LETTERS

Palindromic assembly of the giant muscle protein titin in the sarcomeric Z-disk

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The Z-disk of striated and cardiac muscle sarcomeres is one of the most densely packed cellular structures in eukaryotic cells[1]. It provides the architectural framework for assembling and anchoring the largest known muscle filament systems by an extensive network of protein–protein interactions, requiring an extraordinary level of mechanical stability. Here we show, using X-ray crystallography, how the amino terminus of the longest filament component, the giant muscle protein titin, is assembled into an antiparallel (2:1) sandwich complex by the Z-disk ligand telethonin. The pseudosymmetric structure of telethonin mediates a unique palindromic arrangement of two titin filaments, a type of molecular assembly previously found only in protein–DNA complexes. We have confirmed its unique architecture *in vivo* by protein complementation assays, and *in vitro* by experiments using fluorescence resonance energy transfer. The model proposed may provide a molecular paradigm of how major sarcomeric filaments are crosslinked, anchored and aligned within complex cytoskeletal networks.

The Z-disk of the sarcomere defines the lateral boundary of sarcomeric units within the myocyte cytoskeleton. In higher vertebrates it anchors and aligns at least three major sarcomeric filament systems, including actin, titin and nebulin[1,2]. It also harbours many smaller protein components, some of which, including α-actinin and telethonin, have been mapped to distinct binding sites at the N terminus of titin[3–7]. Their presence, proper sorting and localization within the Z-disk region are critical for myofibril assembly and for the maintenance of an intact Z-disk structure[8]. Components of the Z-disk are also involved in signalling processes that may regulate muscle development and degradation, as well as in linking contractile functions of muscle sarcomeres to membrane systems such as the sarcoplasmic reticulum or the T-tubules associated with excitation–contraction coupling[2,8].

The very N-terminal region of titin comprises a domain topology that has been predicted to consist of two immunoglobulin-like domains, referred to as Z1 and Z2 (ref. 9). Co-localization studies, two-hybrid interaction screens and pull-down assays have demonstrated that they interact with the N-terminal region of telethonin at the Z-disk periphery[3,4]. However, although the interaction with telethonin has been considered as a 'cap' (hence the alternative name 'T-cap'), or a 'bolt'[3], it is not known how titin–telethonin binding affects the overall architecture of myofibrils and their associated functions. The physiological importance of this interaction has been supported by evidence linking mutations in the N-terminal regions of titin, telethonin and the telethonin-binding site of muscle LIM protein (MLP) to different familial forms of limb-girdle muscular dystrophy, as well as hypertrophic and dilated cardiomyopathy[10–12]. These findings, together with complementary data from animal models, indicate the possible existence of a link between the titin–telethonin–MLP interaction and mechanical stress sensor pathways[10].

Depending on the specific isoform of titin, 200–700 N-terminal residues of multimeric titin filaments are located within and cross over most of the Z-disk of striated muscle sarcomeres[2,3,6]. However, no data are yet available on the molecular nature of the titin–titin association. Here we have determined the crystal structure of its N-terminal region in complex with the titin-binding domain of telethonin (Fig. 1, Table 1 and Supplementary Fig. S1). The latter domain is sufficient to localize telethonin to the Z-disk of cardiac myofibrils (Supplementary Fig. S2). Contrary to previous expectations[3] and previous structural findings on other immunoglobulin-like domain-containing proteins (see Supplementary Information), our data reveal an antiparallel assembly of two titin molecules mediated by telethonin, indicating that telethonin might have a key role in titin assembly and Z-disk anchoring.

In the complex, the two N-terminal immunoglobulin-like domains of titin, Z1 and Z2 are in an extended conformation and are connected by a short three-residue linker. The two domains have similar structures (root-mean-square deviation (r.m.s.d.) = 0.66 Å, for all common main-chain atoms) and sequences (40 of 98 residues are identical). In each of the two titin molecules, domains Z1 and Z2 are almost equally translated by 48 Å and rotated by 53° and 61° with respect to each other, generating a superhelical coil arrangement of each titin N terminus. The second component of the complex, telethonin, forms a unique elongated structure with a central five-stranded antiparallel β-sheet that is extended by two exposed

Table 1 | Refinement statistics

Resolution (Å)	15.0–2.45
R_{work}/R_{free} (%)	23.2/26.5
Number of atoms	
Protein	3,726
Ion	25
Water	179
B factors (Å^2)	
Protein	49.7
Ion	51.2
Water	48.6
R.m.s.d.	
Bond lengths (Å)	0.011
Bond angles (°)	1.370

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wing-shaped β-hairpin motifs (A–B, C–D). The two motifs are related by an approximate two-fold symmetry (rotation of 179.4°), generating a nearly perfect palindromic arrangement (Figs 1a and 2a–d). They can be superimposed with an r.m.s.d. of 0.38 Å, and in the corresponding structural alignment 11 of 19 matching residues are similar or identical.

The peculiar symmetry of the telethonin structure allows it to mediate titin–titin assembly (Fig. 1b). The four nearly identical titin–telethonin interfaces, I–IV, are formed from two intermolecular antiparallel β-sheets. Within each interface, the long, invariant β-strand G of one of two titin immunoglobulin-like domains interacts with one of the four β-strands (A, B, C or D) of the two wing-like hairpins from telethonin. Hence, each telethonin hairpin (A–B, C–D) provides the core for one of the two (3–2–3)-stranded, antiparallel titin–telethonin–titin β-sheets. The two β-sheets are separated by the telethonin core β-sheet that is flanked by the two short Z1–Z2 linkers of each sandwiching titin molecule. Taken together, the data on the titin–telethonin complex provide a novel concept exemplifying how immunoglobulin-like domain-containing proteins may act as receptors for protein ligands, such as telethonin. In contrast to other immunoglobulin-like receptors[13,14], the protein–protein interface of the titin–telethonin sandwich complex is formed by main-chain-mediated intermolecular β-sheet interactions. In terms of general principles of complex formation of biological molecules, the titin–telethonin complex reveals an unprecedented analogy to palindromic or pseudopalindromic protein–DNA complexes[15]. Future data are needed to determine whether this type of palindromic complex is unique to the titin–telethonin interaction or whether it establishes a new principle of protein–protein interactions. The extensive network of interactions indicates that the N terminus of titin, after complex formation with telethonin, provides a rigid anchoring scaffold rather than adding to the molecular elasticity that has been observed in several titin segments[2]. Indeed, such an anchoring structure that is resistant to external mechanical forces seems to be a prerequisite for elastic movements of other parts of titin, specifically within the sarcomeric I-band, under active muscle contraction–relaxation conditions, without the danger of uncontrolled disintegration. The bridging structure of telethonin indicates that it might be essential for the functional integrity of the titin filament in mature myofibrils.

To validate our structural data, we first designed an *in vitro* fluorescence resonance energy transfer (FRET) experiment, in which we introduced four site-specific donor–acceptor fluorophore pairs into the two titin molecules to measure residue–residue distances within the complex in solution (Fig. 3C). The FRET distances of all four donor–acceptor pairs well reflect those observed in the crystal structure (Fig. 3D). Because the FRET data can be neither modelled into a putative parallel titin–titin arrangement nor explained by other stoichiometries, they provide independent and unambiguous evidence for an antiparallel arrangement of the two titin molecules in the titin–telethonin complex in solution.

Subsequently, we performed two types of fluorescence imaging experiments to validate our structural findings of the antiparallel titin–telethonin complex under *in vivo* conditions. In the first approach, we used COS cells to test whether the titin–telethonin complex observed structurally can also form under *in vivo* conditions in the absence of a pre-existing sarcomeric filament system (Fig. 3A, B).

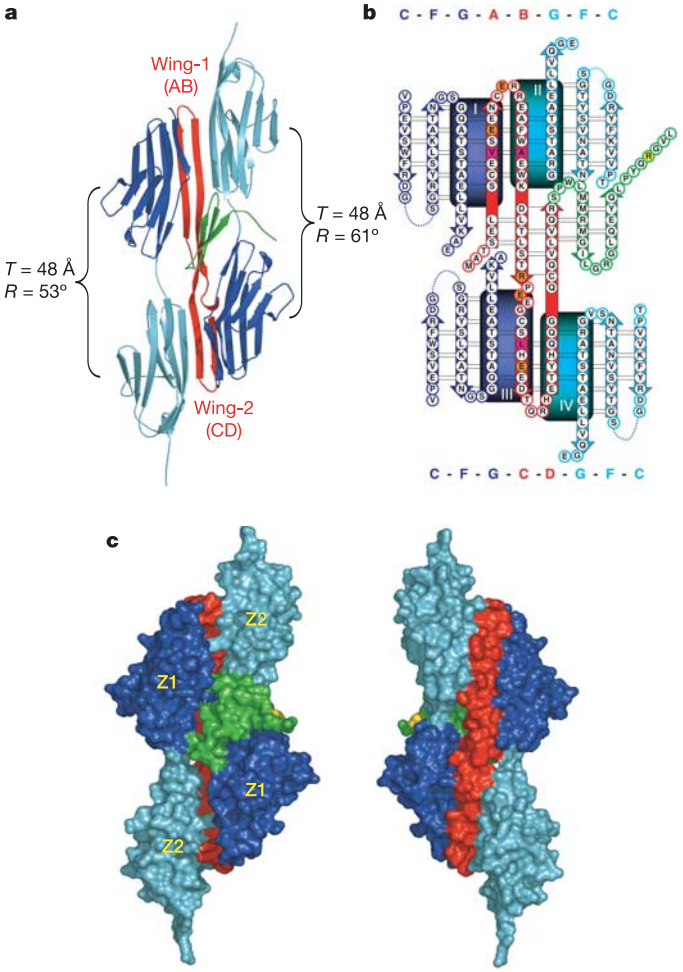

Figure 1 | Structure of the palindromic titin–telethonin–titin complex. Colour codes: blue, titin immunoglobulin-like domain Z1 (residues 1–98); cyan, titin immunoglobulin-like domain Z2, including the Z1–Z2 linker (99–196); red, telethonin(1–59); green, telethonin(60–90). **a**, Ribbon representation. **b**, Schematic representation of the β-sheet structure in the titin–telethonin (2:1) complex. Arg 87 of telethonin, which is linked to dilated cardiomyopathy, is coloured yellow[10]. The β-sheet hydrogen bonds are depicted by lines. For clarity, only those parts of the two immunoglobulin-like tandem repeats from titin that are involved in interactions with telethonin are shown. **c**, Surface presentation of the titin–telethonin–titin complex in two orientations, rotated by 180° relative to each other.

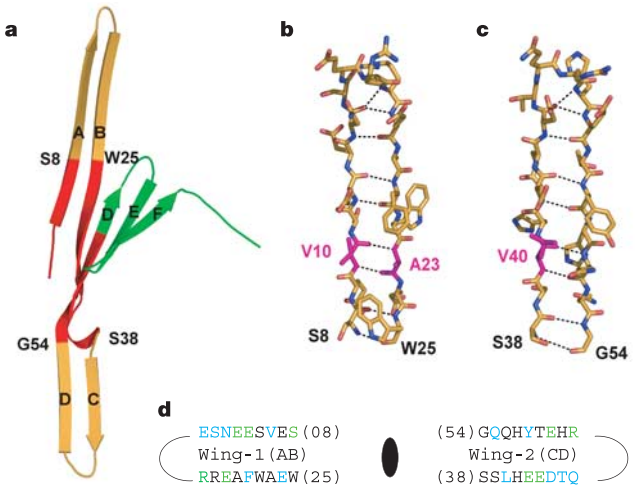

Figure 2 | Telethonin-mediated assembly and Z-disk anchoring of titin filaments. **a**, Ribbon presentation of the structure of telethonin, indicating the residue numbers of the β-hairpin wing boundaries. Colours as in Fig. 1. **b**, **c**, Stick presentations of the two β-hairpin wings of telethonin. Colours as in **a**, except those that have been mutated into prolines (pink) for validation purposes. **d**, Sequence representation of the two β-hairpin wings, indicating how the repeated sequence motif translates into a palindromic structural relation. Identical and similar residues are coloured in green and blue, respectively.

We employed a yellow fluorescence protein (YFP) reconstitution assay using two YFP half-domains (YN, YC)[16]. After transfection of COS cells with constructs resulting in a titin–telethonin complex, fluorescence from reconstituted YFP was detected only for the YN–titin–telethonin–titin–YC system in contrast to the YN–titin–telethonin–YC–titin system, thus fitting an antiparallel assembly of two titin molecules only (Fig. 3A). The data were confirmed by immunoblot assays of lysates from transfected COS cells (Fig. 3B).

To examine the correct targeting of telethonin to the endogenous titin N terminus within the sarcomeric Z-disk *in vivo*, we used neonatal rat cardiac myocytes (NRCs), which express all sarcomeric components (Supplementary Fig. S2). To allow comparison of titin–telethonin binding under *in vitro* conditions, in a test cell line (COS cells) without sarcomeres and in sarcomere-containing muscle cells, we introduced several structure-based single-residue mutations in telethonin. In a first experiment series, we changed several residues involved in the titin–telethonin interface and tested their ability for titin–telethonin complex formation *in vitro* (Supplementary Fig. S3). None of them indicated abolition of the interaction, most probably because of the high stability of the complex formed. However, three

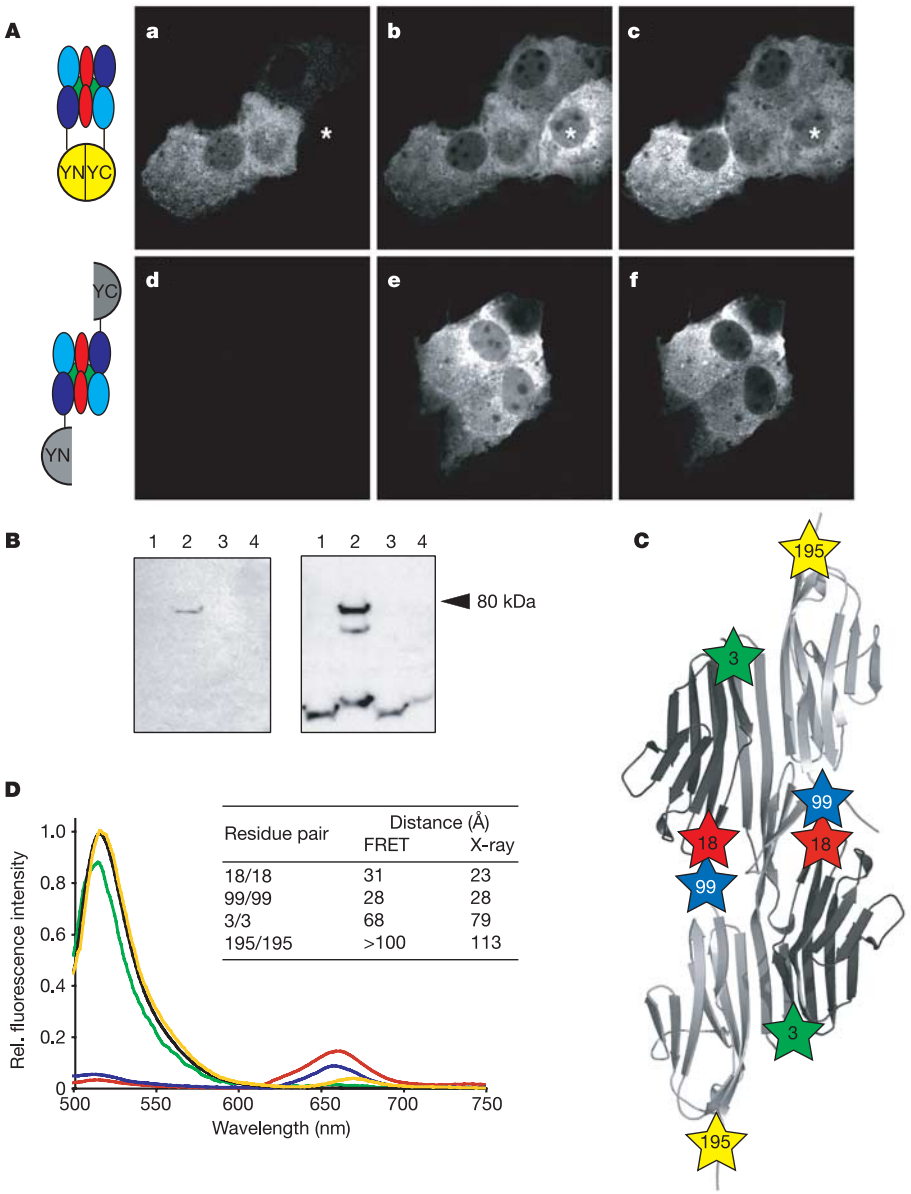

Figure 3 | Evidence for the formation of the palindromic titin–telethonin complex *in vivo* and *in vitro*. **A**, YFP reconstitution experiments in co-transfected COS cells. Top: N-terminal and C-terminal halves of YFP (YN, YC) were fused to opposite termini of the titin tandem immunoglobulin-like constructs YN–titin and titin–YC. Bottom: YN and YC were fused to the N terminus of the titin tandem immunoglobulin-like construct, yielding YN–titin and YC–titin. **a**, **d**, Intrinsic YFP fluorescence; **b**, **e**, detection of HA-epitope-tagged telethonin (residues 1–90), with an anti-HA antibody; **c**, **f**, YC detection, with a GFP-specific antibody. The asterisk in **c** indicates a COS cell expressing only one of the two split-GFP constructs, on the basis of the absence of intrinsic YFP fluorescence in **a**. **B**, Western blot analysis of whole COS cell extracts, using an anti-HA antibody for telethonin detection (left) and a GFP antibody for the detection of split-GFP fusion constructs (right). COS cells were co-transfected with the HA-tagged titin-binding segment of telethonin (1–90; lanes 1 and 2), the C-terminal segment of telethonin (91–167; lanes 3 and 4) as well as either YN–titin and YC–titin (lanes 1 and 3) or YN–titin and titin–YC (lanes 2 and 4), respectively. **C**, **D**, FRET distance analysis of four titin residue pairs (identified by colour in **C**) from the titin–telethonin complex. The observed FRET distances (**D**, inset) can only be fitted with an antiparallel arrangement of the two titin molecules in the titin–telethonin complex. The spectrum of the donor-labelled titin(C195) mutant has been used as a reference (in black).

telethonin variants, in which the local hydrogen bond pattern of one of two the β-hairpin wings was disrupted by proline mutations, lost their capacity for binding the titin N terminus *in vitro* (Supplementary Fig. S3) and in COS-cell YFP complementation assays (Supplementary Fig. S4). In accordance with the molecular data, the same mutants were found to be unable to target correctly to the sarcomeric Z-disk when transfected into NRCs (Supplementary Fig. S2), indicating the abrogation of complex formation with sarcomeric titin. Taken together, the data indicate that the structural integrity of both pseudopalindromic telethonin wings might be critical for titin–telethonin assembly, regardless of whether telethonin assembles with the titin Z1Z2 domains only, as shown in COS cells, or via N-terminal titin within the sarcomeric Z-disk of intact myofibrils (Supplementary Table 1).

Our model implies that there is two-fold symmetry in the assembly of the N terminus of titin, in agreement with previous electron microscopy data[17]. Several arguments have been put forth indicating that titin–actin thin filaments might exist in a 2:1 ratio within the Z-disk[6,17,18]. This stoichiometry could reconcile spacing considerations to match the 28/13 symmetry observed in actin thin filaments, allowing orthogonal α-actinin crosslinks at 195-Å intervals and satisfying the tetragonal lattice symmetry viewed along the filament axis as well as the estimated Z-repeat distances in titin in the order of 120 Å or less[18,19]. An antiparallel titin–titin arrangement may be plausible because of the localization of the Z-links in the Z-disk centre as previously suggested[7,18]. However, the titin–telethonin complex structure does not provide direct information about the origin of the two titin molecules that may belong to the same sarcomeric unit or to an adjacent sarcomere. The latter model, however, would inevitably lead to relative shifts of titin filaments outside the Z-disk areas in the range of hundreds of Ångströms, which would be in conflict with several imaging studies displaying titin as aligned filaments[3,6,20]. The only reconcilable model therefore depicts the N termini of two titin strands as being derived from the same sarcomere.

Within the context of the Z-disk, our structure of the titin–telethonin complex provides an unexpected atomic model for the association of titin molecules at their very N termini, indicating that telethonin might act as a titin–titin crosslinker (Fig. 4). Its molecular architecture, along with evidence from binding and imaging data

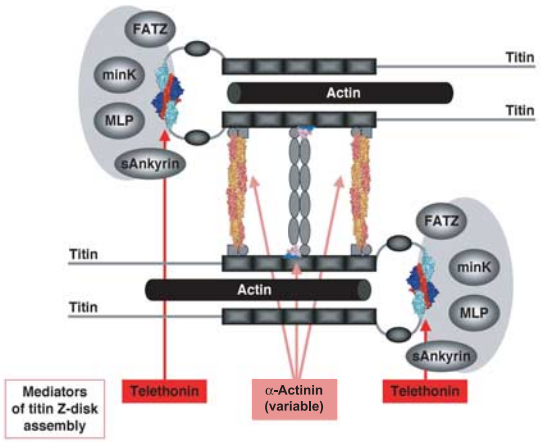

Figure 4 | Model outlining the involvement of the titin–telethonin complex in the architecture of the sarcomeric Z-disk. Titin filaments are assembled by a dual Z-disk bridging system, by α-actinin rods on a variable number of titin Z-repeats (three bridges are shown), and by telethonin by means of the N-terminal immunoglobulin domains Z1 and Z2. The titin N-terminus/telethonin complex forms a core that interacts with several ligands both inside and outside the sarcomeric Z-disk, including MLP, sAnkyrin, the β-subunit of the potassium channel (minK) and the γ-filamin/ABP-L, α-actinin and telethonin binding protein of the Z-disc (FATZ).

both here and in previous papers[3,4], indicates that binding is very tight and may even be irreversible in the absence of signals that would weaken or degrade the interaction. The temporal delay in the proper localization of telethonin and its selective disappearance in neurogenic atrophy[21] might indicate that telethonin turnover is regulated, either intrasterically or by as yet unknown alterations in the telethonin structure. In support of this, there is accumulating evidence indicating that the observed titin–telethonin assembly might interact with other protein components[22–25] that generally seem to be more mobile than titin and telethonin[7,26].

The proposed titin–titin linker function of telethonin is analogous to that of the actin–titin linker of α-actinin[6]. However, in comparison with the α-actinin rod structure, the two terminal β-hairpin wing motifs in telethonin provide a much shorter linker, leading to a sandwich-type rather than to a rod-type linker model. In this complex, even though the shortest distance between the two titin N termini is only in the range of 4–15 Å, there are no direct specific titin–titin interactions. Thus, in structural terms, the function of telethonin is to tether the two titin N termini in close proximity to each other, unlike the α-actinin linker that provides a spacer of more than 200 Å (refs 6, 27). By unravelling the molecular basis of telethonin-mediated titin assembly, an overall picture is emerging on how the protein networks in the sarcomeric Z-disk may contribute to titin assembly and anchoring through at least two ligands (α-actinin and telethonin). This structure resists the mechanical forces generated in active muscle sarcomeres[28] and may feed back to the Z-disk stretch sensor machinery[7,10]. Our data explain how some serious hereditary muscle diseases may be associated with the disruption of molecular interactions that connect and anchor sarcomeric filaments in the Z-disk by bridging mediators.

METHODS

Preparation of the titin–telethonin complex. A titin construct encoding domains Z1 and Z2 (1–196) and several telethonin variants comprising the full-length sequence (residues 1–167) or the N-terminal titin-binding region (1–90) were cloned, expressed and purified as described previously[29]. In telethonin, Cys 8, Cys 15, Cys 38, Cys 57 and Cys 127 (1–167 only) were mutated into serine residues. Production of the seleno-L-methionine (SeMet)-incorporated telethonin is described in Supplementary Information.

Fluorescence imaging by *in vivo* complementation. Neonatal rat cardiomyocytes were prepared as described previously[30]. For transfection assays, the pCMV-5 plasmid or the pEGFP plasmids (Clontech) were used. Telethonin was cloned bearing an N-terminal T7-tag sequence (MTGGQQMGR) or a carboxy-terminal green fluorescent protein (GFP) tag, because N-terminal GFP tags were found to act as dominant-negative proteins and to disrupt myofibrils. Transfection of cells was performed 1 day after plating with a standard liposome carrier system (Escort III) in accordance with the manufacturer's instructions (Sigma). At 24–48 h after transfection, cells were fixed in 4% paraformaldehyde/PBS for 10 min and stained with different antibodies as described previously[30]. For the protein complementation experiments, titin(Z1Z2) complementary DNA was cloned by polymerase chain reaction and fused to either the N- or C-terminal region of YN(1–172) or YC(156–239) of YFP. COS-1 cells were co-transfected with haemagglutinin (HA)-tagged telethonin(1–90) or telethonin(91–163)–HA, together with either YN–titin(Z1Z2) and YC–titin(Z1Z2) or YN–titin(Z1Z2) and titin(Z1Z2)–YC. Cells were fixed 2 days after transfection and stained as described previously[30].

FRET analysis. Four different single cysteine-containing versions of the titin N terminus (Cys 3, Cys 18, Cys 99 and Cys 195) were used for labelling with Alexa488 (donor) and Cy5 (acceptor). For fluorescence measurements, the titin–telethonin complexes of the four mutants and wild-type titin(Z1Z2), as a negative reference, were mixed in the following molar ratios: protein:acceptor, 10:1; protein:acceptor, 1:40; protein:donor, 10:1; protein:donor, 1:40; protein:acceptor:donor, 1:50:1. The labelled probes were separated by gel filtration. To determine the concentration of each dye bound to the donor–acceptor sample adducts the absorbance was measured for $\lambda = 230$–900 nm. The fluorescence spectra were scanned for $\lambda = 500$–800 nm, with an excitation wavelength $\lambda_{ex} = 494$ nm. All experiments were performed in the dark. The energy transfer efficiency, E, of FRET was calculated as a function of the donor–acceptor distance (R_{AD}); $E = 1/(1 + (R_{AD}/R_0)^6)$, where R_0 is a DA-pair-specific constant, the Förster radius ($R_0 = 49$ Å) for the donor–acceptor pair used. Corrected fluorescence

spectra were used to determine the quenching effect of the donor emission intensity when an acceptor was present within the FRET distance. The spectrum of the donor-labelled Z1Z2(C195) mutant (Z1Z2-D) was used as a reference. *E* values were determined directly from the fluorescence intensity: $E = 1 - I_{AD}/I_D$, where I_{AD} is the fluorescence intensity at 524 nm of the acceptor–donor labelled sample and I_D is the fluorescence intensity of Z1Z2-D at 524 nm.

X-ray structure determination. Crystals of the titin–telethonin(1–90) complex were grown by vapour diffusion, mixing equal volumes of about 15 mg ml^{-1} protein solution and mother liquid containing 5% w/v 8,000 kDa poly(ethylene glycol) (PEG 8000) and 100 mM sodium citrate buffer pH 4.45. Crystals were first grown in clusters of thin plates and were then used for macroseeding. In the seeding step, 6 mg ml^{-1} protein solution was mixed with 7.5% w/v PEG 35000, 100 mM sodium citrate buffer pH 4.45 and 200 mM Mg_2SO_4. X-ray data were collected on the tunable wiggler beamline BW6 (MPG-ASMB/DESY, Hamburg) and beamline X11 (EMBL/DESY, Hamburg). The X-ray structure was determined with the use of experimental phases from a selenomethionine version of the complex. Details of X-ray data acquisition, processing and structure determination are described in the legend to Supplementary Table S2.

Received 3 June; accepted 18 October 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank D. Fürst for the gift of antibodies; R. Kühnemuth for discussions on the FRET experiments; M. Forster for involvement in expression and purification tests; G. Burenkov for assistance during data collection at beamline BW6 at MPG-ASMB/DESY; E. Mandelkow and M. von Bergen for making the fluorimeter at MPG-ASMB/DESY available; and E. Ehler for the preparation of neonatal rat cardiomyocyte cultures. N.P. and S.L. were supported by the EU research and training network CAMKIN to M.W. and M.G., respectively. During involvement at the project, O.M. was supported by an EU Marie-Curie postdoctoral fellowship.

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