



UNIVERSITY OF LEEDS

This is a repository copy of *Hypertrophic cardiomyopathy mutations in the calponin-homology domain of ACTN2 affect actin binding and cardiomyocyte Z-disc incorporation*.

White Rose Research Online URL for this paper:
<http://eprints.whiterose.ac.uk/100865/>

Version: Accepted Version

Article:

Haywood, NJ, Wolny, M, Rogers, B et al. (4 more authors) (2016) Hypertrophic cardiomyopathy mutations in the calponin-homology domain of ACTN2 affect actin binding and cardiomyocyte Z-disc incorporation. *Biochemical Journal*, 473 (16). pp. 2485-2493. ISSN 0264-6021

<https://doi.org/10.1042/BCJ20160421>

© 2016 The Author(s). This is an author produced version of a paper published in *Biochemical Journal*. Uploaded in accordance with the publisher's self-archiving policy. The final version of record will be available at: <http://dx.doi.org/10.1042/BCJ20160421>.

Reuse

Unless indicated otherwise, fulltext items are protected by copyright with all rights reserved. The copyright exception in section 29 of the Copyright, Designs and Patents Act 1988 allows the making of a single copy solely for the purpose of non-commercial research or private study within the limits of fair dealing. The publisher or other rights-holder may allow further reproduction and re-use of this version - refer to the White Rose Research Online record for this item. Where records identify the publisher as the copyright holder, users can verify any specific terms of use on the publisher's website.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.



eprints@whiterose.ac.uk
<https://eprints.whiterose.ac.uk/>

Hypertrophic Cardiomyopathy Mutations in the calponin-homology domain of ACTN2 affect actin binding and cardiomyocyte Z-disc incorporation.

Natalie J. Haywood, Marcin Wolny, Brendan Rogers, Chi H. Trinh, Yu Shuping, Thomas A. Edwards and Michelle Peckham

Astbury Centre for Structural Molecular Biology and the School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, UK;

Corresponding author:

E-mail: m.peckham@leeds.ac.uk

School of Molecular and Cellular Biology

Faculty of Biological Sciences

University of Leeds

Leeds

UK

LS2 9JT

Phone: 44 (0)1133434348

Fax: 44 (0)1133434228

Short Title: mutations in ACTN2

ABSTRACT

α -actinin 2 (ACTN2) is the only muscle isoform of α -actinin expressed in cardiac muscle. Mutations in this protein have been implicated in mild to moderate forms of hypertrophic cardiomyopathy (HCM). We have investigated the effects of two mutations identified from HCM patients; A119T and G111V, on the secondary and tertiary structure of a purified actin binding domain of ACTN2 by circular dichroism and X-ray crystallography, and show small but distinct changes for both mutations. We also find that both mutants have reduced F-actin binding affinity, although the differences are not significant. The full length mEos2 tagged protein expressed in adult cardiomyocytes shows that both mutations additionally affect Z-disc localisation and dynamic behaviour. Overall, these two mutations have small effects on structure, function and behaviour, which may contribute to a mild phenotype for this disease.

6 KEY WORDS

α -actinin, familial hypertrophic cardiomyopathy, crystal structure, actin, cardiomyocytes, imaging

Summary statement

We have discovered that two mutations in the actin binding domain of α -actinin 2, which cause hypertrophic cardiomyopathy, have minor effects on its structure, ability to bind actin and integrate into Z-discs, providing a potential disease mechanism.

INTRODUCTION

Familial hypertrophic cardiomyopathy (HCM) is a cardiovascular disorder affecting between 1 in 200 to 1 in 500 people in the general population [1, 2]. It is commonly characterized by a hypertrophic left ventricle, and an impaired diastolic relaxation of the heart [3]. Currently well over 1000 mutations in a range of sarcomeric proteins have been identified that cause HCM, with over 500 mutations in the gene for β -cardiac myosin heavy chain (*MYH7*) alone [4]. Mutations in *MYH7* and cardiac myosin-binding protein C cause about 70% of the familial HCM cases [5]. Mutations in several proteins found in the Z-disc have been implicated in HCM [6], including the recent discovery of mutations in α -actinin-2 (*ACTN2*) [7]. *ACTN2* is one of four genes encoding isoforms of α -actinin, two of which (*ACTN1* and *ACTN4*) encode non-muscle isoforms. The third gene (*ACTN3*) is co-expressed with *ACTN2* in skeletal muscle, while only *ACTN2* is expressed in cardiac muscle [8].

All four isoforms of α -actinin consist of an N-terminal actin binding domain (ABD) comprising two calponin homology (CH) domains, connected via a flexible linker, which allows for conformational flexibility in the ABDs, to a rod domain (Fig. 1A). The rod domain contains 4 spectrin repeats (triple helix coiled-coil bundles; SR1-4). The C-terminus of the protein contains a calmodulin-like domain (CaM) composed of 4 EF hands, a well characterised Ca^{2+} binding motif [9]. The rod domain is 24 nm long, and both acts as a stiff spacer and is responsible for the formation of an anti-parallel homodimer. However, the EF hands in striated muscle specific isoforms of ACTN have lost their ability to bind Ca^{2+} and binding to actin is instead regulated by phosphatidylinositol phosphate (4,5) P_2 (PtdIns(4,5) P_2 or PIP $_2$) to the ABD [10-12]. Binding of PIP $_2$ has been shown to activate *ACTN2* by promoting the 'open' conformation of the molecule (Fig. 1B), and increasing its ability to bind to Z-disc titin motifs [13]. Thus *ACTN2* is important in cross-linking actin and titin filaments in the Z-disc [14]. A crystal structure for human *ACTN2* was recently reported, confirming that this molecule forms an anti-parallel heterodimer (as diagrammed in Fig. 1B) with actin binding sites (ABDs) at either end, enabling the protein to bundle and cross-link actin filaments [13].

Eight distinct mutations in *ACTN2* have been reported [7], yet despite its pivotal role in the Z-disc, very little has been done to understand how these mutations lead to disease, in contrast to mutations in non-muscle isoforms such as *ACTN4* [15, 16]. For example, the kidney disease causing mutation, *ACTN4*-K225E, increased the binding affinity of *ACTN4* for actin, leading to increased actin filament aggregation, providing an explanation for the disease [16].

Two mutations for the actin binding domain on *ACTN2* have been described; G111V ([17], which was associated with an HCM patient with a type of hypertrophy classified as 'sigmoidal' thought to be more typical of Z-disc linked disease; and A119T [7], as one of 3 mutations picked up in a screen of Australians with HCM. The original family reported to have an association of the A119T mutation with HCM, had a variable phenotype, with some patients only developing heart failure at the age of 75, and others showing heart failure at ages 36-44 [7]. G111V was only reported for a 31 year old male [17], with no family history of HCM. While he did have significant myopathy it is possible that there were other undetected mutations that may have contributed to his disease. It is not known how these mutations affect the properties of *ACTN2*.

To determine how these two mutations might contribute to HCM, we have expressed and purified the actin binding domain (ABD) comprising the two calponin homology domains from human *ACTN2*, together with two mutant variants A119T (AT) [7] and G111V (GV) [7, 17] and determined their structure, and actin binding affinity. In addition, we investigated the effects of the mutations on the localisation and dynamic behaviour of full length *ACTN2* tagged with mEos2 in cardiomyocytes.

EXPERIMENTAL

Cloning of α -actinin-2 constructs.

The DNA coding sequence for human α -actinin-2 (ACTN2) was purchased from OriGene as a GFP fusion protein in a pCMV6-AC-GFP plasmid (RG208531). The full length open reading frame was subcloned into a modified pEGFP-N1 plasmid, in which the mEos2 [18] replaced eGFP, the *NotI* site following mEos2 was replaced with a *Sall* site, the *XhoI* site in the multiple cloning site (MCS) had been deleted and a novel *NotI* site introduced upstream of mEos2 to enable cloning of the cDNA for ACTN2 between the *BamHI* and *NotI* site in frame with mEos2 α -actinin-2. ACTN2-mEos2 was then subcloned into the Adeasy pdc315 plasmid using *EcoRI* and *Sall*. After confirmation that the sequence was correct (Sanger sequencing (GATC Biotech)), this plasmid was then used to generate adenovirus for expression in adult rat cardiomyocytes as described [19]. mEos2 was a kind gift from Michael Davidson and Loren Looger [18]

To generate the pGEX6p-1 plasmid with ACTN2 CH-domains, DNA for amino acids 19 to 266 was amplified by PCR. *EcoRI* and *Sall*/stop sites were introduced using the primers (5' and 3' respectively). The PCR products were subcloned into a TOPO vector, and sequenced. The TOPO vectors were then digested with *EcoRI* and *Sall* and the cDNA for the CH domains ligated into pGEX-6p-1, in frame with glutathione transferase (GST).

Generation of mutants

Overlap PCR was used to generate point mutations. Short fragments containing the desired mutations were subcloned into the pdc315 ACTN2-mEos2 plasmid to replace the wild type sequence and generate mutant full length constructs for expression using the adenovirus system. Overlap PCR was also used to generate mutant constructs for expression using the pGEX6P1 plasmid. All plasmids were Sanger sequenced (GATC Biotech) to check that the ACTN coding sequences were correct.

Expression and purification of CH domains

Wild type (WT) α -actinin binding domain (ABD) and its mutants (A119T; G111V) were expressed in *E. coli* BL21 Rosetta 2 (Novagen). Overnight small volume (5 ml) cultures were used to inoculate 400 ml LB media. Cultures were grown for 3h in 37 °C, 220 RPM shaking until reaching OD₆₀₀ ~ 0.6 when they were induced with 0.5 mM IPTG and grown for additional 3h in the same conditions. Harvested pellets were resuspended in the lysis buffer (PBS, 1 mM DTT, 1 mM EDTA, 200 ug/ml Lysozyme, 0.1% Triton X-100, 1 protease inhibitors tablet/50 ml) and stored in -80 °C.

Proteins were purified using GST-tag affinity chromatography on SuperGlu resin (Generon). Thawed lysates were sonicated (6 cycles of 10sec on/off pulses), centrifuged (30000g, 20 min) and applied onto the equilibrated column. Column was washed 5 times with PBS and bound protein was incubated with prescission protease in cleavage buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5) overnight in 4 °C on a roller. After cleavage 8 x 1 ml fractions were collected and the column was regenerated using 15 mM reduced glutathione. The purity of the fractions was assessed by SDS-PAGE. Pooled fractions were either dialysed against CD buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.4) for CD measurements, or against F-actin buffer (50 mM KCl, 10 mM MOPS, 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, pH 7.0), for actin binding assays. Alternatively purified protein in the cleavage buffer was used for protein crystallisation at a concentration of 10 mg/ml.

Actin binding assays

Skeletal muscle actin was polymerised from stocks of G-actin, purified from rabbit muscle as described [20]. Actin (~70 μ M) in buffer A (2.0mM Tris pH 8.0, 0.2 mM ATP, 0.2 mM CaCl₂, 1 mM DTT, 0.5 mM NaN₃) was mixed on ice with exchange buffer (3 mM MgCl₂, 11 mM EGTA) in a 10:1 ratio and after 5 minutes an equal volume of polymerisation buffer (300 mM KCl, 12 mM MgCl₂, 1 mM EGTA, 120 mM MOPS, pH 7.0) was added. After 2h incubation actin was dialysed into F-actin (polymerisation) buffer for 72h in 4 °C.

Polymerised actin (5 μ M) was incubated for 1h at room temperature with increasing concentrations of actin binding domain and its mutants (2.5 – 60 μ M) and spun-down,

51000 rpm, 4 °C, 30 min. Supernatant was collected and protein was precipitated using TCA while the pellet was resuspended in Laemmli buffer. Samples were applied on 15% SDS-PAGE gel and stained with Coomassie blue. Image-Lab BioRad software package was used to perform densitometry analysis. Control gels with actin or actin binding domain in 1-60 μ M concentration range were used for normalization of obtained data.

CD spectroscopy

CD measurements were performed on an APP Chirascan CD spectropolarimeter with a 0.1 cm path length quartz cuvette in CD buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.4). Data were collected every 1 nm with 30 s averaging time, each measurement being an average of two repeated scans. Data presented are averaged data collected from at least two separate protein preparations. Thermal measurements were performed in a temperature range from 10 to 85 °C with 0.7 °C/min heating rate, data acquisition every 5 °C and 20 s averaging time. The mean residue molar ellipticity of proteins was calculated as described previously [21]. Protein concentration was measured by absorption at 280 nm. Absorption coefficients were obtained from ProtParam software.

Crystallisation

Crystals were obtained using the hanging drop, vapour diffusion method with crystallisation conditions consisting of different percentages and molecular weight polyethylene glycol (PEG). Solution conditions were wild type (WT) 30% PEG1500 (v/v), G111V mutant 28% PEG2000 (v/v) and A119T mutant 24% PEG3350 (v/v). The wild type and mutant crystals grew at 18°C and belonged to the space group *P1*.

Data collection and processing

Crystals were transferred to the crystallization buffer with the addition of 25% PEG400 (v/v) prior to flash freezing at 100 K. All α -actinin-2 data were recorded to a resolution range of between 1.9 to 2.0 Å from single crystals at 100 K on the macromolecular crystallography beamline stations I02 and I04 at the Diamond Light Source. The diffraction images were integrated, scaled and reduced using two different set of programs implemented as part of the CCP4 program suite [22] or Xia2 [23]. Amongst the data collected two different unit cell parameters were obtained $a=38.7\text{\AA}$, $b=47.5\text{\AA}$, $c=70.3\text{\AA}$, $\alpha=80.8^\circ$, $\beta=80.8^\circ$, $\gamma=76.5^\circ$ for WT1 (AaB6I) and $a=38.3\text{\AA}$, $b=46.6\text{\AA}$, $c=70.2\text{\AA}$, $\alpha=73.8^\circ$, $\beta=80.2^\circ$, $\gamma=75.4^\circ$ for WT2 (AaD2I), G111V mutant and A119T mutants; the differences being a change in the α angle of 7°. Five percent of the reflections from each of the wild type data were selected at random and excluded from the refinement using the program FREERFLAG [24] and constituted the R_{free} set. The R_{free} set for G111V and A119T were copied from the dataset for WT2. The data processing statistics are shown in Table 1.

Structure determination

Two crystal structures were obtained for WT ABD (WT1 and WT2, which refer to crystals from particular wells; AaB6I and AaD2I respectively), and one each for the two mutants. The crystal structure of WT2 was determined by molecular replacement (MR) using the program PHASER [25] with the human α -actinin-3 structure (PDB code 1WKU, [26] as the search model (94% sequence identity). One single MR solution was obtained from PHASER and after initial rounds of rigid body and restrained refinements using REFMAC5 [27], iterative cycles of manual model building using both $2F_o-F_c$ and F_o-F_c maps and refinement were carried out using COOT [28] and REFMAC5 respectively. Water molecules were manually added in COOT for peaks over 3.5σ in the F_o-F_c map, and where appropriate hydrogen bonds could be made to surrounding residues or other water molecules. Refinement was judged complete when the R factor had converged and no significant interpretable features remained in the F_o-F_c map. Similarly the WT1 structure was also determined by MR using PHASER with a single monomer of the refined structure of WT AaD2I as the search model. One single MR solution was obtained from PHASER and iterative cycles of manual model building and refinement carried out using COOT and REFMAC5. The refined WT2 structure was used as a starting model for the refinement of both the mutants in REFMAC5. The side chains of the mutated residues G111V and A119T

were built into their respective F_o-F_c maps and iterative cycles of manual model building and refinement carried out using COOT and REFMAC5. All structural validations were carried out with MOLPROBITY [29]. All refinement statistics are shown in Table 1. Coordinates and structure factor amplitudes for the structures have been deposited in the Protein Data Bank (www.pdb.org), and have been assigned PDB 5A36, 5A38, 5A37 and 5A4B.

Cardiomyocyte culture and Photoactivation experiments

Adult rat cardiomyocytes were isolated, and cultured as described [19] in glass bottomed dishes (Iwaki) coated with laminin. The cardiomyocytes were prepared from isolated rat hearts using a Langendorff retrograde perfusion technique with a collagenase-containing solution [30] by the Cardiovascular group at Leeds, using 20 week old male Wistar rats and kindly provided to us. 2 separate experiments were performed for WT, G11V and A119T, and data from cells from both experiments used in the analysis. Virus was added to the cells in glass bottomed culture dishes (Ibidi) at an MOI of ~50 as described [19] and the cells were incubated overnight before imaging.

The following day, the culture dishes were placed on an heated stage (37 °C) of a confocal (Zeiss LSM510), and imaged with the Plan-achromat x63, n.a.1.4, oil objective, using LSM Meta software and the Multi-Time Series (MTS) macro. First, 5-7 cells in the dish were selected and their positions recorded. Next the zoom set to x6, and for each cell, the image rotated so that the Z-discs in the cell were either vertical or horizontal in the frame. Once this was done, a separate configuration file was saved for each cell, and this was then loaded into the MTS macro. Next, for each cell, a small box or region of interest (ROI) (~140 x 140 (6.6 μ m) pixels; see figure 6 for approximate position of the ROI prior to photoactivation) was positioned over the centre of the cell to select a region that would be illuminated with 405nm light during the experiment. ROIs were individually positioned for each cell, and the positions saved in the MTS macro. The ROIs were slightly wider than three Z-discs. The 'bleaching' protocol was then set up such that the cells were imaged once before illuminating the box with 405nm light (10 cycles, 22% laser power). The cells were then imaged every 150 seconds for at least 12 further frames, using 488nm light (1-5% laser power) and 543 nm light (50% laser power). Laser intensities were chosen to minimise any bleaching, while providing sufficient signal to noise, without the need for averaging. This experiment is similar to a photobleaching experiment, except that in this case the 405nm light permanently switches 'green' fluorescent mEos2 molecules to 'red' within the ROI, Fig. 6). The dynamic behaviour of the switched molecules was then measured from the decrease in red fluorescence in the initial 'photoconverted' region with time. This decrease reports on movement of ACTN2-Eos away from the region of photoconversion.

Additionally, cells were cultured on laminin coated glass coverslips as described [19], fixed using fresh warm 4% paraformaldehyde, which was added to culture medium (50:50) for 15 minutes. Cells were permeabilised using Triton-X 100 (0.5% in PBS) and co-stained for myosin using the A4.1025 antibody [19]. Fixed cells were imaged using a Deltavision deconvolution microscope equipped with a Roper CoolSnap Hq CCD camera, and using the 100x 1.4 N.A. Plan Apo lens. Identical image capture settings were used for all images.

Parallel cell cultures were used to make proteins samples for SDS gels, which were then transferred to polyvinylidene fluoride membrane for use in western blots, using antibodies to actin (as a loading control: Santa Cruz sc4778), ACTN (A5044; Sigma) and mEos (Badrilla). Secondary anti-HRP antibodies, and Immobilon Western Chemiluminescent HRP substrate (Millipore) were used to detect antibody binding.

RESULTS AND DISCUSSION:

EFFECTS OF MUTATIONS IN THE ABD ON ITS SECONDARY STRUCTURE AND THERMAL STABILITY, AND ABILITY TO BIND TO F-ACTIN IN VITRO

Purified wild-type and mutant actin-binding domains (ABDs; AA19-266) from α -actinin-2 (ACTN2) had a similar secondary structure as shown by circular dichroism (Fig. 2A). All three constructs have almost identical far UV spectra with a strong α -helical signal (Fig. 2A). The total helical content (measured by the molar ellipticity value at 222nm) is similar for WT and both mutant constructs, suggesting that the mutations do not have a strong effect on secondary structure.

Thermal denaturation experiments showed that the thermal stability for both mutants was lower than that for the WT construct (Fig. 2B). The G111V mutation had the greatest effect on stability with its melting temperature ($T_m=56.8$ °C) being 5.5 °C lower than that for WT (62.3 °C). The A119T mutation has an intermediate effect with T_m (60.8 °C) only slightly less than WT (Fig. 2B). These results indicate that although the mutations do not affect the secondary structure of the ABD, they lower its stability, with G111V mutation having the stronger effect.

Both G111V and A119T mutations reduced the binding affinity of the ABDs to F-actin. The K_d was increased ~ 2 fold for the A119T mutant, and ~ 1.2 fold for the G111V mutant constructs compared to the wild type ABD (Fig. 2C). However although we always observed a similar trend for these values, they were not significantly different.. G111V lies just outside one of the three stretches of amino acid residues thought to mediate actin binding (residues 41-50, 116-140 and 145-165, reviewed in [31] and A119T lies within one of these stretches. The measured K_d for the WT construct of 7.5 μ M, is comparable to that measured for other ACTN actin binding domains (~ 1 μ M for smooth muscle ACTN (ACTN1), and 5 μ M for *Acanthamoeba* ACTN (39)).

EFFECTS OF MUTATIONS IN THE ABD ON ITS STRUCTURE

Given the small effects on secondary structure, we went on to crystallise the WT and mutant ABDs to determine what effect, if any, the mutations had on tertiary structure. We were successful in obtaining crystals for the WT and both mutant ABDs and were able to solve their structures to a resolution of between 1.9 to 2.2 Å (Table 1). The ABD was in the 'closed' conformation in all the structures, as found for other structures of the ABDs for the second striated muscle isoform ACTN3 [26], and the two non-muscle isoforms ACTN1 [32].

In the closed conformation that we observed, there is extensive contact between the CH1 and CH2 domains, and the residues R243 and W145 stack together (Fig. 3A), as found for other ACTN ABDs [26, 32, 33]. The stacking of R243 and W145 is similar to the stacking between the equivalent lysine residue at position 255 in the CH2 domain of ACTN4 with tryptophan (135) in the closed conformation. Interestingly, the crystal structure of the K255E mutant of the ACTN4 ABD also showed a closed structure, even though this mutation was predicted to promote an open conformation [33]. Although the ACTN2 ABD may adopt a more open conformation, in which the two CH domains become more separated, when it binds to actin [34] currently there is not clear demonstration that this does happen.

Intriguingly, one of our two structures for the wild type construct contains an additional N-terminal α -helix, upstream of the first CH1 domain not seen previously (Fig. 3A). From our structures of the other WT and the two mutants that do not have this N-terminus helix, this may suggest that the N-terminus is highly flexible. The appearance of this helix for one of our structures may suggest that in this case, the crystal packing stabilises the helix by adjacent molecules. This helix is not thought to be involved in binding to actin but it could be involved in binding to one of the many other Z-disc proteins known to bind to α -actinin (reviewed in [31]). Muscle LIM protein is known to bind close to this region, and the Q9R

mutation in ACTN2 has been shown to interfere with its ability to bind muscle LIM protein [35]. Aligning this structure of the WT ACTN2 with that for the almost full length ACTN2 recently published [13] shows how this N-terminal helix points away from the CH domains, but that otherwise our structure super-imposes well (Fig. 3B).

Super-imposing the structures for the two mutants onto the wild type structure for the ABD of ACTN2 derived from a crystal structure for the almost full length protein (residues 34-892 were assigned in the deposited model [13]) shows only minor differences in the region of overlapping structure (Fig. 4A-D). However, while continuous densities are observed for the loop region at residues 110-120 in both the wild type (WT; Fig. 4B) and the A119T mutant structure (Fig. 4D), this region is disordered for G111V mutant between residues 112 and 117 (Fig. 4C). For both the WT and A119T mutant structure, Val112 is orientated towards the interior of the molecule packing the side chain into a hydrophobic region. For the G111V mutant, the mutant residue Val111 now occupies this same hydrophobic region and thus reorients Val112 and the subsequent residues away from the molecule resulting in a more flexible conformer for this loop region. This larger alteration to the structure for G111V may help to explain the decreased thermal stability for this mutation observed by circular dichroism (Fig. 2B).

The CH domains of ACTN are thought to have three regions important in binding to actin: residues 41-50, 116-140 and 145-165 (reviewed in [31]). G111V is just outside of one of these regions, and although this mutation appears to introduce some instability into the subsequent loop containing this binding region, it may not have a large effect on actin binding affinity. A119T is within the binding region itself, and its alterations to the structure may be expected to have a small effect on binding. These observations may explain why, despite the small changes in structure, we observed a larger effect of the G111V mutation on thermal stability and tertiary structure, while A119T had a larger effect on actin binding.

MUTATIONS IN THE CH DOMAINS IN ACTN2 REDUCE INCORPORATION INTO Z-DISCS IN CARDIOMYOCYTES.

Wild type, A119T and G111V mEos-2 tagged human ACTN2 all localised to Z-discs in adult cardiomyocytes (Fig. 5A). However, the integration of mutant ACTN2 appeared somewhat weaker than that for WT ACTN2, and there was a greater tendency for mutant isoforms to form aggregates outside the Z-discs. Expression levels of the mutant isoforms were similar to those of WT ACTN2 (Fig. 5B). Therefore, it is unlikely that the change in localisation observed for the mutant isoforms is a result of large differences in expression levels.

In addition, both mutant isoforms affected the dynamic behaviour of ACTN2. After photoactivation of a small region of mEos2-tagged ACTN (Fig. 6A), we found that the rate of fluorescence decay from the central region of the 'photoswitched' area was reduced for both G111V and A119T mutants compared to wild type ACTN2 (Fig. 6B-D), and this change was significant for G111V. This suggests that mutant ACTN that does incorporate into the Z-disc is less able to leave (Fig. 6D). Taken together, these data suggest that the exchange of mutant protein within the the Z-disc is aberrant for both mutants compared to wild type ACTN2.

These data shows that both mutations appear to reduce the ability of the protein to incorporate normally into Z-discs. The small reduction in actin binding affinity we observed *in vitro* for the isolated CH domains for the two mutant may help to account for the reduction in incorporation of the full length mEos2-tagged ACTN into Z-discs in cells, although the changes we observed *in vitro* were small and non-significant. If the mutant full length proteins are less able to incorporate into the Z-discs, then the unincorporated protein may be more likely to aggregate as we observed. As ACTN2 is important for structural integrity and mechanosensing of the Z-disc, reduced incorporation could lead to reduced force transmission in cardiac muscle, thus providing a potential mechanism for development of disease.

In the full length protein, the C-terminal EF3/4 domain of ACTN2 would be expected to be more important in localising ACTN2 to the Z-disc, as it binds tightly to the Z repeat (ZR7) of titin (Fig. 2B). However, the slightly weaker actin binding affinity that we measured for both mutants does appear to affect the dynamic incorporation of ACTN2 into Z-discs. The binding of ACTN2 to ZR7 depends on the binding of PtdIns(4,5)P₂ (PIP2) to the ABD, which results in the release of the EF3/4 domain from the neck (Fig. 2), allowing the EF3/4 domain to bind to ZR7. PIP2 is thought to bind to a triplet of residues R163, R169 and R192 [26]. The binding affinity of the neck domains for ZR7 in the presence of PIP2 is ~10 fold higher (K_d of 380nM [13]) than in its absence, and over 10 fold higher than the binding affinity of the CH domains for actin. PIP2 binding mutations in ACTN do not affect F-actin binding [13], and it is unlikely that the ACTN mutations we tested here affect PIP2 binding. We speculate that perhaps there is some kind of mechanical strain between the actin and titin binding sites in ACTN2, when it is localised to the Z-disc, which promotes the exchange of ACTN2 between Z-discs. A reduced actin binding capability may reduce this strain, thereby reducing the rate and extent of exchange of ACTN molecules in the Z-disc.

As discussed in the introduction, the two mutations we have investigated here result in a relatively mild phenotype, are likely to be benign, and in common with many HCM mutations in sarcomeric proteins, may take many years to show themselves as an obvious phenotype [4]. Our data suggest that both G111V and A119T mutations affect the ability of ACTN2 to function normally. Although these mutations have only small effects on structure and behaviour, this is consistent with their mild phenotype, and thus they are likely to be genuine disease mutations.

Acknowledgements

Natalie Haywood and Yu Shuping were Masters students in the Faculty of Biological Sciences at Leeds. Marcin Wolny was supported by a BBSRC grant to MP (BB/I007423/1). The Wellcome Trust funded the CD instrument (WT094232). Brendan Rogers is funded by the BBSRC White Rose DTP in Mechanistic biology. We thank the MX beamline scientists at Diamond Light Source Ltd for their assistance in collecting the ACTN2 data.

Author contribution:

Natalie Haywood generated the crystals, and helped to solve the structure. Yu Shuping generated the expression constructs, and performed some of the early imaging and actin binding experiments. Marcin Wolny expressed and purified constructs, performed CD and actin binding assays. Brendan Rogers expressed and purified constructs and performed additional binding assays. Chi Trinh advised on the crystallography and solved the structures Thomas Edwards advised on the protein purification and crystallography Michelle Peckham helped to generate the constructs, wrote the paper and performed some of the imaging. All the authors commented on the paper.

REFERENCES

- 1 Maron, B. J., Gardin, J. M., Flack, J. M., Gidding, S. S., Kurosaki, T. T. and Bild, D. E. (1995) Prevalence of hypertrophic cardiomyopathy in a general population of young adults. Echocardiographic analysis of 4111 subjects in the CARDIA Study. Coronary Artery Risk Development in (Young) Adults. *Circulation*. **92**, 785-789
- 2 Maron, B. J. and Maron, M. S. (2015) The 20 advances that have defined contemporary hypertrophic cardiomyopathy. *Trends in cardiovascular medicine*. **25**, 54-64
- 3 Wigle, E. D. (1990) Impaired left ventricular relaxation in hypertrophic cardiomyopathy: relation to extent of hypertrophy. *Journal of the American College of Cardiology*. **15**, 814-815
- 4 Colegrave, M. and Peckham, M. (2014) Structural implications of beta-cardiac myosin heavy chain mutations in human disease. *Anat Rec (Hoboken)*. **297**, 1670-1680
- 5 Maron, B. J. and Maron, M. S. (2013) Hypertrophic cardiomyopathy. *Lancet*. **381**, 242-255
- 6 Bos, J. M. and Ackerman, M. J. (2010) Z-disc genes in hypertrophic cardiomyopathy: stretching the cardiomyopathies? *Journal of the American College of Cardiology*. **55**, 1136-1138
- 7 Chiu, C., Bagnall, R. D., Ingles, J., Yeates, L., Kennerson, M., Donald, J. A., Jormakka, M., Lind, J. M. and Semsarian, C. (2010) Mutations in alpha-actinin-2 cause hypertrophic cardiomyopathy: a genome-wide analysis. *Journal of the American College of Cardiology*. **55**, 1127-1135
- 8 Beggs, A. H., Byers, T. J., Knoll, J. H., Boyce, F. M., Bruns, G. A. and Kunkel, L. M. (1992) Cloning and characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1 and 11. *The Journal of biological chemistry*. **267**, 9281-9288
- 9 Kretsinger, R. H. (1976) Calcium-binding proteins. *Annu Rev Biochem*. **45**, 239-266
- 10 Fukami, K., Furuhashi, K., Inagaki, M., Endo, T., Hatano, S. and Takenawa, T. (1992) Requirement of phosphatidylinositol 4,5-bisphosphate for alpha-actinin function. *Nature*. **359**, 150-152
- 11 Young, P., Ferguson, C., Banuelos, S. and Gautel, M. (1998) Molecular structure of the sarcomeric Z-disk: two types of titin interactions lead to an asymmetrical sorting of alpha-actinin. *The EMBO journal*. **17**, 1614-1624
- 12 Young, P. and Gautel, M. (2000) The interaction of titin and alpha-actinin is controlled by a phospholipid-regulated intramolecular pseudoligand mechanism. *The EMBO journal*. **19**, 6331-6340
- 13 Ribeiro Ede, A., Jr., Pinotsis, N., Ghisleni, A., Salmazo, A., Konarev, P. V., Kostan, J., Sjoblom, B., Schreiner, C., Polyansky, A. A., Gkoukoulia, E. A., Holt, M. R., Achmann, F. L., Zagrovic, B., Bordignon, E., Pirker, K. F., Svergun, D. I., Gautel, M. and Djinic-Carugo, K. (2014) The Structure and Regulation of Human Muscle alpha-Actinin. *Cell*. **159**, 1447-1460
- 14 Luther, P. K. (2009) The vertebrate muscle Z-disc: sarcomere anchor for structure and signalling. *Journal of muscle research and cell motility*. **30**, 171-185
- 15 Weins, A., Kenlan, P., Herbert, S., Le, T. C., Villegas, I., Kaplan, B. S., Appel, G. B. and Pollak, M. R. (2005) Mutational and Biological Analysis of alpha-actinin-4 in focal segmental glomerulosclerosis. *Journal of the American Society of Nephrology : JASN*. **16**, 3694-3701
- 16 Weins, A., Schlondorff, J. S., Nakamura, F., Denker, B. M., Hartwig, J. H., Stossel, T. P. and Pollak, M. R. (2007) Disease-associated mutant alpha-actinin-4 reveals a mechanism for regulating its F-actin-binding affinity. *Proceedings of the National Academy of Sciences of the United States of America*. **104**, 16080-16085
- 17 Theis, J. L., Bos, J. M., Bartleson, V. B., Will, M. L., Binder, J., Vatta, M., Towbin, J. A., Gersh, B. J., Ommen, S. R. and Ackerman, M. J. (2006) Echocardiographic-determined septal morphology in Z-disc hypertrophic cardiomyopathy. *Biochemical and biophysical research communications*. **351**, 896-902

- 18 McKinney, S. A., Murphy, C. S., Hazelwood, K. L., Davidson, M. W. and Looger, L. L. (2009) A bright and photostable photoconvertible fluorescent protein. *Nat Methods*. **6**, 131-133
- 19 Wolny, M., Colegrave, M., Colman, L., White, E., Knight, P. J. and Peckham, M. (2013) Cardiomyopathy mutations in the tail of beta-cardiac myosin modify the coiled-coil structure and affect integration into thick filaments in muscle sarcomeres in adult cardiomyocytes. *The Journal of biological chemistry*. **288**, 36260
- 20 Pardee, J. D. and Spudich, J. A. (1982) Purification of muscle actin. *Methods in enzymology*. **85 Pt B**, 164-181
- 21 Greenfield, N. J. (2006) Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. *Nat Protoc*. **1**, 2527-2535
- 22 Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. M., Krissinel, E. B., Leslie, A. G., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterton, E. A., Powell, H. R., Read, R. J., Vagin, A. and Wilson, K. S. (2011) Overview of the CCP4 suite and current developments. *Acta crystallographica. Section D, Biological crystallography*. **67**, 235-242
- 23 Winter, G. (2010) xia2: an expert system for macromolecular crystallography data reduction. *J. Appl. Cryst.* **43**, 186-190
- 24 Brunger, A. T. (1997) Free R value: cross-validation in crystallography. *Methods in enzymology*. **277**, 366-396
- 25 McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. and Read, R. J. (2007) Phaser crystallographic software. *J Appl Crystallogr.* **40**, 658-674
- 26 Franzot, G., Sjoblom, B., Gautel, M. and Djinic Carugo, K. (2005) The crystal structure of the actin binding domain from alpha-actinin in its closed conformation: structural insight into phospholipid regulation of alpha-actinin. *Journal of molecular biology*. **348**, 151-165
- 27 Murshudov, G. N., Vagin, A. A. and Dodson, E. J. (1997) Refinement of Macromolecular Structures by the Maximum-Likelihood Method. *Acta Cryst.* **D53**, 240-255
- 28 Emsley, P. and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Cryst.* **D60**, 2126 - 2132
- 29 Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. and Richardson, D. C. (2010) Structure of protease-cleaved Escherichia coli α -2-macroglobulin reveals a putative mechanism of conformational activation for protease entrapment. *Acta Cryst.* **D66**, 12-21
- 30 McCrossan, Z. A., Billeter, R. and White, E. (2004) Transmural changes in size, contractile and electrical properties of SHR left ventricular myocytes during compensated hypertrophy. *Cardiovascular research*. **63**, 283-292
- 31 Sjoblom, B., Salmazo, A. and Djinic-Carugo, K. (2008) Alpha-actinin structure and regulation. *Cellular and molecular life sciences : CMLS*. **65**, 2688-2701
- 32 Borrego-Diaz, E., Kerff, F., Lee, S. H., Ferron, F., Li, Y. and Dominguez, R. (2006) Crystal structure of the actin-binding domain of alpha-actinin 1: evaluating two competing actin-binding models. *Journal of structural biology*. **155**, 230-238
- 33 Lee, S. H., Weins, A., Hayes, D. B., Pollak, M. R. and Dominguez, R. (2008) Crystal structure of the actin-binding domain of alpha-actinin-4 Lys255Glu mutant implicated in focal segmental glomerulosclerosis. *Journal of molecular biology*. **376**, 317-324
- 34 Joseph, C., Stier, G., O'Brien, R., Politou, A. S., Atkinson, R. A., Bianco, A., Ladbury, J. E., Martin, S. R. and Pastore, A. (2001) A structural characterization of the interactions between titin Z-repeats and the alpha-actinin C-terminal domain. *Biochemistry*. **40**, 4957-4965
- 35 Mohapatra, B., Jimenez, S., Lin, J. H., Bowles, K. R., Coveler, K. J., Marx, J. G., Chrisco, M. A., Murphy, R. T., Lurie, P. R., Schwartz, R. J., Elliott, P. M., Vatta, M., McKenna, W., Towbin, J. A. and Bowles, N. E. (2003) Mutations in the muscle LIM protein and alpha-actinin-2 genes in dilated cardiomyopathy and endocardial fibroelastosis. *Molecular genetics and metabolism*. **80**, 207-215
- 36 Engh, R. A. and Huber, R. (1991) Accurate bond and angle parameters for X-ray protein structure refinement. *Acta Crystallographica Section A*. **47**, 392-400

37 Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr.* **66**, 12-21

Tables

Table 1. Data collection, processing and refinement statistics for wild type ACTN2 and the mutants G111V and A119T.

Figure Legends

Figure 1. Domain structure of ACTN2 **A:** The domain structure of α -actinin, showing the positions of the different domains. The positions of the two hypertrophic cardiomyopathy mutations in the actin-binding domain studied here are indicated. The actin-binding domain (ABD) is made up of 2 CH (calponin homology) domains. The calmodulin like (CaM) domain contains two pairs of EF hands (EF12 and EF34). Numbers refer to amino acid residues for human skeletal ACTN2. The two main expression constructs used in this study are shown below. **B:** The molecule forms an anti-parallel homodimer with a 'closed' conformation in the absence of PtdIns (4,5) P₂ (PIP2), in which the CaM domains interact with the ABD domain. On the binding of PIP2, the molecule adopts an open conformation which allows ACTN2 to bind to actin via its ABD, and to the Z-repeat 7 (ZR7) of titin via its second calponin homology domain (EF34) in the Z-disc.

Figure 2. Analysis of the secondary structure, thermal stability, and actin binding ability of the WT and mutant ACTN2 actin binding domain (ABD: CH1-CH2) construct. **A)** Far UV spectra show similar spectra for WT and the two mutant ABDs. Data shown are the mean values for 3-4 spectra (\pm S.E.M) for each construct. **B)** Thermal denaturation profiles of the WT and two mutant ABDs, together with the values at which half the protein has melted (T_m). Data shown are the mean values (\pm S.E.M) for 3 (WT, A119T) or 2 (G111V) experiments. **C)** The binding curves for the WT and mutant ABD and actin. Data shown are the mean values (\pm S.E.M.) for at least 5 separate experiments. The mean K_d values (\pm S.E.M) are shown in the legend. **D)** Example gels for the pelleted F-actin together with co-pelleted WT ABD, and one of the mutant ABD (A119T) constructs. Arrow shows decreasing concentration of ABD. The gels include a lane without ABD as indicated.

Figure 3. Crystal structure obtained for the WT ABD of α -actinin-2 (ACTN2) and a comparison between this structure and that obtained for the almost full length protein reported recently [13]. **A)** Our crystal structure of the WT ABD of ACTN2 with the CH1 domain shown at the bottom, and the CH2 domain at the top. Red indicates the identified actin binding regions, and cyan the PIP2 binding region. The structure contains an additional resolved N-terminal α -helix (I21-P33). The stacking of R243 and W145 between CH1 and CH2 is indicated. **B)** Superposition of the WT1 ABD of α -actinin-2 (blue) onto the ABD of the full length α -actinin-2 (red) (PDB code 4D1E, [10]).

Figure 4. Effects of mutations in the actin-binding domain on structure. **A)** Overlay of the WT1 (blue), WT2 (cyan), G111V (magenta) and A119T (green) mutant ABD of α -actinin-2 structures. **B-D)** $2F_o - F_c$ electron density contoured at 0.9σ for the loop regions 110-120 for WT and the two mutant constructs.

Figure 5. Expression of wild type and mutant isoforms of mEos2-tagged ACTN2 in adult cardiomyocytes. **A:** Cells were co-stained for myosin heavy chain. Arrows for the mutant isoforms in the ACTN2 mEos2 images shows the presence of aggregated protein in addition to Z-disc localisation. These images are representative images obtained for 3 separate experiments. Scale bar: 10 μ m. **B.** Western blots for adult cardiomyocytes expressing mEos2-tagged ACTN2. Anti-mEos2 bands show comparative expression levels for all three constructs. Anti-ACTN2 did not detect an additional band for the mEos2-tagged protein, due to low levels of expression. Anti-actin shows similar loading for each sample.

Figure 6: Dynamic behaviour of wild type and mutant mEos2-tagged ACTN2 in live adult cardiomyocytes. **A)** A small region of the cell (indicated by the white box in the pre photo-activation images (Pre-PA)) is illuminated with 405nm light, which results in the

photo-conversion of 'green' mEos2-ACTN2 in this region to red, as shown in the adjacent image at +3 minutes after photo-conversion. The red-fluorescent protein can then diffuse away from the initial site of activation (as shown in subsequent frame at +14 minutes). Examples for wild type and the two mutant constructs are shown. **B)** The change in fluorescence in the central region of photo-activation is plotted against time to show the decay of fluorescence. (Values shown are the means \pm S.E.M. where for WT, n=8; AT mutant, n=9; and GV mutant, n=10) **C)** Box and Whisker plot for measurements of tau, for the rate of change of fluorescence in B for wild type (WT) and the mutant constructs. Mean \pm S.E.M values are overlaid on the individual measurements. **D)** Box and Whisker plot for measurements of the plateau levels of fluorescence for wild type (WT) and mutant constructs, calculated from the fits to the curve in **B)**. Mean \pm S.E.M values are overlaid on the individual measurements. (n=8 cells for WT, 9 for AT and 10 for G111V, and measurements were obtained from cells from 2 separate experiments for each construct). Levels of statistical significance: $p < 0.05$ (*).

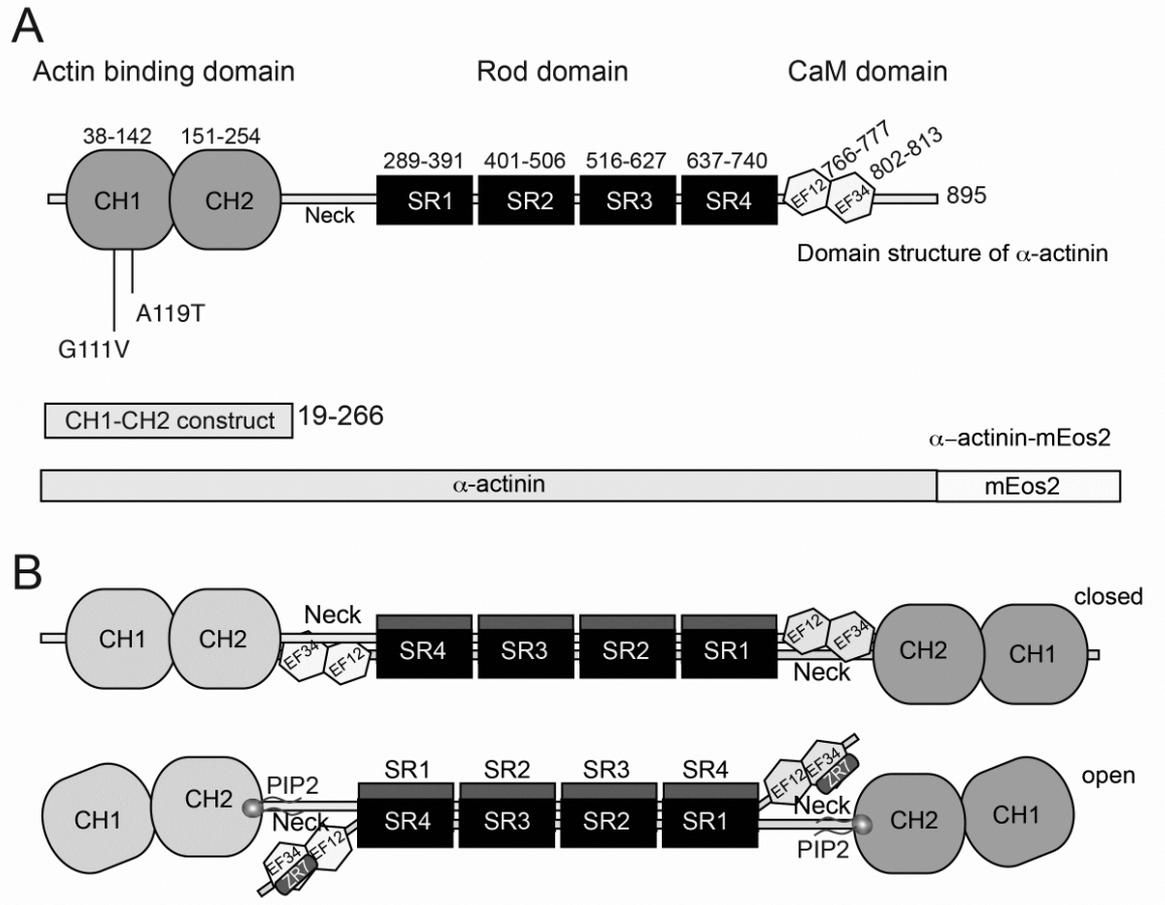


Figure 1

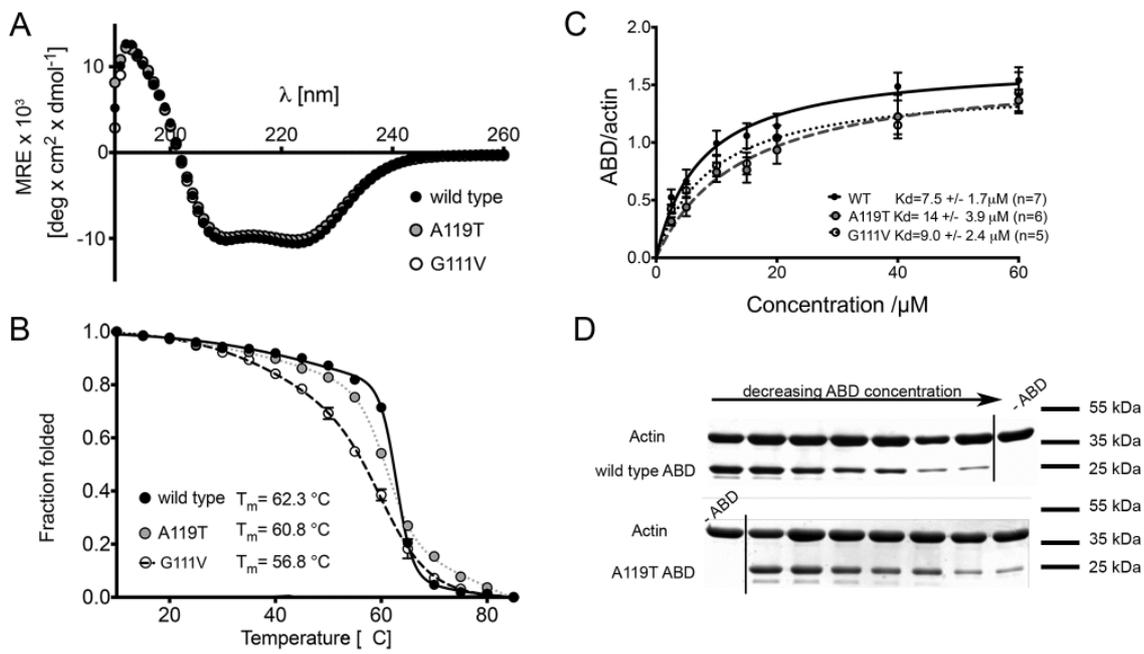


Figure 2.

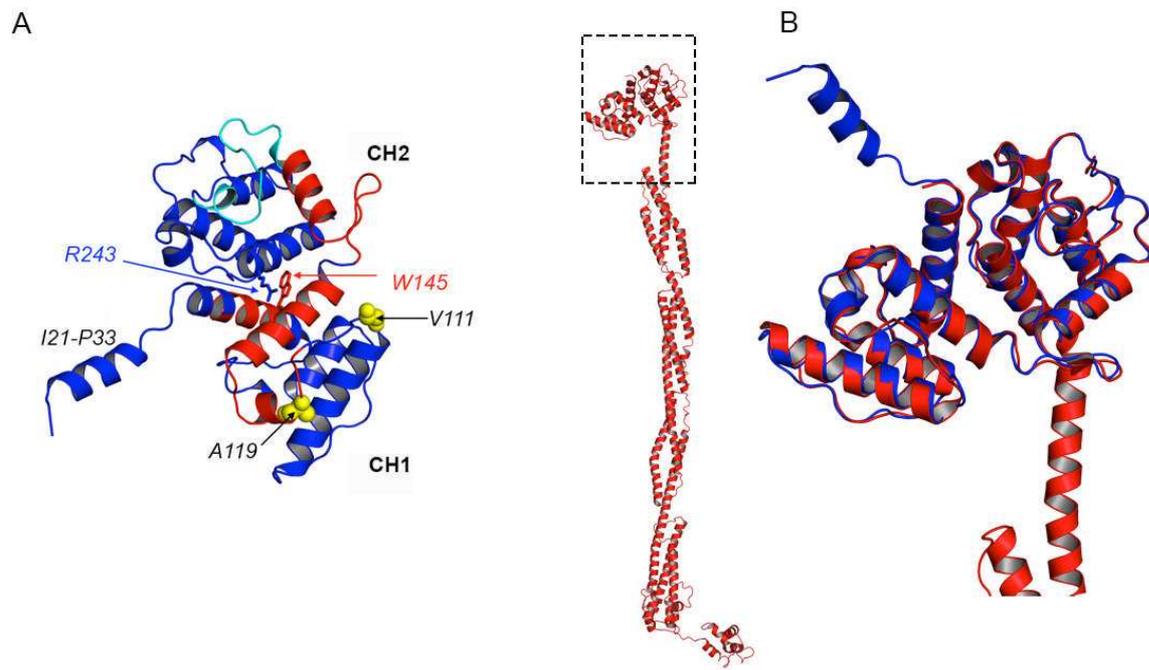


Figure 3

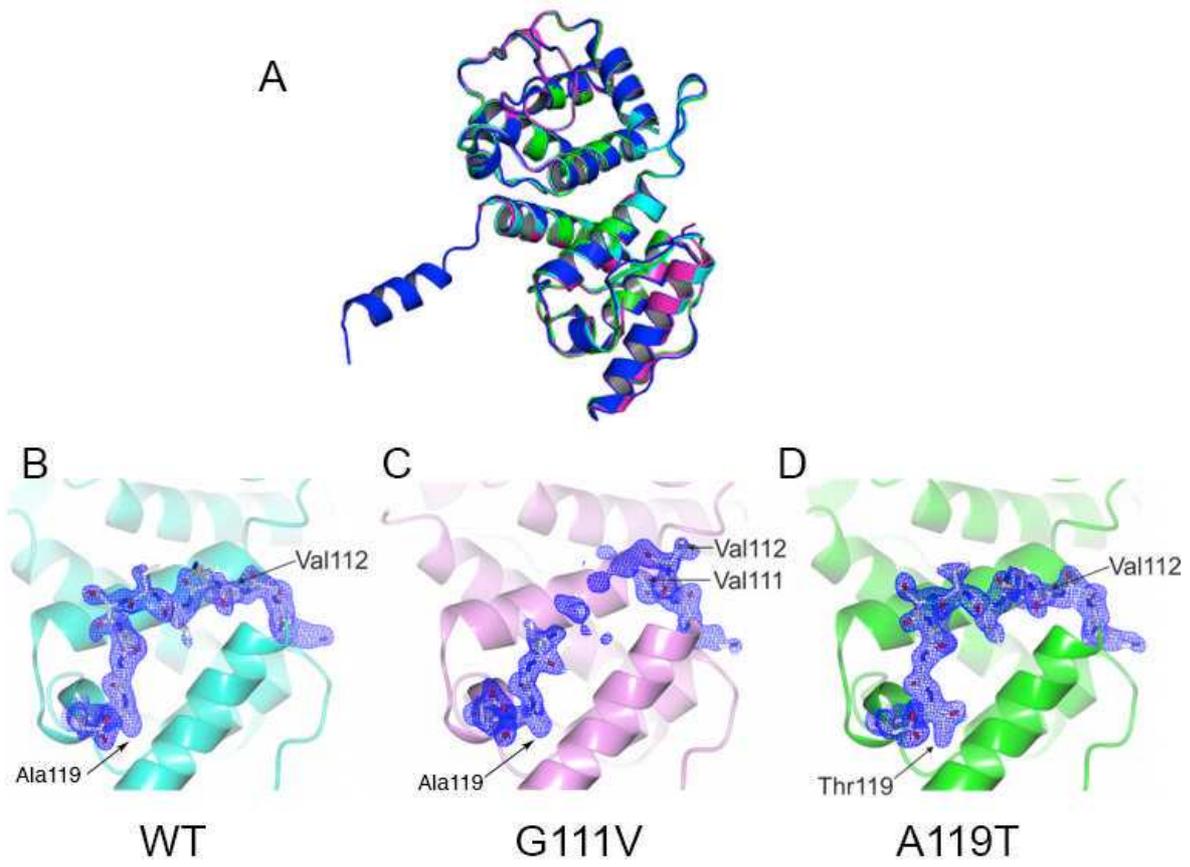


Figure 4

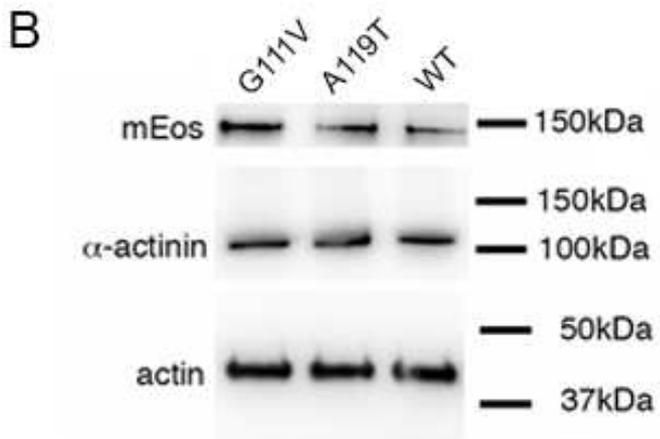
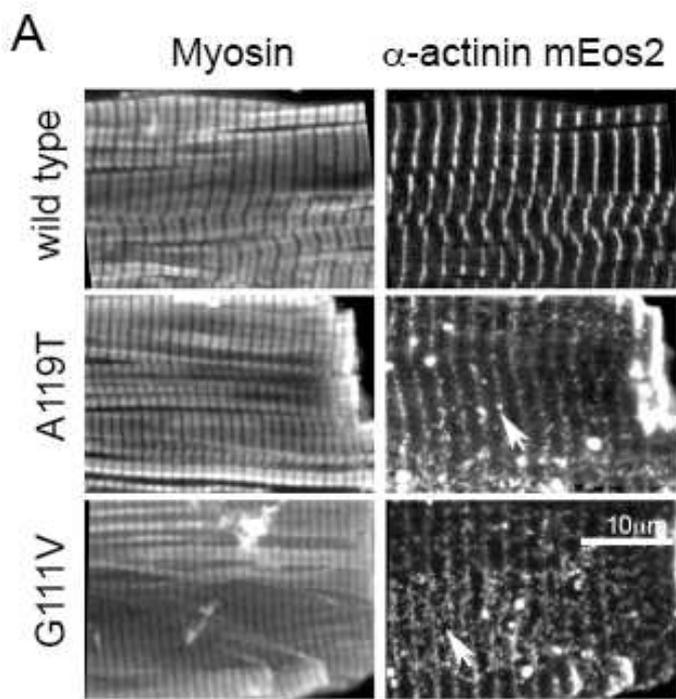


Figure 5

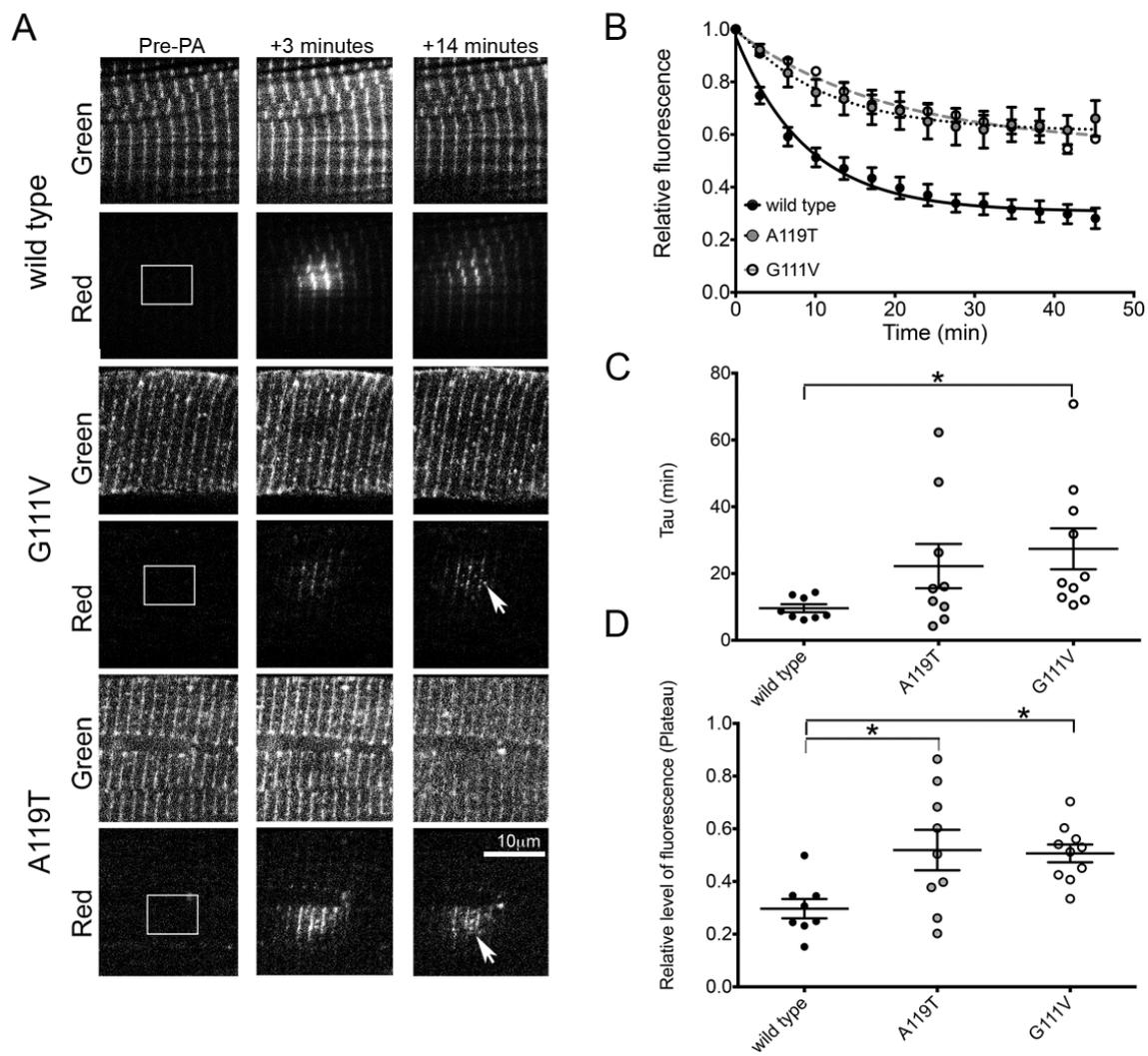


Figure 6

Table 1. Data collection, processing and refinement statistics for wild type ACTN2 and the mutants G111V and A119T.

	WT1	WT2	G111V mutant	A119T mutant
Source	Diamond i04	Diamond i04	Diamond i04	Diamond i02
Wavelength (Å)	0.9464	0.9464	0.9796	1.0000
Resolution range (Å) *	68.80–2.00 (2.05–2.00)	67.00–1.90 (2.00–1.90)	67.14–1.88 (1.98–1.88)	66.68–2.01 (2.06–2.01)
Space group	<i>P</i> 1	<i>P</i> 1	<i>P</i> 1	<i>P</i> 1
Unit-cell parameters (Å)	<i>a</i> =38.7, <i>b</i> =47.5, <i>c</i> =70.3 α =80.8, β =80.8, γ =76.5	<i>a</i> =38.3, <i>b</i> =46.6, <i>c</i> =70.2 α =73.8, β =80.2, γ =75.4	<i>a</i> =38.2, <i>b</i> =46.5, <i>c</i> =70.4 α =73.7, β =80.1, γ =75.5	<i>a</i> =38.4, <i>b</i> =46.6, <i>c</i> =69.9 α =73.8, β =80.0, γ =75.1
No. of observed reflections	67208	63654	71584	58524
No. of unique reflections	31249	33050	34585	28251
Redundancy	2.2 (2.0)	1.9 (2.0)	2.1 (2.1)	2.1 (2.1)
Completeness (%) *	96.4 (82.2)	93.6 (94.0)	94.2 (91.1)	95.0 (95.1)
$\langle I/\sigma(I) \rangle$ *	5.8 (1.5)	7.2 (2.1)	7.6 (2.0)	6.2 (2.2)
R_{merge} (%) §*	12.2 (58.3)	9.0 (51.7)	7.6 (41.2)	7.7 (31.7)
R_{pim} (%) ¶*	12.1 (48.1)	7.4 (38.7)	7.1 (39.1)	6.5 (27.4)
Resolution range for refinement (Å)	68.80–2.00	67.00–1.90	67.14–1.88	66.68–2.01
<i>R</i> factor (%)	21.7	19.2	17.8	19.8
R_{free} (%) †	26.7	23.1	21.6	23.4
No. of protein non-H atoms	3723	3597	3547	3588
No. of water molecules	117	201	307	201
R.m.s.d bond lengths (Å) ξ	0.012	0.009	0.007	0.011
R.m.s.d bond angles (°) ξ	1.4	1.3	1.1	1.4
Average overall <i>B</i> factor (Å ²)				
Protein	30.5	26.4	20.7	23.3
Water	28.5	27.2	25.5	24.9
Ramachandran analysis, the percentage of residues in the regions of plot (%) ‡				
Favoured region	98.0	98.7	98.2	98.4
Outliers	0	0	0	0
PDB code	5A36	5A38	5A37	5A4B

*Values given in parentheses correspond to those in the outermost shell of the resolution range.

$$\S R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$

$$\yen R_{pim} = \sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$$

† R_{free} was calculated with 5% of the reflections set aside randomly.

ξ Based on the ideal geometry values of Engh and Huber [36].

‡ Ramachandran analysis using the program MolProbity [37].