In Vivo Function of the Chaperonin TRiC in α-Actin Folding during Sarcomere Assembly

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In Brief
TRiC-deficient zebrafish feature specific defects in sarcomere and neurite formation. Berger et al. demonstrate a role for TRiC as a multiprotein scaffold positioned at the sarcomere’s Z-disk, where it enhances the processing of skeletal muscle α-actin. Accordingly, TRiC causes aggregation of myopathic α-actin variants in nemaline myopathy.

Highlights
- In zebrafish, TRiC loss causes specific defects in sarcomere and neurite formation
- TRiC only enhances the folding of skeletal α-actin at the sarcomeric Z-disk
- ATP binding by Cct5 is required for folding of α-actin, but probably not tubulin
- TRiC function is required for myopathic actin to form rods in nemaline myopathy
**In Vivo Function of the Chaperonin TRiC in α-Actin Folding during Sarcomere Assembly**

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**SUMMARY**

The TCP-1 ring complex (TRiC) is a multi-subunit group II chaperonin that assists nascent or misfolded proteins to attain their native conformation in an ATP-dependent manner. Functional studies in yeast have suggested that TRiC is an essential and generalized component of the protein-folding machinery of eukaryotic cells. However, TRiC’s involvement in specific cellular processes within multicellular organisms is largely unknown because little validation of TRiC function exists in animals. Our in vivo analysis reveals a surprisingly specific role of TRiC in the biogenesis of skeletal muscle α-actin during sarcomere assembly in myofibers. TRiC acts at the sarcomere’s Z-disk, where it is required for efficient assembly of actin thin filaments. Binding of ATP specifically by the TRiC subunit Cct5 is required for efficient actin folding in vivo. Furthermore, mutant α-actin isoforms that result in nemaline myopathy in patients obtain their pathogenic conformation via this function of TRiC.

**INTRODUCTION**

In eukaryotes, folding of mis- or unfolded proteins is aided by the TCP-1 ring complex (TRiC, also called chaperonin containing TCP-1 [CCT]), a cylindrical structure of two back-to-back rings, each comprised of eight paralogous subunits (CCT1–8) (Gao et al., 1992; Muñoz et al., 2011; Yaffe et al., 1992). Driven by binding and hydrolysis of ATP, TRiC changes its conformation to encapsulate and fold the substrate polypeptides (Joachimiak et al., 2014; Meyer et al., 2003). However, despite the considerable structural data that exist for this complex, the exact mechanism by which TRiC facilitates the folding of its substrates is not fully understood.

In addition to actin and tubulin, the two main substrates of TRiC, it has been estimated that 5%–10% of the proteome of mammalian cells interact with TRiC (Hein et al., 2015; Yam et al., 2008). TRiC loss-of-function mutations in *S. cerevisiae* result in lethality, demonstrating the essential function of TRiC (Stoldt et al., 1996). However, the extent to which TRiC contributes to the folding of its substrates in multicellular, metazoan animals is less explored, and recent in vivo studies indicate that TRiC might have specific functions within higher animals, such as autophagy, cell division, and cell migration (Lundin et al., 2008; Pavel et al., 2016). TRiC has also been associated with multiple diseases, including retinal dystrophy (Migneshi et al., 2016), myocardial infarction (Erdmann et al., 2013) and dysfunction (Melkani et al., 2017), sensory neuropathy (Pereira et al., 2017), Parkinson’s disease (Sot et al., 2017), and Huntington’s disease (Behrends et al., 2006; Tam et al., 2006), suggesting that TRiC components may well be involved in specific cellular processes within distinct tissues.

**RESULTS**

A Missense Mutation in the Cct5 Subunit of TRiC Leads to Skeletal Muscle Defects

To study sarcomere assembly in vertebrates, we systematically isolated and characterized mutants that carry defects in sarcomere formation within zebrafish larvae. As part of this approach, we mapped the unresolved mutant *tf212b*, which was previously identified in a genetic screen for mutants with reduced birefringence (Granato et al., 1996). Birefringence is the diffraction of polarized light by the pseudo-crystalline array of the muscle’s myofibril and, therefore, a direct measure of myofibril integrity (Berger et al., 2012). Under bright-field microscopy, *tf212b* homozygotes appeared grossly normally (Figure 1A). However,
Figure 1. A Missense Mutation in cct5 Causes Muscle Impairment within cct5<sup>422T2b</sup>

(A) At 3 dpf, cct5<sup>422T2b</sup> mutants appear similar to their siblings under bright-field conditions (n = 6 per genotype).

(B) Representative images of 3-dpf-old cct5<sup>422T2b</sup> mutants under polarized light show a reduction in birefringence (quantified in C).

(C) Quantification of birefringence followed by normalization to 3-dpf-old siblings revealed a highly significant reduction in cct5<sup>422T2b</sup> homozygotes through 7 dpf. Data are mean ± SEM; ****p < 0.0001 homozygotes versus siblings of the same stage by one-way ANOVA with Tukey's post hoc test; n = 3.

(D) SSLP-based mapping linked the phenotype-causing mutation to cct5 on chromosome 24. SSLP positions are shown in centimorgans and million base pairs according to the zebrafish genome assembly. Scale bar, 1 Mbp.

(E) Sequencing identified a missense mutation in exon 9 of cct5 (G422V).

(F) G422 (pink) forms part of the ATP-binding pocket (surface representation, green) of human CCT5. The glycine carbon is only 3.3 Å away from the adenosine nitrogen (stick representation, blue).

(G) Labeling of the sarcolemma with mCherryCaaX and the myofibril with Lifeact-GFP confirmed a reduced amount of myofibrils within cct5<sup>422T2b</sup> homozygotes (n = 4 per genotype).

(H) H&E-stained sagittal sections of 6-dpf-old larvae confirmed the reduced amount of myofibrils in cct5<sup>422T2b</sup>; arrows mark enlarged regions of the sarcoplasm (n = 8 per genotype).

(I) Representative transmission electron micrographs depicting organized sarcomeres, marked by brackets, in 3-dpf siblings and cct5<sup>422T2b</sup> mutants (n = 3 per genotype).

(J) Immunohistochemistry with antibodies against actin (red) and α-Actinin (green) enabled quantification of the sarcomere width that was not altered in cct5<sup>422T2b</sup> (n = 3 per genotype).

(K) At the 18-somite stage, regularly spaced actinin-positive Z-bodies indicate the presence of pre-myofibrils in siblings and cct5<sup>422T2b</sup> homozygotes (n = 4 per genotype).

(L) Maximal force generation of cct5<sup>422T2b</sup> were significantly impaired compared with siblings. Data are mean ± SEM; ****p < 0.0001 by Student’s t test; n = 9.

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polarized light revealed a highly significant reduction in birefringence that persisted to 7 days post-fertilization (dpf), indicating a severe reduction in the amount of myofibrils (Figures 1B and 1C). Mapping of tf212b linked the phenotype-causing mutation to the gene encoding the TRiC subunit Cct5 on chromosome 24 (Figure 1D). Subsequent sequencing identified a missense mutation in cct5 resulting in a glycine-to-valine substitution at position 422, which constitutes part of the conserved ATPase domain (Reissmann et al., 2012; Figures 1E and 1F). The crystal structure of the human ortholog CCT5 (Pereira et al., 2017), which is 87% identical to zebrafish Cct5, shows that the distance between the G422 carbon and adenosine nitrogen of ADP is only 3.3 Å (Figure 1F) and is therefore unlikely to accommodate a valine substitution. Furthermore, molecular dynamics simulations on the wild-type (WT) and G422V mutant protein resulted in structural changes of the ATP binding pocket, a reduction in interaction energy, and ejection of nucleotide on the nanosecond timescale (Figures S1A–S1E). This analysis implies that the G422V mutation results in ATPase-deficient Cct5 protein.

The G422V-harboring cct5 mutant was subsequently named cct5<sup>tf212b</sup>. Knockdown of cct5 by two independent morpholinos, both validated for their functionality, resulted in a reduction of birefringence comparable with cct5<sup>tf212b</sup> homozygotes (Figures S2A–S2G). Furthermore, a second mutant allele of cct5, cct5<sup>tf2972tq</sup>, which carries a single retroviral insertion in cct5 (Amsterdam et al., 2004), failed to complement the birefringence reduction of cct5<sup>tf212b</sup> (Figure S2H). In addition, both cct5 mutants were significantly ameliorated by injection of full-length cct5 mRNA, confirming that the phenotype-causing mutation of cct5<sup>tf212b</sup> resides within cct5 (Figures S2I and S2J).

**The Skeletal Muscle Pathology of cct5<sup>tf212b</sup> Mutants Results in Muscle Weakness**

The reduced amount of myofibril in cct5<sup>tf212b</sup>, indicated by birefringence, was confirmed in the Tg(acta1:mCherryCaaX) and Tg(acta1:Lifeact-GFP) transgenic background, in which mCherryCaaX highlights the sarcolemma together with t-tubules and Lifeact-GFP directly marks actin thin filaments (Figure 1G; Berger et al., 2014). Accordingly, sagittal sections stained with H&E revealed the enlarged sarcoplasm evident within cct5<sup>tf212b</sup> mutants (Figure 1H). However, transmission electron microscopy (TEM) and immunohistochemistry with antibodies against α-Actinin and actin revealed the typical pattern of striation and preserved sarcomere length within the residual myofibril of cct5<sup>tf212b</sup> mutants (Figures 1I and 1J; data not shown). At the 18-somite stage, immunolabeling of Z-bodies with antibodies against α-Actinin within cct5<sup>tf212b</sup> mutants indicated preserved formation of the pre-myofibril, the first step leading to myofibrillogenesis (Sanger et al., 2010; Figure 1K).

To quantify the physiological effect of reduced myofibril formation in cct5<sup>tf212b</sup>, 5-dpf-old cct5<sup>tf212b</sup> homozygotes and siblings were subjected to mechanical force measurements using a force transducer (Li et al., 2013). Isometric force analysis was performed with entire larvae at various larva lengths to identify the optimal muscle length for maximal active force quantification. After single-twitch stimulation, the generation of maximal active force was significantly reduced in the cct5<sup>tf212b</sup> mutant group compared with siblings (Figure 1L). To assess whether the reduced muscle force was based on defective motor neurons, contractions were analyzed using single-twitch stimulations with different pulse duration because short-duration pulses are motor neuron-dependent (Abramsson et al., 2013). The obtained results revealed a superimposable relationship between the two groups, indicating unaltered excitation responses and fully functional motor neurons within cct5<sup>tf212b</sup> mutant larvae (Figure 1M).

In addition to the trunk muscle, the head musculature of cct5<sup>tf212b</sup> mutants was also affected, as indicated by the highly significant reduction in the length of the hyohyoideus muscle marked by the transgenic line Tg(−503unc:GFP) (Berger and Currie, 2013; Figure S3A). In accordance with the reduced head musculature, Alcian blue staining revealed cartilage abnormalities in cct5<sup>tf212b</sup>, preventing mutants from closing their jaw, suggesting that death of cct5<sup>tf212b</sup> mutants at 11 dpf was caused by starvation (Figures S3B and S3C).

Collectively, these analyses reveal that cct5<sup>tf212b</sup> mutants possess a global skeletal muscle pathology, characterized by a severe reduction in the amount of myofibrils formed within individual muscle fibers. The residual sarcomeric units that do form within mutant fibers appear normal in spacing and length, but the overall reduction in myofibrils results in a severe reduction in skeletal muscle-dependent force generation.

**Mutations in Other TRiC Subunits Also Cause Impaired Myofibril Assembly**

To assess whether the myofibril phenotype of the cct5 mutants is subunit-specific, a number of distinct TRiC subunit mutants were analyzed. In previous large-scale gene trap screens, mutations in subunits Cct1 (tct1<sup>13356Ti</sup>), Cct2 (cct2<sup>12769Ti</sup>), Cct5 (cct5<sup>2972tq</sup>), and Cct8 (cct8<sup>3062ti</sup>) have been identified, although detailed phenotypic information is missing on all these mutants (Amsterdam et al., 2004; Petzold et al., 2009). Birefringence analysis of all four mutants revealed a highly significant birefringence reduction in each mutant (Figure 1N). Similar to cct5<sup>tf212b</sup>, muscle phenotypes of all analyzed cct mutants were evident on H&E-stained cross-sections, which revealed increased amounts of endomysial connective tissue (Figures S4A–S4E). Accordingly, TEM analysis of all gene trap mutants revealed a reduced amount of myofibrils with otherwise preserved sarcomere organization (Figures S5A–S5D). These results indicate that mutations in three other TRiC subunits result in a muscle phenotype highly similar to that evident in cct5<sup>tf212b</sup>.

Because the gene trap alleles and the cct5<sup>tf212b</sup> missense mutation likely result in partial loss-of-function phenotypes, we sought to examine full null mutations. The cct3<sup>sa1761</sup> mutant

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(M) Pulse duration and active force relationships are similar in both groups, suggesting unchanged motor neuron excitation. Data are mean ± SEM; n = 10.

(N) The insertion mutants cct1<sup>13356Ti</sup>, cct2<sup>12769Ti</sup>, cct5<sup>2972tq</sup>, and cct8<sup>3062ti</sup> all feature a highly significant reduction in birefringence. Data are mean ± SEM; ***p < 0.001 and ****p < 0.0001 by Student’s t test; n = 3.

See also Figures S1–S5.
Figure 2. Loss of TRiC Subunits Causes Lack of Functional TRiC at Sarcomeric Z-disks

(A) cct3sa1761 harbors an essential splice site mutation in cct3 (red arrow).

(B) RT-PCR using oligonucleotides targeting regions marked by green arrows in (A) generated a 334-bp amplicon with WT embryos and a single 415-bp amplicon with cct3sa1761 homozygotes. Amplicons were identified by sequencing.

(C) Western blot analysis using antibodies against human CCT3 revealed epitope loss in cct3sa1761 homozygotes (GAPDH served as a loading control).

(D) CRISPR/Cas9-mediated deletion of 14 bp from exon 5 of cct4 causes a frameshift evoking premature stop codons in cct4/C014.

(E) Western blot analysis using antibodies against CCT4 revealed epitope loss in cct4/C014 homozygotes (GAPDH served as a loading control).

(F) At 3 dpf, the birefringence was significantly reduced in cct3sa1761 and cct4/C014 mutants compared with siblings. Data are mean ± SEM; ****p < 0.0001 by Student’s t test; n = 3.

(G) GFP fluorescence of Tg(cry:mCherry,-600unc:cct3GFP) localized to sarcomeric Z-disks as identified by co-localization with mCherryCaaX-positive t-tubules introduced by Tg(acta1:mCherryCaaX) in siblings and cct3sa1761 homozygotes (arrows) (n = 5 per genotype).

(H) Expression of the Cct3-GFP fusion protein ameliorated the birefringence of cct3sa1761 homozygotes with high significance. Data are mean ± SEM; ****p < 0.0001 by one-way ANOVA with Tukey’s post hoc test; n = 3.

(I) The striated pattern obtained with antibodies against Cct4 in siblings was severely compromised in cct3sa1761 (n = 5 per genotype).

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TRiC Assemblies at Sarcomere Z-disks, with Loss of Individual Subunits Abolishing Z-disk Localization

To identify the cellular localization of TRiC within skeletal muscle cells, the transgenic line Tg(cry:mCherry,-600unc:ctt3GFP) was generated, in which the α-A-crystallin promoter reports transgene expression by driving mCherry expression in the lens, and GFP-tagged Cct3 expression is directed to the musculature by the unc-45b promoter (Berger and Currie, 2013). To prevent GFP from interfering with TRiC formation and function, GFP was integrated in the apical domain of the Cct3 subunit in a manner similar to studies performed in yeast, in which this tag location preserved CCT function (Pappenberger et al., 2006). In both siblings and rescued cct3sa1761, Cct3-GFP protein was localized to Z-disks, which were indicated by mCherryCaaX-positive t-tubules marked in the Tg(cry:mCherry,CaaX) line (Figure 2G). Expression of GFP-tagged Cct3 in the musculature significantly ameliorated the muscle integrity deficits of transgenic cct3sa1761 homozygotes, as evaluated by birefringence analysis, indicating that the fusion protein was functional and integrated normally into TRiC (Figure 2H). The TRiC localization was also confirmed by immunohistochemistry with antibodies against CCT4 performed in siblings (Figure 2J). However, Cct4 localization was severely compromised in cct3sa1761 mutants, indicating that TRiC was not assembled at the Z-disk in Cct3 deficiency. To validate the subunit specificity of this finding, cct4-14 was crossed to Tg(cry:mCherry,-600unc:ctt3GFP). Localization of Cct3-GFP to Z-disks was not found in cct4-14 homozygotes, and the fluorescence signal was severely reduced compared with the robust signal detected in siblings, suggesting that TRiC was not assembled at the sarcomere after deletion of only one of the eight TRiC subunits (Figure 2J). Accordingly, antibodies against Cct5 and α-Actinin showed that Cct5 was confined to actinin-positive Z-disks in the skeletal muscle of siblings but not cct4-14 homozygotes (Figure 2K). However, expression of transgenic Cct3-GFP in the partial loss-of-function mutant cct5-212b showed that GFP-tagged TRiC in siblings as well as homozygous cct5-212b was localized to the sarcomere’s Z-disk, indicating that TRiC assemblies in cct5-212b (Figure 2L).

Next, TEM was performed to examine sarcomere structure within cct null mutants. Similar to the myofibril organization in other TRiC mutants, residual sarcomeres in cct3sa1761 and cct4-14 were organized (Figure 2M; Figure S5E). However, in contrast to cct5-212b homozygotes, abundant electron-dense aggregates were detected, extending outside of sarcomeres specifically at Z-disks, where previously GFP-tagging and immunohistochemistry analyses indicated that TRiC was localized. Collectively, these analyses suggest that loss of individual subunits abolishes TRiC assembly at the sarcomere’s Z-disk, which, in turn, causes protein aggregation and arrest of sarcomere assembly.

TRiC Function Is Required for Efficient Folding of Skeletal Muscle α-Actin

To analyze whether the documented muscle phenotypes could be related to a reduction of functional α-actin, morphodown of the two orthologs of human ACTA1 was undertaken to reduce, but not eliminate, the levels of Acta1 in zebrafish. Administration of the acta1ATG morpholino, which simultaneously targets translation starts of both acta1a and acta1b, induced a highly significant reduction of birefringence in WT larvae (Figure 3A). The myofibril reduction induced by acta1ATG administration was also apparent in the transgenic background of Tg(acta1:mCherry,CaaX) and Tg(acta1:lifeact-GFP) (Figure 3B). Importantly, residual myofibril organization remained intact after acta1ATG administration (Figure 3C). Therefore, based on the analyzed myofibril reduction and organization, simultaneous
knockdown of acta1a and acta1b resulted in a muscle phenotype similar to that evident in cct mutants.

Folding of skeletal muscle myosin has been suggested to be accelerated by TRiC (Srikakulam and Winkelmann, 1999). Accordingly, along with the main substrates actin and tubulin, skeletal muscle myosin was co-immunoprecipitated from 5-dpf-old WT larvae with antibodies against human CCT5 (Table S1). However, administration of the small molecule EMD57033, which refolds myosin (Radke et al., 2014) and significantly ameliorates the birefringence reduction resulting from unfolded myosin in unc45b morphants (Figures S6A and S6B; Etard et al., 2015), did not show a significant effect on the birefringence of cct3sa1761 homozygotes (Figure S6C).

These results collectively indicate that deficiencies in myosin folding might not be the main effector of the phenotype of cct mutants.

To test whether TRiC loss of function leads to defects specifically in folding of α-actin, GFP-tagged human α-actin (ACTA1-GFP), which has been shown to incorporate into sarcomeres (Sztal et al., 2015), was transiently expressed under control of the muscle-specific unc-45b promoter. Fluorescence of ACTA1-GFP in siblings resulted in the expected striation pattern, indicating integration of ACTA1-GFP into the myofibril (Figure 3D). In contrast, cct3sa1761 myofibers presented with a uniform fluorescence, indicating that the majority of ACTA1-GFP was not integrated into the residual myofibril, which suggests that ACTA1-GFP integration into sarcomeres is enhanced by Cct3 activity. Furthermore, GFP-positive aggregates were not detected, neither in siblings nor in cct3sa1761 homozygotes, indicating that ACTA1-GFP does not aggregate, even at high levels elicited by transgenic overexpression.

TRiC Function Is Required for Nemaline Rod Formation

Nemaline myopathy is a human muscle disorder that is diagnosed by the presence of nemaline rods, rod-shaped electron-dense aggregates that form inside myofibers (North et al., 2014). D286G substitution in ACTA1 has been suggested to cause nemaline rod formation in patients suffering from nemaline myopathy (Ravenscroft et al., 2011). In both mouse and zebrafish, expression of ACTA1D286G-GFP within muscle fibers results in incorporation of the transgenic fusion protein into the myofibril and formation of nemaline rods (Ravenscroft et al., 2011; Sztal et al., 2015). Accordingly, expression of ACTA1D286G-GFP within siblings caused formation of long, rod-shaped nemaline rods and a striated GFP fluorescence pattern resulting from the incorporation of ACTA1D286G-GFP into the sarcomeric background of Tg(acta1: mCherryCaaX) and Tg(acta1:lifeact-GFP) (n = 4 per genotype).

Mutant ACTA1D286G-GFP formed rod-shaped structures (arrowhead) in siblings, whereas, in cct3sa1761 homozygotes, exclusively amorphic aggregates of various sizes (arrowhead) were observed (4 larvae per genotype, each with 5 myofibers analyzed) (Figure 3E). This result revealed that TRiC function is required for nemaline rod formation resulting from the expression of disease-causing skeletal muscle α-actin variants.

ATP Binding by Cct5 Is Specific for Skeletal Muscle α-Actin but Not Tubulin Processing

The other proposed main substrates suggested for TRiC besides actin are α- and β-tubulin (Yaffe et al., 1992), which are the main...
components of microtubules that are essential for cellular functions, including growth and maintenance of neuronal neurites. The zebrafish cct3 mutant has been reported previously to have impaired differentiation of retinal ganglion and tectal neurons (Matsuda and Mishina, 2004), and missense mutations in human CCT2 have been associated with retinal dystrophy and macular degeneration (Minegishi et al., 2016). Accordingly, H&E-stained sections revealed retinal degeneration in cct3sa1761 accompanied by abundant apoptotic cells, as identified by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Figure 4A). Interestingly, all other analyzed TRiC mutants are characterized by a similar retinal defect, except for cct5tf212b, in which the retina appears to develop normally (Figure 4B; Figures S6D–S6G).

Because neuron viability depends on neurites to innervate target tissues (Dubey et al., 2015), and retinal axons connect to the tectum, immunostaining was performed with antibodies against acetylated α-tubulin. This analysis detected a severe reduction of neuronal neurites within the tectum of cct3sa1761, in which the retina appears to develop normally (Figure 4B; Figures S6D–S6G).

The DISCUSSION section introduces the concept that in vivo analysis of cct3 or cct4 loss of function in zebrafish revealed that loss of one Cct subunit likely prevents assembly of TRiC, a result predicted by the crystal structure of yeast TRiC but lacking in vivo validation until our observations. Despite the absence of assembled TRiC, cct3sa1761 and cct4/C014 homozygotes develop into 5-day-old larvae, which is surprising given the proposed absolute requirement of TRiC for the folding of many substrates (Lopez et al., 2015), including β- and γ-actins, that build the general cytoskeletal architecture of the cell. Our studies of GFP-tagged ACTA1 show impaired folding of skeletal muscle α-actin in cct3sa1761, confirming actin as a substrate for TRiC. Importantly, however, pre-myofibril formation is not abolished in cct4/C014, and residual organized sarcomeres are still formed in cct3sa1761 and cct4/C014 that completely lack Cct3 or Cct4 protein. Assuming that TRiC function is largely abolished by the removal of critical Cct subunits, these results demonstrate that skeletal α-actin can fold into its native conformation and assemble into thin filaments without TRiC function in vivo. Similarly, tubulin-based microtubules were detected in residual neuronal axons of the tectum, indicating that α- and β-tubulin can also fold independent of functional TRiC.

Analysis of GFP-tagged Cct3 and immunohistochemistry demonstrates that TRiC localizes to sarcomeres’ Z-disks. Therefore, cct5tf212b, which carries a missense mutation in the ATP-binding pocket, is the only analyzed mutant with a muscle-specific phenotype. This result suggests that ATP binding by the Cct5 subunit is specifically required for skeletal muscle α-actin and not tubulin folding by TRiC, providing an indication of how distinct protein folding activities of TRiC may be regulated.
that TRiC not only plays a role in enhancing the folding of \( \alpha \)-actin but might also act as a scaffold for other proteins to confer actin processing to the Z-disk. During translation, nascent \( \alpha \)-actin is bound by the co-chaperone prefoldin and subsequently transported to TRiC for folding (Vainberg et al., 1999). Interestingly, the co-chaperone Bag3 has recently been reported to also bind TRiC (Fontanella et al., 2010). Bag3 localizes to the Z-disk, where it binds to the actin-capping protein CapZ, which stabilizes thin filaments together with nebulin (Hishiya et al., 2010; Pappas et al., 2006). Combined with our analysis, these results collectively suggest a mechanism of actin processing in which prefoldin delivers nascent actin to TRiC, which ensures effective folding of \( \alpha \)-actin and subsequently passes native \( \alpha \)-actin on to its binding partner Bag3. Bag3 binds to CapZ, which, together with nebulin, stabilizes assembling thin filaments. When assembled, thin filaments are capped by tropomodulin-4 and integrated into sarcomeres (Berger et al., 2014), thereby extending the myofibril.

The important role TRiC plays in the processing of skeletal \( \alpha \)-actin is also reflected by results showing that nemaline rod formation by ACTA1\(^{T286G} \)GFP was only detected in siblings but not in Cct3-deficient mutants that feature loss of functional TRiC at the Z-disk. This important finding suggests that TRiC may act as a central control point in the pathogenesis of \( \alpha \)-actin-associated nemaline myopathies. Considering that the molecular bases of many types of nemaline myopathy remain unknown (North et al., 2014), these results indicate TRiC as a potential modifier and therapeutic target for \( \alpha \)-actin-associated nemaline myopathies.

Direct binding of actin to Cct5 has been suggested for the open conformation of TRiC (Llorca et al., 1999), which is in contrast to a more recent study that proposes functional partitioning of TRiC, in which substrates are bound only by the subunits CCT6, CCT8, and CCT3, which locate opposite to the ATP binding subunits CCT4, CCT2, and CCT5 (Joachimiak et al., 2014). The significant role of ATP binding by Cct5 for substrate folding was confirmed by the phenotype of the zebrafish mutant cct5\(^{T212b} \). Interestingly, however, cct5\(^{T212b} \) mutants were uniquely characterized by a muscle-specific phenotype and not the degeneration of the retina or neuronal axons evident in other cct alleles, suggesting that Cct5 possesses substrate-specific regulatory properties, with folding of \( \alpha \)-actin but not tubulin being affected in cct5\(^{T212b} \). In light of a recent study revealing that CCT5 does not directly bind to actin (Joachimiak et al., 2014), one could speculate that the defects in skeletal muscle actin folding in cct5\(^{T212b} \) are caused by impaired binding of ATP by Cct5 resulting in an impaired TRiC conformation change rather than by direct binding of actin to Cct5. Interestingly, a mutation in human CCT5 has been reported to be associated with sensory neuropathy (Bouhouche et al., 2006). This reported H147R mutation of CCT5 affects the flexibility of the equatorial domain of CCT5 and leaves ATP hydrolysis by CCT5 unaffected (Pereira et al., 2017; Sergeeva et al., 2013). Although not directly examined, it has been speculated that the sensory neuropathy evident in patients may well result from altered tubulin and microtubule processing (Pereira et al., 2017; Sergeeva et al., 2013). By contrast, the G422V substitution in zebrafish Cct5 we describe in this study likely disrupts ATP binding and affects actin but not tubulin folding. Collectively, these studies suggest a model whereby distinct conformational changes of the CCT5 subunit may regulate the substrate specificity of TRiC. More research is clearly needed, however, to unravel the exact molecular function of CCT5 in regulating the folding of specific substrates by TRiC.

In conclusion, genetic dissection of TRiC function in zebrafish identified a highly unexpected role for this complex in coordinating sarcomere assembly during vertebrate skeletal muscle formation and revealed TRiC as a potential modifier of nemaline myopathy. Our observations suggest a model in which TRiC acts as a multiprotein scaffold positioned at the Z-disk of the forming sarcomere, where it enhances the folding and processing of skeletal muscle \( \alpha \)-actin required for efficient thin filament and sarcomere assembly.

**EXPERIMENTAL PROCEDURES**

**Quantification of Birefringence**

The Abrio LS2.2 microscope unit was used to automatically image larvae under unbiased polarized light conditions as reported previously (Berger et al., 2013). Images of larvae were analyzed using the software ImageJ, which enabled selection of the first 20 somites of imaged larvae and measurement of the mean of all gray values of the pixels within this selected region. To facilitate comparison of the birefringence from different mutants, gray values were normalized to control siblings, which were set to 100%. To normalize values of siblings, measured gray values \( (A_1 \text{ to } A_0) \) of each larva were multiplied by 100 and divided by the average of all measured gray values of siblings using \( A_x \times 100/(\sum_i^{n-1} A_i/n) \). To normalize values of mutants, measured gray values \( B_0 \text{ to } B_0 \) of each larva were multiplied by 100 and divided by the average of the measured gray values of the siblings using \( B_x \times 100/(\sum_i^{n-1} A_i/n) \). For morpholino and rescue analyses, 10 or 20 larvae were analyzed per genotype \( (n = 10 \text{ and } n = 20, \text{ respectively}) \). For mutant analyses, normalized values of 6 larvae from the same clutch were averaged, and three independent clutches were analyzed \( (n = 3) \).

**Statistical Analysis**

All data are presented as mean \( \pm \) SEM. SEM was calculated utilizing error propagation. Statistical significance between two groups was determined by Student’s t-test, and significance between multiple groups was calculated by one-way ANOVA with post hoc Tukey’s test. Statistical calculations were performed with the software Prism (GraphPad).

**Force Measurement**

The 5-dpf larval preparations were mounted with aluminum clips between a fixed hook and a force transducer on a micrometer screw for length adjustment. The preparations were held in 3-(N-morpholino)propanesulfonic acid (MOPS)-buffered physiological solution at 22°C and stimulated via two Platinum electrodes. The maximal active force was determined using single-twitch stimulation. Muscles were also stimulated at optimal length where the maximal force was achieved, using different pulse durations (0.01 to 0.5 ms) to examine the excitability of the neuromuscular activation pathway. Although entire larvae were analyzed, the muscle cross-sectional areas of 5-dpf-old larvae were measured to exclude that the difference in active force generation stems from smaller muscles. Importantly, although cct5\(^{T212b} \) mutants showed a myofibril reduction, cross-sectional areas of cct5\(^{T212b} \) homozygotes and their siblings were similar \( (0.0326 \pm 0.0004 \text{ mm}^2 \text{ and } 0.0330 \pm 0.0008 \text{ mm}^2, \text{ respectively}) \).

**Actin Knockdown**

The morpholino acta1ATG (5’-tctgtgctcgctacactttgat-3’) was designed fully complementary to the translation start of acta1b and with two mismatches against acta1a and ordered from Gene Tools. Zebrafish embryos were injected at the 1-cell stage into the yolk with 1.4 nL of morpholino solution as described.
previously (Berger et al., 2011). As controls, isogenic embryos from the same clutches were injected with injection solution (0.1% phenol red in water) and equivalent concentrations of standard control morpholinol (5’-ccttactcctgtcataccttata-3’). The birefringence of morphants was normalized to larvae treated with injection solution.

**Data and Accessibility**

The grey values obtained by the birefringence assay have been deposited at Mendeley Data and are available at https://doi.org/10.17632/fd3fnnr2zr.1.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.12.069.

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**AUTHOR CONTRIBUTIONS**

J.B. performed the experiments. S.B. performed histology, M.L. and A.A. conducted force measurements. A.S.J. initiated the simple sequence length polymorphism (SSLP) analysis. A.G.S. and N.B. modeled human CCT5. J.B. and P.D.C. conceived the experiments and wrote the manuscript, which was edited by all authors.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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