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ADVANCED REVIEW

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Walking with giants: The challenges of variant impact assessment in the giant sarcomeric protein titin

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Abstract

Titin, the so-called "third filament" of the sarcomere, represents a difficult challenge for the determination of damaging genetic variants. A single titin molecule extends across half the length of a sarcomere in striated muscle, fulfilling a variety of vital structural and signaling roles, and has been linked to an equally varied range of myopathies, resulting in a significant burden on individuals and healthcare systems alike. While the consequences of truncating variants of titin are well-documented, the ramifications of the missense variants prevalent in the general population are less so. We here present a compendium of titin missense variants—those that result in a single amino-acid substitution in coding regions—reported to be pathogenic and discuss these in light of the nature of titin and the variant position within the sarcomere and their domain, the structural, pathological, and biophysical characteristics that define them, and the methods used for characterization. Finally, we discuss the current knowledge and integration of the multiple fields that have contributed to our understanding of titin-related pathology and offer suggestions as to how these concurrent methodologies may aid the further development in our understanding of titin and hopefully extend to other, less well-studied giant proteins.

This article is categorized under:

Cardiovascular Diseases > Genetics/Genomics/Epigenetics
Congenital Diseases > Genetics/Genomics/Epigenetics
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KEYWORDS

disease, missense variant, protein, sarcomere, titin

1 | INTRODUCTION

The giant protein titin (alias connectin; Maruyama, 1976) is the largest single-chain protein in nature (Granzier & Labeit, 2004; Labeit et al., 1990, 1992; LeWinter & Granzier, 2010; Loescher et al., 2022). While the best-known components of the sarcomere, the basic contractile unit of striated muscle, are those that make up the thin and thick filaments

(Z. Wang et al., 2021), titin is a crucial element from one end of the sarcomere to the other, to the point of being described as the "third filament" (Herzog, 2018; Herzog et al., 2012; Lindstedt & Nishikawa, 2017). As a molecular ruler, titin determines the length of the sarcomere and those of its various sub-regions (Luis & Schnorrer, 2021; Tonino et al., 2017). As a molecular spring, titin's I-band region provides passive force to the sarcomere, preventing the overextension of the muscle (Anderson & Granzier, 2012; W. A. Linke et al., 1998, 1999). As a stress sensor, titin measures and communicates changes in the forces impacting the sarcomere to downstream signaling pathways and effecting transcriptional and regulatory changes (Sheetz, 2021; Solís & Russell, 2021). As a molecular scaffold, titin provides binding points at its diverse signaling hubs, playing a role in both local signaling cascades and the greater cytoskeletal architecture (Crocini & Gotthardt, 2021; Lieber & Binder-Markey, 2021; R. J. van der Pijl, Domenighetti, et al., 2021). As a dynamic cellular component, it modulates muscle properties on both long and short timescales, with a range of post-translational modifications that control transient muscle stiffness (Solís & Russell, 2021; Steinberg, 2013), and longer-term control of stiffness through changing isoform ratios in the make-up of the muscles (Crocini & Gotthardt, 2021; Loescher et al., 2022).

With such an integral role in cardiac and skeletal muscles, it is unsurprising that damage to the titin protein in the form of mutations is associated with the development of muscle disease (Chauveau, Rowell, & Ferreiro, 2014; Eldemire et al., 2021; LeWinter & Granzier, 2013). A panoply of both skeletal and cardiac myopathies have been linked to variants in titin, and the burden that these conditions place on both individuals and health systems worldwide has led to considerable interest in understanding the pathomechanisms that connect the mutation to the phenotype. However, while more progress has been made in identifying highly damaging truncating and frameshift variants (Chauveau, Rowell, & Ferreiro, 2014; Fomin et al., 2021; Herman et al., 2012), there have been far fewer missense variants identified and characterized, despite being more common. We present here an overview of the current knowledge regarding missense variants in titin and their associated pathologies, and how they reflect their localization in titin domains, in sarcomeric regions, and in specific tissues. We will also discuss the different approaches that have been used to identify pathogenic missense variants—variants that cause or increase the risk of developing a particular disease—and the implications that the current body of evidence has for clinical diagnosis.

2 | PROPERTIES OF TITIN

Titin has been described as the molecular backbone that forms the foundation of the sarcomere, the contractile unit of striated muscle tissue (Bailey et al., 2002) (Figure 1). Each molecule extends over 1 micrometer in length, across half of the sarcomere, overlapping with antiparallel titin filaments at each end. Titin is present in all sub-regions of the

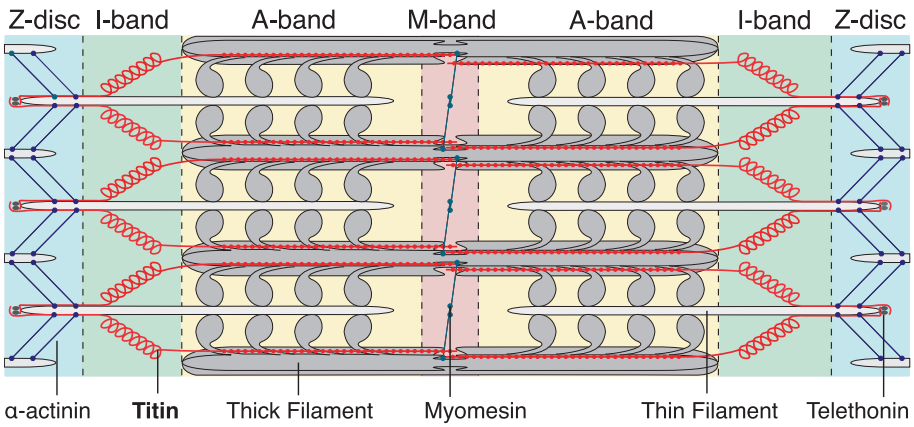

FIGURE 1 Schematic representing titin (red), its key structural binding partners, and the other major components of the sarcomere. The core of the sarcomere is composed of the myosin thick filaments and actin thin filaments, which together undertake the cross-bridge cycle that allows for contraction and relaxation of the sarcomere, and thus of the striated muscle. Two antiparallel titin molecules overlap at the center of the sarcomere, the M-band; they are bound to the thick filament in the A-band, and to the thin filament at the Z-disc, through their interactions with alpha-actinin 2. The titin molecules extend through the Z-disc into the adjacent sarcomere, where they are anchored by telethonin. The I-band region of titin is elastic and provides passive force to the sarcomere, preventing the cross-bridge cycle from overextending the muscle; different isoforms of titin have different levels of elasticity.

sarcomere: the Z-disc, where thin filaments are cross-linked by alpha-actinins; the I-band, where the thin filaments progress into the sarcomere; the A-band, where the motor domains of the bundled myosin molecules in the thick filament are found; and the M-band, where thick filaments are cross-linked at the center of the sarcomere. Titin, while extremely large, contains only three types of domains, these being I-set immunoglobulin (Ig) domains, fibronectin type-III (Fn3) domains, and the single protein kinase domain(Lange et al., 2020; Loescher et al., 2022). The many Ig and Fn3 domains of titin are not only extremely similar in terms of structure but often of sequence as well, with invariant residues consistently located at the same structural locations. The Ig domains are classified as the I-set type, which have many defining structural features and residues at key positions (J.-H. Wang, 2013).

The various isoforms of titin are all expressed from the 364-exon TTN gene, whose full protein product encompasses 35,991 amino acid residues, arranged into 169 Ig domains, 132 Fn3 domains, the kinase domain, and extensive unstructured repeat regions (see Figure 3). The inferred complete (IC) isoform (Laddach et al., 2017), a theoretical protein containing all exons in the titin gene, does not exist in nature, and as such all titin proteins in the body exclude some of the 364 exons present. The three main isoforms of titin in adults are the N2BA, N2A, and N2B giant isoforms; at the protein level, the difference between these giant isoforms is found in the I-band region, where the TTN gene encodes three regions of tandem Ig domains separated by varying lengths of unstructured repeats and signaling hub regions, which are differentially spliced to make the different isoforms (see Figure 2). Ig25 and Ig26 are spliced out from the giant isoforms N2BA, N2A, and N2B; these are expressed in the novel exon isoforms novex-1 and novex-2, respectively. Finally, the "tiny titin", novex-3, includes only 46 exons (Kellermayer et al., 2017); however, one of these, exon 48, is not found in any of the giant isoforms. While these are the most well-established isoforms of titin, many others are present and continue to be discovered in different physiological contexts; two examples include foetal cardiac titin (FCT) (Lahmers et al., 2004), which is found in neonatal and foetal heart muscle, and the recently discovered *Cronos* titin isoform (J. Zou et al., 2015), which is up-regulated in developing cardiomyocytes.

2.1 | Classification of skeletal myopathies

The concept of the "titinopathy," a disease of the muscle linked with damaging variants in titin, is one that has become increasingly relevant to the diagnosis of myopathy as more and more damaging variants are identified. Titin variants

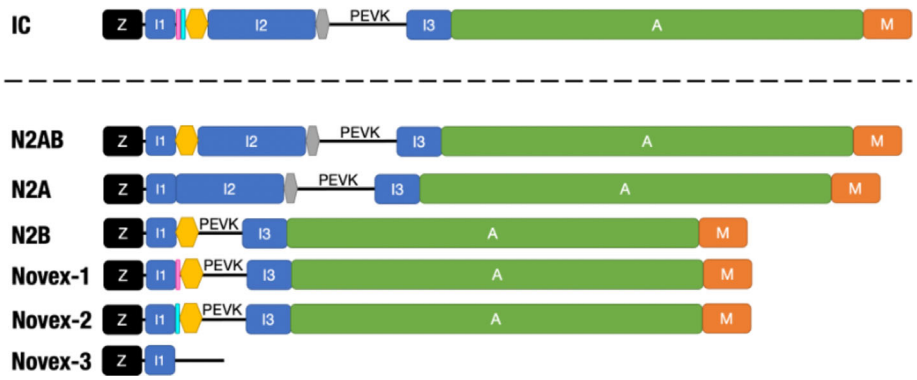

FIGURE 2 Major isoforms of titin in adults. The inferred complete isoform, titin-IC, contains all exons in the titin gene. The key differences in exon composition for the giant isoforms N2AB (canonical isoform), N2A (predominant in skeletal muscle), N2B (predominant in cardiac muscle), novex-1 and novex-2 are found in the I-band (blue). All giant isoforms contain a region of tandem Ig domains, from domains Ig10 to Ig24; Ig25 (magenta) and Ig26 (cyan) are spliced out from the isoforms N2BA, N2A, and N2B, and are only expressed in the novel exon isoforms novex-1 and novex-2, respectively. The proximal Ig domains are followed by the "N2B region" (yellow): domains Ig27 and Ig28, connected by a long linker of almost 30 residues, precede a unique, unstructured sequence of around 600 residues in length, followed by Ig29 and Ig30. This N2B region is absent in the predominant skeletal isoform, N2A; instead, what follows Ig30 is a second Ig tandem region, from domains Ig31 to Ig82, found in the N2BA and N2A isoforms only. Also absent from the N2B isoform is the subsequent region, from Ig83 to Ig86, known as the "N2A region" (gray), which also includes a long, 112-residue insertion sequence between Ig83 and Ig84. The PEVK sequence is an elastic, repeating, unstructured region named for the abundance of proline, glutamate, valine, and lysine residues in its constitution. In the N2BA isoform, the PEVK sequence contains almost 2000 amino acids, spanning 31 repeats; by contrast, in the N2B isoform, the PEVK sequence is considerably shorter, with only 186 residues. Finally, in all giant isoforms, a third and final region of closely linked Ig domains follows the PEVK region, from Ig87 to Ig107. After this distal Ig tandem region, the titin molecule moves into the A-band (green).

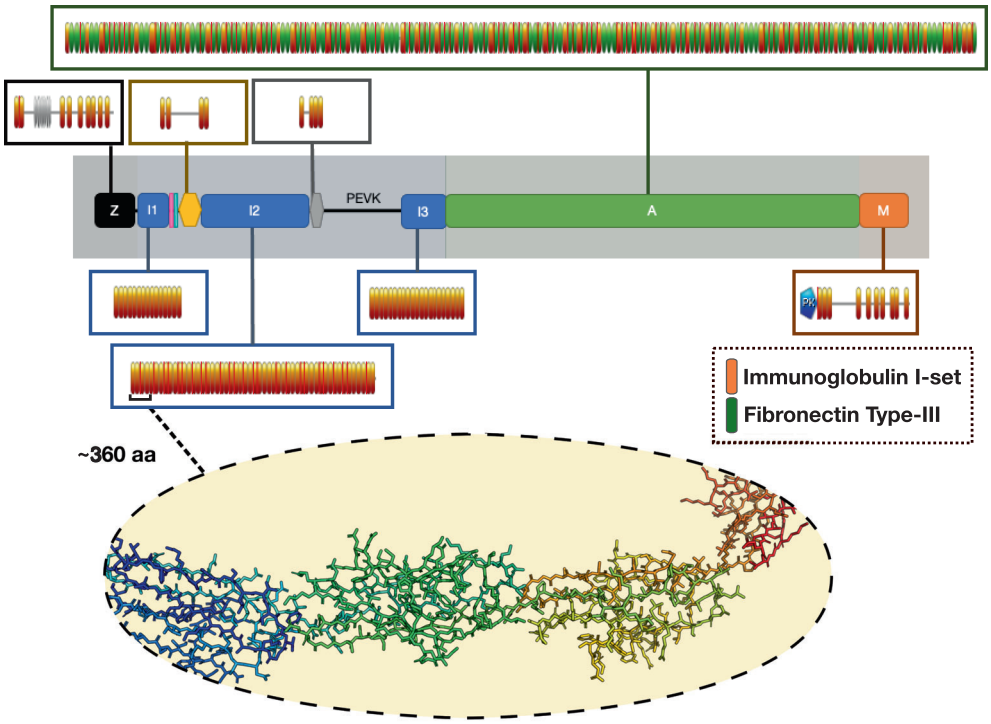

FIGURE 3 Domain- and molecular-level view of the inferred complete isoform of titin. Arrangement of folded globular domains in the main organizational regions of titin is represented schematically (orange: Immunoglobulin I-set (Ig); green: Fibronectin Type-III (Fn3); Titin Kinase represented by blue pentagon). The Z-repeat sequences in the Z-disc region, which only form single alpha-helices, have been denoted in gray. Titin regional schematic (center) is arranged according to the color scheme described for Figure 2. Inset, partial molecular-level view of domains Ig31-Ig34 visualized in PyMol (Schrödinger, 2015). Construction of schematic representation of domains performed using PROSITE (Hulo et al., 2008).

have been associated with a broad range of neuromuscular pathologies with distinct phenotypic characteristics (Savarese et al., 2020), consistent with the size and number of possible disruptions that may be linked back to the titin molecule. More recently, it has been suggested that titinopathies, rather than being a collection of distinct, albeit partially overlapping disorders, in fact represent a continuum of phenotypes associated with a single condition. This interpretation has yet to be implemented in any clinical setting, but several authors have provided evidence in support of this framework (Huang et al., 2021; Oates et al., 2018; Rees et al., 2021).

The skeletal muscle diseases linked to titin can broadly be split into muscular dystrophies and congenital myopathies. The former refers to the gradual, progressive degeneration of muscle fibers, leading to worsening health, while the latter refers to those conditions that are associated with early-onset, characteristic damage to the muscle (Tubridy et al., 2001), although sometimes the distinction is blurred by slowly progressing myopathies (Shieh, 2013). The most well-documented associations with missense variants in titin are those with muscular dystrophies. Tibial muscular dystrophy (TMD) (Udd & Hackman, 2005) has been associated with multiple variants in the M-band, particularly the Ig169 domain (Hackman et al., 2002); homozygous and compound heterozygous variants in these exons are associated with additional, more severe phenotypes, including limb-girdle muscular dystrophy type 2J (LGMD2J) (Hackman et al., 2008). Another well-established association with titin is that of hereditary myopathy with early respiratory failure (HMERF) (Pfeffer & Chinnery, 1993), which has been shown to result from missense variants in the Fn3-119 domain (Palmio et al., 2019). Several titin variants associated with HMERF were originally identified in patients diagnosed with myofibrillar myopathy (MFM), a muscular dystrophy with considerable phenotypic overlap with HMERF (Pfeffer et al., 2014; Uruha et al., 2015).

Congenital myopathy phenotypes associated with titin variants include but are not limited to, centronuclear myopathy (CNM) (Ceyhan-Birsoy et al., 2013), congenital fiber-type disproportion (CFTD) (Rees et al., 2021), multi-minicore disease (MmD) (Chauveau, Bonnemann, et al., 2014), central core disease (CCD) (Rees et al., 2021), type 1 predominance (T1P) (Rees et al., 2021), and childhood-juvenile onset Emery-Dreifuss–like phenotype without cardiomyopathy (Cid et al., 2015). Typically, these conditions present with significant clinical overlap; diagnosis of individual conditions

is mainly achieved by the identification of characteristic sub-cellular structures in the affected muscle fibers. For example, MmD and CCD are collectively known as "core myopathies" for the presence of cores, or distinctive circular structures, within the sarcomere (Chauveau, Bonnemann, et al., 2014). There can be a considerable heterogeneity in severity and pathological consequences of the conditions even under the same diagnosis; other proteins that are linked with these same conditions may also modulate the clinical presentation.

2.2 | Classification of cardiac myopathies

Cardiomyopathies, or diseases primarily affecting the myocardium, are typically considered as secondary to skeletal myopathies in the context of titin missense variants. While a considerable body of work has demonstrated that truncating titin variants play a role in conditions such as dilated cardiomyopathy (DCM) (Jordan et al., 2021; Mazzarotto et al., 2020; Owens & Day, 2021), there are comparatively few associations between missense variants and cardiomyopathies that have been widespread or definitive enough to attract the same attention as the congenital titinopathies. Even so, a great many missense variants have been put forward as potentially causative of these conditions, and they continue to be actively studied.

The classification of cardiomyopathies remains a subject of considerable debate (Elliott et al., 2008; Maron et al., 2006; Richards et al., 2015). At the simplest level, genetically inherited cardiomyopathies are partitioned into DCM, hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), and arrhythmogenic cardiomyopathy (ACM). Each of these is so named for the observed consequences to the heart structure: HCM, the most common cardiomyopathy, causes a thickening of the heart muscle walls, and consequently a constriction of the heart chambers, notably the ventricles. DCM, the second-most common, causes an enlargement of the heart, where the heart chambers expand as the muscle walls stretch. RCM does not involve stretching or compressing of the heart muscle, but rather a loss of elasticity and a rigidity of the heart muscle walls, restricting the cardiac cycle. ACM (Reuter et al., 2021) does not typically involve a dysfunction of the cardiac blood pump, but rather is characterized by arrhythmia; it is typically diagnosed as arrhythmogenic right ventricular cardiomyopathy (ARVC), although cases of ACM involving the left ventricle have also been reported (Corrado et al., 1997; Sen-Chowdhry et al., 2008).

While the above classification covers most cardiomyopathy-related diagnoses, more recent observations have identified cardiomyopathies which are considered separate pathologies. Most relevant to titin is left ventricular noncompaction (LVNC) (Towbin et al., 2015), a developmental disorder affecting the heart muscle associated with the left ventricle, leading to a dysfunctional, characteristically "spongy" myocardium. Furthermore, within the different sub-classifications of cardiomyopathy there is a considerable heterogeneity in consequences, from fatal pathology to asymptomatic, and is likely to be heavily influenced by non-genetic factors. Attempts to refine the classification of cardiomyopathies have been conducted in the past, including the MOGE(S) classification system (Arbustini et al., 2013; Westphal et al., 2017), where individual cardiomyopathies are given a multi-character code based on their morpho-functional properties, organ system involvement, genetic history, genetic etiology, and optionally the stage of progression. However, to our knowledge this system has seen only limited uptake in a clinical setting, and will not be used when describing cardiomyopathies here.

2.3 | Truncating variants and myopathy

While the scope of this work does not include the contributions of truncating, frameshift, splice-site, and nonsense variants to disease, it is worth establishing their significance in genetic contributions to muscle disease, as a lens through which to view those of missense variants. Truncating titin variants, commonly abbreviated as TTNtvs, are thought to be the most important single genetic contributor to DCM (Akinrinade et al., 2019; Fomin et al., 2021; Herman et al., 2012; Huynh, 2022; McAfee et al., 2021), as well as similar conditions such as peripartum cardiomyopathy (PPCM) (Iorgoveanu et al., 2021; Spracklen et al., 2021). They have also been implicated in congenital skeletal myopathies both with (Chauveau, Bonnemann, et al., 2014; Rees et al., 2021; Rich et al., 2020; Roberts et al., 2015) and without cardiac involvement (Ceyhan-Birsoy et al., 2013; Hackman et al., 2008; Rees et al., 2021). While the strength of evidence in support of this association is considerable in reported work (Jordan et al., 2021; Mazzarotto et al., 2020; Owens & Day, 2021), the incidence of TTNtvs in the general population is around 2%–3%, higher than the incidence of DCM (Roberts et al., 2015); thus, the presence of a TTNtv alone does not necessarily imply the development of DCM.

That a TTNtv, which represents a significant alteration to a vital sarcomeric component, can be found in healthy individuals with no apparent ill effects, has important implications for the mechanisms of pathogenicity not only of TTNtvs but of missense variants as well. A TTNtv located in only one allele of the titin gene may be insufficient alone to produce pathogenic consequences; the presence of healthy isoforms expressed from the wild-type allele may rescue the functional integrity of the muscle, even if malformed titin is also integrated into the sarcomere. Pathology may thus result from haploinsufficiency rather than complete loss of function, where the loss of correct-length titin protein results in the destabilization of the sarcomere. However, it is difficult to determine in such cases whether the TTNtv in question has a dominant effect, or if pathology is only produced in tandem with environmental or other genetic factors such as co-inheritance *in trans* with a missense variant, which has been observed in a cohort of congenital myopathy patients (Rees et al., 2021).

Two notable papers (Fomin et al., 2021; McAfee et al., 2021) that have recently detected and quantified the presence of TTNtvs in the hearts of DCM patients offer an insight into the pathomechanisms that may result from TTN variations, both truncating and missense. The first used quantitative methods to compare the abundance of full-length and truncated titin in DCM explant hearts, demonstrating both the decrease in full-length titin, indicative of haploinsufficiency, as well as the active presence of truncated titin, indicating the integration of both forms of titin in the muscles (McAfee et al., 2021). The second study also found the discrepancy between levels of titin protein in DCM hearts with TTNtvs, but also demonstrated the aggregation of unfolded, truncated titins within the cells, rather than incorporation into the sarcomere, which the authors suggest could result in a proteotoxic response (Fomin et al., 2021). Thus haploinsufficiency, proteotoxicity, and integration of malformed titins together form a tricast of potential mechanisms that all may influence the consequences, not only of TTNtvs, but also of missense variants. Indeed, recent work has implicated a proteotoxic response as a consequence of a missense variant in Z-disc titin, albeit as a result of unbound cytoplasmic telethonin (Jiang et al., 2021).

3 | PRIORITIZATION OF VARIANTS

For a variant to be associated with a given pathology, it must first be identified in an individual with that condition. With the advent of next-generation sequencing (NGS) technologies, it is now possible to perform large-scale genomic sequencing on sizable cohorts of patients, rather than single genes or single individuals, greatly increasing the capacity for disease-associated variants to be identified. However, while many such variants are identified from genetic testing of individuals, only a small proportion of these are then proposed as such in the scientific literature, and fewer still are considered as causative of disease with any degree of certainty.

The issue of validation, or how those variants identified within patients can be confirmed to play a causative role in the development of disease, is not one that is unique to titin. However, the size of titin as a protein, and the corresponding number of possible variants that can exist exacerbates this issue considerably. Even so, there exist many techniques that can be used to gather evidence in support of the pathogenicity of a variant, or to investigate the functional consequences of its inclusion in titin.

3.1 | Determining pathogenicity of variants

The ascertainment of pathogenicity of protein variants is never simple, and much guidance has been produced to aid researchers in their classification (Richards et al., 2015), but the problems that plague the annotation of variants in smaller proteins is magnified greatly when considering those in the giant titin. For example, rare variants—typically defined as those with a population minor allele frequency (MAF) below 1%—are considered first when attempting to identify likely candidate genes to link to a given condition. However, titin is significantly larger than any other single protein in the human proteome; consequently, the likelihood that any given individual will carry a rare variant in titin is all but certain (Gigli et al., 2016), many such variants will be present in healthy controls (Roberts et al., 2015), and the majority of these variants will be missense variants of unknown significance (Campuzano et al., 2015). Thus, any attempt to associate titin with a muscle disease will always produce many potential candidate variants—both common and rare—which must be filtered based on previous knowledge of likely disease-causing variants.

The danger of considering variants as potentially pathogenic based solely on their rarity has been discussed by many authors (Laddach et al., 2017). One notable paper (Manrai et al., 2016) identified several missense variants in

sarcomeric proteins associated with hypertrophic cardiomyopathy which, while rare when considering the genetics of the whole population, were far more common in certain ethnic sub-populations, greatly decreasing the likelihood that they were pathogenic. In titin specifically, a study of truncating variants in DCM patients (Haggerty et al., 2019) found no enrichment of truncated titin protein in the hearts of patients with African ancestry, unlike the patients with European ancestry who showed a strong association. Moreover, several of the missense variants in titin reported as pathogenic in the literature are present in unaffected individuals in the general population; notably, the ARVC-associated variant H10092Y (Taylor et al., 2011) is present in about 1 in 250 individuals in the gnomAD databases 2.1.1 and 3.1.2 (Lek et al., 2016), increasing to around 1 in 100 individuals in the Finnish sub-populations (Table 1) This does not necessarily preclude their role in disease, but must be considered in any assessment of their pathogenicity.

Another issue related to the number of variants in titin is that the true causative variant may be overlooked in favor of one with a perceived greater likelihood of pathogenicity. For example, the rare missense variant R34091W, identified in the protein kinase domain of titin in 2 Swedish families (Nicolao et al., 1999), was proposed as causative of HMERF based on its reduction of binding affinity of the kinase domain for nbr1 (Lange et al., 2005). Subsequent studies identified a strong association between variants in a different part of titin with the same condition, and a re-evaluation of the original patients found that they too had variants in this region (Hedberg, Melberg, et al., 2014). Thus, R34091W is no longer considered causative for this disease in isolation.

The patterns of inheritance in diseases associated with titin variants are complicated by the multifaceted etiology of these diseases (Weintraub et al., 2017). In general, disease-associated titin variants can be grouped into three main patterns of inheritance: heterozygous, homozygous, and compound heterozygous. Heterozygous variants present with dominant inheritance and are causative of disease without any other genetic contribution. Homozygous variants present with recessive inheritance and require inheritance in both alleles to cause disease. Compound heterozygous variants are those that are causative for disease when inherited with another compound heterozygous variant in the other allele. Further complicating this picture is the variable penetrance of different variants and their interaction with environmental effects; some variants may not be disease-causing alone but may exacerbate the phenotype of a disease caused by another variant or external factor (Evilä et al., 2014).

Beyond the difficulties with the assessment of genetic data, the environmental component of the diseases in question must be emphasized. Myopathies of the cardiac and skeletal muscles, while they possess a heritable component, are strongly influenced by external and environmental factors, including obesity (Olivotto et al., 2013) and level of physical activity (Reineck et al., 2013). These factors can modulate the disease or the specific pathology alongside genetic factors, and the delineation of the relative contributions of each factor in disease prognosis is in its infancy. Thus, just as many of those with ostensibly damaging variants in titin appear in perfect health, many of those who suffer from the conditions associated with missense variants in titin and the other sarcomeric proteins will not have any clear genetic issues. The purpose of this reiteration is to remind the reader that, while the underlying causes of these conditions cannot be solved through genetic factors alone, understanding the relative risks imposed by different genetic burdens is vital to the protection of long-term health.

3.2 | Next-generation sequencing and linkage studies

By far the greatest quantity of known variants are those that have arisen from what will hereafter be referred to as in silico methodologies—that is to say, those that are proposed from studies of data only. While sequencing is a necessary step in identifying novel variants, NGS and other high-throughput strategies allow for the capture of the full genetic landscape of the individual or cohort. Notably, whole-genome or whole-exome sequencing (WGS/WES) will highlight many more variants than can be related to the pathology in question; for example, healthy individuals who participated in the UK Biobank study carried a median of 1695 rare autosomal variants (MAF <1%) across all proteins, of which 379 were rare missense variants (van Hout et al., 2020). This is particularly relevant for titin, as other variants in the genome notwithstanding, there will almost certainly be many variants in the gene itself that do not have any bearing on the individual's condition, given that many such variants are also present in healthy controls (Roberts et al., 2015).

Typically, a range of bioinformatic and computational tools and pipelines will be used to automatically prioritize those variants with the highest likelihood of playing a role in the disease state. These begin with the elimination of common population variants by comparison with known variant databases, such as gnomAD (Karczewski et al., 2020; Lek et al., 2016), ExAC (Karczewski et al., 2017), and 1000 Genomes (The 1000 Genomes Project Consortium, 2015). If a variant is found to exist in healthy, ethnically matched controls, or has a high population or sub-population incidence

TABLE 1 Allele frequencies of reported variants in this review in gnomAD-2.1.1, both from the global population and from reported sub-populations.

Variant	Global	AFR	FIN	NFE	SAS	EAS	AMR	ASJ	Other
V54M	4.25E-05					5.53E-04	2.82E-05		
A82D									
A178D									
E238Q									
Q466R	2.39E-05	3.69E-04							
R740L									
A743V									
W976R									
V1034M	8.16E-04		1.91E-03	1.12E-03	6.86E-04		2.54E-04		1.11E-03
T2014A	1.45E-04					2.05E-03			
T2896I									
S4116Y									
G4714D	8.23E-05			1.56E-05		1.08E-03			
S4780N	8.93E-05			7.82E-06	1.31E-04	1.03E-03			
L4854F	4.02E-06						2.90E-05		
C5054R									
Y9275C	6.43E-05			1.41E-04					
R9744H	1.78E-05	4.14E-05		3.12E-05					
R9848Q	1.21E-05				3.27E-05	1.11E-04			
A9980T	6.03E-04			7.82E-06	3.00E-04	8.06E-03			1.41E-04
H10092Y	4.04E-03	2.89E-04	8.83E-03	6.01E-03	2.71E-03		5.09E-04	1.16E-03	2.94E-03
S13702P									
A13715E									
R14640C	2.82E-05			6.23E-05					
N16133K									
N16429K									
W16471C	3.88E-04	4.14E-05	4.83E-04	6.51E-04			1.42E-04	9.74E-05	8.47E-04
Y16686C									
C17051R	4.03E-06				3.27E-05				
L18237P	6.43E-04	2.07E-04	1.20E-04	1.13E-03			6.22E-04		8.43E-04
A18983T	9.32E-05			1.81E-04			5.68E-05		1.41E-04
P19288R									
I19517T	4.03E-06			8.92E-06					
A19938T	8.07E-06			8.92E-06	3.27E-05				
N19955I									
A21147T	5.39E-04		4.03E-05	2.62E-04	3.30E-03		1.15E-04	2.94E-04	1.14E-03
A21877S	1.02E-04			5.41E-05	5.91E-04				1.67E-04
V22232E									
G24621R	8.07E-06				3.27E-05	5.61E-05			
R25480P									
G27849V									
R28118H	3.63E-05			4.46E-05		5.60E-05	8.71E-05		

TABLE 1 (Continued)

Variant	Global	AFR	FIN	NFE	SAS	EAS	AMR	ASJ	Other
R29293C	1.93E-03			1.64E-04	1.63E-04	2.60E-02			8.42E-04
S29303G									
E29590Q	4.02E-06			8.88E-06					
L30639P									
P30723S	6.18E-05		1.22E-04	1.03E-04					1.43E-04
K31268T	4.79E-04		1.92E-03	6.33E-04					7.02E-04
W31429R									
P31709H									
P31709R									
C31712R	4.11E-06			9.10E-06					
C31712Y									
C31712W									
W31729C									
W31729L									
W31729R									
P31732L	1.21E-05			1.78E-05	3.27E-05				
A31784V									
N31786K									
G31791D									
G31791R									
G31791V									
R31847P									
P33415L									
R33903H	2.42E-04	8.27E-05	7.64E-04	6.26E-05	3.27E-05	2.00E-03			
W34072R	8.11E-06			1.78E-05					
V35643I	6.07E-05			6.24E-05		4.61E-04			
M35859T	1.77E-03	8.26E-05		8.81E-04	8.82E-03	5.12E-05	2.54E-04	8.02E-03	2.52E-03
T35915P									
W35930R									
H35946P									
I35947N	4.01E-06			8.85E-06					
L35956P									

Note: A label of "." denotes variants not found in any individuals in the respective ethnic groups. All variant numbering in this review has been aligned to the inferred complete isoform (Ensembl Transcript ID: ENST00000589042.5; NCBI Reference Sequence: NM_001267550.2). Sub-population ethnicities are given as follows: AFR African; FIN Finnish; NFE European (excluding Finnish); SAS South Asian; EAS East Asian; AMR Latin American; ASJ Ashkenazi Jewish.

in population databases (in other words, common variants with MAF >1%), it is unlikely to be strongly associated with disease, and can be excluded from further analysis. Additionally, pathogenicity prediction tools can be used to remove from consideration those variants that do not display features indicative of known damaging variants. Various tools exist that allow for all these approaches to coalesce into a single, efficient pipeline, such as ANNOVAR (K. Wang et al., 2010), which was used by Begay et al. (2015) to filter titin variants in DCM patients to prioritize those most likely to be associated with the condition.

While sequencing alone can prioritize those variants most likely to play a role in pathogenesis, it cannot validate the causal role of any given variant in a pathology alone. The most common method to support the causative role of a variant in disease is to perform linkage or segregation analysis. Here, a family or body of related individuals both with

and without the condition are sequenced, and the pattern of inheritance between the variant of interest and the disease are compared. For segregation analysis, different models of inheritance patterns are fitted to the linkage data to ascertain the link between the condition and variant. Where the variant co-segregates with the disease—present in all affected family members and absent in all unaffected family members—this can be used as evidence of the pathogenicity of the variant. However, segregation analysis is not always possible or unreliable due to availability of family history or variable penetrance of the variant in question. In such cases, functional assessments of variant impact, whether in vivo or in vitro, are necessary to determine the effects of the variant and validate its role in disease.

3.3 | Experimental characterization

The experimental characterization of variants in titin, hereafter referred to collectively as "in vitro" methodologies, involves the attempt to elucidate the biophysical or cellular consequences of the variant at the local level, and to then translate these changes to the expected result at the global, organismal level. In general, this requires the establishment of a parameter, such as thermal or mechanical stability, that can be associated with both the wild-type and variant titin, and thus quantitatively compared between the two. Given the sheer size of the titin protein, expressing the entire protein in vitro is impossible; typically, only the domain in which the variant is found, or a tandem including the domains either side of it, are expressed. The wild-type and variant constructs can thus be compared independently of the remainder of the titin protein.

The first in vitro methodologies were chiefly concerned with the binding properties of titin to other proteins and its disruption by variants of interest; yeast 2-hybrid assays (Y2H) (Fields & Song, 1989) were used to assess the binding of titin to alpha-actinin 2 (ACTN2) (Itoh-Satoh et al., 2002; Satoh et al., 1999), telethonin (TCAP) (Itoh-Satoh et al., 2002), and four-and-a-half LIM domains protein 2 (FHL2) (Matsumoto et al., 2006). As Y2H has been criticized for a high incidence of false positives, the relevance of these changes for pathogenic potential, absent further validation, remains unclear. A broad range of other techniques to quantify and compare binding ability, which are considered more reliable indicators of protein–protein interactions, have been used in more recent experimental characterizations: co-immunoprecipitation (co-IP) assays (Arimura et al., 2009), isothermal titration calorimetry (ITC) (Rudloff et al., 2015), glutathione S-transferase (GST) pulldown assays (Hastings et al., 2016) and Förster resonance energy transfer (FRET) microscopy (Hastings et al., 2016).

For variants in domains with no known binding partners, measurements of the stability of purified recombinant protein have been used to characterize the damage done by the variant to the local structure. Native polyacrylamide gel electrophoresis (PAGE) can indicate unfolded protein as decreased electrophoretic mobility through the gel reflects a more partially unfolded domain; bulkier, less compact monomers move more slowly than fully folded ones. Intrinsic fluorescence analysis allows for the detection of naturally occurring fluorophores in a domain; the detection of greater fluorescence intensity in the mutant versus the wild-type spectrum indicates that intrinsic fluorophores, such as tryptophan, are more solvent-exposed, and thus protein fold is less compact. Nuclear magnetic resonance (NMR) approaches can detect the presence or absence of different fold states within a mixed sample. Studies of titin domain thermal destabilization have used methods such as differential scanning fluorimetry (DSF) (Rees et al., 2021) and circular dichroism (CD) (Rees et al., 2021; Rudloff et al., 2015) spectroscopy to uncover the melting temperatures at which the domains unfold, which can be compared between wild-type and mutant systems. Mechanical methods measure the force that must be exerted on a domain to cause unfolding; single-molecule atomic force microscopy (AFM) (Anderson et al., 2013; Zuo et al., 2021) has been used to probe the destabilization of domains by missense variants. While protein denaturation methods and protocols vary widely, experiments using chemical, thermal, and mechanical methods on titin domain Ig86 have demonstrated that the unfolding rates were consistent (Kelly & Gage, 2021). Thus, comparison across different methods is possible; however, re-folding rates differ slightly.

In addition to experimental characterization of variant domains alone, mutated titin constructs may be expressed in cultured cells, allowing for characterization in a context closer to that of the native environment. An example of this approach is found in a paper by Rees et al. (2021), where GFP-tagged multi-domain tandem constructs of both wild-type and missense variant domains were expressed in neonatal rat cardiomyocytes and skeletal myocytes. The authors compared the expression, localization, and solubility of wild-type and missense-containing constructs under confocal microscopy, noting that the variant systems showed mis-localization of the titin protein, as well as evidence of aggregation. These demonstrate how characterized features such as solubility and cellular localization can be used to indicate the functional impact of a variant in addition to changes to binding affinity and stability.

3.4 | Expression in model organisms

The use of model organisms allows for the pathogenicity of a variant to be tested within a living system—should the phenotype of the human carrier be mirrored in the model upon introduction of the variant, a causal relationship between variant and pathology can be established. However, due to the considerable investment of time and resources required, in vivo techniques still only account for a minority of studies on titin missense variants in the published literature. The main animal models developed thus far to investigate the properties of titin and its associated variants are mouse and fish (medaka, zebrafish) models (for a recent review, see Marcello et al., 2022). The majority of these models have large deletions in the coding region of titin, but exonic point mutations in zebrafish and medaka models have produced an MD and a HCM-like phenotype, respectively.

One of the difficulties in studying a living variant system is that, while laboratory mice are a useful system for studying gene variants, the phenotypes they exhibit are often markedly different from those of human patients with the same variant. This is exemplified by a recently published, long-term study by Jiang et al. on the disease mechanisms of the Z-disc variant A178D (Jiang et al., 2021). Despite previous work demonstrating the capacity of this variant to destabilize its Ig domain, resulting in aggregation and loss of binding to TCAP, no mechanism was offered as to how this could lead to the observed DCM/LVNC phenotype in humans (Hastings et al., 2016). To investigate this, a knock-in mutant for A178D in mice was developed using CRISPR-Cas9. While the mice homozygous for A178D had a mild DCM phenotype, those heterozygous for the variant showed no phenotypic difference to wild-type mice, despite the dominant inheritance pattern for this variant in humans.

This tolerance of even known damaging mutations is often found for studies using laboratory mice, and in many ways is to be expected; laboratory mice generally live short lives with few stressors which would exacerbate damage to the sarcomere. Even where a different phenotype is produced, it may be a weaker or less noteworthy pathology; for example, the variant T2850I, which has been linked to ARVC, produces only a mild diastolic stiffness phenotype in laboratory mice (Bogomolovas et al., 2016). For this reason, Jiang et al. implemented a long-term approach where the mice were subjected to challenge conditions. The first challenge condition, aging of mice to 1 year old, did not provoke a difference to wild-type mice. A second challenge condition, infusion with isoprenaline/phenylephrine (Iso/PE) to induce adrenergic challenge, resulted in a hypertrophic response that aggravated the cardiac decline; this allowed for the difference between the variant and wild-type mice to be studied.

4 | VARIANT IMPACT BY SARCOMERE REGION

4.1 | Missense variants in the Z-disc

The Z-disc region of the sarcomere represents the connection between two subsequent sarcomeric units; the characteristic Z-shape that marks the region comes from the cross-linking of anti-parallel actin thin filaments with alpha-actinin. Various other proteins localize to the Z-disc (Wadmore et al., 2021), building up a complex network of structural and signaling functions around the lateral cross-links. Titin is one such protein, anchored to the Z-disc through its interactions with telethonin and alpha-actinin (Bertz et al., 2009) (see Figure 4).

Given the unusually strong binding between titin and telethonin (Lee et al., 2006), as well as the likely role the complex plays in the integrity of the sarcomere (P. Zou et al., 2006), several missense variants in Ig1 and Ig2 that impact the binding between the two proteins have been identified and studied in detail. The most extensively studied of these variants, A178D, was first identified through linkage analysis of a three-generation family with DCM and LVNC (Hastings et al., 2016); the variant was demonstrated to cause destabilization and unfolding of the Ig2 domain as well as loss of binding to telethonin. Interestingly, despite the loss of the titin-telethonin interaction, the integrity of the sarcomere appeared to be relatively unaffected when the variant was expressed in transgenic mice (Jiang et al., 2021); rather, the pathology appeared to result from the abundance of unbound, unfolded telethonin in the cytoplasm. As telethonin is typically unfolded when not bound to titin, the study suggested that the transgenic mice experienced a proteotoxic response if unable to clear the unfolded protein. In support of this hypothesis, RNA-seq analysis indicated up-regulation of proteasomal and heat-shock proteins, suggesting a response to an excess of unfolded protein, as well as the presence of telethonin in the pulldown fraction of a tandem ubiquitin binding entity (TUBE) assay.

Other variants that have been found in the Ig1-Ig2 region include V54M and A82D. V54M is found in Ig1 and, like A178D, decreases the binding of the Ig1-Ig2 tandem to telethonin in vitro, and is also linked with DCM (Itoh-Satoh

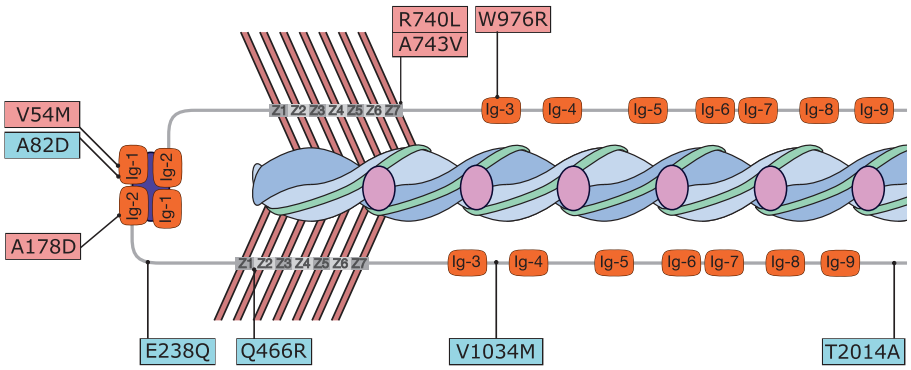

FIGURE 4 Titin domains and reported disease-associated missense variants (red = cardiomyopathy-associated, blue = skeletal myopathy-associated) in and adjacent to the Z-disc. The characteristic Z-shape that marks the region comes from the cross-linking of anti-parallel thin filaments (Actin dimer in light blue; tropomyosin in light green; complex of troponins in light pink) with alpha-actinin (maroon). The N-terminal region of titin passes through the Z-disc and extends through to the other side, where the initial domains Ig1 and Ig2 form a complex with telethonin (purple) with extremely strong binding (Bertz et al., 2009; Lee et al., 2006). Two parallel titin proteins are cross-linked with a single telethonin, forming a dimer (P. Zou et al., 2006). A disordered region of the protein separates Ig2 and Ig3 as titin passes through the Z-disc, including a succession of up to seven Z-repeat sequences of around 45 residues each in length, which are necessary for binding to alpha-actinin. Seven Ig domains (Ig3-Ig9), with long linkers of at least 50 residues between them, conclude titin's presence in the Z-disc.

et al., 2002). V54M has also subsequently been extensively characterized computationally, with its effects on titin-telethonin binding probed using molecular dynamics simulations on the solved crystal structure of titin Ig1-Ig2 complexed with telethonin (Thirumal Kumar et al., 2017). The simulations indicated that V54M destabilized the Ig1 domain, and also that it decreased the binding affinity of Ig1-Ig2 for telethonin. A82D (Ig1) was found co-inherited *in trans* with the truncating mutation R34807Sfs*7; interestingly, it is found at the exact structural position in Ig1 that A178D is located at in Ig2 (Rees et al., 2021). While no experiment has been performed to assay the binding of telethonin to A82D-titin, it has been demonstrated that the variant substantially lowers the thermal stability of Ig1 and renders the mutant domain insoluble. However, the patient in which this variant was first identified was diagnosed with CFTD and had no evidence of cardiomyopathy or cardiac involvement at age 37 when the study was conducted.

Outside of the Ig1-Ig2 tandem, there are several other missense variants that have been linked with disease. In the Z-repeat region, two variants close to one another were found separately in cardiomyopathy patients, and alter the binding of titin to alpha-actinin 2—R740L and A743V. Intriguingly, while A743V decreased the binding affinity of titin to alpha-actinin in Y2H assays (Itoh-Satoh et al., 2002), R740L had the opposite effect, increasing the binding affinity instead (Satoh et al., 1999). Moreover, while A743V was linked to DCM, R740L was linked to HCM. This curious difference demonstrates the degree to which variants located only a few residues apart can be associated with surprisingly different pathologies. Another DCM-linked variant, W976R, is located in Ig3 and was found via linkage analysis in a single family with the condition (Gerull et al., 2002).

Finally, several variants have been found in the unstructured regions between Ig domains in the Z-disc. Genetic analysis of a family with a LGMD phenotype identified the variants E238Q and Q466R, both in the region between Ig2 and Ig3, *in cis* within an affected mother and her son, but absent from the unaffected son (Peddareddygari et al., 2022); intriguingly, a known pathogenic variant in calpain-3 (CAPN3:R489Q) was also found in these patients, but did not co-segregate with the condition, despite localizing to sub-cellular regions more closely associated with LGMD than the Z-disc of titin. Two more variants have been reported via genetic sequencing of two cohorts of neuromuscular disease (NMD) patients: V1034M was found in the long linker between Ig3 and Ig4 (Vasli et al., 2012), compound heterozygous with the A-band variant A18983T; T2014A was identified between Ig9 and Ig10 (Huang et al., 2021), at the very periphery of the Z-disc and I-band, *in cis* with the M-band variant R33903H and compound heterozygous with the splice-site variant c.1800+1G>A. It should be emphasized that E238Q, Q466R, T2014A, and V1034M were prioritized solely through bioinformatics pipelines, and their functional consequences have not been experimentally confirmed; this, alongside their location in unstructured regions and identification in patients with muscular dystrophies (MD) rather than with cardiomyopathy, suggests that their disease associations should be treated with caution. Indeed, T2014A

occurs in around 1 in 500 individuals of East Asian ethnicity in gnomAD, thus is relatively common in healthy individuals.

4.2 | Missense variants in the I-band

The I-band region of titin is often characterized as "the elastic region" (von Castelmur et al., 2008; W. Linke, 2000); unlike the other regions of titin, it is mostly not anchored to the greater structure of the sarcomere and can be subdivided into multiple different regions, including three Ig domain tandem regions (see Figures 2, 3, 5). The differential splicing of the *TTN* gene in the I-band region distinguishes the major isoforms of titin from one another (Freiburg et al., 2000; Labeit & Kolmerer, 1995; Luis & Schnorrer, 2021). In brief, the Ig domains and PEVK region of the I-band adopt a folded, bent arrangement at rest, which straightens and extends as the sarcomere relaxes, including the unfolding of the PEVK; this connection allows the sarcomere to stretch without compromising structural integrity in vertebrate striated muscle, limits the movement of the thin and thick filaments relative to one another, and provides minor passive force to contraction (Lieber & Binder-Markey, 2021).

Within the extensible regions, many variants have been proposed as associated with various diseases, chiefly cardiomyopathies. The most notable of these is T2896I in Ig19, in the first Ig tandem region. It was originally prioritized through whole titin exon sequencing of 38 families with ARVC phenotypes (Taylor et al., 2011), where it co-segregated fully with the disease in a single family of 26 individuals (8 affected carriers) and was selected for further analysis in vitro. The variant and WT domains were compared by various methods. The authors conducted analysis of T2896I and WT domains with native PAGE and intrinsic fluorescence, suggesting that the mutant folded into a less compact state than WT; heteronuclear Single Quantum Correlation (HSQC) NMR analysis showed the sum of two spectra in the tested sample, indicating a combination of correctly folded and partially unfolded structures. Finally, a tandem Ig construct of Ig16-Ig22 experienced a greater degree of proteolysis when incubated with trypsin where mutant T2896I Ig19 was used instead of WT. Of the other ARVC-linked variants found in the original patient cohort, one other was found in the elastic I-band: Y9275C in Ig78, in the second, intermediate Ig tandem. However, unlike T2896I, penetrance in this variant was incomplete, and it has not been characterized further.

Following on from these assays, subsequent studies have further characterized the T2896I variant through a combination of in silico, in vitro, and in vivo methods, looking at its effects on the single domain, the longer titin chain, and the organism itself. Anderson and Granzier (2012) used atomic force microscopy (AFM) to demonstrate that constructs of 5 tandem mutant Ig19 domains (5-mers) required lower force to unfold than wild-type 5-mers, but that re-folding rates were not significantly affected. They also conducted a trypsin digestion assay, concluding that the mutant domain was more susceptible to degradation than the wild-type. Conversely, Bogomolovas et al. (2016) did not detect any significant difference in degradation between wild-type and mutant protein introduced into living mouse skeletal muscle

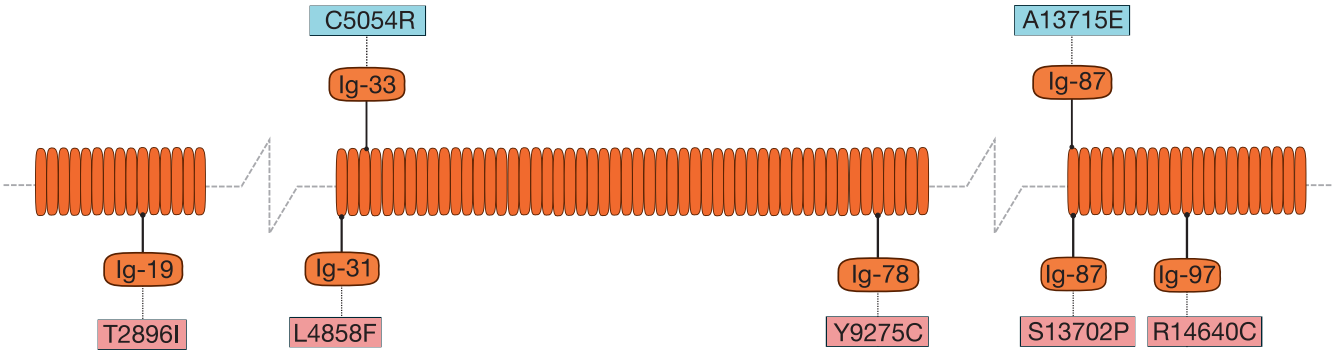

FIGURE 5 Titin domains and reported disease-associated missense variants (red = cardiomyopathy-associated, blue = skeletal myopathy-associated) in the I-band tandem regions. The transition between the Z-disc and I-band regions of titin is marked by a short, unstructured region of titin between Ig9 and Ig10, the first domain in the I-band. A region of closely arranged tandem Ig domains connected by short linkers then follows, from domains Ig10 to Ig24. The second Ig tandem region, from domains Ig31 to Ig82, is composed of closely arranged Ig domains from Ig31 to Ig82, in blocks of around 5–6 domains joined by short, 2–3 residue length linkers, themselves connected by longer linkers of 5–6 residues. This region is not found in the N2B isoform and is only present in the N2BA and N2A isoforms. The third Ig tandem region, from Ig87 to Ig107, follows the PEVK region in all giant isoforms.

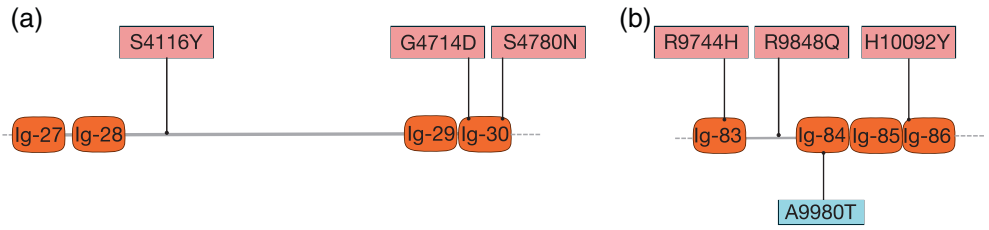

FIGURE 6 Titin domains and reported disease-associated missense variants (red = cardiomyopathy-associated, blue = skeletal myopathy-associated) in the a) N2B and b) N2A regions. The N2B region is found in the N2B and N2BA isoforms and functions both as an extensible region and as a signaling hub for proteins such as heat shock proteins and members of the four-and-a-half LIM domain protein family (FHL). The N2A region is found in the N2A and N2BA isoforms and acts as a signaling hub for proteins such as the ANKRD family.

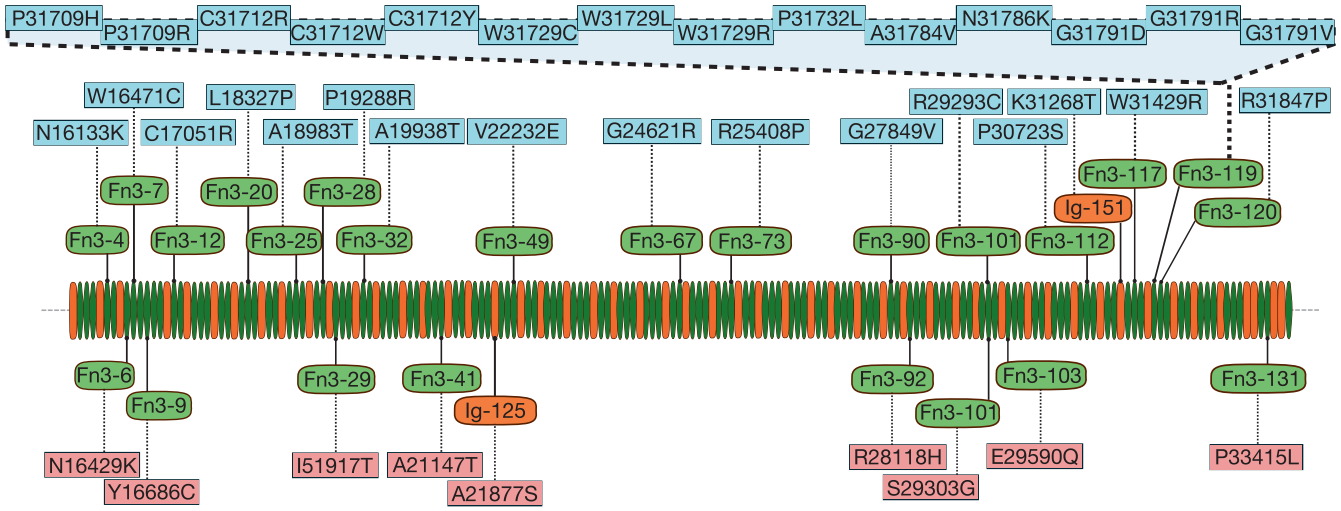

FIGURE 7 Titin domains and reported disease-associated missense variants (red = cardiomyopathy-associated, blue = skeletal myopathy-associated) in the A-band. The section of titin in this part of the sarcomere is closely associated with the myosin thick filaments, and is distinct in its composition, being mostly comprised of tandems of 2–3 Fn3 domains (green) interspersed by individual Ig domains (orange), all closely connected by short linkers to form characteristic super-repeats of 7 (Ig-Fn3-Fn3-Ig-Fn3-Fn3-Fn3) or 11 (Ig-Fn3-Fn3-Ig-Fn3-Fn3-Fn3-Ig-Fn3-Fn3-Fn3) domains.

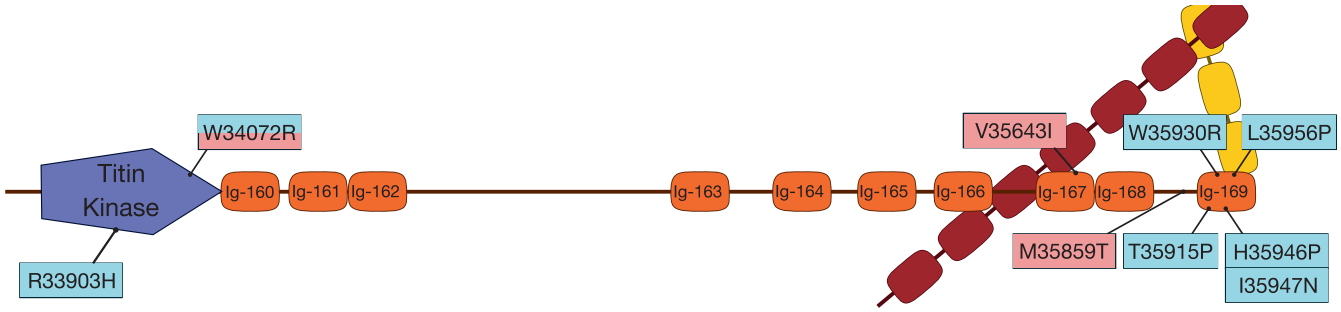

FIGURE 8 Titin domains and reported disease-associated missense variants (red = cardiomyopathy-associated, blue = skeletal myopathy-associated) in the M-band. Here, the C-terminal domains of titin overlap with those of the antiparallel titin molecules from the opposite end of the sarcomere. Titin binds to its fellow giant sarcomeric protein obscurin (yellow) and myomesin (dark red) to form a ternary complex. The kinase domain of titin at the A/M-band junction acts as a signaling hub that monitors the mechanical stresses undertaken by the sarcomere. Note that W34072R (titin kinase) is designated as associated with both skeletal and cardiac myopathies, as it is cardiomyopathy-linked if co-inherited with a cardiac-expressed truncation.

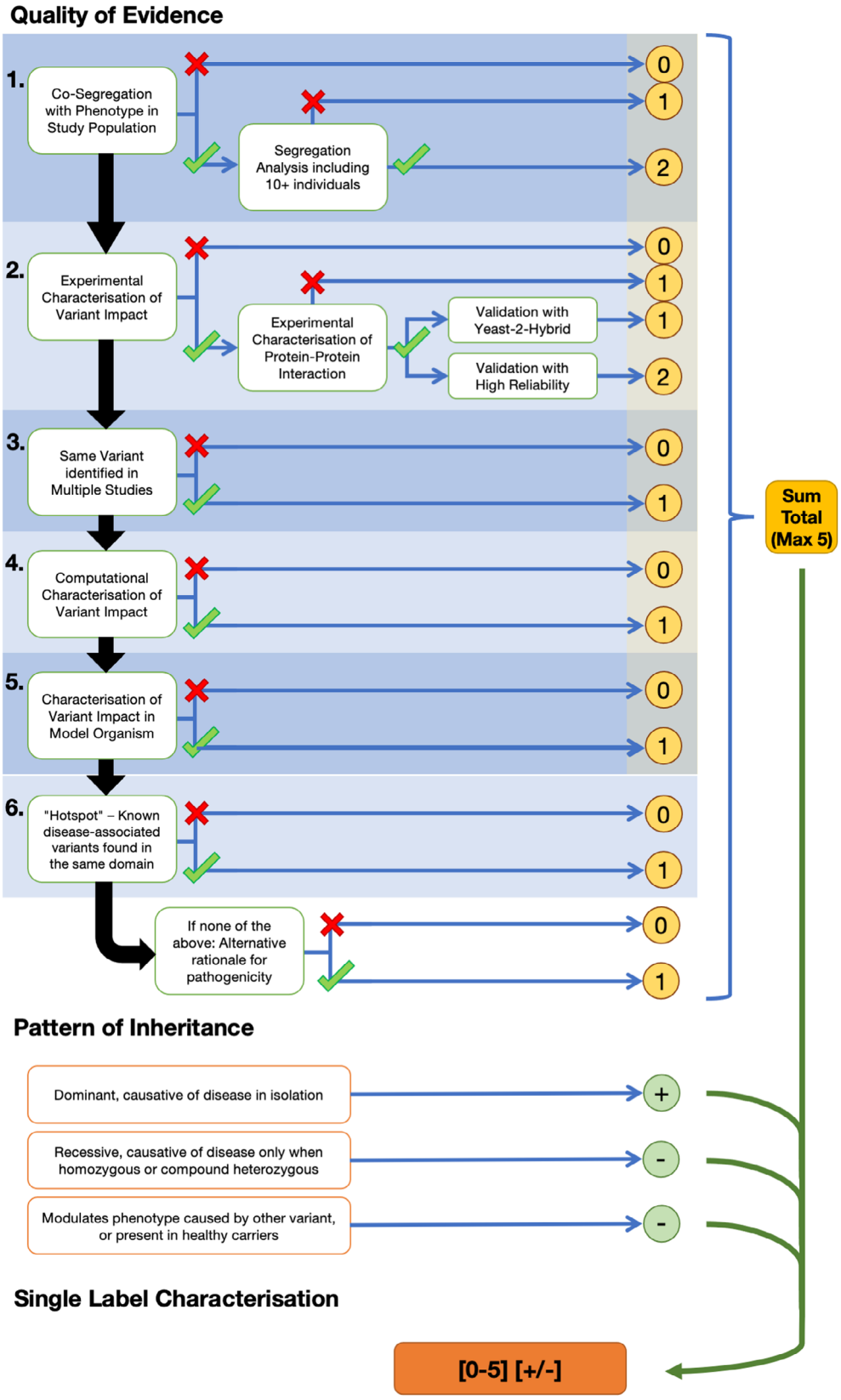

FIGURE 9 Legend on next page.

tissue by electroporation. However, they found that the T2896I variant decreased the melting temperature of the Ig19 domain by around 11°C using differential scanning fluorimetry (DSF). The structure of the mutant domain was solved using x-ray crystallography, and HSQC NMR was used to quantify chemical shifts between mutant and WT; the authors concluded that the variant was causative of a direct increase in the internal flexibility of the domain but did not alter its global structure. X-ray crystallography was again used to solve the structure of the Ig18-Ig20 tandem, which was then used to assess the conformational dynamics of the titin chain using MD simulations, showing that the interface-buried T2896I was likely to alter the flexibility of the chain and thus the relative orientation of the domains. Finally, transgenic mouse models were generated and compared with wild-type mice; while the T2896I KI mice indicated an increase in diastolic stiffness based on echocardiography, they did not present with the full ARVC phenotype. The variant has since been further characterized using MD simulation and in silico stability prediction tools, which suggest that any variants at the T2896 position may be potentially damaging through the same mechanism (Fleming et al., 2021).

The cardiomyopathy most linked to variants in the elastic I-band is DCM; the association has been reported across multiple studies worldwide. The first studies to report the connection were performed on East Asian populations; S4780N was found in Japanese patients with familial DCM along with other variants in the Z-disc and N2A region (Itoh-Satoh et al., 2002), while G4714D was described in an analysis of Chinese families with DCM (X. Liu et al., 2008). Interestingly, both variants localize to Ig30, a domain that borders the N2B region of the I-band, and is present in all three of the N2A, N2B, and N2BA giant isoforms. A third variant, L4854F, was found nearby in Ig31, co-segregating with a severe DCM phenotype when double heterozygous with the laminin A variant K219T in a single Italian family (Roncarati et al., 2013). It should be noted that this variant, located in Ig31, would not be expressed in the predominant cardiac N2B isoform, suggesting that it would play a reduced, likely modulatory role in cardiomyopathy.

An extensive study of TTN missense variants in 147 DCM patients revealed 348 missense variants in N2B and N2BA isoforms across the cohort (Begay et al., 2015), which were filtered down to 44 priority variants using computational tools. These 44 variants were then filtered based on segregation analysis, giving 5 variants that co-segregated with the disease in the I-band and A-band. Two of the co-segregating variants, S13702P (Ig87) and R14640C (Ig97) were found in the I-band, both in the third Ig tandem. However, S13702P showed discordant segregation, with 1 obligate carrier, and R14640C was co-inherited with the A-band variant E29590Q. These two variants were then further characterized by a subsequent study using AFM and chemical denaturation (Zuo et al., 2021). Both variants reduced the overall thermodynamic stability of their respective domains but did not cause unfolding under physiological conditions. However, R14640C was found to substantially decrease the mechanical stability of the domain, reducing unfolding force from an average of 187 pN to 145 pN, while S13702P only slightly destabilizes its domain, from an average of 180 pN to 167 pN. Taken together, these results indicate that, despite being prioritized together, S13702P is either neutral or close to, while R14640C likely has a much stronger role in DCM pathogenesis, together with E29590Q.

More recently, variants in the elastic I-band linked to skeletal myopathies have been identified and characterized (Rees et al., 2021). The recessive variant C5054R, located in Ig33, is linked with a CFTD phenotype; the variant is not found in the N2B isoform, and the patient showed no sign of cardiomyopathy. Conversely, the variant A13715E, linked with a type 1 fiber predominance (T1P) phenotype, is found in Ig87, which is present in the N2B isoform; the patient here did have cardiomyopathy. The authors observed similar trends where truncating variants compound heterozygous with single splice site or nonsense variants in the Ig31-82 region did not produce cardiomyopathy but did produce localized skeletal myopathy, albeit without a full segregation of the phenotype.

FIGURE 9 Flowchart describing annotation of titin missense variants with single label pathogenicity descriptor, as provided in Table 2. Each label consists of a Quality of Evidence score (0–5) and a Pattern of Inheritance (+/−). The Quality of Evidence score is a summation of scores from each of six categories of evidence typically presented for validation of the disease association, with a maximum value of five. The score should be read as an indication of the reliability of the evidence presented in support of the association between the given variant and disease: a score of 0 would indicate no meaningful evidence to support this classification; a score of 1–2 would indicate the need for further evidence to definitively label the variant as disease-associated; a score of 3 or higher indicates sufficient evidence to consider the disease association as validated, with a score of 4–5 denoting multiple studies or populations in which the variant is reported. If the score for a variant is equal to 0 after summation of all six categories, an alternative rationale for pathogenicity may be put forward, and the Quality of Evidence score set as 1; this is for scenarios where none of the six categories apply, but preponderance of other evidence is weighted towards favoring the disease association. For example, a known phenotype is exacerbated in the presence of both the variant in question and a second, causative variant. A "hotspot", or pathogenic hotspot domain, is here defined as a domain in which multiple variants have been found and validated as being associated with the same disease; this includes Fn3-119 and Ig-169 in titin.

4.2.1 | Missense variants in the N2B-specific region

The N2B region is a short, mostly disordered region found in the N2B and N2BA isoforms (see Figure 6a); with that said, its importance both as an extensible region and a signaling hub has long been known and more recently experienced renewed interest (R. J. van der Pijl, Domenighetti, et al., 2021; Watanabe et al., 2002). Modifications to this region can decrease (e.g., phosphorylation by PKA and PKG) or increase (e.g., the formation of disulphide bridges between cysteine residues) titin-based passive stiffness of the sarcomere. It is also the target for several interaction partners, including the heat shock proteins Hsp90 and αB-crystallin, and members of the four-and-a-half LIM domain protein family (FHL) (R. J. van der Pijl, Domenighetti, et al., 2021).

The interaction between the N2B region and the FHL proteins is thought to be the basis of pathogenicity for the best-characterized variant in the N2B region, S4116Y. First identified in a Japanese patient with HCM (Itoh-Satoh et al., 2002), it was then characterized in vitro in a subsequent paper looking at several HCM- and DCM-linked variants in titin (Matsumoto et al., 2006). A Y2H assay indicated that S4116Y significantly increased the binding strength of the N2B region to FHL2; another study demonstrated that the variant also decreased the phosphorylation of the N2B region by MAPK/Erk2 (Raskin et al., 2012). The authors concluded that the binding of FHL proteins is an essential part of the stress-sensing function of the N2B region and demonstrated the consequences on the cardiac stress response in ex vivo knock-out studies in FHL1 in mice. However, the increase in binding affinity reported in Matsumoto et al. was not reproduced when a co-immunoprecipitation assay was used to assess the variant impact on N2B binding to FHL1 and FHL2; in both cases, the binding affinity was found to substantially decrease (Lange, 2005) compared to the wild-type. Thus, the link between S4116Y and other titin variants with HCM remains contentious, and should be treated with caution.

4.2.2 | Missense variants in the N2A-specific region

The N2A region is, like the N2B region, only present in certain isoforms (in this case N2A and N2BA) and acts as a signaling hub, with many interaction partners and post-translational modifications that modulate its behavior (Hessel & Linke, 2021; R. J. van der Pijl, Domenighetti, et al., 2021) (see Figure 6b). However, it is described as an inelastic part of the I-band (Bennett et al., 1997), and there is some debate over whether certain described interactions are indeed reflected in vivo (R. J. van der Pijl & Ottenheijm, 2021). The most noteworthy of these is the assertion that N2A binds directly to the actin thin filament, modulated by calcium binding; however, even more recent studies have drawn conflicting conclusions from the experimental data. Kelly et al. (2021) argue that Ig86 alone binds actin in a calcium-dependent manner; Stronczek et al. (2021) were unable to find binding sites for either calcium or F-actin in the Ig84-86 tandem; Tsiros et al. (2022) argue that only constructs that contain Ig83 and with at least 3 Ig domains can bind F-actin, and that the calcium-dependent binding property is lost when Ig86 is not present. Outside of actin binding, the N2A region binds to chaperones, methyltransferases, and proteases, thought to protect the region from damage and to trigger a response in the event of mechanical stress being detected. Most notably, the ANKRD protein family cross-links adjacent titin molecules by dimerization; a trimeric complex of CARP1, titin, and F-actin has also been suggested (R. van der Pijl, van den Berg, et al., 2021).

Like S4116Y in the N2B region, variants in the N2A region have also been reported in patients with HCM, in this case, postulated to act through the disruption of the binding of titin to CARP1, a protein product of the ANKRD1 gene. Genetic sequencing of 384 HCM patients for variants in titin or ANKRD1 identified 5 mutants, 2 in the N2A region of titin and 3 in ANKRD1 (Arimura et al., 2009). The first of these, R9744H, was found in Ig83; the second, R9848Q, was found in the unique sequence between Ig83 and Ig84. A Co-IP assay demonstrated that the binding of titin to CARP1 was increased by both titin variants, and functional studies in vitro demonstrated that the subcellular localization of Myc-tagged CARP1 was altered. A third variant in the N2A region, H10092Y (Ig-86), was found in the same study of ARVC families that first identified T2896I in the I-band (Taylor et al., 2011); it showed incomplete penetrance, and as mentioned in Section 3.1, is present in about 1 in 250 individuals in gnomAD, increasing to around 1 in 100 individuals in the Finnish sub-populations (Table 1). Subsequently, a re-examination of the family in question identified a variant in desmoglein-2 that co-segregated with ARVC (Costa et al., 2021), likely settling this variant as neutral. Similarly, A9980T (Ig-84) was identified in two siblings with titinopathy and asymmetric facial and limb weakness; it too has a high MAF in gnomAD, present in around 1 in 1000 non-Finnish European individuals, and was reported alongside the variants A19938T and R29293C in the same individuals (Sasaki et al., 2020).

4.3 | Missense variants in the A-band

The A-band is the region of the sarcomere where the motor domains of the myosin thick filaments are located, and where the active contraction of the sarcomere takes place through the cross-bridge muscle contraction cycle (Fitts, 2008). Unlike the regions of titin found in other regions, the A-band section of titin contains both Fn3 and Ig domains (see Figure 7) and is closely associated with the myosin thick filaments, thus unlike the I-band titin region does not extend in response to stretch (Granzier & Labeit, 2004), although a role in thick filament mechanosensation has recently been proposed (Park-Holohan et al., 2021). Given the great length of the A-band relative to the other regions of the sarcomere—183 of titin's 302 total domains are in this region—it follows that many titin missense variants fall within this region by sheer coincidence, accompanied by a corresponding variation in the evidence for pathogenicity and types and severities of pathologies recorded.

Fn3-119, a single fibronectin type-III domain located near to the C-terminal end of the A-band, is the most extensively characterized domain in titin in relation to a single condition, with clear genetic evidence for pathogenicity in many families worldwide. Over 10 missense variants in this one domain have been linked to hereditary myopathy with early heart failure (HMERF), with five variants characterized in vitro (Hedberg, Toledo, et al., 2014). The first such variant identified, C31712R, is a fully penetrant variant with autosomal dominant inheritance, and has been identified in a wide range of studies and different populations. It was first found to co-segregate with the disease in the 3 unrelated Swedish families tested (Ohlsson et al., 2012); another study, published separately but almost concomitantly, found the same variant in 3 British families, again co-segregating with observed pathology (Pfeffer et al., 2012). It has subsequently been reported in a number of studies, in different ethnic groups: in individuals of British, Swedish, Finnish, Italian, and Argentinian ancestry (Palmio et al., 2014); in two individuals, one of Native Canadian and one of Spanish ancestry (Toro et al., 2013), with the distinct ancestral haplotype of the Native Canadian patient suggesting independent origins in different ethnic groups; in two separate studies of myofibrillar myopathy (MFM), associated with HMERF pathology related to that condition (Pfeffer et al., 2014; Uruha et al., 2015); in a Chinese family in 2015 (Yue et al., 2015), confirming this variant as having a worldwide distribution; in one individual in a British cohort for a study of clinical applications of magnetic resonance imaging (MRI) (Bugiardini et al., 2018); in one Afghan, one Spanish, and two unrelated Italian individuals, as well as four individuals in a family of Russian ancestry (Palmio et al., 2019); in a Portuguese patient (Morais et al., 2020); in a Chinese patient (Huang et al., 2021). Finally, the variant domain has been experimentally characterized in vitro, and was found to greatly decrease the solubility and folding of the Fn3-119 domain, where the wild-type domain and those with common variants did not (Hedberg, Toledo, et al., 2014). Thus, C31712R is considered the TTN missense variant with the greatest consensus as being directly causative of disease.

Alongside C31712R, many other variants in Fn3-119 have been linked to HMERF. Variant W31729L was identified in 20 HMERF patients in a single Japanese family and found to co-segregate with the disease in the 9 family members used for linkage analysis (Izumi et al., 2013). Two more substitutions at the same position, W31729R, and W31729C, were found in British and German individuals, respectively, in another study, alongside P31709R in a French family (Palmio et al., 2014). W31729C was also found in another individual in the above British MRI cohort (Bugiardini et al., 2018). P31709R, W31729R, and W31729C were characterized in vitro in the same study that characterized C31712R, and likewise decreased solubility and folding of the Fn3-119 domain (Hedberg, Toledo, et al., 2014). W31729R was also identified in another study of Chinese titinopathy patients (Huang et al., 2021). The previously mentioned studies describing C31712R in patients with MFM and HMERF pathology also identified the variant G31791D, as well as three more mutations at the same positions as these two mutations (C31712Y, G31791R, G31791V) (Uruha et al., 2015); one also identified N31786K as a potential variant, albeit with no segregation analysis (Pfeffer et al., 2014). N31786K was subsequently identified in a single Iranian patient (Palmio et al., 2019); this study also described the variant P31709H, at the same position as the previously described P31709R, in one Portuguese and one Filipino-Caucasian patient, as well as another example of the W31729C variant in an unrelated Portuguese patient, and the novel variant A31784V in two French families and one Argentinian patient. Finally, a third variant at the same position as C31712R and C31712Y was recently reported; C31712W was identified in a Korean family with HMERF manifesting in old age (Yeo et al., 2021).

While almost all the above variants are thought to be fully penetrant, causative variants, the variant in Fn3-119 that has been most extensively characterized besides C31712R has much more uncertainty over its pathogenicity. P31732L was the second identified variant in Fn3-119 after C31712R, in a study that used a blind bioinformatics pipeline to prioritize likely causative genetic variants in a cohort of patients with neuromuscular diseases (Vasli et al., 2012). Like C31712R, P31732L has been identified in multiple patients since its original description, including an Italian family

(Palmio et al., 2014), two French-Portuguese families (Palmio et al., 2014), and sporadic cases in British (Pfeffer et al., 2014), Chinese (Yue et al., 2015), and Japanese (Sano et al., 2022) individuals; however, several of these studies made note of the incomplete penetrance observed in these cases, particularly in comparison to C31732R and other fully penetrant variants. Palmio et al. (2014) observed that the variant was neither fully dominant nor fully recessive, producing a severe phenotype when homozygous but an unclear one when heterozygous. Similarly, Pfeffer et al. (2014) noted that P31732L was also found in the affected patient's unaffected brother. They concluded that the variant may be neutral, of variable penetrance, or late-onset, but ruled out its role as a fully penetrant, dominant variant. However, two separate studies have found that this variant reduces the thermal stability and solubility of the Fn3-119 domain (Hedberg, Toledo, et al., 2014; Rees et al., 2021), as with the fully penetrant variants mentioned above. In the later of these two studies, P31372L was homozygous in the two affected individuals (Rees et al., 2021) while heterozygous family members were asymptomatic, supporting the argument that the severe phenotype requires homozygosity or compound heterozygosity. However, the observed phenotype in these patients was a congenital myopathy with features of HMERF, rather than HMERF in its canonical form.

Outside of the well-established Fn3-119, there are many other variants in the A-band associated with both skeletal and cardiac myopathies. Interestingly, several of these variants occur at the same positions as variants in other domains associated with other conditions, and even represent identical amino acid substitutions; this suggests that there is a similar mechanism that produces different phenotypes depending on the position of the domain within titin. For example, N16133K (Fn3-4) is associated with MmD and disrupts the solubility and thermal stability of the Fn3-4 domain in vitro (Rees et al., 2021); it is located at the same structural position as N16429K (Fn3-6), which was reported to co-segregate with atrioventricular block (AVB) in a 3-generation Chinese family (G. Liu et al., 2020), as well as the previously mentioned N31786K (Fn3-119). While not the same residue substitution, this position is also the location of N19955I (Fn3-32), which was found compound heterozygous with L30639P (Fn3-111) in a Chinese infant with titinopathy (Yu et al., 2019); they are both also compound heterozygous for the splicing variant c.44282-2A>G (inherited *in cis* with N19955I, *in trans* with L30639P). Interestingly, an in vitro expression assay showed no difference in expressed isoform ratios, while confirming a significant decrease in full-length titin compared to healthy controls. W16471C (Fn3-7), C17051R (Fn3-12) and G27849V (Fn3-90) are three more MmD-associated variants, all of which were found to decrease the thermal stability and/or solubility of their respective domains in vitro (Rees et al., 2021); each of them is located at the same structural position as the Fn3-119 variants W31729C, C31712R, and G27849V, respectively. W31429R (Fn3-117) was identified in a Spanish family with TMD (Evilä et al., 2017), at the same structural position as W31729R (Fn3-119). Finally, while not at the exact same structural position in the two domains, P31732L (Fn3-119) and P33415L (Fn3-131) are located at positions only one residue apart in the same loop region of the fibronectin fold and represent the same residue substitution; however, they differ in that P33415L is an exposed surface residue whereas P31732L is mostly buried, and that P33415L is associated with the cardiac ARVC phenotype (Taylor et al., 2011), whereas P31732L is associated with HMERF and congenital myopathy.

The remaining known variants are linked with a variety of both skeletal and cardiac myopathies, located throughout the A-band. L18237P (Fn3-20) and R25480P (Fn3-73) are two variants associated with centronuclear myopathy (CNM) (Rees et al., 2021); both have been found to destabilize their respective domains in vitro. A18983T (Fn3-25) was identified in a patient with MD (Vasli et al., 2012), compound heterozygous with the previously mentioned V1034M at the Z-disc; it has yet to be experimentally characterized. K31268T (Ig151) was identified through WES of a single Estonian family; it co-segregates with inguinal hernia in that family and was not found in ethnically matched population controls (Mihailov et al., 2017). Two further variants associated with MmD not yet mentioned here are R31847P (Fn3-120) and V22232E (Fn3-49), both of which destabilize their domain in vitro (Rees et al., 2021). The same individual in which V22232E was found has been described in two separate studies (Chauveau, Bonnemann, et al., 2014; Rees et al., 2021); interestingly, it is noted that this variant is implicated in heart disease as well as the regular MmD phenotype. Recently, several variants have been reported on the basis of bioinformatics methods alone; these should not be treated as definitive absent future experimental validation. P19288R (Fn3-28) and G24621R (Fn3-67) (Huang et al., 2021) were identified as damaging by PolyPhen-2 (Adzhubei et al., 2010) and SIFT (Ng & Henikoff, 2003); P19288R was found alongside the frameshift variant K4230Nfs*17, and G24621R with the truncating variant Y4418*. A19938T (Fn3-32) and R29293C (Fn3-101) were reported alongside the previously mentioned A9980T (see Z-disc) (Sasaki et al., 2020); however, as with A9980T, R29293C has a high incidence in population data (found in around 1 in 500 individuals of East Asian ethnicity in gnomAD).

Two TMD-linked variants have been found in the A-band, despite the strong association of that condition with variants in the M-band. The first, W31429R, has already been mentioned (Evilä et al., 2017); however, it has subsequently

been reported in a Spanish patient with TMD and DCM (Lopez-Bravo et al., 2021). In the former study, it was found to be compound heterozygous with the truncating variant R21209*, whereas in the latter, it was reported homozygous; this distinction may have some bearing on the differences in phenotype. Similarly, the second variant, P30723S (Fn3-112), was identified in a patient compound heterozygous for this variant and the strongly TMD-linked "FINmaj" indel (see M-band) (Evilä et al., 2014). The patient's father, who carried P30723S only, was asymptomatic; consequently, the authors argue that the phenotype of TMD is exacerbated, rather than caused, by these missense variants in conjunction with truncating variants, resulting in greater severity of disease.

For cardiac variants, the single TTN variant associated with RCM, Y16686C (Fn3-9), was found to co-segregate with the condition in a single Jewish family (Peled et al., 2014). The variants I19517T (Fn3-29), A21147T (Fn3-41), and A21877S (Ig125) were prioritized computationally as associating with ARVC in the same study that identified the previously mentioned P33415L (Taylor et al., 2011); however, the variants did not fully segregate with the pathology within the patients' families. Several DCM-linked variants that have been prioritized by bioinformatics approaches also fall within the A-band, namely R28118H, S29303G, and E29590Q; these were identified in a previously mentioned large-scale DCM study (see I-band) (Begay et al., 2015), and do show co-segregation within families, though they lack additional experimental validation.

4.4 | Missense variants in the M-band

The C-terminal region of titin is located in the M-band, at the very center of the sarcomere (Lange et al., 2020) (see Figure 8). Here, the antiparallel myosin molecules that make up the thick filament overlap; while the heads of the molecules extend into the A-band to bind the actin thin filaments, the tails are held in position at the M-band by the members of the myomesin family. Titin, too, is anchored at the M-band, with its C-terminal domains overlapping with those of the antiparallel titin molecules from the opposite end of the sarcomere. In this way, the full sarcomere can be thought of as a symmetrical construct, with two titin proteins extending from either Z-disc to meet at the M-band. Titin binds to its fellow giant sarcomeric protein obscurin or obscurin-like 1 to form a ternary complex with myomesin, forming a link both to the thick filaments and to the outer sarcolemma and sarcoplasmic reticulum (Fukuzawa et al., 2008). Moreover, the presence of the kinase domain of titin at the border between the A- and M-band regions is thought to act as a signaling hub that monitors the mechanical stresses undertaken by the sarcomere (Lange et al., 2005; Sheetz, 2021; Solís & Russell, 2021).

While the M-band has historically received less attention than other regions of the sarcomere despite the importance of its structural and signaling functions, many of the earliest associations between skeletal myopathies and titin missense variants were made in this region, particularly with respect to tibial muscular dystrophy (TMD) and its links to the final domain in titin, Ig169 (also known as M10). The first such association was found in 2002, where genetic testing of Finnish patients identified a non-frameshift indel of four consecutive substitutions (EVTW>VKEK at position 35,927) (Hackman et al., 2002). This variant, labeled FINmaj, completely co-segregated with TMD with LGMD2J in the 81 individuals from 12 unrelated families tested. The authors attempted to find a comparable mutation in a French family with TMD with LGMD2J; they were able to isolate the variant L35956P, which again co-segregated with the phenotype within that family. Similar studies later identified the variant I35947N in a Belgian family (Van den Bergh et al., 2003), and H35946P in an Italian family (Pollazzon et al., 2010).

These four mutations were biophysically characterized in vitro by Rudloff et al. (2015) to compare the magnitude of their effects on the single Ig169 domain. As Ig169 binds to obscurin's N-terminal Ig1 domain at the M-band, the binding affinity of mutant Ig169 for OBSCN-Ig1 was compared to wild-type Ig169 using ITC, as well as comparisons of stability, expression, and aggregation. The FINmaj variant had the greatest effect on the domain, producing severe misfolding, aggregation, the lowest expression, and an abolition of binding to OBSCN-Ig1. The L35956P variant also produced severe misfolding and a loss of OBSCN-Ig1 binding, as well as lowered expression, but did not show aggregation. While the H35946P variant was stable at room temperature, it unfolded at body temperature; otherwise, like L35956P, it had lowered expression, loss of OBSCN-Ig1 binding, but soluble expression. Finally, I35947N showed no difference in stability from WT, as well as normal binding affinity for OBSCN-Ig1, normal expression, and no aggregation.

A further study of the Finnish founder mutation FINmaj found that most patients expressed a classic TMD phenotype, whereas only a minority expressed additional phenotypes such as LGMD2J (Udd et al., 2005); a study of a 5-generation Chinese family with LGMD2J identified the variant W35930R, which co-segregated fully with the disease in that family (Zheng et al., 2016). Interestingly, unlike the other variants mentioned, co-segregation of I35947N with

TMD was not complete; an asymptomatic family member also carried the variant, albeit with a sub-clinical condition only discovered after subsequent muscle biopsy (Van den Bergh et al., 2003). A later study also identified I35947N in another Belgian family with TMD (Evilä et al., 2017); here, the variant was compound heterozygous with the truncating variant Q22507*. This study also identified the variant T35915P in a Tunisian family with TMD, compound heterozygous with the frameshift variant E28338Hfs. Notably, not only were both variants found together with truncating variants, but they also had incomplete penetrance; family members with only one variant and without compound heterozygosity were not affected. Thus, the diversity in phenotype resulting from Ig169 variants may arise in part from truncating or frameshift variants that work in concert with them.

Aside from Ig169, the other element of M-band titin that has received the most interest is the kinase domain. The mechanical stretching of the sarcomere induces conformational change in the kinase, allowing for the binding of the zinc-finger protein nbr2, precipitating a signaling cascade that leads to the activation of the serum response transcription factor SRF (Lange et al., 2005). Disruption of this pathway would likely lead to disease as transcriptional changes in response to stress would be altered; however, the variant first identified as doing so, R34091W, was found in two Swedish families with HMERF (Nicolao et al., 1999), whose relatives would later be confirmed to also carry the A-band Fn3-119 variant P31732L, which has already been noted as strongly linked to HMERF (Hedberg, Toledo, et al., 2014). This, combined with the relatively high allele frequency of R34091W in non-Scandinavian European populations, has led to its re-classification as benign, although it has been suggested that it may contribute to the phenotypic penetrance of the otherwise incompletely penetrant P31732L variant (see A-band) in monoallelic-bimutational inheritance (Lange et al., 2014). The only other variant in the kinase domain that has been validated is W34072R, which was originally identified in a patient with MmD-HD (with cardiomyopathy), compound heterozygous with the frameshift variant E2989fs* (Chauveau, Bonnemann, et al., 2014). It was found to decrease the thermal stability of the kinase domain compared to wild type. Additionally, it was identified a second time in an unrelated patient with non-specific congenital myopathy (Rees et al., 2021) without cardiomyopathy, compound heterozygous with the truncating variant R7796*. Finally, the variant R33903H was identified in a titinopathy patient *in cis* with the previously mentioned T2014A (see Z-disc) and compound heterozygous with the splice-site variant c.1800+1G>A (Huang et al., 2021); as with T2014A, R33903H is relatively common in East Asian populations, also occurring in around 1 in 500 individuals in gnomAD, thus is unlikely to have much role in the disease.

While several cardiomyopathy-associated variants have been reported in the M-band, they are not as comprehensively studied as the variants in Ig-169 and the titin kinase domain, and their associations with disease have not been validated. In the same family in which the previously mentioned ARVC-linked variant A21877S (see A-band) was identified, the M-band variant M35859T was also reported in the long linker between Ig168 and Ig169 (Taylor et al., 2011). As with A21877S, M35859T has incomplete penetrance with ARVC; moreover, its location in an unstructured region and its high population frequency (around 1 in 100 individuals of South Asian ethnicity in gnomAD) indicate it is unlikely to relate to disease. A recent paper that aimed to identify individuals in the early stages of familial HCM prior to diagnosis (Luo et al., 2022) reported the variant V35643I (Ig-167) in a Hui Chinese family with a history of the disease. In a cohort of 18 asymptomatic individuals from this family, the authors report significant echocardiographic differences between those carrying this variant ($n = 6$) and wild-type individuals ($n = 12$); they suggest that these differences reflect future onset of HCM, and that such screening techniques can provide early diagnosis of the condition. This is intriguing, as there are no other reports of variants associated with HCM in M-band titin, and relatively few variants in the whole of titin linked to that condition; follow-up studies on this cohort will hopefully shed light on this hypothesis.

5 | LOCATION-FUNCTION RELATIONSHIPS

The vastness of titin and the diversity of its molecular contexts should make it apparent that a variant in one domain or region may be associated with a very different phenotype than one in another. Efforts to map the etiology of various myopathies with respect to the locations of the associated variants in titin have been mixed (Chauveau, Rowell, & Ferreiro, 2014; Eldemire et al., 2021; LeWinter & Granzier, 2013; Tharp et al., 2019); while there are extremely strong associations between the development of TMD and HMERF and variants in the very specific modules Ig169 and Fn3-119, respectively, most other conditions are associated with many variants spread across titin.

The reverberations of the pathogenic missense mutation to the local domain structure, the dynamics of the titin chain, and ultimately health of the patient carrying the variant, reflect several factors related to its location in titin.

Depending on the structural location of the variant in its parent Fn3 or Ig domain, the domain may still fold correctly, or partially or even fully unfold in physiological conditions, especially if the variant disrupts the hydrophobic core of the domain. Alternatively, if it occurs on the domain surface, where it is less likely to affect domain stability, it may still disrupt or destabilize interactions with native binding partners. While the relationship between a variant's capacity to destabilize its parent domain and its pathogenicity to the carrier is not fully understood, the destabilization of certain domains, most notably Ig169 and Fn3-119, has clear and reproducible consequences (Hedberg, Toledo, et al., 2014; Rudloff et al., 2015). These potential ramifications of the location of variants in titin are discussed below.

5.1 | Isoforms

A clear consequence of the location of the missense variant in the titin sequence is whether it is expressed in a particular isoform. Notably, the Ig31-82 tandem, N2A region, and most of the PEVK region are absent from the N2B isoform, while the N2B region is absent from the N2A isoform (Laddach et al., 2017); thus, the consequences of pathologies may be modulated by the expression patterns of isoforms in different tissues. For example, the variant W34072R (titin kinase) has been identified in two individuals; in the first, where the variant was found compound heterozygous with E2989Efs*4, the patient presented additionally with cardiomyopathy (Chauveau, Bonnemann, et al., 2014), whereas the second, compound heterozygous with R7796*, did not (Rees et al., 2021). It is notable that the former truncating variant occurs prior to the N2B region in the TTN sequence, while the latter occurs after, potentially explaining this discrepancy. Similar considerations may play a role in the often very restricted effects of striated muscle diseases, such as TMD, and also the variability between cases of the same condition (Udd & Hackman, 2005).

The N2B isoform, predominant in cardiac muscle, is shorter than the N2A and N2BA isoforms; it is expected that missense variants associated with cardiomyopathies would not occur in regions which N2B lacks. This is not always the case; for example, the DCM-linked variant L4854F (Ig31) and the ARVC-linked variant Y9275C (Ig78) are both located in the Ig31-82 tandem region. However, L4854F was found as a double heterozygote with LMNA variant K219T (Roncarati et al., 2013), and Y9275C presents with incomplete penetrance (Taylor et al., 2011); hence, it is feasible that both variants have only a modulatory role in disease progression. These variants would be present in affected hearts in the N2BA isoform, but as the expression ratios of the N2B and N2BA isoforms in hearts can vary significantly between species, between individuals, and between muscles (Cazorla et al., 2000; Trombitás et al., 2001), identifying the proportion of titins affected by the variants in question is not simple. In mammals, the N2BA isoform is predominant in atrial muscle, but the N2B isoform represents most titin in the left ventricle (Freiburg et al., 2000). However, the murine models typically used for study of cardiomyocytes have far less N2BA isoform in their muscle than larger mammals, and consequently murine myocardium tends to be much stiffer (Cazorla et al., 2000).

It should be clarified that, when discussing the differential expression of titin isoforms, the designation of N2A, N2B, and N2BA reflects the earliest elucidations of titin's presence in muscle, where the separation of isoforms by the presence or absence of the key signaling hub regions contributed to their identification (Freiburg et al., 2000; Labeit & Kolmerer, 1995). More recent research has shown that alternative splicing can diversify the available isoforms through adding or removing specific exons (Savarese et al., 2018); moreover, it has been shown that certain muscles will upregulate the expression of certain titin exons in transcripts relative to others. Notably, the predominant isoforms in the foetal and neonatal myocardium are longer, more extensible isoforms such as foetal cardiac titin (FCT), which are replaced during development with the shorter, less extensible N2B isoform (Lahmers et al., 2004; Opitz et al., 2004). Thus, the muscle tissue in one region of the body may incorporate far more of the translated titin with the mutated region than another. Some authors have suggested that muscle stiffness may be modulated dynamically through titin expression, especially as a response to heart disease (Makarenko et al., 2004; Nagueh et al., 2004; Neagoe et al., 2002); this further complicates the association of variants with specific conditions or time of onset. Future studies that incorporate the analysis of transcriptomic data, particularly the comparison of expression of missense variant exons in affected and unaffected muscles, would further elucidate this issue.

5.2 | Shared structural positions

As the 302 individual domains in titin are composed of only 3 distinct domain folds, it is not uncommon to see similar or identical residues at the same structural position in different domains, with similar contributions to the stability of

the domain fold. Intriguingly, several missense variants identified in titin represent the exact same amino acid substitution at the same domain position, differing only in the domain in question. Notably, there is the A-band domain Fn3-119, strongly associated with HMERF; of the 13 currently described Fn3-119 missense variants associated with HMERF, 5 have seen the exact same residue substitution at the same structural position in 5 other Fn3 domains in titin, associated with other conditions.

While it is not surprising that the same substitution at the same structural position would cause the same damage to the structure, questions remain over whether the mechanisms governing the pathogenicity of the variant itself can be directly compared. For example, the variants A82D and A178D in Ig1 and Ig2, respectively, represent the same residue substitution at identical structural positions. In both cases, a buried hydrophobic core residue is replaced by a hydrophilic, charged residue, destabilizing the domain core. The A82D variant was found to decrease the melting temperature of the Ig1 domain by around 37°C in vitro (Rees et al., 2021), and the A178D variant to decrease the binding affinity of titin for TCAP (Hastings et al., 2016). However, while the A178D variant has been associated with the cardiomyopathies DCM and LVNC, the A82D variant is associated with the skeletal myopathy CFTD, without cardiac involvement, and is co-inherited with the truncating variant R34807Sfs*7.

Both Ig1 and Ig2 are expressed in all major isoforms of titin, including the "tiny titin" novex-3 isoform, and both are involved in the intransient interaction with TCAP that anchors titin to the Z-disc. However, most interactions between TCAP and titin involve the Ig2 domain, with no similarly extensive bond networks stabilizing the interaction between Ig1 and TCAP (Bertz et al., 2009; P. Zou et al., 2006), potentially explaining the discrepancy. This is thought to be a consequence of the directionality of muscle stretch, as the titin-TCAP complex can bear remarkably heavy loads applied to the C-terminal end both in silico (Thirumal Kumar et al., 2017) and in vitro, but not when the same loads are applied to the N-terminus (Bertz et al., 2009). In summary, the destabilization of adjacent domains, with similar physiological roles, in an identical manner, does not imply that the consequent pathology or pathomechanisms will apply to both.

5.3 | HCM/DCM phenotype

A curious observation of missense variants in the Z-repeat region of the Z-disc concerns the two cardiomyopathies, HCM and DCM. As previously mentioned, the HCM-linked variant R740L reportedly increases the binding affinity of the Z-repeat region for ACTN1 in vitro (Satoh et al., 1999), whereas the purported DCM-linked variant A743V decreases it (Itoh-Satoh et al., 2002). The increase in binding affinity for a particular binding partner is a consistent trait of reported HCM-linked variants; the N2B-region variant S4116Y increases the affinity of the N2B region for FHL2 (Matsumoto et al., 2006), and the N2A-region variants R9744H and R9848Q increase the affinity of the N2A region for CARP (Arimura et al., 2009). By contrast, the two other DCM variants that are linked with changes in binding affinity, V54M in Ig1 and A178D in Ig2, both decrease the affinity of titin for TCAP (Hastings et al., 2016; Itoh-Satoh et al., 2002).

While no firm conclusions may be drawn from the small sample size of variants in titin, it is interesting to consider the implications in light of the pathologies in question. HCM is characterized by the thickening and expansion of the heart muscle, leading to a decreased volume of the heart chambers, whereas DCM is characterized by a stretching and elongation of the heart muscle, leading to an increased volume of the heart chambers (Elliott et al., 2008). In many respects, the pathologies resemble opposite extremes, alike the opposing consequences of the Z-repeat variants. The idea for stabilizing mutations to be pathogenic remains a subject of ongoing study (Gerasimavicius et al., 2022), but has yet to be brought into focus for titin. It should be reiterated, however, that the majority of HCM-linked genetic variants reside in the myosin thick filament and its binding partners, and there are inconsistencies in the genetic and biomedical evidence provided for the role of titin in HCM; for example, the HCM-associated variant S4116Y has been characterized as both strengthening (Matsumoto et al., 2006) and diminishing (Lange, 2005; R. J. van der Pijl, Domenighetti, et al., 2021) the interaction between titin N2B and FHL2.

6 | IMPLICATIONS FOR DIAGNOSIS

An emerging diagnostic paradigm is one that seeks to address the current classification of variants into categories based on evidence for pathogenicity, and the limitations that arise from titin variants that seem to differ in damage caused

based on their molecular context. Most titin missense variants that are strongly associated with disease possess an autosomal dominant inheritance pattern (Akinrinade et al., 2019; Savarese et al., 2016); this is to be expected, as more tenuous disease associations require more evidence to demonstrate the pathogenic quality of the variant satisfactorily. Less attention, therefore, has traditionally been paid to recessive alleles, which may be common in the general population, but go unnoticed except in the presence of another variant. However, incomplete phenotypic penetrance does not preclude the role of a variant in disease, whether causative or modulatory. Increasingly, variants in titin are reported whose phenotypic effects are only demonstrable in concert with other variants (Evilä et al., 2014, 2017; Roncarati et al., 2013), and this list is likely to expand as we continue to seek the genetic basis for inherited myopathies without a clear monogenetic association.

In a recent study of congenital myopathy patients (Rees et al., 2021), it was found that most of the patients were compound heterozygous for two or more titin variants, often one missense and one truncating; in this mutational context, the damaging consequences of a misfolded region of titin can be seen in earnest at the organismal level. The authors suggested, therefore, that destabilizing titin missense variants in compound heterozygosity with truncating variants are sufficient to cause disease. This paradigm must be explored further, but offers an intriguing insight into some of the disease associations discussed here. For example, the TMD-associated domain Ig-169 contains multiple variants, but not all these variants are equivalent; I35947N is noticeably less damaging, both in experimentally characterized properties (Rudloff et al., 2015) and in patterns of inheritance (Evilä et al., 2017; Van den Bergh et al., 2003), compared to other characterized variants. It is also the only one of these variants in which a truncating mutation was found alongside the missense, and indeed, the disease phenotype co-segregated only with those individuals who were compound heterozygous for both the missense and truncating variants.

This issue is one that is especially pertinent for titin. Not only is it larger than any other protein in the human genome, and thus likely to include multiple variants on both alleles (Chauveau, Rowell, & Ferreiro, 2014; LeWinter & Granzier, 2013), but it is also a gene of many functions. Any one variant in titin may have minimal interplay with another, through the compartmentalization of the gene into domains, the different isoforms expressed in different muscle tissues, and the fixed position of the protein in the sarcomere. Consequently, the standard approach for identification of pathogenic variants, where variants are labeled based on their likelihood of being causative for disease independently of any other genetic factors, risks eliminating key information that distinguishes the consequences of genetic variants with the same disease associations. For example, the online database for pathogenic variants, ClinVar (Landrum et al., 2016, 2018), lists 11 single amino acid substitutions in titin as definitively pathogenic; notably, these include the incompletely penetrant variants I35947N (Ig-169) and P31732L (Fn3-119) in the same category as fully penetrant variants in these same domains. While these are certainly disease-associated and well-characterized, the clear differences between these variants and those more damaging variants are lost, giving an unclear representation of damaging titin variants.

To compensate for this weakness in the annotation of titin variants and those of other giant proteins and genes, we have additionally labeled the variants reported in the literature (Table 2) with a simple key that broadly categorizes the variants based on the strength and nature of the disease association. The variants are ranked from 0 to 5 on the evidence presented in their characterization, based on the quantity and quality of meaningful evidence to support classification. They are then further annotated with either a "+" (disease-causing with full penetrance) or "−" (modulatory role or recessive action) where possible, indicating the most likely role played by the variant based on that evidence. The evidence metric is additive, such that multiple sources of validation are considered cumulatively rather than separately.

The purpose of this annotation should be to give a brief but recognizable indication of the reliability of the disease associations described. We consider any variant with an Evidence value of 3 or greater to be of close to certain pathogenicity; those variants with a value of 4–5 reflect those that have been additionally validated by multiple studies or different methods. Variants with a value of 2 should be treated with caution, while those with a value of 1 should be treated as requiring further validation. A value of 0 indicates that no evidence supports this disease association despite the reported pathogenicity. This approach is limited by the quantity of data on titin missense variants currently reported in the literature; future sequencing studies that record the presence of recessive missense variants in titin-linked pathologies would lay the groundwork to delineate the combinations of variants that are causative for disease. Encouragingly, the existence of titin-specific databases of variants, including TITINdb (Laddach et al., 2017) for protein variants and CardioDB (Roberts et al., 2015) for genetic data, will aid in the processing of these large quantities of data in future.

TABLE 2 Missense variants in titin reported in the literature, with relevant annotations described in this review.

Variant	Domain	Associated condition	Validation	Penetrance	Co-inheritance	Label	Reference
V54M	Ig-1	DCM	(1) Y2H PPI assay (1) MD simulation	Unknown		2+	Itoh-Satoh et al. (2002), Thirumal Kumar et al. (2017)
A82D	Ig-1	CFTD	(2) Thermostability assay (1) Cellular expression, solubility, localization	Unknown	R34807Sfs*7	3−	Rees et al. (2021)
A178D	Ig-2	DCM, LVNC	(2) Segregation analysis with 10+ individuals (2) GST PPI assay (1) in vivo murine model	Full (8 affected, 14 total)		5+	Hastings et al. (2016), Jiang et al. (2021)
E238Q	NA	LGMD	(1) Segregation analysis	Full (2 affected, 3 total)	Q466R	1−	Peddareddygari et al. (2022)
Q466R	NA	LGMD	(1) Segregation analysis	Full (2 affected, 3 total)	E238Q	1−	Peddareddygari et al. (2022)
R740L	NA	HCM	(1) Y2H PPI assay	Unknown		1+	Satoh et al. (1999)
A743V	NA	DCM	(1) Y2H PPI assay	Unknown (2 affected, 2 total)		1+	Itoh-Satoh et al. (2002)
W976R	Ig-3	DCM	(2) Segregation analysis with 10+ individuals	Incomplete (11 affected, 35 total; 4 unaffected carriers)	R21201*	2−	Gerull et al. (2002), Herman et al. (2012)
V1034M	NA	MD	(0) Association only	Unknown (2 affected, 3 total)	A18983T	0	Vasli et al. (2012)
T2014A	NA	Possible titinopathy	(0) Association only	Unknown	R33903H, c.1800+1G>A	0	Huang et al. (2021)
T2896I	Ig-19	ARVC	(2) Thermostability assay (1) MD simulation (1) in vivo murine model	Full (7 affected, 11 total)		4+	Taylor et al. (2011), Anderson et al. (2013), Bogomolovas et al. (2016), Fleming et al. (2021)
S4116Y	NA	HCM	(1) Y2H PPI assay	Unknown		1+	Itoh-Satoh et al. (2002), Matsumoto et al. (2006)
G4714D	Ig-30	DCM	(1) Segregation analysis	Full (2 affected, 2 total)		1+	Liu et al. (2008)
S4780N	Ig-30	DCM	(0) Association only	Unknown		0	Itoh-Satoh et al. (2002)
L4854F	Ig-31	DCM	(1) Segregation analysis	Incomplete/severe only (5 affected, 16 total)	LMNA:K219T	1−	Roncarati et al. (2013)
C5054R	Ig-33	CFTD	(2) Thermostability assay (1) Cellular expression, solubility, localization	Unknown	Y24599Mfs*12	3−	Rees et al. (2021)
Y9275C	Ig-78	ARVC	(0) Association only	Unknown		0	Taylor et al. (2011)

(Continues)

TABLE 2 (Continued)

Variant	Domain	Associated condition	Validation	Penetrance	Co-inheritance	Label	Reference
R9744H	Ig-83	HCM	(2) Co-IP PPI assay (1) ex vivo histopathology (1) Multiple studies	Unknown		4+	Arimura et al. (2009), Lopes et al. (2013)
R9848Q	NA	HCM	(2) Co-IP PPI assay (1) ex vivo histopathology	Unknown (2 affected, 2 total)		3+	Arimura et al. (2009)
A9980T	Ig-84	Novel titinopathy	(0) Association only	Unknown (2 affected, 3 total)	A19938T, R29293C	0	Sasaki et al. (2020)
H10092Y	Ig-86	ARVC	(0) Association only	Unknown		0	Taylor et al. (2011), Costa et al. (2021)
S13702P	Ig-87	DCM	(1) Segregation analysis (0) Mechanostability assay (1) in silico assessment	Incomplete (2 affected, 3 total)		2−	Begay et al. (2015), Zuo et al. (2021)
A13715E	Ig-87	T1P	(2) Thermostability assay (1) Cellular expression, solubility, localization	Unknown (2 affected, 2 total)	R11022*	3−	Rees et al. (2021)
R14640C	Ig-97	DCM	(1) Segregation analysis (1) Mechanostability assay (1) in silico assessment	Full (2 affected, 3 total)	E29590Q	3−	Begay et al. (2015), Zuo et al. (2021)
N16133K	Fn3-4	MmD	(2) Thermostability assay (1) Cellular expression, solubility, localization	Unknown	R5308*	3−	Rees et al. (2021)
N16429K	Fn3-6	AVB	(1) Segregation analysis	Full (5 affected, 7 total)		1+	Liu et al. (2020)
W16471C	Fn3-7	MmD	(2) Thermostability assay (1) Cellular expression, solubility, localization	Full (2 affected, 2 total)	R17337*	3−	Rees et al. (2021)
Y16686C	Fn3-9	RCM	(2) Segregation analysis with 10+ individuals	Full (5 affected, 13 total)		2+	Peled et al. (2014)
C17051R	Fn3-12	MmD	(2) Thermostability assay (1) Cellular expression, solubility, localization	Full (2 affected, 2 total)	Q32711Lfs*22	3−	Rees et al. (2021)
L18237P	Fn3-20	CNM	(2) Thermostability assay (1) Cellular expression, solubility, localization	Unknown	D32441Ffs*1, W35453*	3−	Rees et al. (2021)
A18983T	Fn3-25	MD	(0) Association only	Unknown (2 affected, 3 total)	V1034M	0	Vasli et al. (2012)

TABLE 2 (Continued)

Variant	Domain	Associated condition	Validation	Penetrance	Co-inheritance	Label	Reference
P19288R	Fn3-28	Possible titinopathy	(0) Association only	Unknown	K4230Nfs*17	0	Huang et al. (2021) \\
I19517T	Fn3-29	ARVC	(0) Association only	Incomplete		0	Taylor et al. (2011)
A19938T	Fn3-32	Novel titinopathy	(0) Association only	Unknown (2 affected, 3 total)	A9980T, R29293C	0	Sasaki et al. (2020)
N19955I	Fn3-32	Novel titinopathy	(1) Muscle biopsy	Unknown	L30639P, c.44282-2A>G	1−	Yu et al. (2019)
A21147T	Fn3-41	ARVC	(0) Association only	Unknown	M35859T	0	Taylor et al. (2011)
A21877S	Ig-125	ARVC	(0) Association only	Unknown		0	Taylor et al. (2011)
V22232E	Fn3-49	MmD-HD	(2) Thermostability assay (1) Cellular expression, solubility, localization	Recessive (1 affected, 2 total)	N34020Tfs*9	3−	Chauveau, Bonnemann, et al. (2014), Rees et al. (2021)
G24621R	Fn3-67	Possible titinopathy	(0) Association only	Unknown	Y4418*	0	Huang et al. (2021)
R25480P	Fn3-73	CNM	(2) Thermostability assay (1) Cellular expression, solubility, localization	Unknown	R14679*	3−	Rees et al. (2021)
G27849V	Fn3-90	MmD	(2) Thermostability assay (1) Cellular expression, solubility, localization	Unknown	R21201*	3−	Rees et al. (2021)
R28118H	Fn3-92	DCM	(1) Segregation analysis (1) in silico assessment	Full (2 affected, 2 total)		2+	Begay et al. (2015)
R29293C	Fn3-101	Novel titinopathy	(0) Association only	Unknown (2 affected, 3 total)	A9980T, A19938T	0	Sasaki et al. (2020)
S29303G	Fn3-101	DCM	(1) Segregation analysis (1) in silico assessment	Full (2 affected, 2 total)	LMNA: c.936G>A	2−	Begay et al. (2015)
E29590Q	Fn3-103	DCM	(1) Segregation analysis (1) in silico assessment	Full (2 affected, 3 total)	R14640C	2−	Begay et al. (2015)
L30639P	Fn3-111	Novel titinopathy	(1) Muscle biopsy	Unknown	N19955I, c.44282-2A>G	1−	Yu et al. (2019)
P30723S	Fn3-112	Novel titinopathy	(1) Segregation analysis	Incomplete (1 affected, 3 total)	FINmaj (35927delins-EVTW>VKEK)	1−	Evilä et al. (2014)
K31268T	Ig-151	IH	(1) Segregation analysis			1−	Mihailov et al. (2017)

(Continues)

TABLE 2 (Continued)

Variant	Domain	Associated condition	Validation	Penetrance	Co-inheritance	Label	Reference
				Incomplete (5 affected, 9 total)			
W31429R	Fn3-117	TMD, DCM	(1) Segregation analysis (1) Multiple studies	Recessive (1 affected, 5 total)	R21209*	2−	Evilä et al. (2017), Lopez-Bravo et al. (2021)
P31709H	Fn3-119	HMERF	(1) Hotspot domain	Unknown		1+	Palmio et al. (2019)
P31709R	Fn3-119	HMERF	(1) Segregation analysis (2) Western blot assay (1) Hotspot domain	Full (3 affected, 6 total)		4+	Palmio et al. (2014), Hedberg, Toledo, et al. (2014)
C31712R	Fn3-119	MFM, HMERF	(2) Segregation analysis with 10+ individuals (1) Multiple studies (2) Western blot assay (1) Hotspot domain	Full (multiple studies)		5+	Ohlsson et al. (2012), Pfeffer et al. (2012), Palmio et al. (2014), Toro et al. (2013), Hedberg, Toledo, et al. (2014), Pfeffer et al. (2014), Uruha et al. (2015), Yue et al. (2015), Palmio et al. (2019), Morais et al. (2020), Huang et al. (2021)
C31712Y	Fn3-119	MFM, HMERF	(1) Hotspot domain	Unknown (2 affected, 2 total)		1+	Uruha et al. (2015)
C31712W	Fn3-119	HMERF	(1) Hotspot domain	Unknown		1+	Yeo et al. (2021)
W31729C	Fn3-119	HMERF	(1) Segregation analysis (2) Western blot assay (1) Hotspot domain (1) Multiple studies	Full (2 affected, 4 total); Full (4 affected, 4 total)		5+	Palmio et al. (), Hedberg, Toledo, et al. (2014), Bugiardini et al. (2018), Palmio et al. (2019)
W31729L	Fn3-119	MFM, HMERF	(2) Segregation analysis with 10+ individuals (1) Hotspot domain	Full (5 affected, 10 total)		3+	Izumi et al. (2013)
W31729R	Fn3-119	HMERF	(2) Western blot assay (1) Hotspot domain (1) Multiple studies	Unknown (1 affected, 1 total)		4+	Palmio et al. (2014), Hedberg, Toledo, et al. (2014), Huang et al. (2021)
P31732L	Fn3-119	CM, HMERF	(2) Segregation analysis with 10+ individuals (1) Multiple studies (2) Western blot assay, thermostability assay (1) Cellular expression, solubility, localization	Incomplete (multiple studies)		5−	Vasli et al. (2012), Palmio et al. (2014), Hedberg, Toledo, et al. (2014), Pfeffer et al. (2014), Yue et al. (2015), Rees et al. (2021), Sano et al. (2022)

TABLE 2 (Continued)

Variant	Domain	Associated condition	Validation	Penetrance	Co-inheritance	Label	Reference
			(1) Hotspot domain				
A31784V	Fn3-119	HMERF	(1) Segregation analysis (1) Hotspot domain	Full (6 affected, 8 total)		2+	Palmio et al. (2019)
N31786K	Fn3-119	HMERF	(1) Segregation analysis (1) Multiple studies (1) Hotspot domain	Full (2 affected, 7 total)		3+	Pfeffer et al. (2014), Palmio et al. (2019)
G31791D	Fn3-119	MFM, HMERF	(1) Hotspot domain	Unknown		1+	Uruha et al. (2015)
G31791R	Fn3-119	MFM, HMERF	(1) Hotspot domain (1) Multiple studies	Unknown		2+	Toro et al. (2013), Uruha et al. (2015)
G31791V	Fn3-119	MFM, HMERF	(1) Hotspot domain	Unknown		1+	Uruha et al. (2015)
R31847P	Fn3-120	MmD	(2) Thermostability assay (1) Cellular expression, solubility, localization	Unknown	R5430*	3−	Rees et al. (2021)
P33415L	Fn3-131	ARVC	(0) Association only	Unknown		0	Taylor et al. (2011)
R33903H	Kinase-1	Possible titinopathy	(0) Association only	Unknown	T2014A, c.1800+1G>A	0	Huang et al. (2021)
W34072R	Kinase-1	CM, MmD-HD	(2) Thermostability assay (1) Cellular expression, solubility, localization (1) Multiple studies	Recessive (1 affected, 2 total); Unknown)	E2989Efs*4/ R7796*	4−	Chauveau, Bonnemann, et al. (2014), Rees et al. (2021)
R34091W	Kinase-1	HMERF	(1) Segregation analysis (1) Functional assays	Full (8 affected, 25 total)	P31732L	2−	Lange et al. (2005)
V35643I	Ig-167	HCM	(1) Segregation analysis	Unknown (6 affected, 18 total)		1+	Luo et al. (2022)
M35859T	NA	ARVC	(0) Association only	Unknown	A21877S	0	Taylor et al. (2011)
T35915P	Ig-169	TMD	(1) Hotspot domain	Recessive (3 affected, 4 total)	E28338fs	1−	Evilä et al. (2017)
W35930R	Ig-169	LGMD2J	(1) Segregation analysis (1) Hotspot domain	Recessive (3 affected, 6 total)		2−	Zheng et al. (2016)
H35946P	Ig-169	TMD, LGMD2J	(1) Segregation analysis (2) Thermostability assay (1) Hotspot domain	Full (5 affected, 7 total)		4+	Pollazzon et al. (2010), Rudloff et al. (2015)
I35947N	Ig-169	TMD, LGMD2J	(1) Segregation analysis (2) Thermostability assay	Incomplete (6 affected, 9 total, 1 unaffected	Q22507*	4−	Van den Bergh et al. (2003), Rudloff et al. (2015), Evilä et al. (2017)

(Continues)

TABLE 2 (Continued)

Variant	Domain	Associated condition	Validation	Penetrance	Co-inheritance	Label	Reference
			(1) Hotspot domain	carrier); Recessive (2 affected, 3 total)			
L35956P	Ig-169	TMD, LGMD2J	(1) Segregation analysis (2) Thermostability assay (1) Hotspot domain	Full (3 affected, 7 total)		4+	Hackman et al. (2002), Rudloff et al. (2015)

Note: Each variant is identified by its residue change, position according to the inferred complete isoform, parent domain, and reported condition with which it is associated. All variant numbering in this review has been aligned to the inferred complete isoform (Ensembl Transcript ID: ENST00000589042.5; NCBI Reference Sequence: NM_001267550.2). We include a summarized notation of validation methods used, as well as the reported penetrance of the variant and the size of the cohort(s) used to make that determination. Any variants co-inherited with the titin variant in question in the affected individuals are included. Finally, a labeling scheme is provided to summarize the confidence of the label of damaging variant, and the magnitude of the pathological consequence; details may be located by reference to Figure 9.

7 | CONCLUSION

The evidence for the role of titin missense variants in disease, while still lagging behind that of truncating variants, is now more pronounced and better understood. Biophysical characterization of variants and their parent domains in vitro has given us an appreciation of how these variants affect their local structure, and the expression of variants in vivo has begun to shed light on the pathomechanisms that have so far been shrouded in uncertainty. The data collected here represent, to the best of our knowledge, the most complete resource for disease-associated titin missense variants, and the evidence in support of those associations. We have also discussed the different validation methodologies, both experimental and computational, that have been used in the past to investigate these variants, and the quantity and quality of evidence that should be required to support a given disease association. Thus, we have identified those variants which may be reliably considered as pathogenic, and those which will necessitate further study to validate their hypothesized mechanisms, and to enable their use in disease-associated variant data sets.

While debates continue over the severity, penetrance, and validity of individual missense variants, the mutational map of titin continues to be filled, and patterns can begin to be drawn. However, the evidence gathered thus far is dwarfed by the evidence that is needed; large regions of titin remain uncharacterized and with functions unknown, and the patterns of isoform expression are not fully understood. The continued unraveling of the veil of titin-linked myopathies depends in large part on our continued ability to generate clear, reliable data on titin missense variants.

AUTHOR CONTRIBUTIONS

Timir G. R. Weston: Conceptualization (lead); data curation (lead); visualization (lead); writing – original draft (lead). **Martin Rees:** Writing – review and editing (equal). **Mathias Gautel:** Supervision (equal); writing – review and editing (equal). **Franca Fraternali:** Conceptualization (supporting); supervision (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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