Structural and Functional Insights into Alpha-actinin Isoforms and their Implications in Cardiovascular Disease

Maya Noureddine[1*], Halina Mikolajek[2], Neil V. Morgan[1], Chris Denning[3], Siobhan Loughna[4], Katja Gehmlich[1,5], Fiyaz Mohammed[6*]

[1]Department of Cardiovascular Sciences , School of Medical Sciences, College of Medicine and Health University of Birmingham, Birmingham, UK

[2] Diamond Light Source Ltd, Harwell Science & Innovation Campus, Didcot, UK

[3] Biodiscovery Institute, University of Nottingham, Nottingham, UK

[4] School of Life Sciences, Faculty of Medicine and Health Sciences, University of Nottingham, Nottingham, UK

[5] Division of Cardiovascular Medicine, Radcliffe Department of Medicine and British Heart Foundation Centre of Research Excellence Oxford, University of Oxford, Oxford, UK

[6]Department of Immunology and Immunotherapy, School of Infection, Inflammation and Immunology, College of Medicine and Health, University of Birmingham, Birmingham, UK

*Correspondence:
Maya Noureddine, mmn207@student.bham.ac.uk
Fiyaz Mohammed, f.mohammed@bham.ac.uk

Summary: This review provides comprehensive insights into alpha-actinin isoforms and their role in cardiovascular disease, specifically macrothrombocytopaenia and hypertrophic cardiomyopathy, using structural modelling approaches.

Abstract:

Alpha-actinin (ACTN) is a pivotal member of the actin-binding protein family, crucial for the anchoring and organisation of actin filaments within the cytoskeleton. Four isoforms of alpha-actinin exist: two non-muscle isoforms (ACTN1 and ACTN4) primarily associated with actin stress fibres and focal adhesions, and two muscle-specific isoforms (ACTN2 and ACTN3) localised to the Z-disk of the striated muscle. Although these isoforms share structural similarities, they exhibit distinct functional characteristics that reflect their specialised roles in various tissues. Genetic variants in alpha-actinin isoforms have been implicated in a range of pathologies, including cardiomyopathies, thrombocytopenia, and non-cardiovascular diseases, such as nephropathy. However, the precise impact of these genetic variants on the alpha-actinin structure and their contribution to disease pathogenesis remain poorly understood. This review provides a comprehensive overview of the structural and functional attributes of the four alpha-actinin isoforms, emphasising their roles in actin crosslinking and sarcomere stabilisation. Furthermore, we present detailed structural modelling of select *ACTN1* and *ACTN2* variants to elucidate mechanisms underlying disease pathogenesis, with a particular focus on macrothrombocytopenia and hypertrophic cardiomyopathy. By advancing our understanding of alpha-actinin's role in both normal cellular function and disease states, this review lays the groundwork for future research and the development of targeted therapeutic interventions.

Key words: muscle alpha-actinin, non-muscle alpha-actinin, actin cytoskeleton, pathogenic variant, structural modelling, macrothrombocytopenia (MTC), hypertrophic cardiomyopathy (HCM)

Abbreviations

ABS: Actin-binding site

ABD: Actin-binding domain

ACTN1-4: Alpha-actinin 1-4

CaM: Calmodulin homology domain

CH: Calponin homology domain

DCM: Dilated cardiomyopathy

GnomAD: Genome Aggregation Database

HCM: Hypertrophic cardiomyopathy

HGMD: Human gene mutation database

Ident: Identity

MAF: Minor allelic frequency

MTC: Macrothrombocytopenia

PIP2: Phosphatidylinositol 4,5-bisphosphate

PIP3: Phosphatidylinositol (3,4,5)-trisphosphate

PolyPhen-2: Polymorphism Phenotyping v2

SAXS: Small-angle X-ray scattering

SIFT: Sorting Intolerant from tolerant

Sim: Similarity

SR: Spectrin repeat

Zr: Z-repeat

Introduction

The actin cytoskeleton represents a highly intricate network primarily composed of actin filaments, which function as a major force-generating mechanism within cells (Svitkina, 2018). These filaments are integral components of the contractile apparatus in muscle cells (Geeves & Holmes, 1999) and contribute to the formation of stress fibres in non-muscle cells (Tojkander et al., 2012). Additionally, the cytoskeleton is crucial for cell motility, signal transduction, and intracellular transport (Abraham et al., 1999). The cytoskeleton is stabilised by various components, including focal adhesions that connect the cytoskeleton to the extracellular matrix (Kumari et al., 2024) and intercellular adherens junctions, which link the cell membrane to the actin cytoskeleton across neighbouring cells (Niessen & Gottardi, 2008).

A critical regulator of the cytoskeleton is the alpha-actinin protein, which belongs to the spectrin superfamily of actin-binding proteins. This family includes both short and long actin cross-linkers, such as alpha-actinins and alpha- and beta-spectrin, respectively (Chiu et al., 2010; Dixson et al., 2003; Sjöblom et al., 2008). Alpha-actinin exists in four isoforms, and the emergence of these isoforms is thought to be linked to a vertebrate-invertebrate split which occurred during evolution (Virel & Backman, 2004). The four isoforms include, non-muscle isoforms ACTN1 and ACTN4, and muscle-specific isoforms ACTN2 and ACTN3. The non-muscle alpha-actinins are essential for crosslinking actin in stress fibres and anchoring them to cell focal adhesions (Barstead et al., 1991; Hsu et al., 2018), while muscle isoforms crosslink actin thin filaments, thereby stabilising the sarcomere (Hsu et al., 2018). Alpha-actinin isoforms are conserved across different species including, human, mice and chicken (Mills et al., 2001), although ACTN3 is notably absent in avian species (Holterhoff et al., 2009). Nonetheless, alpha-actinin isoforms exhibit unique association kinetics with myofibers, displaying differential

turnover dynamics when analysed using fluorescence recovery after photobleaching (FRAP) (Hsu et al., 2018). Specifically, ACTN4 shows fast recovery, ACTN1 exhibits intermediate recovery, and ACTN2 and ACTN3 demonstrate slow recovery, with the relatively rapid recovery of the non-muscle isoforms potentially attributed to their calcium sensitivity (Hsu et al., 2018). In addition, the functional diversity of alpha-actinin isoforms is well-characterised, with distinct expression profiles across various cell types. Non-muscle alpha-actinins are ubiquitously expressed in diverse tissues, whereas muscle alpha-actinins are specifically localised to striated muscle. For example, ACTN1 co-localise with actin filaments in the cytoplasm and cell membrane of surface-activated platelets (O'Sullivan et al., 2021). Similarly, ACTN4 maintains cytoplasmic structural organisation of the cytoskeleton and exhibits unique functions in kidney tissues (Weins et al., 2007) and cancer invasion (Knight et al., 2000). In contrast, the sarcomeric isoforms ACTN2 and ACTN3, are primarily expressed in striated muscle tissues and act as key components at the Z-disks of the contractile apparatus. ACTN2 is abundant in both skeletal and cardiac muscle fibres, while ACTN3 is specifically found in glycolytic skeletal muscles (Mills et al., 2001). Advances in whole-genome sequencing and genome-wide association studies have facilitated the identification of numerous missense and nonsense variants of alpha-actinin associated with cardiovascular and non-cardiovascular diseases. Despite the rapid pace of these discoveries, significant gaps remain in understanding how these variants compromise alpha-actinin structure and function, as well as the mechanisms by which they contribute to disease pathogenesis. This review aims to explore the functional and structural characteristics of the four alpha-actinin isoforms, underlining their roles in actin crosslinking and sarcomere stabilisation (sections 1, 2 and 3). It also highlights the implications of genetic variants of alpha-actinin isoforms in thrombocytopenia (*ACTN1*) and cardiomyopathies (*ACTN2*), examining how these

variants disrupt normal cellular functions (section 4). Finally, this review evaluates the pathogenicity of identified ACTN1 and ACTN2 variant using bioinformatic *in silico* tools (section 5) and assesses the structural impact of these missense variants through molecular modelling approaches (section 6), offering insights into the molecular mechanisms governing disease pathogenesis. Understanding these mechanisms is crucial for guiding future research directions and developing novel therapeutic strategies targeting alpha-actinin-related diseases.

1. Functions of alpha-actinins

1.1. Functions of non-muscle alpha-actinin isoforms

Non-muscle alpha-actinins, ACTN1 and ACTN4, are crucial regulators of the actin cytoskeleton, maintaining cell shape, and serving as scaffolds that interact with various cytoskeletal and transmembrane proteins. The non-muscle isoforms demonstrate varying localisation patterns across different cell types. For instance, ACTN1 is mainly associated with focal adhesions and adherens junctions (Kovac et al., 2018), whereas ACTN4 is predominantly found in stress fibres, suggesting divergent functional roles. Both isoforms dynamically link actin in stress fibres and stabilise the non-muscle contractile apparatus. Non-muscle alpha-actinins bind to β_1 integrin receptor, a transmembrane protein located at focal adhesions, thereby anchoring the actin cytoskeleton to the plasma membrane and facilitating the transmission of mechanical contractile forces (Otey et al., 1990; Roca-Cusachs et al., 2013). Notably, ACTN4 plays a pivotal role in sensing extracellular matrix stiffness during focal adhesion maturation (Meacci et al., 2016). Furthermore, non-muscle isoforms contribute to cell motility by coordinating contractile forces generated at focal adhesions, and may also participate in focal adhesion disassembly, which permits cell movement (Ye et al., 2014). They also significantly contribute to adherens junctions

through their association with alpha-catenin, thereby indirectly linking the actin cytoskeleton to these junctions (Knudsen et al., 1995; Nieset et al., 1997).

1.1.1. Role of ACTN1 in platelet function and development

The actin cytoskeleton is crucial for platelet production, circulation, activation, and aggregation, in response to vascular damage. As a major actin crosslinking protein, ACTN1 is essential for platelet activation (O'Sullivan et al., 2021). In surface-activated platelets, ACTN1 localises to various sites, including the cytoplasm, cell membrane, and actin filaments. It is also associated with actin nodules formed during the early adhesion of platelets, thereby enhancing the stability of platelet aggregates (Poulter et al., 2015). In addition, ACTN1 contributes to megakaryocyte maturation by influencing endomitosis, a process by which pro-megakaryoblasts increase their ploidy (O'Sullivan et al., 2021). Overexpression of ACTN1 has been associated with impaired cytokinesis due to inhibited actin turnover and increased actin accumulation (Mukhina et al., 2007). ACTN1 also participates in the formation and fission of proplatelets and the release of platelets (O'Sullivan et al., 2021).

1.1.2. Implications of ACTN4 in cancer progression

The non-muscle isoform ACTN4 is implicated in cancer metastasis through its involvement in cell motility and localisation within the actin-rich dorsal ruffles (Araki et al., 2000). Supporting this, elevated expression of ACTN4 in colorectal cancer specimens correlated with increased cell motility, suggesting a role in promoting lymph node metastasis (Honda et al., 2005). ACTN4 has also been proposed as an oncogene candidate and a potential marker for predicting poor outcomes in patients with chemotherapy-resistant tumours (Yamamoto et al., 2009). This isoform exhibits dynamic localisation between the nucleus and cytoplasm, with cytoplasmic

localisation linked to an infiltrative phenotype in breast cancer (Honda et al., 1998). Conversely, the functional significance of nuclear ACTN4 remains uncertain, although previous research has suggested that ACTN4 may serve as a nuclear receptor coactivator to promote breast cancer cell proliferation (Khurana et al., 2011).

1.1.3. Functional significance of ACTN4 in renal physiology

ACTN4 is uniquely expressed in the podocytes of the kidneys (Kaplan et al., 2000). Podocytes are highly differentiated epithelial cells attached to the glomerular basement membrane via integrin receptors (Pavenstädt et al., 2003). The foot processes of the podocytes are interconnected through adherens junctions and possess a dense cortex of actin filaments beneath their cell membranes (Drenckhahn & Franke, 1988). ACTN4 plays a crucial role in stabilising actin filaments, which are essential for maintaining the architecture of complex podocytes (Feng et al., 2015). In addition, ACTN4 may serve as a linker between actin filaments and other proteins in the adherence junction, such as alpha and beta II spectrin (Lehtonen et al., 2005).

Research using ACTN4 knockout mice has demonstrated that these mice exhibit glomerular collapse, altered podocyte morphology, and the development of focal segmental glomerular sclerosis (Kos et al., 2003). Another study revealed that ACTN4 knock out mice displayed reduced podocyte adherence to the glomerular basement membrane, attributed to decreased binding affinity between integrins and the cytoskeleton (Dandapani et al., 2007). Further investigation of a missense variant, K255E, in mouse models showed signs of proteinuria and abnormalities in podocyte effacement (Cybulsky & Kennedy, 2011). Additionally, ACTN4 protein aggregates were identified in an altered localisation pattern, eventually leading to podocyte proteotoxicity (Cybulsky & Kennedy, 2011). These findings underscore the significant role of ACTN4 in kidney disease, warranting further investigation.

1.2. Functional characteristics of muscle alpha-actinin isoforms

The muscle isoforms of alpha-actinin, ACTN2 and ACTN3 are highly expressed in sarcomeric muscle, acting as major stabilisers of the contractile apparatus. ACTN2 is widely expressed in both cardiac and skeletal muscles, whereas ACTN3 is specially localised to glycolytic skeletal muscle fibres (Mills et al., 2001). Additionally, both muscle isoforms exhibit low expression levels in the brain, specifically in the grey matter, with ACTN3 displaying even lower expression than ACTN2 (Mills et al., 2001). This expression is suggested to either regulate cytoskeletal remodelling or provide structural support for N-methyl-D-aspartate (NMDA) receptors in the brain (Michael Wyszynski et al., 1997).

ACTN2 localises specifically at the Z-disk of the sarcomere, where it is essential for organising thin filaments by anchoring and cross-linking actin and titin filaments from adjacent sarcomeres. This process stabilises the contractile muscle apparatus by forming a lattice-like structure between these filaments (Good et al., 2020; Sjöblom et al., 2008). Beyond its structural role, ACTN2 regulates the transactivation activity of various receptors (Huang et al., 2004) and ion channels, such as calcium-activated K^+ channels (Lu et al., 2009). ACTN2 also plays a regulatory role in the organisation of sarcomeric cytoskeleton by linking the cytoskeleton to several transmembrane proteins (Otey & Carpen, 2004; Sjöblom et al., 2008). Moreover, recent studies propose novel role for ACTN2 in regulating mitochondrial organisation and function (Zech et al., 2022), serving as a scaffold for mitochondrial messenger RNAs (Ladha et al., 2020).

ACTN3 is primarily expressed in fast-twitch skeletal muscle fibres (type II), suggesting a specific function in fast muscle contraction (North et al., 1999). It plays an integral role in generating sarcomeric force (Baltazar-Martins et al., 2020), and regulating muscle tension and length during contraction (Houweling et al., 2018). Numerous studies have focused on assessing

its modifying effect on muscle function, strength, and size in athletes and the elderly (Delmonico et al., 2007; Walsh et al., 2008). To identify additional roles of ACTN3, a termination variant, R577X, has been widely studied. This common loss of function variant has a high minor allelic frequency of 0.439, as retrieved from the Genome Aggregation Database (GnomAD). Studies have shown that individuals with the homozygous genotype (XX) exhibit ACTN3 deficiency; however, this deficiency does not manifest as an overt disease phenotype, suggesting potential redundancy in ACTN3 expression (Mills et al., 2001; Zouhal et al., 2023). This implies compensatory regulation by other alpha-actinin isoforms, particularly ACTN2 (MacArthur et al., 2007; J. T. Seto et al., 2011). This compensation is thought to result from the high structural and functional similarity between the two isoforms (North et al., 1999).

Additionally, a systematic review highlighted studies that assessed prevalence differences among sprint and endurance athletes, with endurance athletes exhibiting a higher XX genotype (El Ouali et al., 2024). This suggests that the R577X polymorphism enhances endurance performance, indicating potential advantages for certain high-performance activities.

It is estimated that around 20% of the population possess the XX genotype in which it is generally tolerated (Domańska-Senderowska et al., 2019); however, it may be associated with quantitative disadvantages Individuals lacking ACTN3 have been shown to display reduced muscle strength (Walsh et al., 2008) and volume (Del Coso et al., 2019), along with decreased grip strength, slower baseline sprint times, and impaired capacity to tolerate muscle strain (Clarkson et al., 2005; Moran et al., 2007; J. T. Seto et al., 2011). Furthermore, ACTN3 deficiency is linked to lower bone mineral density (Yang et al., 2011), potentially increasing the susceptibility to contraction-induced damage (Jane T. Seto et al., 2011) and muscle injuries (Watsford et al., 2010). The loss of ACTN3 also correlates with decreased glycogen

phosphorylase activity, altered calcium handling (Quinlan et al., 2010), and a shift in muscle metabolism towards an aerobic pathway (MacArthur et al., 2007). As these quantitative disadvantages are not impairing the overall muscle function, they are not sufficient to cause evolutionary selection pressure.

In summary, alpha-actinin isoforms display distinct localisation and function. Non-muscle isoforms are primarily found in the actin cytoskeleton, with ACTN1 involved in platelet function, and ACTN4 playing important roles in cancer progression and renal physiology. Conversely, muscle isoforms are located in sarcomeric muscle, where ACTN2 contributes to Z-disk stability and ACTN3 plays important functions in fast-twitch skeletal muscle.

2. Structural components of alpha-actinin

2.1. Alpha-actinin domain structure

Non-muscle and muscle alpha-actinin isoforms are highly conserved in structure, with a sequence identity of 84% and 80%, respectively (Figure S1, S2), as calculated using the Ident & Sim software (Stothard, 2000). Despite these similarities, they have evolved to regulate actin filaments in distinct ways, influenced by tissue-specific modifications and binding of specific ligands (Hsu et al., 2018). All alpha-actinin isoforms exist physiologically as anti-parallel dimers, where they cross-link actin filaments at both ends (Figure 1A). All isoforms also share a common structural domain topology crucial for force generation, comprising an N-terminal actin-binding domain (ABD), a flexible neck region, a central rod module, and a C-terminal calmodulin homology (CaM) domain (Figure 1B). High-resolution crystal structures have been determined for the ABD of all human alpha-actinin isoforms, including ACTN1 (E. Borrego-Diaz et al., 2006), ACTN2 (Haywood et al., 2016), ACTN3 (Franzot et al., 2005), and ACTN4 (Feng et al., 2020). Conversely, the full-length structure has been resolved for human and

Entamoeba histolytica ACTN2 via X-ray crystallography (Pinotsis et al., 2020; Ribeiro Ede et al., 2014) and for chicken ACTN1 using cryo-electron microscopy (Liu et al., 2004).

2.1.1. Actin-binding domain: Actin ligand binding

The ABD of alpha-actinins play an essential role in binding and cross-linking actin thin filaments in both muscle and non-muscle cells. It comprises two consecutive calponin homology domains (CH1 and CH2). Each CH domain consists of approximately 110 residues forming a compact globular domain (Broderick & Winder, 2002). Each individual CH domain comprises four principal alpha-helices (designated A, C, E, and G), 11 to 18 residues each, forming the domain core (Figure 1C) (Djinovic Carugo et al., 1997; Franzot et al., 2005). These alpha helices are connected via long loops and are interspersed by three short helices (Broderick & Winder, 2002). Helices C and G are parallel to each other and sandwiched between an N-terminal helix A (Franzot et al., 2005). In addition, helix A packs against helices C and G in a perpendicular orientation, tightly interacting with helix G and partially burying helix C (Djinovic Carugo et al., 1997).

2.1.2. Neck region: Imparting flexibility and stability

Alpha-actinin features an alpha-helical neck region, consisting of approximately 24 residues, which connects the ABD to the central rod module. The flexibility of the neck region determines the nature and properties of the ABD, enabling it to adopt structural conformational changes (Sjöblom et al., 2008; Ylänne et al., 2001). In addition, the neck region is important for maintaining the stability of the alpha-actinin dimer through intermolecular interactions with the CaM domain, specifically the EF34 hand (Ribeiro Ede et al., 2014).

2.1.3. Central rod module: Conferring structural integrity

The central rod module of alpha-actinin encompasses four spectrin repeats (SR1-SR4), each ranging from 106 to 122 residues in length, connected by inter-spectrin helical linkers of approximately 10 residues (Figure 1C) (Liem, 2016). The inter-spectrin repeat domain links adjacent spectrin repeats, thereby maintaining their integrity without any breaks, discontinuities, or changes in the secondary structure between spectrin repeats (Grum et al., 1999). Each spectrin repeat consists of three alpha-helices (designated A, B, and C), where helices A and C are parallel and helix B is antiparallel (Figure 1C) (Grum et al., 1999). These three helices form a non-straight domain, wrapping around each other to form a left-handed supercoil (Grum et al., 1999).

Spectrin repeats play an important role in the formation of an exceptionally strong anti-parallel homodimer in a zipper-like manner (Speicher et al., 1992). The antiparallel assembly of opposite spectrin repeats forms an alpha-actinin dimer, stabilised by direct polar interactions (Djinović-Carugo et al., 1999). Approximately 38 residues per monomer, distributed across different spectrin repeats, are present at the dimer interface (Djinović-Carugo et al., 1999). Additionally, the monomer surface is buried upon dimer formation, forming a 90° twist along the long axis of the alpha-actinin central rod (Broderick & Winder, 2002). This twist forms a curved interface that is crucial for stabilising the rod structure (Djinović-Carugo et al., 1999). Furthermore, alpha-actinin dimer of all isoforms exhibit a high mechanical stability when exposed to shear-stretching forces, with a rupture force of ≥60 pN required to potentially rupture the dimer (Zhang et al., 2024). This force is relatively high compared to another actin filament crosslinker protein, filamin, which has a reported rupture force of 14 pN (Zhang et al., 2024). Therefore, this further underscores the key role of alpha-actinin as a strong crosslinker for actin filaments.

Beyond dimerisation, spectrin repeats are integral to the formation and stability of the alpha-actinin rod domain. Non-polar and polar interactions in spectrin repeats, including both intrahelical and interhelical contacts, contribute to the stabilisation of the alpha-helical fold. Intrahelical interactions are mediated between residues within the same alpha-helix, whereas interhelical contacts involve residues from the three alpha-helices of the same spectrin repeat. This arrangement stabilises the coiled-coil assembly of the spectrin repeat, contributing to its tertiary structure (Djinović-Carugo et al., 1999). In addition, spectrin repeats form a rigid connector between the two actin-binding domains positioned at the ends of the rod-like structure (Sjöblom et al., 2008; Ylänne et al., 2001). They facilitate the cross-linking of actin filaments (Clark et al., 2002) by anchoring conserved actin-binding head domains (Djinović-Carugo et al., 1999). During muscle contraction, the cytoskeletal architecture is preserved by providing docking surfaces for signal transduction and cytoskeletal proteins (K. Djinovic-Carugo et al., 2002). Additionally, spectrin repeats interact with various ligands, including N-methyl-D-aspartate receptor subunits (M. Wyszynski et al., 1997), alpha-catenin (Pradhan et al., 2001), integrins (Otey et al., 1990), L-selectin (Pavalko et al., 1995), and intercellular adhesion molecules (Carpén et al., 1992). These interactions are essential for generating multiprotein assemblies, contributing to the development of the actin cytoskeleton architecture and cytoplasmic domains of integrins (Kristina Djinovic-Carugo et al., 2002).

2.1.4. Calmodulin homology domain: Regulating actin bundling activity

The C-terminal Calmodulin homology (CaM) domain is composed of a pair of interacting EF hands (EF12 and EF34). EF-hands feature two short alpha-helices connected by a loop region, forming a helix-loop-helix configuration (Figure 1C) (Drmota Prebil et al., 2016; Sjöblom et al., 2008). EF-hands form a globular domain that is involved in intracellular calcium binding

(Atkinson et al., 2001). Notably, the calcium-binding properties of EF-hands differentiate between muscle and non-muscle alpha-actinin isoforms, as not all EF-hands can chelate calcium (Liem, 2016). In muscle isoforms, ACTN2 and ACTN3, the EF hands are rendered non-functional due to a loss of the calcium-chelating side chain, allowing actin to bind independently of calcium (Mills et al., 2001). This inability to bind calcium may result from mutations in residues critical for calcium coordination, that occurred during evolution (Noegel et al., 1987). Such genetic adaptations likely serve to prevent structural conformational changes that could compromise muscle integrity during calcium-induced contractions (Blanchard et al., 1989).

In contrast, the ACTN1 and ACTN4 non-muscle isoforms can exist as either calcium-sensitive or calcium-insensitive isoforms arising from the alternative splicing of two exon variants encoding part of the EF12 domain (Foley & Young, 2013). Calcium-sensitive isoforms are broadly expressed across various tissues. The calcium-sensitive ACTN1 is present in platelets (Rosenberg et al., 1981), whereas the calcium-insensitive ACTN1 is expressed in smooth muscle tissues (Foley & Young, 2013). Calcium-sensitive non-muscle isoforms bind calcium through their EF hand, inducing a conformational shift in the CaM domain from a closed to an open state, that alters its interaction with the neck domain (Atkinson et al., 2001). This change modifies the orientation of the actin-binding domains within the α-actinin dimer, ultimately affecting its capacity to effectively cross-link F-actin (Lehne and Bogdan 2023). In addition, calcium binding stabilises the loop region connecting the EF hand helices, reducing their mobility (Yamniuk & Vogel, 2004) and exposing alpha helices and hydrophobic residues for interactions with other ligands (Ikura, 1996; Yap et al., 1999). Collectively, this suggests that calcium acts as an allosteric regulator of α-actinin's F-actin bundling activity.

In conclusion, all isoforms of alpha-actinin share a similar domain structure, which includes: (1) an actin-binding domain; (2) a central rod responsible for the formation of an anti-parallel dimer formation; and (3) a calmodulin homology domain which possess different characteristics between isoforms.

3. Alpha-actinin ligand binding dynamics

The major ligand-binding partner of alpha-actinin is actin, where all isoforms cross-link actin filaments via their actin-binding domain located at both ends of the alpha-actinin dimer. In addition, non-muscle alpha-actinins interact with several cytoskeletal and regulatory proteins, including CapZ (Papa et al., 1999), zyxin (Beckerle, 1997; Crawford et al., 1992), and intercellular adhesion molecule-2 in focal adhesions and cell-cell contacts (Carpén et al., 1992; Heiska et al., 1996). The C-terminus of ACTN4 interacts with MAGI-1, a tight junction protein, thereby linking the cell membrane to the cytoskeleton (Patrie et al., 2002). Additionally, ACTN1 is associated with signalling molecules, such as protein kinases, including mitogen-activated protein kinase (Christerson et al., 1999) and protein kinase N (Mukai et al., 1997).

Similarly, sarcomeric alpha-actinin ACTN2 engages with PDZ domain proteins, including alpha-actinin-associated LIM protein and the Z-band alternatively spliced PDZ motif (Pomiès et al., 1999; Zhou et al., 1999). ACTN2 binds to other cytoskeletal and sarcomeric proteins, such as myopalladin (Bang et al., 2001), myotilin (Salmikangas et al., 1999), and muscle LIM proteins (Mohapatra et al., 2003). ACTN2 also plays an important role in linking the membrane and sarcomere by interacting with dystrophin complex (Hance et al., 1999) and vinculin (McGregor et al., 1994). ACTN2 serves as a ligand for titin via its C-terminal CaM domain (Jalan-Sakrikar et al., 2012; Ribeiro Ede et al., 2014; Young & Gautel, 2000). In addition, the EF34-hand motif

of ACTN2 interacts with palladin and calcium-calmodulin-dependent protein kinase II (Beck et al., 2011).

3.1. Phospholipid regulation of alpha-actinin function

Phospholipids, including phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3), are cellular membrane phospholipids generated after the phosphorylation of phosphatidylinositol (Katan & Cockcroft, 2020). Phospholipids are cleaved by phospholipase C (PLC) into two secondary messengers: (1) inositol 1,4,5-trisphosphate and (2) diacylglycerol (Falkenburger et al., 2013). Both PIP2 and PIP3 play essential roles in regulating the remodelling of the actin cytoskeleton through modulation of alpha-actinin dynamics (Fraley et al., 2005). PIP3 is thought to regulate the functions of non-muscle isoforms. For instance, PIP3 decreases the affinity between ACTN1 and β-integrins, thereby reducing actin binding (Otey et al., 1990). In contrast, PIP3 has been reported to exert an opposite effect on ACTN4 by increasing its binding affinity with actin (Michaud et al., 2009). PIP3 also disrupts the detachment of migrating cells at focal adhesions, thereby increasing their turnover (Fraley et al., 2005). Moreover, both PIP2 and PIP3 appear to exhibit reciprocal functions in modulating alpha-actinin proteolysis by calpains, thus, regulating the exposure of a cleavage site within the CH2 domain (Sprague et al., 2008). For example, PIP3 increases cleavage by enhancing flexibility of the neck region, whereas PIP2 reduces cleavage and stabilises this region (Corgan et al., 2004).

Furthermore, the binding of PIP2 to alpha-actinin triggers conformational changes in the CaM domain (Atkinson et al., 2001; Joseph et al., 2001; Young et al., 1998). The aliphatic chain of PIP2 extends to the EF34-hand, facilitating its release from the neck region (Franzot et al., 2005). This, in turn, induces a major conformational rearrangement of EF34, permitting its

binding to different ligands (Beck et al., 2011), including titin, a giant elastic filament that spans half of the sarcomere (Herzog, 2018). The Z-disk portion of titin consists of consecutive immunoglobulin domains and 45-residue repeating Z-repeat (Zr) modules (Gautel et al., 1996). It has also been shown that the interaction between EF34-hand and the first and last titin Z-repeats (Zr1 and Zr7) regulates conformational changes in EF34 (Atkinson et al., 2001; Joseph et al., 2001; Ohtsuka et al., 1997).

Further insights into alpha-actinin regulation revealed that the binding of PIP2 to the ABD directly influences its actin-binding activity. For instance, one study demonstrated that the loss of PIP2 results in impaired binding between alpha-actinin and actin (Fraley et al., 2003). This regulatory mechanism of PIP2 requires spatial proximity to the ABD of alpha actinin. Consequently, several studies have aimed to identify the specific PIP2 binding sites. Using a PLC inhibition assay, researchers mapped the PIP2 binding site to residues N168-H184 in ACTN1 (Fukami et al., 1996). Similarly, a solid-phase binding assay identified residues T172-K188 in ACTN3 as being involved in the bundling activity of PIP2 (Fraley et al., 2003). These residues in both alpha-actinin isoforms map to a loop that connects the two helices of the CH2 domain. In addition, a triad of positively charged residues present in the CH2 domain (R170, R176, and R199) forms a platform for PIP2 to bind with ACTN3 (Figure 2A) (Franzot et al., 2005).

3.2. Conformational changes induced by phospholipid binding

Docking of PIP2 to alpha-actinin triggers conformational changes in the ABD, allowing actin binding (Ribeiro Ede et al., 2014). This interaction induces structural rearrangement in the CH domains, specifically altering the loop that links these domains and disrupting their interactions (Franzot et al., 2005). As a result, the CH1 and CH2 domains separate and adopt an open

conformation that facilitates actin binding (Franzot et al., 2005). In contrast, in the native closed state, the N-terminus of CH1 interacts closely with the C-terminus of CH2, maintaining a more compact structure (E. Borrego-Diaz et al., 2006; Franzot et al., 2005).

Several studies have identified residues crucial for regulating of the open and closed conformations of alpha-actinin. For example, residues K255 and W147 in ACTN4 form a hinge-like connection that is crucial for maintaining the closed state of CH1 (Weins et al., 2007). K255 resides at the interface between the two CH domains. A mutant ACTN4 protein incorporating a disease-associated missense variant, K255E, exhibited a six-fold increase in actin affinity, suggesting that this variant disrupts the hinge-like connection with W147 and destabilises the CH1-CH2 interface (Weins et al., 2007). This destabilisation promotes a transition to an open conformation, exposing additional actin-binding sites and preventing the rapid turnover of ACTN4 during cytoskeletal remodelling (Weins et al., 2007). Similar mechanisms are observed in other alpha-actinin isoforms, where residues at the CH1/CH2 interface contribute to conformational stability. For instance, interactions between residues W128 and K236 in ACTN1 (E. Borrego-Diaz et al., 2006), and between W135 and R243 in ACTN2 have been observed (Haywood et al., 2016). These findings suggest a shared mechanism among different alpha-actinin isoforms for preserving conformational stability at the CH1-CH2 interface (Figure 2B).

3.3. Identification and Characterisation of actin-binding sites

The CH1 and CH2 domains of alpha-actinins play important roles in binding actin thin filaments, yet they vary in their amino acid sequences and affinities (Way et al., 1992). The CH1 domain, recognised as the primary actin-binding site (E. Borrego-Diaz et al., 2006), demonstrated reduced affinity for actin in isolation compared to its enhanced affinity in conjunction with the CH2 domain (Way et al., 1992). Other studies have shown that isolated CH2 cannot

445 independently bind actin (Galkin et al., 2010; Iwamoto et al., 2018). Therefore, the CH2 domain
446 may serve to regulate the binding of the CH1 domain to actin (Young & Gautel, 2000).
447 Additionally, the CH2 domain functions as a locator, positioning the CH1 actin-binding domains
448 and contributing to stabilising the ABD while preventing clashes with actin (Galkin et al., 2010;
449 Young & Gautel, 2000).

450 Further work has established that the CH1 and CH2 domains contain multiple actin-binding sites
451 that become accessible after transitioning to an open conformation (Joseph et al., 2001). Several
452 studies have identified three actin-binding sites (ABS) in alpha-actinin. The first actin-binding
453 site (ABS-1) is found in the N-terminal helix of CH1, the second actin-binding site (ABS-2) is
454 localised in the C-terminal helix of CH1, and the third actin-binding site (ABS-3) is situated in
455 the N-terminal helix of CH2 (Bresnick et al., 1991; Hemmings et al., 1992; Kuhlman et al., 1992;
456 Levine et al., 1992). ABS-3, which consists of loop linker residues which connect the two CH
457 domains (Franzot et al., 2005), demonstrated a higher affinity for actin binding than ABS-1
458 (Corrado et al., 1994).

459 Using actin co-sedimentation assays, residues 89-115 within the CH1 domain of chicken alpha-
460 actinin were identified as essential for actin-binding activity (Bresnick et al., 1990). Using the
461 same assay, other studies pinpointed the significance of residues 120-134 in chicken alpha-
462 actinin (Kuhlman et al., 1992) and residues V108-F134 in chicken ACTN1 (Hemmings et al.,
463 1992) for actin binding. The use of NMR spectroscopy on chicken ACTN3 identified two actin-
464 binding sites, encompassing residues R48-S57 and I153-T172 (Levine et al., 1992). Furthermore,
465 another study mapped previously identified residues on the crystal structure of human ACTN1-
466 ABD, showing that ABS-1 is an isolated site compared to ABS-2 and ABS-3, which are more
467 adjacent (Emma Borrego-Diaz et al., 2006). The buried residues of CH1 may become more

accessible to actin upon the reorientation of the CH domains conformation from open to closed states (E. Borrego-Diaz et al., 2006). The low resolution cryo-EM structure of the ACTN3-ABD bound to actin provided valuable insights into the docking modes (Galkin et al., 2010). At this resolution it was possible to fit the actin and the CH1 domain into the cryo-EM density maps, but identifying key residues involved in ligand binding proved challenging. Furthermore, the superimposition of a previously determined crystal structure of ACTN3-ABD onto the ACTN3-CH1/actin cryo-EM complex revealed steric clashes between the CH2 domain and actin (Galkin et al., 2010), underscoring the critical importance of a specific spatial arrangement of CH domains for effective actin binding.

To summarise, the approaches used thus far to characterise the actin-binding sites of alpha-actinin have not yielded definitive results. Therefore, it is crucial that future investigations employ structural approaches, such as X-ray crystallography or cryo-EM, to determine high resolution structures of alpha-actinin/actin complex structures and elucidate precise docking modes.

In conclusion, the actin-binding domain of the alpha-actinin isoforms share similar functions, primarily facilitating actin binding. This process is regulated by phospholipids, which trigger conformational changes that expose three specific actin-binding sites within the domain, enabling interaction with actin.

4. Literature review on the implications of alpha-actinin variants in disease pathogenesis

Genetic variants of alpha-actinin isoforms have been implicated in a wide spectrum of diseases, encompassing both cardiac and non-cardiac conditions. Specifically, *ACTN1* variants are associated with bleeding disorders, while *ACTN2* variants are linked to various forms of

cardiomyopathy. Additionally, genetic variants in other alpha-actinin isoforms, such as *ACTN4* and *ACTN3*, are associated with nephropathies (Meng et al., 2019) and schizophrenia (Rodríguez-López et al., 2018), respectively. This section will focus on the available literature of the genetic implications of *ACTN1* and *ACTN2* variants in cardiac diseases, specifically macrothrombocytopenia and hypertrophic cardiomyopathy.

4.1. Phenotypic effects of *ACTN1* variants in bleeding disorders

Missense variants of *ACTN1* are notably associated with congenital bleeding disorders, attributable to the critical role of ACTN1 in platelet formation and function. Variants of *ACTN1* are linked to inherited platelet disorders, immune thrombocytopenia, and macrothrombocytopenia (MTC). The majority of *ACTN1* variants are associated with MTC, a disease characterised by enlarged platelets and reduced platelet counts (Kunishima & Saito, 2006). For instance, six *ACTN1* missense variants (Q32K, R46Q, V105I, E225K, R738W, and R752Q) were identified in 13 Japanese families with MTC using Sanger sequencing (Kunishima et al., 2013). Another study reported 10 *ACTN1* missense variants in 11 MTC patients, including novel variants, such as D22N, R46W, G251R, D666V, T737N, G764S, and G769K (Bottega et al., 2015). Notably, the R46Q variant has been identified in a large six-generation French family with MTC, genetically underlying its evidence of pathogenicity (Guéguen et al., 2013). This variant has been associated with significant phenotypic effects, including abnormal organisation of the cytoplasm and giant heterogeneous granules in platelets, as observed using electron microscopy of bone marrow smears (Guéguen et al., 2013). Further characterisation of this variant using COS-7 cells has revealed discrete disorganisation of ACTN1 and actin filaments (Guéguen et al., 2013). Additionally, the *ACTN1* L395Q variant, identified in a thrombocytopenic child and mother, demonstrated shortened and disorganised actin filaments in

variant-transduced CHO cells (Yasutomi et al., 2016). Subsequent investigations using actin co-sedimentation assays indicated that both variants enhanced binding affinity to actin and increased filament bundling (Murphy et al., 2016). Further investigations of the role of ACTN1 in platelet function involved the use of ACTN1 knock out mice model. The model exhibited reduced platelet count, impaired homeostasis, and mitochondrial dysfunction in platelets (Huang et al., 2023).

In summary, *ACTN1* has been established as a disease gene for bleeding disorders. While the evidence of pathogenicity of individual variants (e.g. D22N and Q32K) might not always be strong (especially in the absence of co-segregation studies), functional studies point at defects in organising the actin cytoskeleton and thereby impairing platelet function. Further functional and structural characterisation of these *ACTN1* variants is necessary to gain a deeper understanding of their role in platelet dysfunction and associated bleeding disorders.

4.2. *ACTN2* variants and their association with cardiomyopathy

Studies have also explored the association between *ACTN2* missense variants and cardiomyopathies. Inherited cardiomyopathies are classified into different types according to dominant structural or functional changes in the heart (Watkins et al., 2011), including hypertrophic cardiomyopathy (HCM), characterised by diastolic dysfunction and non-dilated left ventricular hypertrophy (Teekakirikul et al., 2019). Other types of inherited cardiomyopathies include dilated cardiomyopathy (DCM) (Mahmaljy et al., 2024), restrictive cardiomyopathy (Brodehl & Gerull, 2022), and arrhythmogenic cardiomyopathy (Krahn et al., 2022). Genetic missense variants in *ACTN2* are mainly associated with HCM and DCM.

A study screening 239 unrelated patients with HCM identified three *ACTN2* variants (G111V, T495M, and R759T) (Theis et al., 2006). Further histological analysis of these variants revealed endocardial and interstitial fibrosis, myocyte disarray, and cardiomyocyte hypertrophy (Theis et al., 2006). A genome-wide analysis of 23 patients with HCM identified several *ACTN2* variants, including four variants predicted to be causative due to an increase in RNA markers of hypertrophy (A199T, T495M, E583A, and E628G) (Chiu et al., 2010). Furthermore, biophysical characterisation of the previously identified *ACTN2* A119T and G111V variants demonstrated decreased thermal stability and reduced binding affinity to actin for both variants (Haywood et al., 2016). Further analysis of adult rat cardiomyocytes following adenoviral transduction revealed the presence of ACTN2 protein aggregates for both variants (Haywood et al., 2016).

Next-generation sequencing identified a novel *ACTN2* variant, M228T, in 11 patients with HCM from a large-generational family (Girolami et al., 2014). Another recent study assessed this variant in a mouse model, revealing that mice homozygous for M228T exhibited embryonic lethality at E15.5 (Broadway-Stringer et al., 2023). Additionally, a rare *ACTN2* variant, T247M, was identified in a patient with HCM (Prondzynski et al., 2019). Human induced pluripotent stem cells (iPSCs) derived from patients and differentiated into cardiomyocytes (iPSC-CMs) showed impaired relaxation and hypercontractility, prolonged action potential duration, and increased calcium sensitivity (Prondzynski et al., 2019), which are characteristics displayed in an HCM phenotype. Furthermore, myofibrillar disarray, multinucleation, and protein aggregation were observed in the iPSC-CM model for this variant (Prondzynski et al., 2019). Further assessment by inhibiting two important protein degradation systems: (1) the ubiquitin-proteasome system and (2) the autophagy-lysosomal pathway, revealed high activation of both systems without direct involvement in ACTN2 degradation (Zech et al., 2022).

In conclusion, *ACNT2* has been established as a certain, but rare disease gene for HCM with numerous variants being reported. However, functional studies have only been done with a very small number of variants. Therefore, further research is needed to evaluate whether the disease mechanisms identified for these variants are relevant to all HCM-associated *ACTN2* variants.

5. Identification and evaluation of pathogenicity of *ACTN1* and *ACTN2* variants using *in silico* tools

In the pursuit of investigating additional *ACTN1* and *ACTN2* variants documented in the literature, the Human Gene Mutation Database (HGMD) was utilised to identify further variants (Stenson et al., 2020). A total of 57 missense variants in *ACTN1* and 95 variants in *ACTN2* were identified. The pathogenicity of these variants was predicted using minor allelic frequency (MAF) values with a cutoff of 1×10^{-4}, retrieved from the GnomAD database (Gudmundsson et al., 2022; Kobayashi et al., 2017). However, given that MAF values alone may not reliably predict pathogenicity, *in silico* tools such as SIFT (Sim et al., 2012) and PolyPhen-2 (Adzhubei et al., 2013) were utilised alongside the MAF data. These tools evaluate the effects of variants on protein function and structure, aiding in the prediction of disease-causing variants. Variants with low MAF scores and those predicted to be deleterious by both SIFT and PolyPhen-2 were classified as potentially pathogenic. The findings for the *ACTN1* and *ACTN2* variants are summarised (Tables S1 and S2). Applying this approach, 32 missense variants in *ACTN1* and 60 variants in *ACTN2* were predicted to be disease-causing.

6. Investigating the molecular basis of *ACTN1* and *ACTN2* variants and their contribution to disease pathogenesis by structural predictions

6.1. Assessing structural impact of *ACTN1* and *ACTN2* variants

The *ACTN1* variants predicted as pathogenic (Table S1) were mapped onto the Phyre2-derived molecular model of human ACTN1 (Figures 3AB). This model was based on the chicken ACTN1 Cryo-EM structure (Liu et al., 2004), which shares high sequence identity (98 %) with human ACTN1 (Stothard, 2000). In addition, the *ACTN2* variants linked to cardiac disease and predicted to be pathogenic (Table S2) were systematically mapped onto the crystal structure of human ACTN2 (Ribeiro Ede et al., 2014) (Figures 4AB). Structural modelling tools were employed to assess the impact of *ACTN1* and *ACTN2* variants on alpha-actinin structure. The selection of *ACTN1* and *ACTN2* variants for structural modelling was guided by a strategic approach that considered domain distribution, functional relevance, and the potential for revealing diseases mechanisms, with two variants per domain being shown as examples. Molecular models of ACTN1 and ACTN2 incorporating disease-linked variants were generated using Phyre2 (Kelley et al., 2015). Due to the structural complexity of alpha-actinin, which contains multiple domains with distinct functions and interactions, accurate full-length modelling proved challenging. Consequently, only individual domains harbouring the disease-linked mutations were modelled, as these domain-specific models offered greater accuracy and reliability. Molecular interactions between wild-type and mutant protein structures were evaluated, facilitating the assessment of the impact of variants on alpha-actinin structure.

6.2. Structural modelling of MTC-linked *ACTN1* variants

6.2.1. Probing the impact of F37C and R46W variants on ACTN1-ABD structure

More than 50% of the identified *ACTN1* variants are linked to MTC and are distributed across the actin-binding, central rod, and EF-hand domains. We examined the impact of *ACTN1* MTC-associated variants F37C and R46W on the actin-binding domain structure. F37 resides at the CH1/CH2 interface adjacent to W128, which forms stacking interactions with K236, thereby

regulating the transition between the open and closed conformations of the ABD (Figure 5A, top panel) (E. Borrego-Diaz et al., 2006). Introduction of F37C variant could potentially disrupt this stacking interaction, impairing the conformational dynamics of the ABD (Figure 5A, bottom panel). Similarly, the impact of R46W was investigated through molecular-based analyses. R46 is situated in a major helix of the CH1 domain, which is crucial for its structural stability (Figure 5B, top panel). Substituting R46 with the bulky aromatic side chain W46, could disrupt intra-helical interactions and destabilise the helical arrangement within the CH1 domain (Figure 5B, bottom panel).

6.2.2. Examining the impact of R320Q and R738W variants on ACTN1 central rod domain structure

Several *ACTN1* variants were identified within the central rod region, prompting a focused assessment of their potential impact on ACTN1 structure. Key variants R320Q and R738W were selected for investigation. R320 resides within the SR1 domain, where it forms a salt bridge with E376, crucial for stabilising the SR1 domain (Figure 5C, top panel). Substituting R320 with a glutamine may disrupt this interaction, potentially compromising the stability of SR1 (Figure 5C, bottom panel). In addition, R738 is positioned in a loop region connecting the SR4 and EF12 domains, known for stabilising the inter-domain interface (Figure 5D, top panel). Introducing a bulky side chain such as W738 in this loop region could potentially destabilise this region (Figure 5D, bottom panel).

6.2.3. Investigating the impact of R752P and G746S variants on ACTN1-EF12 domain structure

Our analysis also revealed *ACTN1* variants residing in the EF-hand domains. Two of these MTC-linked variants, R752P and G764S, were subjected to molecular modelling-based analysis to assess their impact on EF-hand domain stability. R752 is located in the helix of EF1 and forms a hydrogen bond interaction with N749, potentially stabilising the region (Figure 5E, top panel). The introduction of P752 at this position would disrupt the interaction and may destabilise the EF12 module (Figure 5E, bottom panel). G764 is located in the loop region between the two helices of the EF1 domain (Figure 5F, top panel). The transition from a glycine to a serine side chain may reduce the conformational flexibility of the loop connecting the EF1 helices, potentially compromising domain stability (Figure 5F, bottom panel).

6.3. Structural modelling of HCM-linked *ACTN2* variants

6.3.1. Evaluating the impact of S147L variant on ACTN2-ABD/actin complex

We employed similar approaches to assess the impact of *ACTN2* HCM-associated variants on ACTN2 structure. These variants are distributed across multiple domains, with the majority located in the actin-binding and central rod domains. The S147L variant was particularly intriguing to investigate, as previous NMR studies identified this residue as part of an actin-binding site (Levine et al., 1992). To evaluate its impact on actin binding, an ACTN2-ABD/actin complex was generated using HADDOCK (de Vries et al., 2010). Given the high conservation of the actin-binding domains between ACTN2 and ACTN3, restraints from a cryo-EM-derived ACTN3/actin complex (PDB ID: 3LUE) (Galkin et al., 2010) was used to model the ACTN2-ABD/actin interaction. Modelling revealed that S147 is slightly distant from the ACTN2-ABD/actin interface, suggesting it might indirectly disrupt actin binding (Figure 6A, top panel). S147 is also positioned within a loop structure connecting the CH1 and CH2 domains, potentially stabilising this region. Introducing the nonpolar L147 residue into a polar

environment is likely to incur energetic penalties, destabilise the region, and possibly compromising ACTN2 ligand binding to actin (Figure 6A, bottom panel).

6.3.2. Assessing the impact of R353W, R398H, and E628G variants on ACTN2 central rod domain structure

A significant portion of HCM-linked *ACTN2* variants were mapped to the central rod domain. Molecular-based investigations were conducted for the variant R353W, which is found in ACTN2-SR1. Analysis of the wild-type ACTN2 structure demonstrated that R353 forms a hydrogen bond with Q349, contributing to the stability of the SR1 domain (Figure 6B, top panel). In contrast, introduction of a bulkier W353 side chain is predicted to disrupt this interaction, as well as clash with the surrounding side chains, exacerbating the destabilisation of SR1 (Figure 6B, bottom panel). Other HCM-associated variants such as R398H and E628G, reside between spectrin repeats. For example, R398 is located between SR1 and SR2, mediating a salt bridge with E467 from SR2, thereby stabilising the SR1/SR2 interface (Figure 6C, top panel). The introduction of the H398 variant results in the loss of this salt-bridge interaction and is predicted to destabilise the SR1/SR2 interface (Figure 6C, bottom panel)). Similarly, E628, localised between SR3 and SR4, forms a salt-bridge contact with R631, thereby stabilising the SR3/SR4 interface (Figure 6D, top panel). The G628 variant is predicted to disrupt this interaction, potentially compromising the stability of the SR3/SR4 interface (Figure 6D, bottom panel).

6.3.3. Probing the impact of R759T and R796C variants on ACTN2-EF12 domain structure

Two additional *ACTN2* HCM-linked variants R759T and R796C were identified in the EF12 domain. R759 extends from the EF1 hand and forms a hydrogen bond with N763, thereby the

stabilising the EF1 motif (Figure 6E, top panel). The introduction of the R759T variant is predicted to disrupt this interaction, and potentially adversely impact the stability of the ACTN2-EF12 domain (Figure 6E, bottom panel). Furthermore, R796 plays a crucial role in stabilising the ACTN2-EF2 hand by establishing a salt bridge with E793 (Figure 6F, top panel). The introduction of C796 at this position is predicted to disrupt this contact which may destabilise the ACTN2-EF12 module (Figure 6F, bottom panel).

In conclusion, our molecular modelling predicts that *ACTN1* and *ACTN2* disease-associated variants compromise the structural integrity of the protein through various mechanisms, including impacts on actin ligand binding, disruption of the regulatory mechanisms of the ABD, and alterations in the stability of core structural domains.

7. Concluding remarks

7.1. Insights of structural modelling into alpha-actinin pathogenic variants

Alpha-actinin family members are crucial regulatory proteins essential for maintaining the structural integrity of the actin cytoskeleton. Genetic variants of alpha-actinin isoforms have been associated with a diverse array of cardiovascular and non-cardiovascular diseases. Elucidating the mechanisms by which pathogenic variants of alpha-actinin contribute to disease is paramount for the development of innovative therapeutic strategies targeting such disorders. Advances in next-generation sequencing technology have significantly expedited the identification of genetic variants within alpha-actinin genes, facilitating the discovery of potential disease-causing variants. Computational stability predictors such as SIFT and PolyPhen-2 have contributed to identifying *bona fide* disease-linked structurally deleterious variants. However, despite their utility, these bioinformatic predictive tools encounter challenges

in accurately predicting the phenotypic outcomes of missense variants, underscoring the necessity for further refinement and the incorporation of complementary methodologies.

One such approach is structural modelling, which moves beyond the limitations of stability predictors by offering more detailed insights into the structural consequences of variants. As evidenced in this review, structural modelling programs, including Phyre2 and AlphaFold (Jumper et al., 2021; Kelley et al., 2015), have generated models of individual alpha-actinin modules containing disease-associated variants. These models have provided valuable insights into potential structural mechanisms underlying variant-induced dysfunction. In addition, structural modelling evaluates the impact of disease-linked variants on protein structure, ultimately categorising them based on their putative mechanistic involvement in disease pathogenesis. Nonetheless, these approaches also exhibit limitations, particularly in their ability to assess broader topological impacts and accurately model domain-domain orientations within multi-domain encompassing proteins (Kelley & Sternberg, 2009). It is equally important to understand how alpha-actin-associated pathogenic variants, which are predicted to preserve overall protein structure, contribute to disease pathogenesis. Such variants may modify the functional properties of alpha-actinin by influencing ligand binding, intracellular trafficking, or post-translational modifications.

7.2. Integrating biophysical characterisation techniques with structural modelling approaches

To address these challenges, there is an urgent need to pivot from purely modelling approaches toward experimentally characterising the structural and functional impact of disease-causing variants on alpha-actinins. Future research should prioritise elucidating the structures of full-length alpha-actinin isoforms encompassing pathogenic variants and alpha-actinin/ligand complexes using X-ray crystallography or cryo-EM approaches. These studies will be crucial for

understanding the molecular basis of variant-induced pathogenicity and illuminating ligand docking modes. Complementary biophysical techniques, including small-angle X-ray scattering (SAXS) and size-exclusion chromatography coupled with multi-angle light scattering, can provide insights into changes in the shape, size, and oligomeric state of mutant proteins. In contrast, thermal denaturation assays can reveal alterations in the structural stability of these mutant proteins. Additionally, actin-sedimentation assays can evaluate the binding affinity of mutant alpha-actinin to actin filaments, offering insights into altered binding dynamics. Notable progress has been made in characterising ACTN2 and its variants through these approaches. For example, SAXS studies have investigated the full-length wild-type ACTN2 in both open and closed conformations (Ribeiro Ede et al., 2014). Thermal denaturation studies have demonstrated that certain variants can reduce stability (Atang et al., 2023; Haywood et al., 2016), corroborating our current structural modelling predictions. Finally, co-sedimentation assays revealed that ACTN2-ABD variants can variably increase or decrease F-actin binding affinity, suggesting that altered actin-binding dynamics may contribute to cardiomyopathy (Atang et al., 2023). However, these investigations have predominantly focused on the actin-binding domain, leaving significant gaps in our understanding of how pathogenic variants affect the full-length alpha-actinin protein. Moreover, only a limited number of pathogenic variants have been characterized, underscoring the need for further research as new variants continue to emerge. Consequently, the ongoing application of biophysical characterization techniques in future studies is paramount to bridge these gaps and pave the way for new research directions, but this must be combined with functional studies at the whole-cell tissue or animal level.

7.3. Evaluating alpha-actinin pathogenic variants through functional studies

The functional characterisation of missense variants in alpha-actinin is crucial for understanding their physiological impact. Both *in vivo* and *in vitro* cell models have played a vital for elucidating the effects of these genetic variants. Various cell systems, including traditionally transfected or virally transduced cell lines and primary cells, have been employed to investigate the altered behaviour of proteins. More recently, genome-edited induced pluripotent stem cell-derived cells, such as cardiomyocytes, have provided a more relevant human cellular context for studying disease mechanisms. These advanced models enable the assessment of how specific *ACTN2* variants influence cardiac functions, such as contraction, electrophysiology, and responses to stress. Additionally, mouse models facilitate the exploration of the whole-organism consequences of alpha-actinin variants (Broadway-Stringer et al., 2023; Cybulsky & Kennedy, 2011), thereby complementing cellular approaches. Integrating structural and functional approaches will significantly advance our understanding of how genetic variants influence alpha-actinin structure and function. This unified knowledge could inform personalised medicine approaches to future therapeutic interventions. As an example, functional work on *ACTN2* T247M variant correctly predicted the beneficial impact of diltiazem, an L-type Ca^{2+} channel blocker, for the HCM patient with this variant (Prondzynski et al., 2019). Moreover, recent advancements in gene editing therapies (Henderson et al., 2024) could potentially be applied to alpha-actinin variants in the future.

Collaboratively, structural modelling, biophysical characterisation and functional studies in cellular models will synergistically lead to better understanding of disease mechanisms, which is a prerequisite for more effective treatments for diseases caused by alpha-actinin variants.

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9. Supplementary information

Supplementary data include figures of the sequence alignment of alpha-actinin isoforms. Supplementary tables summarise the identified variants of alpha-actinin-1 and 2.

Figure legends:

Figure 1: Alpha-actinin domain architecture and structure. (A) Schematic representation of the alpha-actinin anti-parallel dimer bound to actin. (B) Schematic representation of the alpha-actinin domain architecture, illustrating the N-terminal actin-binding domain (ABD) comprised of two calponin homology (CH) domains interconnected by a flexible loop linker. The structure includes a flexible neck region, a central rod module encompassing four spectrin repeats (SR1-SR4) separated by inter-spectrin repeat (Inter SR) regions, and a C-terminal calmodulin (CaM) homology domain characterised by two EF-hand motifs (EF12 and EF34). (C) Crystal structure of human ACTN2 (Ribeiro Ede et al., 2014), highlighting the relevant domains. The CH2 domain is depicted with four alpha-helices designated as A, C, E, and G (left panel). The SR2 module consists of three alpha helices labelled A, B, and C (middle panel). Each hand within the EF34 motif form a helix-loop-helix configuration (right panel). All structural representations were generated using PyMOL (Schrödinger, 2020).

Figure 2: Ligand binding and regulatory mechanism of alpha-actinin-ABD. (A) Ribbon diagrams of ACTN1-ABD (E. Borrego-Diaz et al., 2006) and ACTN3-ABD (Franzot et al., 2005), emphasising the phosphatidylinositol 4,5-bisphosphate (PIP2) binding regions. The PIP2 binding site for ACTN1 is delineated by N168-H184 (blue) (left panel), while the binding site for ACTN3 encompasses T172-K188 (green) and a triad of arginine residues (R170, R176, and R199) (right panel). (B) Key residues, including tryptophan and arginine/lysine that stabilise the CH1-CH2 interface and modulate the open-closed conformation are illustrated for ACTN1-ABD (Borrego-Diaz et al., 2006) (left panel), ACTN2-ABD (Ribeiro Ede et al., 2014) (middle panel) and ACTN4-ABD (Feng et al., 2020)

(right panel). Close-up views of stacking interactions are provided in boxes. (C) Ribbon diagram of ACTN3-ABD, highlighting the actin binding sites: ABS-1 (R48-S57, red), ABS-2 (V122-F148, cyan), and ABS-3 (I153-T172, orange).

Figure 3: Mapping of *ACTN1* missense variants associated with bleeding disorders. (A). Schematic representation of ACTN1, illustrating the distribution of *ACTN1* pathogenic missense variants across its structural domains. (B) Mapping of *ACTN1* pathogenic variants onto the Phyre-derived human ACTN1 structure. The association of these variants with various bleeding disorders is colour-coded, with the values in parentheses indicating the number of variants identified for each disorder. Abbreviations for disease conditions: MTC, macrothrombocytopenia; ITP, immune thrombocytopenia; IPD, inherited platelet disorder.

Figure 4: Mapping of *ACTN2* missense variants linked with skeletal and cardiac myopathies. (A). Schematic representation of ACTN2, demonstrating the distribution of *ACTN2* pathogenic missense variants across its structural domains. (B) Mapping of *ACTN2* pathogenic variants onto the crystal structure of human ACTN2 (Ribeiro Ede et al., 2014). The association of these variants with different types of myopathies is colour-coded, with the values in parentheses corresponding to the number of variants identified for each disease. Abbreviations for disease conditions: HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; LVNC, left ventricular noncompaction; DM, distal myopathy; CM, cardiomyopathy (non-specified); RCM, restrictive cardiomyopathy; ACM, arrhythmogenic cardiomyopathy; PF-ACM, primary fibrotic atrial cardiomyopathy; MsCD, multiple structured core disease.

Figure 5: Examining the impact of MTC-related missense variants on ACTN1 structural domains. (A) Ribbon diagram of ACTN1-ABD, illustrating that F37 is located at the CH1-CH2 interface, adjacent to W128, which forms a stacking interaction with K236, crucial for regulating the open-closed mechanisms of the CH1-CH2 interface (top panel). Introduction of the C37 variant is predicted to disrupt this stacking interaction, potentially impairing this regulatory mechanism (bottom panel). (B) Ribbon diagram of ACTN1-ABD, showcasing that R46 stabilises a major helix of the CH1 domain (top panel). Introduction of the bulky W46 variant is anticipated to disrupt this helical arrangement (bottom panel). (C) Ribbon diagram of ACTN1-SR1, indicating that R320 forms a salt bridge with E376 (green dashed line) (top panel). Introduction of the Q320 variant is predicted to disrupt this interaction (bottom panel). (D) Ribbon diagram of ACTN1-SR4-EF12, where R738 is localised to the loop between the SR4 and EF12 domains (top panel). Introduction of the bulky W738 variant is predicted to disrupt the loop conformation between these two domains (bottom panel). (E) Ribbon diagram of ACTN1-EF12, illustrating that R752 forms a hydrogen bond with N749 (black dashed line) (top panel). Introduction of the P752 variant is predicted to disrupt this interaction (bottom panel). (F) Ribbon diagram of ACTN1-EF12, where G764 resides in the loop region between the two helices of EF1 (top panel). Introduction of the S764 variant is predicted to disrupt the loop conformation (bottom panel). Close-up views of the relevant interactions are provided in boxes.

Figure 6: Assessing the impact of HCM-related *ACTN2* variants on ACTN2 structural domains. (A) Ribbon diagram of the Haddock-derived molecular model of the ACTN2-ABD/actin complex, with S147 mapped near the actin-binding interface (top panel). Introduction of the L147 variant is likely to incur energetic penalties, adversely affecting actin binding (bottom panel). (B) Ribbon diagram of ACTN2-SR1, where R353 forms a hydrogen bond with Q349 (black dashed line) (top panel). Introduction of the bulky W353 variant is predicted to disrupt this interaction (bottom panel). (C) Ribbon diagram of ACTN2-SR1-SR2, illustrating that R398 forms a salt bridge with E467 (green dashed line) (top panel). Introduction of the H398 variant is expected to eliminate this contact (bottom panel). (D) Ribbon diagram of ACTN2-SR3-SR4, indicating that E628 mediates a salt bridge with R631 (green dashed line) (top panel). Introduction of the G628 variant is predicted to disrupt this interaction (bottom panel). (E) Ribbon diagram of ACTN2-EF12, where R759 forms a hydrogen bond with N763 (black dashed line) (top panel). Introduction of the T759 variant is predicted to disrupt this interaction (bottom panel). (F) Ribbon diagram of ACTN2-EF12, showing that R796 forms salt bridge with E793 (green dashed line) (top panel). Introduction of the

C796 variant is predicted to eliminate this interaction (bottom panel). Close-up views of the relevant interactions are provided in bo

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