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Identification of the *actc1c* cardiac actin gene in zebrafish

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ABSTRACT

Zebrafish is rapidly becoming a key model organism for studying a variety of biological processes from molecules to organisms. Interactions involving actin, a contractile protein and part of the cytoskeleton, are regulated by actin binding proteins in the majority of physiological processes in eukaryotic cells. To understand the contribution of actin proteins to the physiological processes of zebrafish, it is important to identify the diverse isoforms of actin encoded by its genome; however, significant sequence identity complicates isoform assignments. Through a combination of human-directed sequence and functional analysis, we have assigned and performed localization of *actc1c*, a previously undesignated cardiac actin gene, and propose an updated assignment of α-actin protein isoform identities in zebrafish.

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1. Introduction

The use of zebrafish (*Danio rerio*) as a developmental model organism for human disease has emerged as a powerful tool. Research regarding the dysregulation or mutation of human genes requires a strong assignment of the corresponding zebrafish gene to translate results between species. An example is our research of the role of cardiac actin in the development of human cardiovascular disease. Zebrafish are an excellent model for cardiovascular work because zebrafish embryos, unlike the mouse, do not require a fully functional cardiovascular system to survive as they can receive oxygen through diffusion from the surrounding environment (Bakkers, 2011). This initial reduced reliance on a functional heart allows us to assess the development of cardiovascular defects, such as a non-beating heart. Finally, the optical transparency of zebrafish embryos permits non-invasive visualization of the developing cardiovascular system using a simple light microscope (Bakkers, 2011).

Our goal is to develop zebrafish as a model to study alterations in cardiac actin proteins; however, the high sequence identity among actin isoforms creates many challenges. In humans, there are six primary actin isoforms (Bertola et al., 2008): α-skeletal (*ACTA1*) and α-cardiac actin (*ACTC1*) classified as striated muscle isoforms; α-smooth (*ACTA2*), and gamma smooth muscle (*ACTG2*) classified as smooth muscle isoforms and lastly beta cytoplasmic (*ACTB*) and gamma cytoplasmic (*ACTG1*) classified as cytoplasmic isoforms (Bertola et al., 2008; Müller et al., 2012). A major distinguishing difference among human isoforms exists at the N-terminus with the β-, γ-, and α-isoforms possessing three, three and four acidic residues, respectively. Importantly, these differences are exploited by commercially-available isoform-specific antibodies. Studies have shown that the number of acidic residues at the N-terminus of actin is correlated with function; for instance, the binding activity of myosin subfragment-1 is increased by the addition of acidic residues (reviewed in (Doyle and Reisler, 2002)). At the same time, the sequence of α-isoforms N-termini in humans possesses a few differences: a conserved threonine to serine change distinguishes the α-smooth isoform, and a variance in the sequence of the acidic amino acids between α-skeletal and α-cardiac isoforms (Table 1).

In our work described below, we found the zebrafish gene associated with zgc:86709 was not given a gene name; therefore, we have assigned the name *actc1c* to this gene. Taking the whole set of genomes available into consideration, we searched for the sequence of the *actc1c* gene using Ensembl to view homologs in a

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Table 1
N-terminal sequences of human α-actin isoforms and zebrafish actin translated genes. Sequence positions are those found before N-terminal processing of the mature actin protein. The human α-actin isoform protein sequences (*top*) refer to UniProt database entries. Sequence names in **bold** possess the canonical α-cardiac N-terminal acidic residue sequence. The clusters of N-terminal acidic amino acids are underlined. The position of variable amino acids outside of the N-termini are listed across the top. Distinguishing variances are highlighted in **bold**. The zebrafish gene names (*bottom*) correspond those in the ZFIN database (accessed June 14, 2016).

Isoform	N-terminal sequence	91	162	167	280	301	360	362	Isoform	UniProt
α-skeletal	MCDEDETTAL	**S**	T	I	T	**M**	**T**	Q	α-skeletal	P68133
α-cardiac	**MCDDEETTAL**	T	T	I	T	L	S	Q	α-cardiac	P68032
α-smooth	MCEEEDSTAL	**S**	T	I	T	L	S	**P**	α-smooth	P62736
ZFIN name										
acta1b	MCDDDETTAL	T	**S**	I	**A**	L	**T**	Q	α-cardiac	
actc1a	MCDDDETTAL	T	T	I	**A**	L	S	Q	α-cardiac	
actc1b	**MCDDEETTAL**	T	T	**V**	**A**	L	**T**	Q	α-skeletal	
acta1a	MCDDDETTAL	T	T	I	T	L	**T**	Q	α-skeletal	
actc1c	MCDDDETTAL	T	T	I	T	L	S	Q	α-cardiac	
acta2	**MCDDEESTAL**	**S**	T	I	T	L	S	Q	α-smooth	

Table 2
Gene-specific primers for generating the probes used for *in situ* hybridization experiments (ISH), and for quantifying relative gene expression (RT-qPCR).

Primer name	Sequence	Application
Actc1c F1	5′ ATGTAATGATGCCCAGGATAC 3′	ISH
Actc1c R1	5′ AAAAAATGTGCACTTTGG 3′	ISH
Acta1b F1	5′CTGCATCTGTCTTCTGTG 3′	ISH
Acta1b R1	5′ ATGGTTTAATGGTCTTCTGG 3′	ISH
Actc1c F2	5′GTCCATCCATCGTCCATAGG 3′	RT-qPCR
Actc1c R2	5′AAATGTGCACTTTGGTGTAAACAT 3′	RT-qPCR
Acta1b F2	5′ATCGGCTGCATCTGTCTTCT 3′	RT-qPCR
Acta1b R2	5′GTCCCAAAGCTGTCCCATAA 3′	RT-qPCR
Rpl8 F	5′ATAGTCTGCTGTCTGGAGGAG 3′	RT-qPCR
Rpl8 R	5′TTGGGGACAAGCCCTGGAGC 3′	RT-qPCR

gene tree generated by the Gene Orthology/Paralogy prediction method pipeline. We were able to view the evolutionary history of the zgc:86709 gene. According to these results, orthologs exist between two species: the cave fish and the spotted gar. A BlastP (NCBI Blast): amino acid database against protein database search of the cave fish zgc:86709 protein sequence compared to zebrafish revealed 100% protein sequence conservation. The remaining α-isoforms expressed significant percentage identity (>98%). Specifically, actc1a, acta1b, acta1a, actc1b, and acta2 had percentage identities of 99.73%, 99.20%, 99.73%, 98.67%, 98.94%, respectively. A BlastP search was also performed between the spotted gar and zebrafish zgc:86709 protein sequence. The results revealed 99.73% protein sequence conservation for *actc1c*. Similar to the cave fish, the percentage identity results were also significant (>98%) between the spotted gar and zebrafish. Specifically, actc1a, acta1b, acta1a, actc1b, and acta2 had percentage identities of 99.47%, 98.94%, 99.47%, 98.94%, 99.20%, respectively.

The partially duplicated genome in zebrafish complicates research aimed at analyzing the effect of knocking-out actin isoforms using clustered regularly interspaced short palindromic repeats (CRISPR) technology, due to potential functional redundancies (Bakkers, 2011). The Zebrafish Information Network (ZFIN) database has assigned isoforms to zebrafish actin genes on the basis of alignments with mammalian orthologs (Sprague et al., 2006); however, caution is necessary as the significant sequence identity between actin isoforms creates challenges with isoform assignments. Also, even slight divergence of the actin sequence from the human canonical sequences of actin isoforms can produce misassignments during machine annotation of actin gene isoforms. To develop a more accurate picture of the actin genes in zebrafish, we combined analysis of the actin genes in the zebrafish genome with available experimental evidence to suggest the most probable zebrafish α-actin genes. Our intention is to propose the genes that most likely produce actin isoform orthologs in zebrafish as a starting point for further functional and quantitative experimental testing, recognizing that the significant sequence identity among actin sequences can mislead both computers and researchers.

2. Methods and results

A Basic Local Alignment Search Tool (BLASTP) search of homologous sequences of actin proteins in the zebrafish compared with that of the human yielded 18 different proteins, with sequence identities up to 99%. BLAST-like Alignment Tool (BLAT) analysis revealed that several of these sequences result from the same gene. After merging duplicates, we identified nine distinct zebrafish actin genes encoding the previous 18 different proteins. An actin sequence located on chromosome 20 that had not been previously assigned was found associated with the zgc: 86709 entry and was designated as *actc1c* (Table 1).

Duplicate genes on chromosome 19 and 20, both called *actc1a*, encode the same zebrafish α-actin protein. These *actc1a* genes are almost identical. Both genes are 6.37 kb long with 9 exons. An alignment of the sequences between the start and stop codons of the genes reveals only 71 nucleotide changes and 7 gaps: five 1 bp, one 4 bp and one 20 bp long. Seven nucleotide changes are found among the 1134 nucleotides of the coding regions (99.4% identity).

The nine zebrafish actin gene product sequences, together with human actin isoforms, were organized into clades in a phylogenetic tree using the Neighbor-Joining method corresponding to cytoplasmic actin proteins, smooth actin isoforms, and α-striated (α-cardiac and α-skeletal) isoforms (Fig. 1). The zebrafish cytoplasmic *actb1* and *actb2* genes are distinguished from the smooth and striated actin isoforms, being most similar to human β-cytoplasmic actin, including the lack of a codon encoding cysteine after the initiator methionine seen among α-actin genes. Of the nine zebrafish genes identified, no clear ortholog of human γ-cytoplasmic actin was included; however, the *acta2* gene product was grouped into a clade with the human α- and γ-smooth actin isoforms. The remaining six zebrafish actin gene products were associated with α-striated actin isoforms in a clade that was separated from the rest with a high level of confidence from the bootstrapping.

An alignment of the human α-actin protein sequences with the six zebrafish putative α-actin proteins revealed significant levels of sequence identity (Table 1). Interestingly, none of the zebrafish α-actin sequences possess the canonical human α-skeletal N-terminal acidic amino acid motif DEDE, while two zebrafish α-actin proteins possess the corresponding DDEE motif found in human α-cardiac actin. The most common zebrafish N-terminal acidic residue motif is DDDE, which is not found in any human sequence.

Aside from the four acidic amino acids at the N-terminus, only

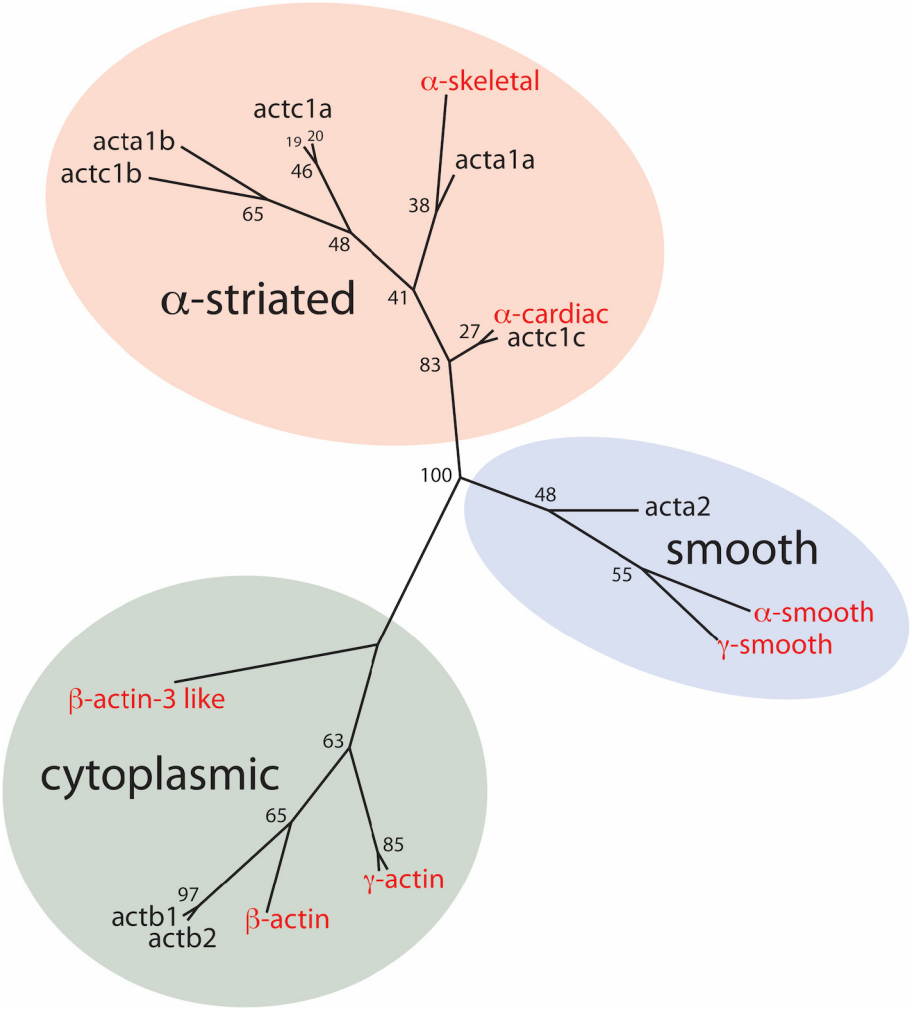

Fig. 1. Phylogenetic tree of zebrafish and human actin proteins. An alignment of actin protein sequences from Table 1 was generated with CLUSTAL Omega and the evolutionary history inferred using the Neighbor-Joining method (Saitou and Nei, 1987). An unrooted tree was produced with HyperTree. The optimal tree with the sum of branch length = 0.19034385 is shown, although the logs of branch distances are shown to provide more space between genes. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The percentage of replicate trees in which associated proteins clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). All positions containing gaps and missing data were eliminated. There were a total of 374 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). Clades representing the three major isoform types of actin are in different colors. Human actin isoforms are shown in red lettering; zebrafish actin gene names from the ZFIN database are shown in black. The chromosome numbers of the duplicate *actc1a* genes are indicated. The *actc1c* gene name was designated in the current work.

eight other positions among the 375 amino acids in the zebrafish or human α-actin protein sequences exhibit substitutions (Table 1), demonstrating the significant conservation of these actin protein sequences. For half of these changes, a change is found in only one of the nine sequences. Of those four changes, three are conserved (T162S, I167V, and L301), whereas one is less conserved (Q362P).

The *acta2* zebrafish α-actin possesses the α-cardiac actin-like DDEE N-terminal acidic residue motif with a characteristic threonine-to-serine change found in α-smooth actin and an α-smooth actin-like Ser-91. That zebrafish gene product was grouped in the α-smooth actin clade, but it is important to note that it is close to the α-cardiac actin. Alternatively, all of the non-N-terminal amino acids of the *actc1c* zebrafish α-actin match those of α-cardiac actin, excluding the presence of the common DDDE N-terminal motif in zebrafish actin sequences. The result is that the *actc1c* gene product is located close to the human α-cardiac actin sequence on the phylogenetic tree.

The remaining four zebrafish α-actin protein sequences, are more closely related to human α-skeletal actin in the phylogenetic tree. All four contain the same zebrafish DDDE N-terminus motif. Subtle conserved changes seem to distinguish this group; namely the presence of Ala-280 and/or Thr-360. Since Thr-360 is present in α-skeletal actin and the product of the zebrafish *acta1a* gene contains a Thr-360 and Thr-280, *acta1a* is situated closer to α-skeletal actin than any other zebrafish actin gene. At the same time, α-skeletal actin does not encode Ala-280 and thus the remaining three genes possessing Ala-280 are defined on a separate branch. Similarly, cardiac and skeletal actin proteins, which exhibit N-terminal acidic amino acids identical to zebrafish muscle actins (DDDE and DDEE) have been identified in medaka *Oryzias latipes* (Kusakabe et al., 1999).

Interestingly, the remaining three actin genes – *actc1a, acta1b, and actc1b* – are the best candidate α-cardiac actin genes in zebrafish. The database naming of these genes as *actc* or *acta* highlights the need for thoughtful designation of actin genes that incorporates more information than sequence similarity alone. Research has shown cardiac-related effects with mutations in *actc1a* or *acta1b*. The S434 mutant of the *actc1a* gene results from a

Y169S amino acid substitution, exhibiting serious cardiac defects. *In situ* hybridization reveals that the *actc1a* gene products are expressed in the heart and somatic muscle (Glenn et al., 2012). The *acta1b* mutant known as *cardiofunk* (*cfk*), resulting from a R177H substitution, also exhibits cardiac defects and *in situ* hybridization shows gene expression in myocardial cells (Bartman et al., 2004; Wen and Rubenstein, 2003). A recent analysis of the zebrafish cardiac transcriptome confirms that *actc1a* and *acta1b* are expressed in the hearts of zebrafish, where *acta1b* is expressed at a higher level in early development and *actc1a* is expressed at higher levels during adulthood (Shih et al., 2015), suggesting developmental regulation of some zebrafish cardiac actin genes. Of note, the transcriptome results demonstrate that the third gene in this group, *actc1b,* is expressed at higher levels in the skeletal muscle of zebrafish, suggesting that this isoform does not function primarily in cardiac muscle. Ultimately, functional characterization like those obtained for *actc1a* and *acta1b* are needed to assign an isoform designation with high confidence.

As noted above, nearly identical *actc1a* genes were found on chromosomes 19 and 20. The S434 mutant studied by Glenn et al. (2012) was mapped only to the *actc1a* gene on chromosome 20. If there are two copies of the *actc1a* gene, one might expect compensation for the S434 mutation. One possibility is that the *actc1a* gene on chromosome 19 is an artifact of genome assembly. To address this issue and provide additional data regarding the assignment of ortholog isoforms, we compared the synteny of the nine zebrafish actin genes shown in Fig. 1 with the three major human α-actin isoforms (Fig. 2).

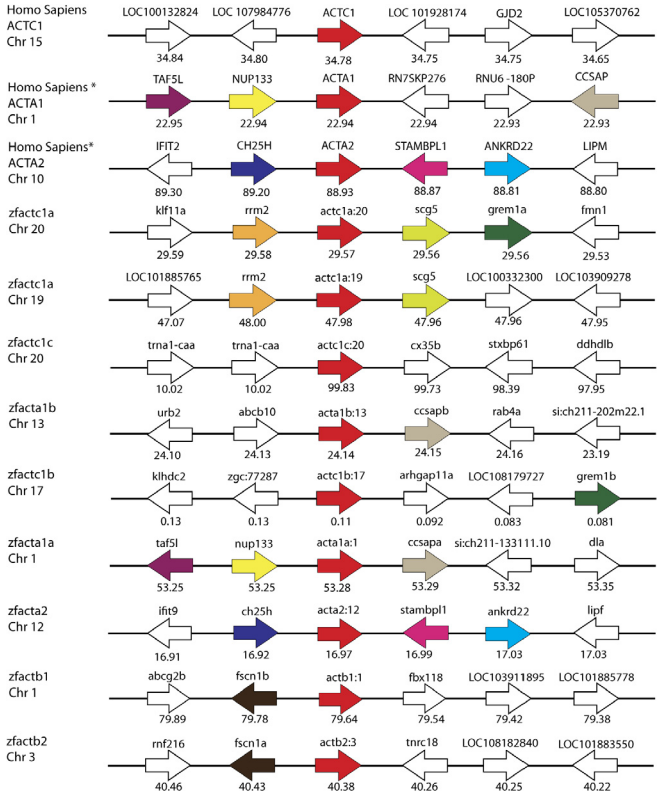

Fig. 2. Syntenic analysis of zebrafish and human actin genes. Presented are two downstream and three upstream genes of the nine zebrafish and three human actin genes described in Fig. 1, with the actin gene in red. Above each arrow is the name of the gene with the start positions of the genes below, in MB. Other genes that find matches among the group are shown in different colors. These analyses were completed using the GRCz10 primary assembly released in September 2014. (*) indicate that some genes for the human *ACTA1* and *ACTA2* genes have not been shown to highlight matching genes found in zebrafish.

The syntenic analysis suggests that the β-actin isoforms in zebrafish resulted from a gene duplication involving both the actin gene and the neighbouring *fscn* genes encoding the actin-binding fascin protein. The syntenic analysis supports the assignment of the zebrafish *acta1a* and *acta2* genes as orthologs of human α-skeletal (*ACTA1*) and α-smooth muscle actin (*ACTA2*), respectively. Interestingly, we see a gene encoding the centriole, cilia and spindle associated protein (*CCSAP*) associated with human *ACTA1* and two actin genes in zebrafish; the *ccsapa* gene linked to *acta1a* and *ccsapb* beside *acta1b*, suggesting that these genes may diverge from a common ancestor, with the *acta1b* gene perhaps resulting from duplication of a region around the *actc1a* gene that included the *ccsap* gene. *In situ* hybridization experiments localizing the products of the *acta1b* gene in both the heart and tail muscle of zebrafish (Glenn et al., 2012) suggests that this actin isoform functions in both tissues.

Are there duplicate *actc1a* genes on chromosome 19 and 20, or is this a result of a genome assembly error? Both *actc1a* genes are between a downstream *rrm2* gene, encoding the ribonucleotide reductase regulatory subunit M2, and an upstream *scg5* gene, encoding secretogranin V. These regions encompass 46,612 bp and 26,412 bp on chromosome 19 and 20, respectively. The structures of the regions are very similar, with a single 20 kb insert starting 267 bp after the *actc1a* stop codon on chromosome 19. However, the common regions are not identical, with sufficient gaps and nucleotide changes to suggest that these are two different regions. A similar study was performed by Venkatesh et al. in which the actin genes were characterized in the Japanese pufferfish *Fugu rubripes* (Venkatesh et al., 1996). In this study, the cardiac actin genes encoded the same protein while differing in their nucleotide sequences and genomic organization. Given the high degree of similarity in the 5' untranslated regions between the two *actc1a* genes, it is likely that both are expressed. Further experiments are required, however, to determine the expression patterns of the two proposed *actc1a* genes and how the presence of the *actc1a* gene on chromosome 19 affects the S434 mutation of the chromosome 20 gene.

Is the product of the *actc1c* gene identified here located in cardiac tissue? To answer this question, we performed whole mount *in situ* hybridization experiments to compare the localization of *actc1c* mRNA to the confirmed cardiac isoform *acta1b* in zebrafish embryos (Fig. 3). We found that both *acta1b* and *actc1c* were localized to the developing heart tissue in the embryos, supporting our sequence-based prediction that the *actc1c* gene encodes a cardiac actin isoform. Next, we compared the relative mRNA abundances of *acta1b* and *actc1c* in cardiac tissue from 36 hpf zebrafish embryos using a previously described heart isolation protocol (Burns and MacRae, 2006) and reverse-transcription quantitative polymerase chain reaction (RT-qPCR; Fig. 4). Results confirmed that both isoforms are expressed at quantifiable levels in embryonic hearts, with approximately 20-fold more *acta1b* than *actc1c*.

3. Discussion

The hallmark N-terminal acidic residue motifs distinguishing the α-actin protein isoforms in mammals are different in zebrafish. Since ZFIN relies on homology with mammalian sequences to assign a gene name, some of the assignments of the zebrafish actin genes may require additional information to be accurate. We have examined the actin sequences and considered experimental evidence to re-assign the most-likely isoform identity for zebrafish actin genes, including assigning the *actc1c* name to a previously undesignated gene and confirming that this gene is expressed in the heart at early developmental stages. While there are no direct orthologs in zebrafish, sequence and syntenic analyses suggest the

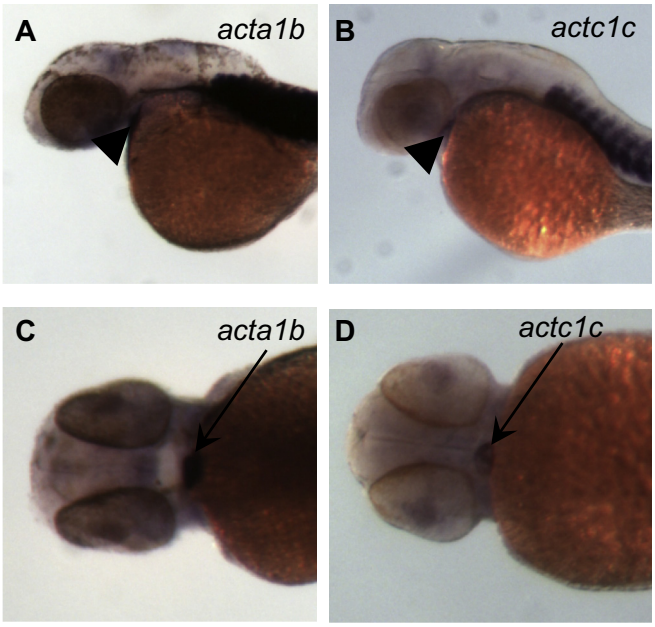

Fig. 3. Localization of *actc1c* expression in 36 hpf zebrafish embryos. (A) Localization of *acta1b* expression at 36 hpf (hours post-fertilization). Note expression in the heart (arrowhead) and significant expression in the tail myotomes. **(B)** Localization of *actc1c* expression at 36 hpf in the heart (arrowhead) and in the tail myotomes, similar to the pattern observed with *acta1b*. **(C)** Ventral view of *acta1b* expression at 36 hpf, showing localization in the developing heart. **(D)** Ventral view of *actc1c* expression at 36 hpf, showing similar localization in the developing heart to *acta1b*. *In situ* hybridization with the new *actc1c* probe was performed four times. To obtain the probes for *in situ* hybridization, total RNA was phenol-chloroform extracted from 20 embryos at 1 dpf using Trizol (Sigma, USA) and reverse-transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystem, USA). The *actc1c* and *acta1b* probe sequences were amplified from the cDNA using designed primers (Table 2) and subcloned into a Topo vector using the Zero Blunt Topo Cloning kit (Life Technologies, USA). Insertion of the sequence into the vector was confirmed by colony PCR and sequencing. The Topo vector containing the *actc1c* and *acta1b* sequence fragments were linearized using XbaI (New England Biolabs, Ipswich) and then transcribed and labelled using SP6 polymerase (Thermoscientific, USA) and a digoxigenin labelling kit (Roche, Germany), respectively. Whole mount *in situ* hybridization was performed using an anti-digoxigenin antibody conjugated to alkaline phosphatase and nitro-blue tetrazolium (NBT) with 5-bromo-4-chloro-39-indolyphosphate (BCIP), (Roche, Germany) for detection.

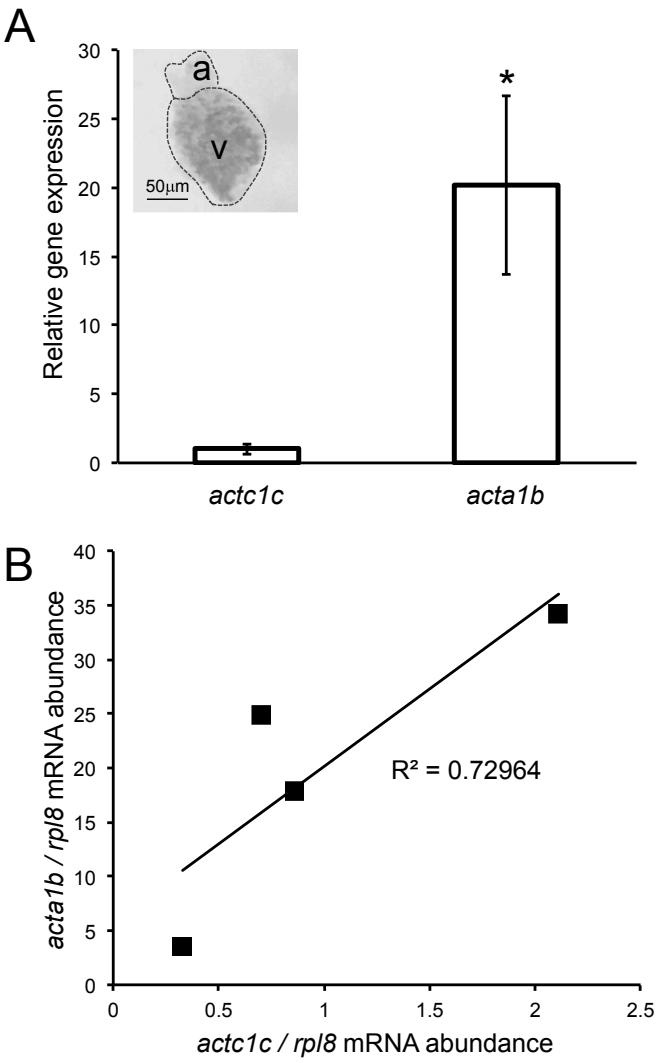

Fig. 4. Comparison of *actc1c* and *acta1b* mRNA abundance in zebrafish hearts at 36 hpf. (A) Gene expression was quantified in isolated hearts (inset showing a = atrium, v = ventricle; scale bar is 50 µm) using RT-qPCR. Hearts were isolated as previously described (Burns and MacRae, 2006), and then RNA was isolated from pairs of hearts using the SingleShot Cell Lysis Kit (Bio-Rad Laboratories, Hercules, CA) at half reaction volumes and following manufacturer's protocol. The isolated RNA was used as template for cDNA synthesis using the iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad) according to manufacturer's instructions such that the final reaction volume contained 40% of the heart lysate. Additional cDNA synthesis reactions that omitted the reverse transcriptase enzyme were included as non-reverse transcribed controls (no-RT). Gene-specific primers for *actc1c*, *acta1b*, and the housekeeping gene *ribosomal protein L8* (*rpl8*; Table 2) were used in separate RT-qPCR reactions containing 1x Power SYBR Green (Life Technologies, Carlsbad, CA), 200 nm each forward and reverse primer, and 1:16 vol template (cDNA, no-RT, or water). Reaction specificity was confirmed with a single-peaked melt curve, and all negative controls failed to amplify. Input values were calculated as the antilog of standard curves generated from serially diluted cDNA (primer efficiency range 93%–113%), averaged across technical replicates (3 for *rpl8*; 4–5 for actin genes), and normalized to *rpl8*. Data is shown as mean ± s.e.m. An * indicates a significant difference, as determined by a Student's t-test ($p < 0.05$; n = 4 pools of 2 hearts each). **(B)** Within sample comparison of mRNA abundance showing a strong positive correlation between *actc1c* and *acta1b* expression.

presence of zebrafish actin isoforms that are more closely related to human isoforms. In particular, *acta2* appears to be a smooth muscle isoform and *acta1a* is closely related to α-skeletal actin. Combined with direct research, the *actc1a* and *acta1b* gene products are likely α-cardiac actin isoforms, while *actc1b* is likely an α-skeletal isoform.

With this information in hand, we can now test the proposed isoform assignments with a combination of functional and expression analyses involving techniques such as comparative fluorescence *in situ* hybridization, RT-qPCR, and CRISPR knockouts. We can target α-actin genes in zebrafish with greater confidence; however, the repeating of *actc1a* on two chromosomes presents challenges when designing morpholinos or CRISPRs that are locus-specific. For *actc1a*, the genomic sequences do not begin to diverge until about 600 bp upstream of the start codon, while the introns and exons encompassing the coding region are nearly identical. As such, coding sequence-disrupting CRISPRs cannot target the different loci. Future studies include experimental evidence to accompany the functional assignment of actin proteins; this is necessary due to the high conservation of the amino acid sequences in the actin proteins. The work presented here permits clarification of the isoform designations of the α-actin genes in zebrafish to aid in studies involving actin genes and proteins.

Editors' note

Please see also related communications in this issue by Segert et al. (2018) and Rafferty et al. (2018).

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