sub-functions of the ancestral gene, both gene copies are required to produce the ancestral gene function, and therefore, both copies are retained in the genome [38]. The UNC-45 gene pair was most likely created by a whole genome duplication event some time after the vertebrate lineage branched from that of a common ancestor to *C. elegans* and *D. melanogaster*. Both genes would have been redundant until mutations accumulated following the duplication event. Duplicate gene pairs have a number of potential fates: become a pseudogene, subdivide the ancestral function at the regulatory or protein level (subfunctionalization), develop a new function (neofunctionalization), or a combination of the above. Both vertebrate *Unc45b* and *C. elegans* UNC-45 are required for myosin motor domain folding and thick filament assembly and are expressed in similar tissues. This suggests that few changes have occurred in the protein domains and regulatory elements responsible for producing the muscle myosin functions of UNC-45 and Unc45b. As the expression pattern and functions of Unc45a appear to have diverged significantly from those of Unc45b in vertebrates, it is likely that expression domain subfunctionalization and neofunctionalization occurred in the Unc45a copy prior to the branching of the bony fish lineage. Had the new Unc45 function not been established after the divergence of lobe and ray-finned fish, either mammals or teleosts, but not
both, would have the gene copy encoding a new Unc45 function. The differential distribution of unc45a and unc45b transcripts suggests that subfunctionalization, which occurred following their duplication, is likely the mechanism for the preservation of Unc45 duplicates in vertebrate genomes.

In conclusion, we have shown that the zygotic-lethal unc45b⁻/⁻; unc45a⁻/⁻ mice display no defects in myocyte function or differentiation suggesting that Unc45a function is not required for myogenesis.

Supporting Information

**Figure S1 Morphology of 4 dpf unc45 mutants.** Wild type siblings (a,a'); unc45a⁻/⁻ (b,b'); unc45b⁻/⁻ (c,c'); and unc45a⁻/⁻ unc45b⁻/⁻ (d',d') mutants. Blood circulates through the hearts of wild type siblings (a,a') and unc45a⁻/⁻ mutant (b,b') but not in those of the unc45b⁻/⁻ (c,c') and unc45a⁻/⁻ unc45b⁻/⁻ (d',d') mutants and absent in wild type siblings (arrowheads). A fully inflated swim bladder is present only in wild type embryos, absent in unc45b⁻/⁻ and unc45b⁻/⁻; unc45a⁻/⁻ mutants, and minimally inflated in unc45a⁻/⁻ mutants (arrows). Somite birefringence is reduced in unc45b⁻/⁻ and unc45b⁻/⁻; unc45a⁻/⁻ mutants compared to wild type and unc45a⁻/⁻ embryos. Apoptoses following a letter denote an increased magnification of the same embryo.

**(TIF)**

**Table S1 UNC-45 sequences used in phylogenetic analysis.**

**DOC**

Acknowledgments

Support for the development of the University of Alberta’s zebrafish facility was provided by Alberta Ingenuity Fund via Dr. Andrew Waskiewicz. Aiah McCorry assisted in maintaining zebrafish stocks, and Layne Myhre commented on the manuscript. The MF20 monoclonal antibody (developed by Stockdale and Fischman) was obtained from the Developmental Studies Hybridoma Bank, under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences.

Author Contributions

Conceived and designed the experiments: SC DP. Performed the experiments: SC. Analyzed the data: SC DP. Wrote the paper: SC DP.

References

1. Hutagalung AH, Landverk ML, Price MG, Epstein HF (2002) The UCS family of myosin chaperones. J Cell Science 115: 3981–3990.
2. Yu Q, Bernstein SI (2005) Ucs proteins: managing the myosin motor. Current Biology 15: R255–257.
3. Verodia I, Ao W, Kim S, Kim C, Pilgrim D (1999) unc45 gene of Caenorhabditis elegans encodes a muscle-specific tetra-tricopeptide repeat-containing protein. Cell Motility Cytoskeleton 42: 163–177.
4. Barral JM, Hutagalung AH, Britner A, Hart PJ, Epstein HF (2002) Role of the myosin assembly protein UNC-45 as a molecular chaperone for myosin. Science 295: 669–671.
5. Epstein HF, Thomson JN (1974) Temperature-sensitive mutation affecting myofilament assembly in C. elegans. Nature 250: 579–580.
6. Walker MG (2003) Pharmaceutical target identification by gene expression analysis. Mini Rev Med Chem 3: 197–205.
7. du SJ, Li H, Bian Y, Zhong Y (2008) Heat-shock protein 90alpha1 is required for organized myofilament assembly in skeletal muscles of zebrafish embryos. Proc Natl Acad Sci U S A 105: 554–559.
8. Geach TJ, Zimmerman LB (2010) Paralysis and delayed Z-disc formation in the Xenopus tropicalis unc45b mutant dicky ticker. BMC Dev Biol 10: 75.
9. Nekovář EF, Zhang PJ, Du S (2010) Knockdown and overexpression of Unc-45b results in defective myofilib organization in skeletal muscles of zebrafish embryos. BMC Cell Biol 11: 70.
10. Liu L, Srikakulam R, Winkelmann DA (2008) Unc45 activates Hsp90-dependent folding of the myosin motor domain. J Biol Chem 283: 13185–13193.
11. Anderson MJ, Pham VN, Vogel AM, Weinstein BM, Roman BL (2008) Loss of unc45a precipitates arteriovenous shunting in the aortic arches. Dev Biol 318: 250–267.
12. Kachur TM, Ao W, Berger J, Pilgrim D (2004) Maternal Unc-45 is involved in cytokinesis and co-localizes with a non-muscle myosin in the early Caenorhabditis elegans embryo. J Cell Science 117: 357–384.
13. Miller DdM, Ortiz I, Blumberg CC, Epstein HF (1983) Differential localization of two myosins within nematode thick filaments. Cell 34: 477–490.
14. Kim J, Lowe T, Hoppe T (2000) Protein quality control genes muscle into shape. Trends Cell Biol 10: 264–272.
15. Maruyama IN, Miller DM, Brenner S (1989) Myosin heavy chain gene amplification as a suppressor mutation in Caenorhabditis elegans. Mol Gen Genet 219: 119–128.
16. Hoppe PE, Waterston RH (1996) Hydrophobicity variations along the surface of the coiled-coil rod may mediate striated muscle assembly in Caenorhabditis elegans. J Cell Biol 135: 371–382.
17. Price MG, Landsverk ML, Barral JM, Epstein HF (2002) Two mammalian UNC-45 isoforms are related to distinct cytoskeletal and muscle-specific functions. J Cell Science 115: 4013–4023.
18. Etard C, Behra M, Fischer N, Hutcherson D, Geiser R, et al. (2007) The UCS factor Stef/Unce45 interacts with the heat shock protein Hspa9a during myoblastogenesis. Dev Biol 308: 133–143.
19. Wohlgemuth SL, Crawford BD, Pilgrim DB (2007) The myosin co-chaperone UNC-45 is required for skeletal and cardiac muscle function in zebrafish. Dev Biol 303: 493–502.
29. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution 28: 2731–2739.

30. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.

31. Thisse C, Thisse B (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. Nature protocols 3: 59–69.

32. French CR, Erickson T, French DV, Pilgrim DB, Waskiewicz AJ (2009) Gdf6a is required for the initiation of dorsal-ventral retinal patterning and lens development. Dev Biol 333: 37–47.

33. Yelick PC, Schilling TF (2002) Molecular dissection of craniofacial development using zebrafish. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists 13: 308–322.

34. French CR, Erickson T, French DV, Pilgrim DB, Waskiewicz AJ (2009) Gdf6a is required for the initiation of dorsal-ventral retinal patterning and lens development. Dev Biol 333: 37–47.

35. Walker MB, Kimmel CB (2007) A two-color acid-free cartilage and bone stain for zebrafish larvae. Biotechnic & histochemistry : official publication of the Biological Stain Commission 82: 23–28.

36. Schilling TF, Piotrowski T, Grandel H, Brand M, Heisenberg CP, et al. (1996) Jaw and branchial arch mutants in zebrafish I: branchial arches. Development 123: 329–344.

37. Winbrodt J, Meyer A, Scharl M (1998) More genes in fish? BioEssays 20: 511–515.

38. Prince VE, Pickett FB (2002) Splitting pairs: the diverging fates of duplicated genes. Nature reviews Genetics 3: 827–837.

39. Vollrath JN (2005) Genome evolution and biodiversity in teleost fish. Heredity 94: 280–294.

40. Hawkins TA, Haramis AP, Etard C, Pedomroni G, Vaughan CK, et al. (2008) The ATPase-dependent chaperoning activity of Hsp90a regulates thick filament formation and integration during skeletal muscle myofibrillogenesis. Development 135: 1147–1156.

41. Chadli A, Graham JD, Abel MG, Jackson TA, Gordon DF, et al. (2006) GCUNC-45 is a novel regulator for the progesterone receptor/hsp90 chaperoning pathway. Molecular and cellular biology 26: 1722–1730.

42. Chadli A, Ehm SJ, Toth DO (2008) GCUNC45 is the first Hsp90 co-chaperone to show alpha/beta isoform specificity. The Journal of biological chemistry 283: 9509–9512.

43. Just S, Meder B, Berger IM, Etard C, Trano N, et al. (2011) The myosin-interacting protein SMYD1 is essential for sarcomere organization. Journal of Cell Science 124: 3127–3136.

44. Hong S-K, Haldin CE, Lawson ND, Weinstein BM, Dawid IB, et al. (2005) The zebrafish kohtalo/trap230 gene is required for the development of the brain, neural crest, and pronephric kidney. Proceedings of the National Academy of Sciences of the United States of America 102: 10473–10478.

45. Krone PH, Sas JB, Lele Z (1997) Heat shock protein gene expression during embryonic development of the zebrafish. Cellular and molecular life sciences : CMLS 53: 122–129.

46. Lele Z, Hartson SD, Martin CC, Whitesell L, Matts RL, et al. (1999) Disruption of zebrafish somite development by pharmacologic inhibition of Hsp90. Developmental Biology 210: 56–70.

47. Shi H, Blobel G (2010) UNC-45/CRO1/She4p (UCS) protein forms elongated dimers and joins two myosin heads near their actin binding region. Proc Natl Acad Sci U S A.

48. Lee CF, Melkani GC, Yu Q, Sugge JA, Kronert WA, et al. (2011) Drosophila UNC-45 accumulates in embryonic blastoderm and in muscles, and is essential for muscle myosin stability. J Cell Sci 124: 699–705.

49. Rizzuto R, Santillan A, Lin Z, Tang T, Lee MK, et al. (2007) Myosin II co-chaperone general cell UNC-45 overexpression is associated with ovarian cancer, rapid proliferation, and motility. The American journal of pathology 171: 1649–1659.

50. Epping MT, Meijer LAT, Bos JL, Bernards R (2009) UNC45A confers resistance to histone deacetylase inhibitors and retinoic acid. Molecular cancer research : MCR 7: 1861–1870.

51. Force A, Lynch M, Pickett FB, Amores A, Yan YL, et al. (1999) Preservation of duplicate genes by complementary, degenerative mutations. Genetics 151: 1531–1545.

52. Lynch M, Force A (2000) The probability of duplicate gene preservation by subfunctionalization. Genetics 154: 459–473.