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The Z-disc protein α -actinin-2 forms a force-activated, directional bond with F-actin

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Abstract:

Force generation in the heart relies on sarcomeres in which myosin II contracts antiparallel arrays of F-actin. Sarcomeres are linked at Z-discs, where the protein α -actinin-2 crosslinks antiparallel F-actin from adjoining sarcomeres and recruits signaling proteins that regulate cardiomyocyte growth. How Z-discs self-assemble and transduce mechanical signals remains unclear. Using optical trap assays, we find that the bond between α -actinin-2 and F-actin selectively strengthens under load applied toward the pointed, (–) end, the direction of myosin force production, whereas binding is dramatically attenuated when load is oriented toward the filament (+) end. Mutations that cause hypertrophic cardiomyopathy (G111V, A119T, T247M) weaken this bond and disrupt directionality, defects that are amplified when multiple α -actinin-2 work together to anchor F-actin against (–)-end directed loads. We further demonstrate that anchoring by wild-type α -actinin-2 assists cardiac myosin II in generating force against an external load, while A119T α -actinin-2 blocks myosin force production. The directional interaction between α -actinin-2 and F-actin offers a plausible mechanism by which the antiparallel organization of actin filaments at the Z-disc, and thereby sarcomeric order as a whole, can emerge spontaneously in response to myosin-generated force. Our data likewise implicate α -actinin-2 as a key mechanosensor in the cardiac sarcomere.

Main Text:

Introduction

Hypertrophic cardiomyopathy (HCM) is a condition marked by progressive, uncontrolled heart muscle growth that, if untreated, can lead to left ventricular occlusion, conduction abnormalities, heart failure, and death. At the cellular level, HCM is characterized by enlarged cardiomyocytes and disorganized sarcomeres—the contractile units composed of alternating bands of thin filaments, primarily filamentous (F)-actin, and thick filaments, chiefly β -cardiac myosin (MYH7). The internal order within sarcomeres is templated by the Z-discs, massive protein assemblies that anchor the barbed, (+) ends of sarcomeric actin and that are physically associated with the signaling pathways responsible for coordinating the CM's shape and size (1-

4). How sarcomeres, which possess almost crystalline order, self-assemble from their molecular components is not well understood. However, myosin-generated force is known to be required for sarcomere formation (5).

Many reported mutations associated with HCM occur in β -cardiac myosin II (MYH7) and associated proteins that regulate CM contractility. Although the details are not fully established, characterized mutations in these proteins appear to converge on an unregulated increase in force production by individual CMs. Intriguingly, mutations in Z-disc proteins, most notably α -actinin-2 (ACTN2), also lead to HCM. Within the Z-disc, two to four α -actinin-2 molecules anchor actin filaments from adjoining sarcomeres in an antiparallel arrangement (6). Z-discs in CMs harboring HCM-associated mutations are often wider and more irregular than those in matched controls, even for mutations that occur in thick filament proteins that are physically distant from the Z-disc (7-10). These findings, along with other data, suggest that mechanosensing by α -actinin-2 may play a key role in organizing the sarcomere and in the mechanotransduction processes that link changes in sarcomeric force production to CM hypertrophy.

To our knowledge the interaction between ACTN2 and F-actin under mechanical load has not been characterized. This knowledge gap limits our understanding of how the Z-disc self-assembles during sarcomere biogenesis and maintains its integrity under myosin-generated contractile forces. Here, we used a single-molecule optical trap assay to determine how mechanical load affects the bond between ACTN2 and F-actin. In addition, we characterized the effects of several HCM-causing mutations (11-14) (G111V, A119T, T247M) located within the actin-binding domain of ACTN2. Finally, we developed a novel assay to assess the ability of wild-type and mutant ACTN2, in conjunction with β -cardiac myosin, to generate and sustain load on F-actin. Together, these data indicate that a mechanosensitive bond between ACTN2 and F-actin may play a central and previously unexamined role in both sarcomere formation and Z-disc-associated mechanosensing.

Results

We used an optical trap assay to characterize the interaction of ACTN2 and F-actin under mechanical load (Fig. 1A). In this assay, two optically trapped beads and the actin filament stretched between them are held stationary, while the microscope stage, and thus the platform beads, is oscillated in a sinusoidal pattern with a peak velocity of 1 $\mu\text{m/s}$, consistent with the actin velocity generated by cardiac myosin II (15,16). Binding of ACTN2 to the actin filament results in a change in force on the optically trapped beads due to stage motion (Fig 1B). When this force exceeds a set threshold of 2 pN, the stage oscillation is paused to maintain a constant force until the binding event ends. After each binding event, the force returns to baseline and the stage resumes its oscillatory motion (see Methods).

We observed a marked anisotropy in binding lifetimes for each filament, such that long-lived binding interactions for a given actin filament were only observed when force was oriented toward one of the two optically trapped beads (Fig. 1B, C). This preferential direction for long-lived events could in principle correspond to load oriented toward either the filament (+) or (–) end. To determine which was the case, we performed a separate assay in which platform beads

were decorated with both ACTN2 and skeletal myosin II. In this experiment, binding lifetimes were markedly increased by myosin-generated force, which is directed toward the filament (–) end (Fig. S1). From this, we infer that the bond between ACTN2 and F-actin is preferentially stabilized by (–)-end directed force. Under the conditions of our assay, approximately 84 to 97% of observed binding events occurred with force oriented towards the (–) end of the actin filament (Fig 1E). This result is intriguing, as it suggests that the intrinsically directional interaction between ACTN2 and F-actin might contribute to orientation of filaments at the Z-disc (see Discussion).

In addition to strongly directional binding, we also observed that wild-type (WT) ACTN2 binding lifetimes increased ~25-fold with force up to ~17 pN, a force that corresponds to the load generated by roughly eight myosin molecules (Fig. 1D). This counterintuitive effect, termed *catch bonding*, was also reported for α -actinin-4 (17-20), a homolog that occurs in non-muscle cells, though the directionality of that interaction was not measured. Our data also indicated that ACTN2 preferentially bound along the periodicity of the actin filament (Fig S2) an effect that may likewise facilitate construction of the Z-disc (see Discussion). We repeated these experiments using a truncated ACTN2 containing the actin-binding domain plus the first two spectrin repeat domains (WT TRUNC). This truncated construct also formed a directional catch bond with F-actin, indicating that ACTN2 dimerization was dispensable for both directionality and catch bond formation.

CMs experience a drop in intracellular pH from approximately 7.4 at rest to ~6.7 under oxygen deprivation or mechanical stress (21,22). To evaluate the effect of intracellular pH on ACTN2 function, we decreased pH to 6.7 and observed a significant decrease in ACTN2 attachment lifetimes (Fig. 1D). This acidification also flattened the force–lifetime curves, with peak binding lifetimes at ~9 pN. These data indicate that the stability of the Z-disc under load may be decreased under physiological stress (see Discussion).

We next repeated these force feedback experiments with three ACTN2 constructs harboring the mutations G111V, A119T, and T247M, each of which is linked to HCM in human patients (11-14) (Fig. 1D). At physiological pH 7.4, G111V (HCM) had statistically insignificant effects on both directionality and the catch bond lifetime. In contrast, T247M (HCM) reduced peak binding lifetimes by more than 60% compared to WT TRUNC and diminished the force-bearing capacity to less than 30% of WT TRUNC levels. A119T exhibited similar or worse impairments. Moreover, the T247M and A119T mutations reduced the fraction of (–)-end directed binding events by ~ 30% (Fig. 1E, Table S2) while increasing the ratio of (+)-end directed binding.

We also quantified the behavior of these mutants under decreased pH. At pH 6.7, all three mutants showed lower binding lifetimes at roughly physiological, ~10 pN loads as compared to WT and WT TRUNC constructs (Fig. 1D). In particular, the G111V mutation, which did not appreciably alter catch bond formation at pH 7.4, showed a reduction in binding lifetimes at forces between 0 and 15 pN ($p = 0.06$), the range of forces that individual thin filaments are likely to experience *in vivo* (see Discussion). Interestingly, the force/lifetime relationships for the wild-type and mutant constructs were more similar at pH 6.7 than at pH 7.4. It is possible that all three mutations lead to a common structural perturbation that affects actin binding. To

summarize, all three ACTN2 mutations that result in HCM also decrease the load stability and directional preference of the bond to F-actin, with one, G111V, specifically affecting the bond at reduced pH (Fig S3, Table S1 and S2).

Actin filaments are crosslinked by two to four ACTN2 dimers in Z-discs. We therefore measured force-dependent F-actin binding lifetimes with varying numbers of ACTN2 molecules per pedestal bead (Fig. 2A,B). Increasing the number of ACTN2 molecules beyond one significantly enhanced attachment lifetimes, force-bearing capacity, and reinforced the directionality of the catch bond (Fig. 2C–E). The A119T mutation, which we used as the example HCM mutation for these experiments, significantly reduced bound lifetimes, diminishing force-bearing capacity and nearly abolishing directional bias (Fig. 3A). For example, an order of magnitude difference in attachment duration across forces was observed between WT TRUNC and the A119T ACTN2 (Fig. 3B, E). In these experiments, 93% of force-bearing events occurred under (–) end-directed force for WT TRUNC complexes, whereas more than 45% of events occurred under (+) end-directed force for A119T ACTN2 (Fig. 3D, S4). An analysis of binding stiffness—indicative of the number of interacting partners—revealed increasing attachment stiffness with force in WT TRUNC, consistent with recruitment of neighboring ACTN2 molecules under (–)-end directed force. In contrast, the A119T mutant exhibited a roughly hyperbolic decay in stiffness with increasing force, suggesting that higher forces promote rapid detachment of binding partners in HCM, an effect predicted to destabilize the Z-disc (see Discussion).

We next assessed the function of WT and A119T ACTN2 in a 3-bead assay that mimics the cardiac sarcomere environment in which cardiac myosin II (β -cardiac myosin II; MYH7) generates force on the actin filament (Fig. 4A, B). In this assay, platform beads are decorated with both ACTN2 and MYH7 such that 2–3 ACTN2 molecules and 5–10 cardiac myosin II molecules (23) interact with a shared actin filament. The addition of WT ACTN2 resulted in a 6-fold increase in bound lifetimes compared to MYH7 alone (142.11 ± 17.07 ms vs. 22.41 ± 3.08 ms; $p < 0.001$). In contrast, we observed a 3-fold *reduction* in attachment lifetimes for MYH7 plus A119T ACTN2 (7.89 ± 0.12 ms; $p < 0.001$) relative to MYH7 by itself, corresponding to a dramatic, 18-fold difference in event lifetimes compared to MYH7 + wild-type ACTN2 (Fig. 4D). Force measurements further revealed that the MYH7 + A119T ACTN2 combination generated significantly lower force compared to either MYH7 alone (0.13 ± 0.02 pN vs. 1.10 ± 0.04 pN; $p < 0.001$) or MYH7 + wild-type ACTN2 (1.34 ± 0.03 pN; $p < 0.001$) despite consistent myosin concentrations across conditions (Fig. 4E). These differences were apparent in all quartiles (e.g. 25th, 50th, 75th, 100th) presenting order of magnitude declines in MYH7-generated force in the presence of WT vs A119T ACTN2, showing a systematic loss of function with the A119T. Thus, the presence of A119T ACTN2 impaired both myosin attachment and force production, whereas wild-type ACTN2 enhanced both.

To further elucidate why A119T failed to enhance the force-generating capacity of cardiac myosin, we performed ensemble averaging by temporally aligning and averaging individual binding events from start to finish (24–28). As expected from prior studies, the MYH7 control experiments exhibited an approximately exponential increase in force during binding events (Fig. 4F). In contrast, the ensemble average for MYH7 + WT ACTN2 showed a more gradual increase in force generation, consistent with ACTN2 slowing MYH7-driven actin

translocation. In contrast, the addition of A119T ACTN2 did not affect the rate of force development in the ensemble averages. Instead, we observed frequent slipping events in which myosin-generated force dropped toward zero (Fig. 4F, insets), an observation consistent with the decreased force production of MYH7 plus A119T ACTN2 compared to MYH7 alone (Fig 4E, G). This behavior indicates that when HCM-associated ACTN2 mutations are present, the actin filament slips during force generation, leading to reduced force output by the sarcomere ensembles—a mechanism by which loss of function could have deleterious effects on muscular force development (see Discussion).

Collectively, these multiscale analyses—from single-molecule to multi-molecule experiments and mini-sarcomere ensembles—highlight several key factors relevant to Z-disc self-assembly and overall sarcomere function. A directional catch bond by ACTN2 selectively anchors the (+) end of F-actin under (–) end-directed forces, as generated by muscle myosin II (Fig. 4H). In contrast, forces directed toward the (+) end of actin promote a weak interaction that quickly dissociates. These effects are amplified when multiple ACTN2 molecules work together to anchor F-actin (Fig. 3). ACTN2 mutations associated with HCM compromise both the sensitivity and directionality of the catch bond (Fig. 1D, 4I). Stiffness measurements indicate that multiple molecules of wild-type ACTN2 work together to anchor F-actin under load, whereas A119T ACTN2 ensembles lack this ability (Fig. 3F). The consequence of these defects, ensembles of A119T ACTN2 fail to provoke filament slippage during force production by MYH7. Taken together, these data indicate that mutations in ACTN2 associated with HCM destabilize the Z-disc (Fig. 4I).

Discussion

Previous studies show that α -actinin-2 and titin are required for the construction of Z-discs and sarcomeres as a whole (29, 30). However, how interactions between these and other proteins result in the almost crystalline order exhibited by mature sarcomeres is not understood. It had been proposed that titin templates the assembly of sarcomeric units into myofibrils (31-33). However, this is unlikely the full explanation, as stress fibers, which occur in cells lacking titin, also exhibit sarcomeric order (34). The formation of Z-bodies does not require titin, and deletion of the titin I-band does not alter Z-disc structure (35, 36). Indeed, the initial stages of myofibril assembly during embryogenesis are independent of titin, which is expressed later in development (37). Finally, although titin spans the Z-disc and M-band, individual titin molecules do not extend between neighboring sarcomeres. Thus, whereas titin is required for sarcomere spacing, it does not seem to be required for the initial patterning of the myofibril.

We propose that a combination of myosin-generated force and a directional, force-sensitive interaction between ACTN2 and F-actin results in a positive feedback loop that helps to drive self-assembly of the Z-disc and, by extension, sarcomeres as a whole. Our data reveal that ACTN2 forms directional catch bonds that crosslink actin filaments, but only when its actin-binding domains are subject to force oriented toward the (–) ends of F-actin, in line with the force imparted by myosin (Fig. 1, S1). This ability to anchor against (–)-end directed load is amplified when multiple ACTN2 molecules work together (Fig. 2). In addition, we show that ACTN2 efficiently anchors F-actin against MYH7-generated force, and cooperates with MYH7 to generate long-lived, force-generating events (Fig. 3). The directional preference of the ACTN2 catch bond (Fig 1D) provides a compelling mechanism for how sarcomeres assemble with the

antiparallel orientation of F-actin emerging from Z-discs to yield the repetitive series of sarcomeric units observed in myofibrils (38-41). Although ACTN2 forms the core of the Z-disc, additional proteins are required for its construction. Understanding how and whether force sensing by titin, nebulin, and muscle LIM protein (MLP) contributes to sarcomerogenesis and mechanical homeostasis provides an important target for future investigations.

Studies from our laboratory show that α E-catenin, vinculin, and talin form directional catch bonds with F-actin (42-44). It is possible that directional and force-regulated interactions may be a ubiquitous feature of the cytoskeleton. In CMs, it is possible that directional catch bonds formed by these, and potentially other, actin-binding proteins may similarly orient F-actin at costameres (talin), and at the intercalated disc (α -catenin, vinculin). However, to our knowledge the force-dependent behavior of α N-catenin and metavinculin, the dominant isoforms of these proteins in the heart, remain to be characterized.

Multiple mutations in ACTN2 are known to result in HCM (45, 46). Of these, A119T and T247M markedly decreased catch bond force sensitivity and directionality. A third mutation, G111V, had minimal impact at physiological pH but shifted force sensitivity at pH 6.7, shortening binding lifetimes within the 0 - 15 pN range likely experienced by thin filaments *in vivo* (Fig. 1). Selecting A119T for more detailed analysis, we found that defects in anchoring and directionality were further amplified in small ensembles compared to WT ACTN2 (Fig. 3). Moreover, A119T interfered with MYH7 force production, resulting in shorter binding events and lower peak forces (Fig. 4). Why this mutant is functionally detrimental, rather than simply ineffective, is unclear. However, stiffness measurements suggest that multiple WT ACTN2 molecules are recruited to anchor F-actin as load increases, whereas for A119T ACTN2, stiffness, and by inference the number of anchoring molecules, is maximal at low force and decreases hyperbolically with load (Fig. 3). This suggests the A119T mutation might cause ACTN2 molecules to resist myosin-generated actin translocation, whereas WT ACTN2 permits efficient translation at low forces but anchors F-actin at higher forces. Rigorously testing this hypothesis requires further study.

Most HCM-causing mutations occur in MYH7 and myosin-binding protein C (MyBP-C) (47, 48). In both cases, available evidence suggests these mutations result in increased force production at the sarcomere and CM levels (48-50). Through mechanisms that are not yet clear, these mutations lead to signaling through calcineurin, protein kinase C (PKC), and other signaling pathways that drive hypertrophy (51, 52). Existing evidence suggests the Z-disc may act as a mechanotransducer that translates myosin-generated force into the activation of these signaling pathways (51, 52). Supporting this view, calcineurin, PKC and the molecules that regulate their activity all localize to Z-discs, and mutations in Z-disc components such as muscle LIM protein (MLP), telethonin (Tcap), and FATZ-2 (calsarcin-1, myozenin-2) cause inherited myopathies (52-60). In addition, some studies indicate that Z-disc force sensing can occur, in part, via an indirect effect on stretch-sensitive Ca^{2+} dynamics (53, 61, 62). Stem-cell derived CMs from a patient heterozygous for T247M ACTN2 exhibited impaired muscle relaxation, prolonged action potential duration, and increased L-type Ca^{2+} currents (59). Thus, mechanosensing by ACTN2 may additionally be coupled to the regulation of Ca^{2+} dynamics, an intriguing finding whose mechanistic basis remains unclear. Regardless of the underlying mechanisms, it is clear that ACTN2 is a key mechanosensor at the Z-disc, and that mutations that disrupt the ACTN2 catch bond change the nature of mechanosensing at the Z-discs that regulates hypertrophy.

The molecular mechanisms by which mutations in ACTN2 lead to pathological hypertrophy are unclear. One possibility is that mutations in ACTN2 that decrease Z-disc stability functionally mirror the effects of increased force production resulting from HCM-associated mutations in MYH7, MyBP-C, and other sarcomeric proteins (48-50). In this scenario, either increased force production or diminished stability of the ACTN2-actin bond could result in microscale damage, structural alterations, or direct mechanosensing involving ACTN2 to activate common, downstream pathways that drive hypertrophy. Such a model is consistent with the increase in Z-disc width in CMs from HCM patients and in stem-cell derived CMs expressing HCM-associated mutations in MYH7. Interestingly, an increase in Z-disc width is also reported in at least some cases of dilated cardiomyopathy (DCM). Although mechanisms for DCM mutations in ACTN2 that explain how ACTN2-mediated mechanosensing may contribute to this disease have not been explored to our knowledge.

In vivo, ACTN2 and the myosin catalytic domain are in tight mechanical communication mediated by the thin filament. Mechanical load slows ADP release from myosin (24, 63-65), an effect that both increases the time that myosin spends bound to actin and contributes to the activation of the thin filament and to the resulting hyperbolic force-velocity profile of muscle (66-68). The ACTN2 mutations studied here weaken the protein's ability to anchor F-actin against load, and are thus predicted to interfere with this bidirectional mechanical communication between the thick filaments and the Z-disc. It is therefore possible that weakened anchoring at the Z-disc alters the forces experienced by myosin. Consequently, well-documented abnormalities in Z-disc morphology that accompany HCM (59, 69-72) may also be functionally connected to the modified force-velocity profiles (70, 73, 74) due to altered mechanosensing and thin filament activation. In line with this reasoning, our comparative mini-sarcomere measurements show that, within the context of this assay, ACTN2 is essential for contractile function: when its mechanosensing role is lost, force production collapses. Consistent with this idea, patients carrying the T247M mutation show depressed thin-filament activation and diminished contractility (59). Future work should examine how the contractile properties of single muscle fibers containing mutations in ACTN2 and related Z-disc proteins alter force production and thin filament activation. Determining whether such mutations modify hypertrophic signaling pathways or activate transcriptional programs in response to sarcomere damage is likewise a high priority. In summary, our study demonstrates that, rather than acting as a passive structural element, ACTN2 is likely an active participant in determining the dynamic properties and mechanosensing capabilities of the cardiac sarcomere.

Methods

DNA Constructs and Protein Expression

Bacterial expression constructs containing either full length *H. sapiens* α -actinin-2 (UniProt: P35609-1) or α -actinin-2 with the EF-hands truncated were assembled using the NEBuilder HiFi Assembly Cloning Kit (New England Biolabs). α -Actinin-2 constructs also contained Halo- and 6xHis tags to facilitate purification, and these tags were appended C-terminally to α -actinin-2 through a GGSG linker. α -Actinin-2 was PCR amplified from an ACTN2-pEGFP plasmid, a gift from Johannes Hell (Addgene plasmid # 52669 ; [http://n2t.net/addgene: 52669](http://n2t.net/addgene:52669) ; RRID:Addgene_52669). For the EF-hand truncation mutant, only amino acids 1-506 of α -actinin-2 were amplified, resulting in a truncation of the region C-terminal to the 2nd spectrin repeat of α -actinin-2. The HaloTag, 6xHis tag, and rest of the expression vector containing an

ampicillin resistance cassette were PCR amplified from a previous construct (T7-LacO-Halo-RGD-STReTCH, Addgene plasmid # 216710). The G111V, A119T, and T247M mutant constructs were produced from the EF-hand truncation mutant construct using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) and the appropriate non-overlapping primers for each mutant.

All purified proteins were expressed in BL21(DE3) chemically competent *E. coli* (New England Biolabs). Bacterial cultures for protein expression were grown at 37 °C to an optical density of 0.6, then induced with 1 mM isopropyl- β -D-thiogalactopyranoside (VWR Scientific) and grown overnight at 18 °C. Cultures were spun down at 6000 $\times$ g for 15 minutes, and bacterial pellets were resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole, pH 8) with a cOmplete EDTA-free protease inhibitor cocktail (11873580001, Roche), 70-150 μ g/mL lysozyme (90082, ThermoFisher), and 7 U/mL DNase I (04536282001, Roche). Resuspended cells were rotated end-over-end for 45 min at 4 °C, lysed with a tip sonicator, and spun at 14,000 $\times$ g for 30 min to clarify the lysate. The supernatant was incubated with 1.5 ml of nickel-nitrilotriacetic acid HisPur Resin (ThermoFisher) for every 500 mL of culture and rotated end-over-end at 4 °C for 2 hours. The solution was transferred to a gravity column and allowed to drain to form the resin bed, which was then washed three times with 5 ml of wash buffer (50 mM sodium phosphate, 600 mM NaCl, and 20 mM imidazole, pH 7.4 with 2 mM β -mercaptoethanol; BME). Protein was eluted by incubating the resin bed with 1 mL of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, and 250 mM imidazole, pH 7.4 and 2 mM BME) for 5 minutes before collecting the eluate, and this process was done four times. The relevant fractions of the eluate were collected, concentrated, and buffer-exchanged into storage buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 2mM β -mercaptoethanol, and 5% sucrose by weight) using Amicon centrifugal filter units (MilliporeSigma) of the appropriate molecular-weight cutoff, then flash-frozen and stored at -80 °C.

Optical Trap Assay

A three-bead optical trap assay was used to determine the duration of strong-binding to an actin filament and the magnitude of force generated by single α -actinin 2 molecules. Briefly, α -actinin 2 was adhered to a nitrocellulose-coated microscope slide where 3 μ m silica microspheres served as pedestals for α -actinin-2 to attach. α -actinin-2 was added at a concentration of [0.5 μ g mL⁻¹] (brackets denoted concentration of added protein) and incubated for 90 seconds. The flow cell was then incubated for 5 minutes with bovine serum albumin [0.5 mg mL⁻¹] to inhibit non-specific interactions between the actin filament and the nitrocellulose-coated coverslip surface. The experimental buffer contained; 1 μ m streptavidin-coated silica microspheres (Bangs Labs Inc.), 50:50 biotin/TRITC-labeled actin, an oxygen scavenger system (29 mM glucose, 1.5 mM glucose oxidase, and 80 units catalase), within a low-salt buffer (62 mM KCl, 1 mM EGTA, 4 mM MgCl₂, and 1 mM DTT, at 25 °C and a pH of 7.4). The experiments were repeated with the addition of HCl to decrease the pH to pH 6.7. This represents the bottom end of the physiological range for cardiomyocytes. At the α -actinin-2 surface density used in this study (0.5 μ g mL⁻¹ added protein), and with a 3 μ m pedestals, we measure single-molecule binding activity on < 1:7 pedestal beads checked.

A three-axis piezo-controlled stage (Mad City Labs, Inc.) was maneuvered to attach a biotin/TRITC-labeled actin filament to two 1 μ m neutravidin-coated silica microspheres (Bangs Labs, Inc.) held in two optical traps (Lumicks C-Trap). Once an actin filament was attached to

the 1 μm microspheres, the filament was extended between the optically trapped microspheres, to apply a pretension of 3-4 pN to the actin filament. The bead-actin-bead assembly was then lowered into close proximity of the 3 μm α -actinin-2 coated pedestal bead. The stage was then oscillated ± 200 -250 nm from the central origin position. At the end of stage's movement, a 100 ms pause occurred where the force balance between the 2 traps was calculated. If the force was ≥ 2 pN (calculation every 200 ms) the stage remained paused for the duration of the binding event. Once force returned to baseline ($F = 0$ pN) the stage would then begin its oscillation procedure. The stage oscillated at a rate of 1 $\mu\text{m}\cdot\text{s}^{-1}$ and the trap stiffness (k_{trap}) was 0.05 to 0.20 pN $\cdot\text{nm}^{-1}$, determined using the equipartition method (75). Force is calculated as the distance the bead traveled multiplied by the trapping stiffness ($F = d \cdot k_{\text{trap}}$). For events < 2 pN, standard three-bead assay techniques were used, as described previously (26). The displacements generated by binding activity were tracked from the output from a quadrant photodiode via interferometry at a sampling rate of 78 kHz. This was down sampled to 5 kHz for binding activity analysis. A total of 12,105 α -actinin 2 binding events were detected across all conditions, sampling 178 different α -actinin 2 -coated 3 μm pedestals, across all single molecule conditions, with a ratio of 1:7 pedestals visited (~ 1245 total visited) displaying single molecule binding activity. An average of 8 flow cells were used for each construct.

To determine the directionality of the interaction between α -actinin 2 and F-actin, an optical trap assay was used as described above. Briefly, the skeletal muscle myosin II concentration used was [$2.5 \mu\text{g}\cdot\text{mL}^{-1}$] and the α -actinin 2 concentration was [$2.5 \mu\text{g}\cdot\text{mL}^{-1}$]. Each protein was incubated for 90 seconds, before following up with 5 minute incubation of BSA, as described above. Here, the myosin molecules progressively pull the bead-actin-bead assembly out of the laser trap, which functions as a linear spring (Fig. S1) thereby progressively increasing the resistive load. As α -actinin-2 binds under myosin generated forces, it experiences mechanical loading in the direction that myosin generates. Brownian captures generate assistive forces in the opposite direction, allowing for a full profile of directionally dependent lifetimes to be measured. The experimental buffer contained; 1 μm streptavidin-coated silica microspheres (Bangs Labs Inc.), 50:50 biotin/TRITC-labeled F-actin, an oxygen scavenger system (29 mM glucose, 1.5 mM glucose oxidase, and 80 units catalase), within a low-salt buffer (62 mM KCl, 1 mM EGTA, 4 mM MgCl_2 , and 1 mM DTT, 100 μM ATP at 25 $^\circ\text{C}$ and a pH of 7.4). These experiments were repeated in the absence of α -actinin-2 to determine the difference in strongly bound lifetimes. Sample sizes for in the absence of α -actinin-2 ($- \text{ACTN2}$) were 433 binding events and in the presence of α -actinin-2 ($+ \text{ACTN2}$) were 1109 binding events. A total of 12 pedestals were sampled for $- \text{ACTN2}$ across 2 flow cells and 27 pedestals across 3 flow cells for $+ \text{ACTN2}$.

To determine how multiple α -actinin-2 interact with a common actin filament a parallel set of experiments at various α -actinin-2 surface densities was implemented [2.0 and $5.0 \mu\text{g}\cdot\text{mL}^{-1}$] for a total of 153 α -actinin-2 binding events sampled over 15 α -actinin-2 coated 3 μm pedestals across 4 flow cells. This was compared to a single molecule α -actinin-2 experiment [$0.5 \mu\text{g}\cdot\text{mL}^{-1}$ added α -actinin-2] which sampled 25, 3 μm pedestals coated with α -actinin-2 for a total of 1429 binding events. These experiments utilized the same feedback conditions of 400nm oscillations, 0.05 to 0.10 pN $\cdot\text{nm}^{-1}$ trap stiffness, an oscillation rate of 1 $\mu\text{m}\cdot\text{s}^{-1}$, and were performed at pH 7.4 at an ionic strength of 95mM. We repeated the assay with the A119T construct at a surface density of ($2 \mu\text{g}\cdot\text{mL}^{-1}$ added A119T α -actinin-2) for a total of 298 binding events sampled across 27 A119T coated 3 μm pedestals and 5 flow cells. This was compared to the WT construct at the same α -actinin 2 surface density.

We then determined the ability of α -actinin-2 to bear load under myosin-generated force. We utilized an α -actinin-2 surface density of [$2 \mu\text{g}\cdot\text{mL}^{-1}$] (brackets denoted concentration of added protein) and β -cardiac myosin surface density of [$10 \mu\text{g}\cdot\text{mL}^{-1}$] flowed into the chamber: α -actinin 2 first, then cardiac myosin. We conducted these cardiac myosin experiments in the absence and presence of full-length WT α -actinin-2, and repeated these with combination of cardiac myosin and the A119T α -actinin-2 construct. The samples sizes of cardiac myosin, WT + cardiac myosin and A119T + cardiac myosin were 402, 1183 and 607 binding events, respectively. A total of 14, 44, and 67 pedestals were sampled for cardiac myosin, WT + cardiac and A119T + cardiac, respectively across 10 flow cells.

Data processing and analysis

Recorded data was analyzed using custom programs within R (v4.04, R Core Team) to automatically detect events, determine peak force, as well as event duration, as previously described elsewhere (23, 27) which are available on GitHub as an R-package (<https://github.com/brentscott93/lasertrapr>). A two-state Hidden–Markov Model (76, 77) was used to identify binding events from running mean and variance transformations of the laser trap data. The windows used to calculate mean and variance had a width of 200 datapoints and progressed with one-quarter overlap over the entire displacement record. The start and end estimations of each binding event were inferred from the original raw data using the output of the Hidden-Markov Model analysis, multiplying the first and last running window position (or indexed data) by the running window width. A changepoint analysis (25) was then applied to a small subset of data surrounding the transition points (2 window widths on either side of the start and end of each binding event). The changepoint analysis was performed by a running mean and variance of the displacement data (50 data point window progressing one data point at a time) to identify the most probable datapoint of the transition in or out of a binding event. The peak displacements in binding events were determined using a 5 ms running mean window that identified the window with the greatest displacement, within the two changepoint-identified transitions (start and end points) of binding events, and returned that window's value as peak displacement (see Fig. 1c. Time off (t_{off}) was the time between the end of one event and the start of the subsequent event. The time spent at peak force (T_{on}) was determined to calculate changes in the detachment rate as a function of load. The starting point of T_{on} began at the greatest displacement window identified within the two changepoint-identified transitions, as described above, and ended at the changepoint-identified end point of the binding event (i.e. detachment). The total time between these two points was then calculated for all single and multi-molecule binding events and used for the calculation of the detachment rate and attachment lifetimes, as previously described (23). Ensemble averaging of the mini sarcomeres was performed by aligning the start of each binding event, synchronizing events by extending all events to the same duration, averaging the resulting positional data (26-28).

Statistical Analyses

The detected events were analyzed using SPSS[®] (version 29.0.0.0). A two-dimensional Kolmogorov-Smirnov was used to detect differences in the single molecule assays across conditions, as described previously (42-44). Differences in the binding event lifetimes, displacements, and forces were found to be non-normally distributed based on a Kolmogorov-Smirnov test of Normality. Therefore, statistical analyses on displacements, forces, and event duration were performed using a non-parametric Kruskal-Wallis ANOVA followed by the appropriate post-hoc tests for the multi-molecule and mini sarcomere assays when warranted.

Comparisons between load orientation binding ratios were performed with two-proportion Z-test (42-44). The level of significance was set at $p < 0.05$ but adjusted for multiple comparisons using a Bonferroni correction.

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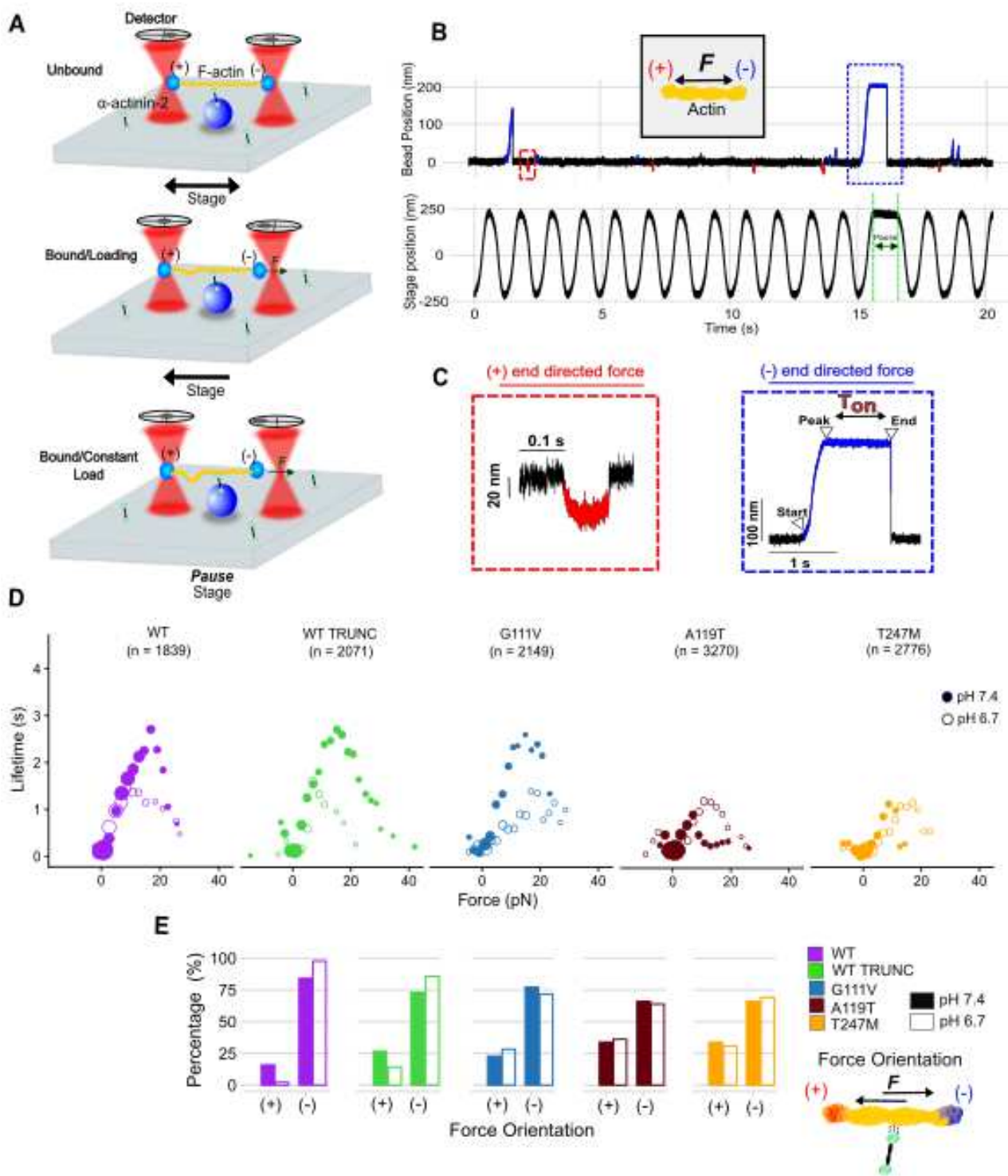
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Figure 1. The directional catch bond by α -actinin-2 is disrupted by HCM. **A)** Optical trap assay. A pedestal bead adhered to the microscope slide is translated left and right by the stage. α -actinin-2 binding to F-actin causes one of the two beads to be pulled from the center of the laser trap, resulting in a restoring force (F). Stage motion halts until α -actinin-2 unbinds, and the force on the trapped beads and the binding duration are recorded. **B)** Representative displacement records. A custom algorithm within R to detect binding events (see Methods), with events colored based on the changepoint transitions. Red signifies (+) end oriented force and blue signifies (-) end directed force events. The algorithm determined the duration of each binding event (t_{on}) based on the time spent from the peak displacement to the end of a binding event for events. Peak force was determined from the peak displacement, for a 5 ms window, multiplied by the corresponding laser trap stiffness. Vertical scale bars = 5 sec and horizontal scales = 100 nm. **C)** Representative (+) and (-) end directed force events. Attachment lifetimes (T_{on}) were taken as the time from peak displacement to the end of the binding event. **D)** Plot of single molecule force-lifetime curves for five ACTN2 constructs (**WT**, **WT TRUNC**, **G111V**, **A119T**, **T247M**). See Table S1 for two-dimensional Kolmogorov-Smirnov test on all comparisons. WT significantly differs from HCM. **E)** Percentage of binding events corresponding with force directed to the filaments (-) or (+) end. See Table S2 for all comparisons. Level of significance set at $p < 0.05$ and Bonferroni corrected for D and E.

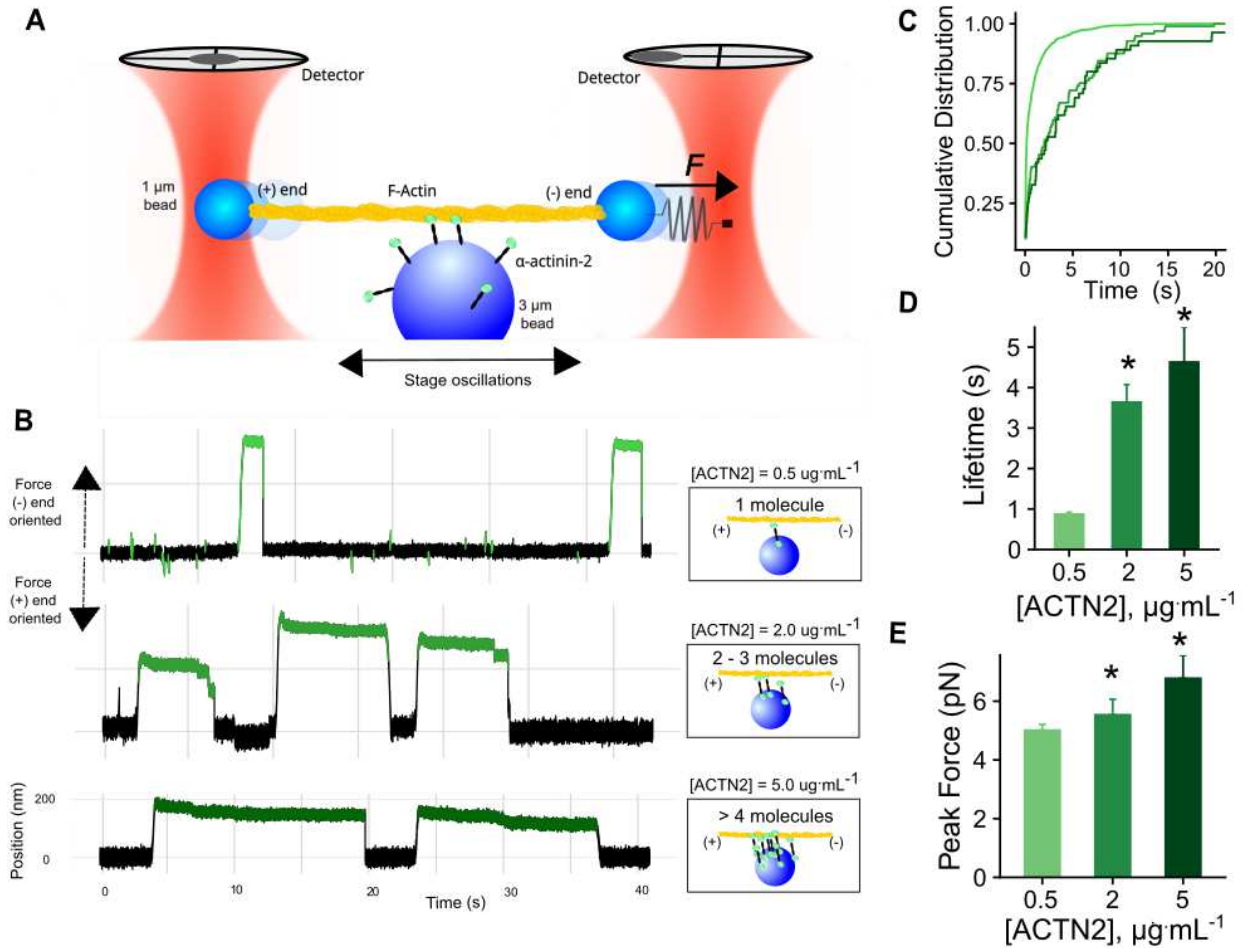


Figure 2. Assembly of sarcomeric Z-disc complexes. Multiple α -actinin-2 anchor filamentous actin under (-) end oriented forces. **A)** Multi-molecule optical trap assay. **B)** Three raw displacement records at 0.5, 2.0, and 5.0 $\mu\text{g mL}^{-1}$ α -actinin-2. As more α -actinin-2 molecules are recruited to a shared F-actin, an increased in binding lifetimes and force bearing capacity occurs and stabilizes the α -actinin-2 – F-actin complex. **C)** Cumulative distributions of binding lifetimes as a function of [ACTN2]. **D)** Bar graphs of mean $\pm$ SEM of binding lifetimes. An increase in [ACTN2] significantly increases binding lifetimes. **E)** Bar graphs of mean $\pm$ SEM of peak forces during α -actinin-2 binding events. Increased [ACTN2] significantly increases forces generated. Significant differences were detected using a non-parametric Kruskal-Wallis ANOVA using SPSS[®]. * $p < 0.05$ significantly different from 0.5 $\mu\text{g mL}^{-1}$.

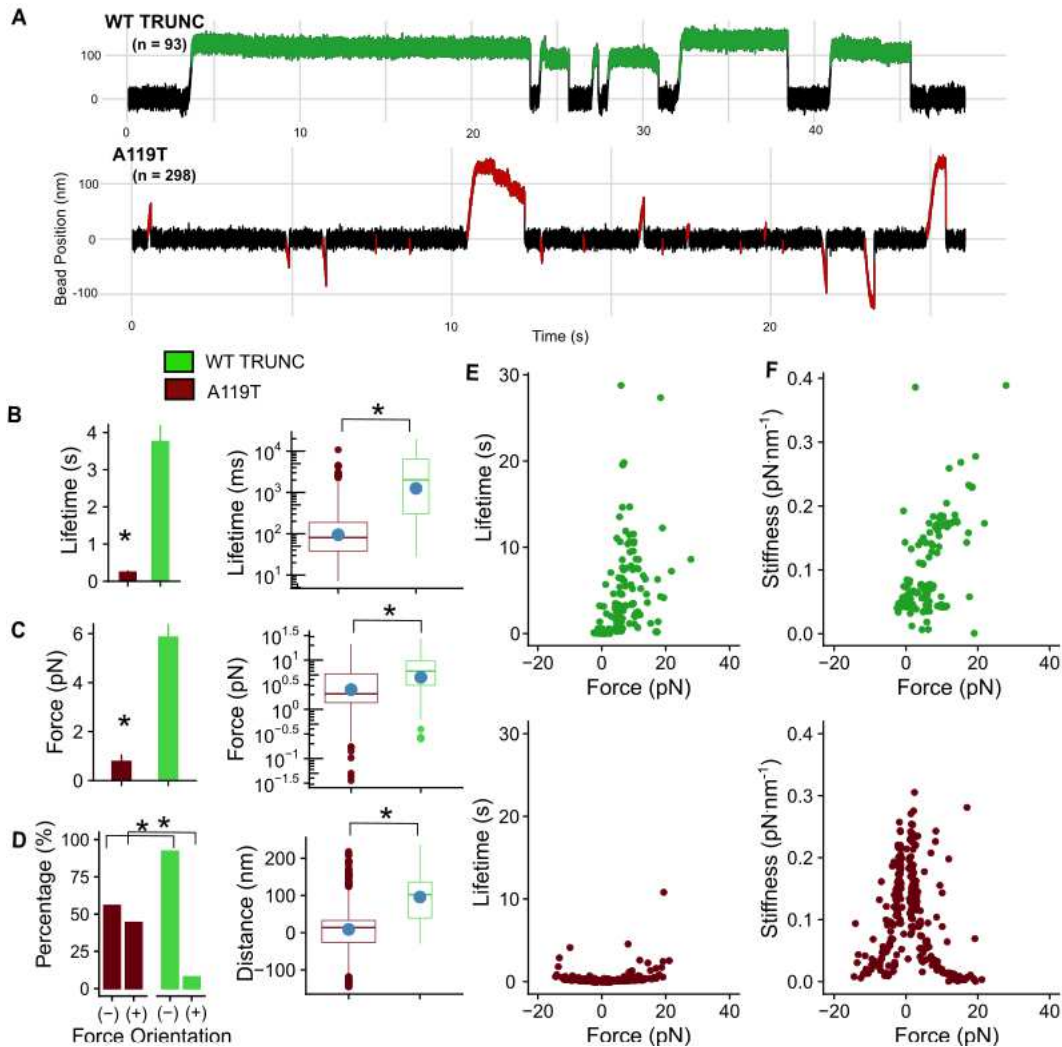


Figure 3. HCM disorder causes failing Z disc by disrupting force bearing and directionality. **A)** Raw displacement records of WT TRUNC and A119T mini Z discs (2 to 3 molecules) under load. **B and C)** Mean $\pm$ SEM and populations of attachment lifetimes and force. **D)** Directionality ratios for (+) end and (-) end directed force bearing events and displacement distributions. A119T disrupts the directional preference of ACTN2 directional catch bond. **E)** Attachment lifetimes vs force. WT increases lifetimes as (-) end directed force increases. A119T bind under bidirectional forces but does not change its attachment lifetimes. **F)** Stiffness of binding events vs force. WT shows an increase in stiffness as force bearing increases. A119T shows a hyperbolic decay as a function of force. * $p < 0.05$, significantly different from WT TRUNC.

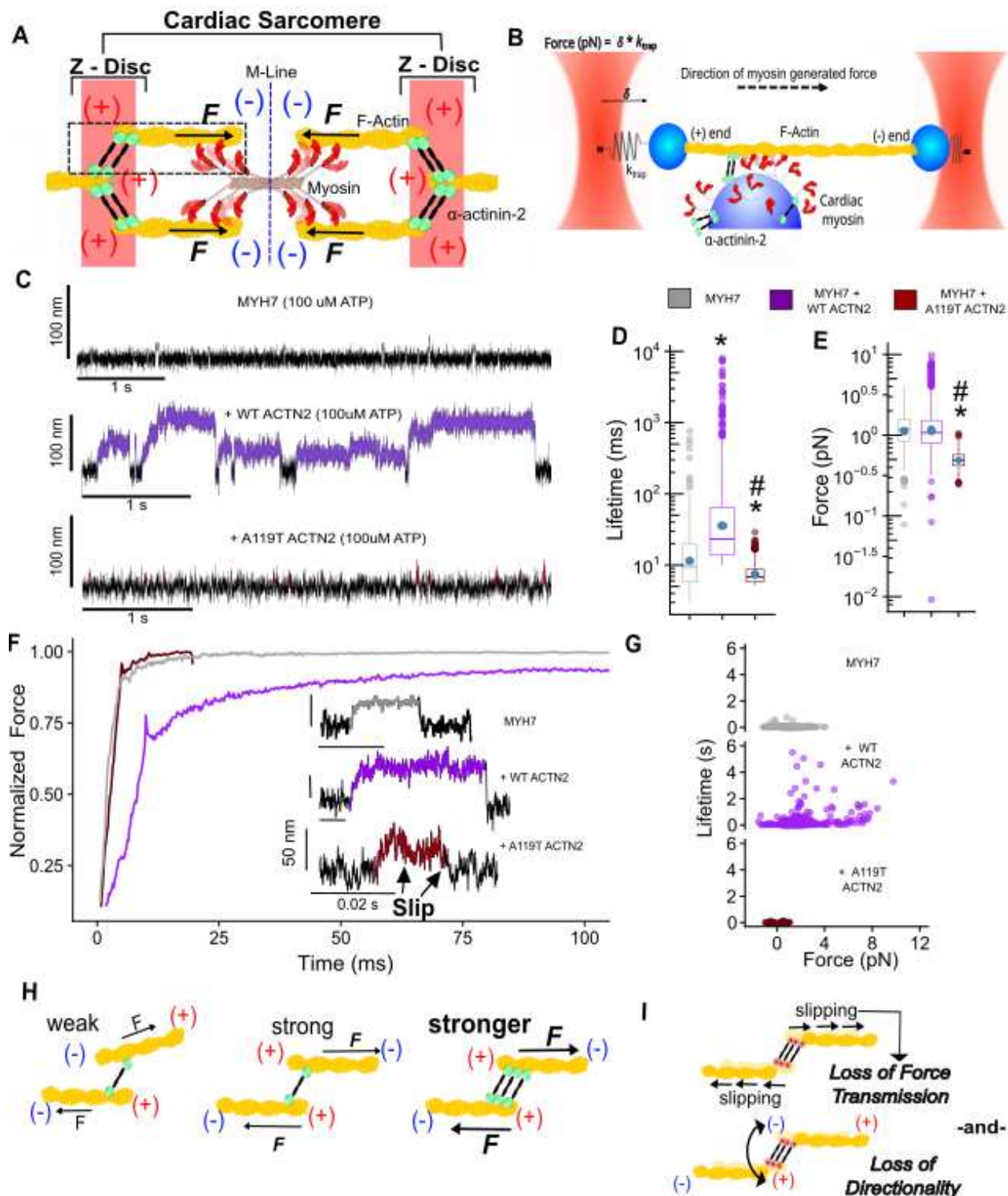


Figure 4. HCM introduces disorder and loss of force transmission due to F-actin - α -actinin-2 slippage under cardiac myosin force generation. A) Within sarcomeres, bipolar myosin II bundles (pink) exert force on the (-) end of actin filaments (blue), which are crosslinked by α -actinin-2 (black/green). Boxed out region shows myosin and α -actinin-2 bind to

892 a common actin filament in sarcomeres. **B)** Optical trap assay recreating the sarcomere
893 interactions on a single actin filament. **C)** Raw displacement records. **D and E)** Mean $\pm$ SEM for
894 attachment lifetimes and force generation. **F)** Ensemble averages from beginning of binding
895 events to the end. Cardiac myosin force generation occurs in an exponential nature. Force
896 generation is increased with +WT ACTN2 due longer attachment lifetime of the anchored
897 filament which allow myosin to perform additional cycling. Replacement with +A119T
898 eliminates F-actin anchoring and force generation begins to slip and creates an unloading
899 regimes as evident in the insets of binding events. Majority of cardiac myosin + A119T events
900 displayed slipping under force generation. **G)** Attachment lifetime vs force. + WT (FL) ACTN2
901 increases the attachment lifetime and force regime generated by the mini sarcomeres.
902 Replacement with A119T molecules destroys sarcomeric function **H)** Model: α -actinin-2 forms a
903 directional catch bond that selectively anchors antiparallel filaments such that the (-) ends point
904 outward, complementary to the orientation of myosin force production (see A, above). As more
905 α -actinin-2 form directional catch bonds the Z-disc reinforces itself with a highly stabilized,
906 strongly bound complex (far right). **I)** Mechanisms of sarcomere disorder introduced by HCM.

Supplementary Files

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