Targeted homozygous deletion of M-band titin in cardiomyocytes prevents sarcomere formation

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Summary

Titin, a multifunctional protein that stretches from the Z-disk to the M-band in heart and skeletal muscle, contains a kinase domain, phosphorylation sites and multiple binding sites for structural and signalling proteins in the M-band. To determine whether this region is crucial for normal sarcomere development, we created mouse embryonic stem cell (ES) lines in which either one or both alleles contained a targeted deletion of the entire M-band-coding region, leaving Z-disk-binding and myosin-filament-binding sites intact. ES cells were differentiated into cardiomyocytes, and myofibrillogenesis investigated by immunofluorescence microscopy. Surprisingly, deletion of one allele did not markedly affect differentiation into cardiomyocytes, suggesting that a single intact copy of the titin gene is sufficient for normal myofibrillogenesis. By contrast, deletion of both alleles resulted in a failure of differentiation beyond an early stage of myofibrillogenesis. Sarcomeric myosin remained in non-striated structures, Z-disk proteins, such as α-actinin, were mainly found in primitive dot-like structures on actin stress fibres, M-band-associated proteins (myomesin, obscurin, Nbr1, p62 and MURF2) remained punctate. These results show that integration of the M-band region of titin is required for myosin filament assembly, M-band formation and maturation of the Z-disk.

Key words: Titin, Muscle, Myofibrillogenesis, Gene targeting

Introduction

Titin, the largest protein so far described, is a long, filamentous molecule found in the sarcomeres of skeletal and cardiac muscle that has many functions (reviewed by Tskhovrebova and Trinick, 2003). A single molecule stretches from the Z-disk (N-terminus) to the M-band (C-terminus) (Fürst et al., 1988; Wang et al., 1991) and titin molecules overlap in the Z- and in the M-band (Gregorio et al., 1998, Young et al., 1998, Obermann et al., 1996). The I-band region of titin is partially elastic and involved in passive tension development in muscle (reviewed by Tskhovrebova and Trinick, 2001; Tskhovrebova and Trinick, 2003). The A-band region of titin is thought to act as a molecular ruler and regulates the length of the thick filament (Labeit et al., 1990). The M-band and Z-disk regions incorporate titin into the transverse anchoring planes of myosin and actin filaments, respectively. The Z-disk and M-band thus have a predominantly mechanical function, withstanding the strain on the contractile filaments during passive stretch and active contraction (Agarkova and Perriard, 2005). Both compartments also contain a large number of non-structural proteins, many of which emerge in signalling pathways involved in stretch-sensing and myocyte growth (reviewed by Lange et al., 2006).

A crucial role of titin is its ability to regulate muscle sarcomere formation. It does this by interacting with many structural components, and possibly by signalling that the structure is in place and functioning normally. The structural components include α-actinin at the Z-disk, myosin and MyBP-C in the A-band and myomesin and M-protein at the M-band (reviewed by Lange et al., 2006). Titin performs its signalling function by interacting with telethonin (also known as t-cap) at the Z-disk, and MURF1 and MURF2, obscurin and DRAL/FHL-2 at the M-band. Some of these proteins are found at both the Z- and M-bands (reviewed by Lange et al., 2006). The variable location of several of these signalling proteins at both the M- and the Z-disk in response to mechanical strain suggests that communication between these two structures may be important in load-dependent maintenance or turnover of the muscle sarcomere.

Furthermore, titin also has a signalling role itself through its titin kinase (TK) domain, which is found at the M-band (Labeit et al., 1992). TK is dually inhibited by a C-terminal autoinhibitory domain, and by an inhibitory tyrosine residue (Y170) (Mayans et al., 1998). TK can phosphorylate telethonin in vitro, and this was suggested to be important for early myofibrillogenesis (Mayans et al., 1998). However, most telethonin is at the Z-disk in skeletal and cardiac muscle, nearly 1 μm away from TK in the M-band (Gregorio et al., 1998; Mues et al., 1998). Therefore, telethonin would need to interact with TK before it becomes incorporated into the Z-disk where it interacts with Z-disk titin (Zou et al., 2006), either in the form of a soluble pool with transient interaction with TK at the M-band, or by interacting with TK before titin and telethonin are incorporated into the muscle sarcomere. More recent work

suggests that both TK activity and, therefore, possibly in vivo phosphorylation of telethonin might be more important for sarcomere maintenance and turnover in adult muscle than for primary myofibrillogenesis, similar to the mechanically sensitive TK interaction with the Nbr1, p62 and MURF2 complex (Lange et al., 2005). In inactive muscles, this complex dissociates, MURF2 translocates to the nucleus and contributes to downregulation of muscle-specific gene expression. A mutation in TK causes an autosomal dominant, late-onset human myopathy (HMERF) by interrupting this pathway (Lange et al., 2005). Deletions and point mutations in human telethonin also result in skeletal and cardiac myopathies (Moreira et al., 2000; Hayashi et al., 2004).

In addition to TK signalling, the M-band region of titin itself plays a key role in the structural integrity of the sarcomere during contraction, and during myofibrillogenesis (Agarkova et al., 2003; Agarkova and Perriard, 2005). Selective deletion of the two M-band coding exons (MEx1 and MEx2) in skeletal muscle using a Cre-*loxP* system (Gotthardt et al., 2003) resulted in transgenic mice that suffered from muscle weakness. This internal deletion removes the mapped binding sites for MURF1/2/3, DRAL/FHL2, myomesin and calpain p94, as well as TK and multiple developmentally regulated phosphorylation sites in the M-band insertions between titin domains M3 and M4, and M5 and M6 (Gautel et al., 1993). However, MEx1 and MEx2 were not completely deleted, and some expression of normal full-length titin persisted until the mice were 10 days old when the activity of the *mck*-promoter driving the Cre-*loxP* system was maximal. Despite this, skeletal muscle sarcomeres ranged from relatively normal to highly disrupted. Cre-mediated homozygous deletion of this region in the heart resulted in early embryonic death, and so myofibrillogenesis in the heart could not be investigated. More recently, this conditional targeting was converted to a constitutive knockout (Weinert et al., 2006), and in this case, early myofibrillogenesis in cardiac cells appeared normal, but sarcomeres failed to expand laterally and eventually disassembled. In a second study, titin was truncated using a gene-targeting approach in a cultured muscle cell line, deleting TK and the downstream sequence in one of the titin alleles (Miller et al., 2003). This mutation resulted in reduced myoblast fusion and disrupted myofibrillogenesis, and similar to the constitutive knockout animals, lateral alignment of sarcomeres was affected. However, in both of these studies, both TK function and a range of other protein interactions, as well as the multiple phosphorylation sites in M-band titin itself are affected, and so the effects cannot solely be attributed to lack of TK itself.

None of these studies could investigate the effect of the complete deletion of TK and all of the downstream sequence on early myofibrillogenesis, especially on myofibrillogenesis in cardiac cells. Therefore, an outstanding question is to what extent sarcomeres can assemble normally if the M-band region of titin is completely missing. To investigate this, we used a gene-targeting strategy in mouse embryonic stem (ES) cells to delete the TK-encoding region from one and both alleles of the titin gene. This was predicted to result in a truncated protein lacking the TK domain and remaining C-terminal sequence. ES cells heterozygous and homozygous for this targeted modification were subsequently differentiated into beating cardiomyocytes to investigate the myofibril structure.

Results

Generation of ES cells heterozygous and homozygous for the TK deletion

The vectors were designed to create a targeted allele in which the region encoding the TK domain of titin was completely deleted. The targeted allele was therefore predicted to produce a mutant titin protein that lacks kinase activity and is also truncated after the deletion position as a consequence of the inserted selection-marker-cassette sequences. The vectors were applied to produce cells heterozygous and homozygous for the targeted modification by sequential transfections. In the initial round of transfection, using the *neo/tk*-containing vector, 14 clones were identified (targeting frequency of approximately 13%) with a single correctly targeted allele (Fig. 1A).

Two of these heterozygous targeted clones (i.e. heterozygous for the TK deletion mutation), NEO^R $GANC^S$ 1-F9 and 5-A8, were confirmed to have a normal karyotype and clone NEO^R $GANC^S$ 1-F9 was further utilised in two different strategies designed to inactivate the remaining wild-type (WT) allele. In the first strategy (Fig. 1B), following transfection with the targeting construct containing the *puro/tk* cassette, 106 puromycin-resistant clones were recovered and screened by Southern blotting. Several targeted clones were identified (targeting frequency of 3.8%) and of these, four clones had been targeted at the second allele (Fig. 2). These homozygous targeted clones (i.e. homozygous for the TK deletion mutation), NEO^R $PURO^R$ $GANC^S$ 1-G6, 1-H6, 2-B11 and 2-C5, were confirmed to have a normal karyotype. In the second strategy (Fig. 1C) the original *neo/tk* cassette was removed from the NEO^R $GANC^S$ 1-F9 cell line by transient Cre expression and consequent recombination between the direct-repeat *loxP* sites flanking the cassette. Following Cre transfection and selection in ganciclovir, 91% of resistant clones were identified with the expected cassette excision as determined from the Southern blot analysis (Fig. 2). This left a single *loxP* site at the deletion position that was also predicted to result in a frameshift mutation in the downstream titin-coding sequence and thus produce a truncated protein. One of these ganciclovir-resistant clones, NEO^S $GANC^R$ 2-A10, was subsequently transfected with the targeting vector containing the *neo/tk* cassette, and of 148 G418-resistant clones screened, one was identified in which the remaining WT allele was targeted. This homozygous targeted clone NEO^R $GANC^S$ 1-D9 was confirmed to have a normal karyotype.

All of the various clones were subsequently expanded, differentiated and analysed by immunocytochemistry.

A single WT copy of a normal titin allele is sufficient for cardiac myofibrillogenesis

Following 10 days of differentiation in hanging-drop cultures, cultures of both WT and cells that were heterozygous for the mutant titin allele contained strongly beating cells by the end of the differentiation period. Overall, the staining patterns for many different sarcomeric proteins were remarkably similar between heterozygous and WT cells (Figs 3, 4). Triple-immunostaining for actin, α-actinin and myosin showed a regular appearance of the myofibrils in WT and heterozygous targeted cells (Fig. 3). Measurements of the sarcomere length measured from the Z-disk repeat in cells immunostained for Z-disk titin, was similar for WT and heterozygous targeted ES cells. Sarcomere length was 1.92+0.02 μm (mean ± s.e.m., n=15, where n=number of cells)

in WT cells and 1.86+0.03 μm (n=15) in heterozygous targeted cells. In addition, the area of cells that immunostained positively for sarcomeric myosin was the same in both WT and heterozygous targeted cells (data not shown).

Next, we investigated whether there was an effect on M-band organisation, because of the deletion of the M-band region of a single titin allele. Results from immunostaining suggest that there was no disruptive effect on M-band organisation. However, sarcomeres appeared a little less ordered and less parallel in heterozygous targeted cells, suggesting that the truncated titin M-band might interfere with later steps of M-band integration. Triple-immunostaining for actin, the M-band epitope of titin (using the T51 antibody) and α-actinin shows the expected alternating pattern of Z- and M-bands in both WT and heterozygous targeted cells. Triple staining for the Z-disk epitope of titin (using the Z1Z2 antibody), myomesin and myosin, again showed the expected alternating staining pattern for Z-disk titin and myomesin, suggesting that myomesin is correctly localised to the M-band in both WT and heterozygous targeted cells (Fig. 3). In addition, double-immunostaining for obscurin and α-actinin (Fig. 4) showed an alternating pattern of staining, because these two proteins organise into the M-bands and Z-disks, respectively, in mature sarcomeres. We only very occasionally observed Z-

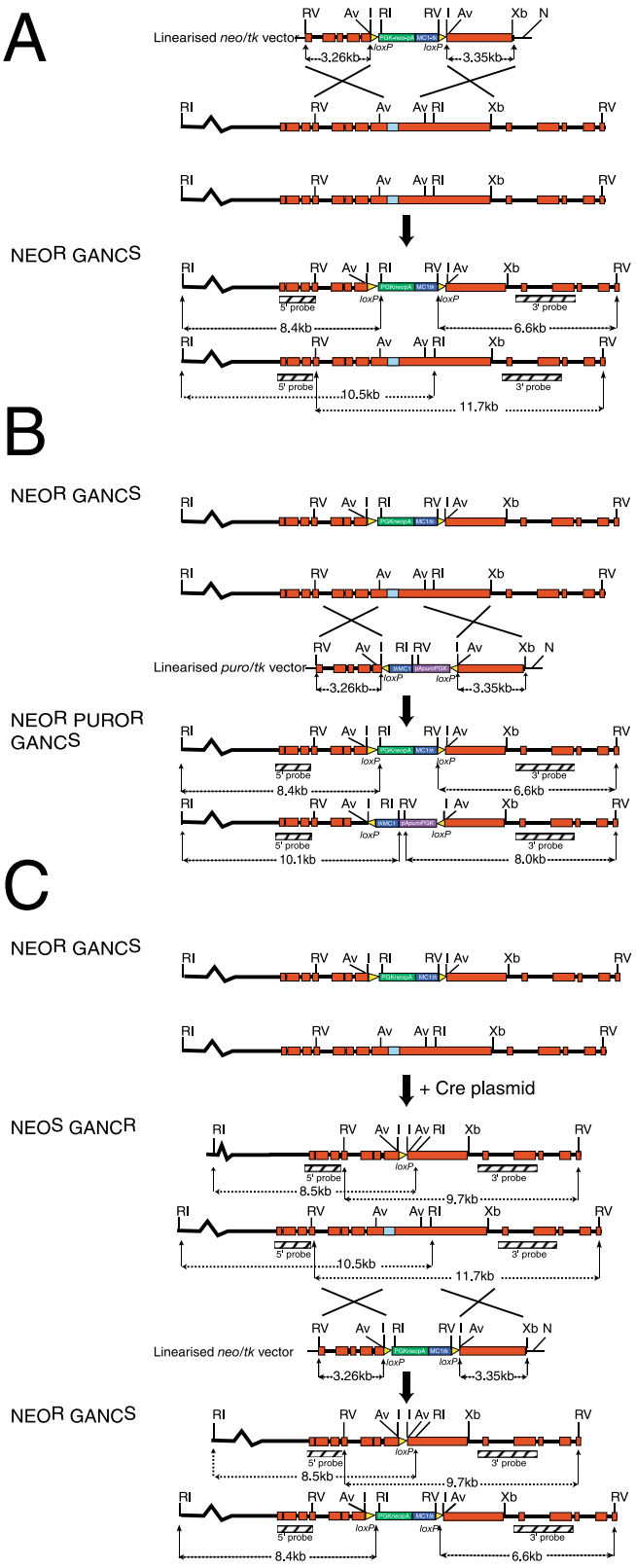

Fig. 1. Generation of ES cells heterozygous and homozygous for a Titin M-band deletion. (A) Generation of an ES cell clone heterozygous for a Titin M-band deletion. A linearised *neo/tk* Titin targeting vector was integrated by homologous recombination into one WT *Titin* allele to generate a heterozygous targeted clone, NEO^R GANC^S 1-F9. (WT – WT cells with two normal chromosomes.) (B) Generation of ES cell clones homozygous for a Titin M-band deletion utilising a *puro/tk* cassette. A linearised *puro/tk* targeting vector was integrated by homologous recombination into the remaining WT *Titin* allele of clone NEO^R GANC^S 1-F9 to generate homozygous targeted clones NEO^R PURO^R GANC^S 2-C5, 2-B11, 1-H6 and 1-G6 (labelled as 2-C5 etc on the figure). (C) Generation of an ES cell clone homozygous for a Titin M-band deletion utilising Cre recombination. Clone NEO^R GANC^S 1-F9 was transiently transfected with the pCAGGS-Cre-IRES*puro* plasmid to excise the *neo/tk* cassette by Cre-mediated recombination between the direct-repeat *loxP* sites flanking the cassette. A resultant clone, NEO^S GANC^R 2-A10, contained a single *loxP* site at the deletion position. In a second round of targeting a linearised *neo/tk* Titin targeting vector was integrated by homologous recombination into the remaining WT *Titin* allele to generate a homozygous targeted clone, NEO^R GANC^S 1-D9. Restriction enzyme sites indicated are: *Ava*I (*AV*), *Eco*RI (RI), *Eco*RV (RV), *I-Sce*I (*I*), *Not*I (*N*) and *Xba*I (*X*). Confirmation that the allele had been targeted was performed by Southern blot analysis of *Eco*RI (see Fig. 2) and *Eco*RV (data not shown) digested genomic DNA, using probes flanking the 5′ and 3′ homology arms, respectively. The sizes of the expected restriction fragments are shown in Fig. 2. Exon sequences are shown as red boxes (but with the kinase-encoding domain in one exon highlighted in pale blue), intron sequences as a thick black line, plasmid vector sequences as a thin black line, selection marker sequences as green (PGK*neo*pA), blue (MC1*tk*) and purple (pA*puro*PGK) boxes, *loxP* sites as yellow triangles, and probe sequences as hatched boxes.

A

Origin of band:	Homozygous targeted NEO^R PURO^R 2-C5, 2-B11, 1-H6 & 1-G6	Homozygous targeted NEO^R CRE 1-D9	Heterozygous targeted CRE 2-A10	Heterozygous targeted NEO^R 1-F9	WILD TYPE
Targeted *neo/tk* cassette	8.4kb	8.4 kb		8.4kb	
Targeted *puro/tk* cassette	10.1kb				
Excised cassette allele		8.5kb	8.5kb		
wt allele			10.5kb	10.5kb	10.5kb

B

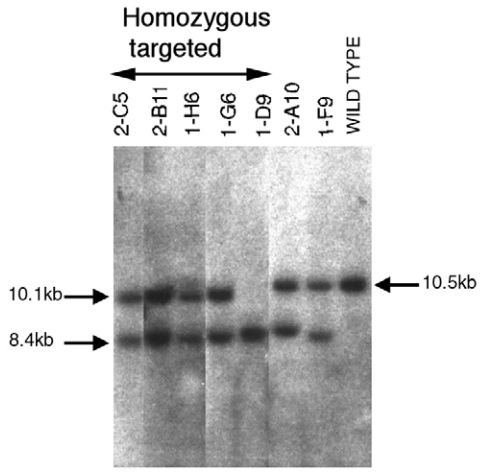

Fig. 2. Confirmation that the alleles had been targeted to generate heterozygous or homozygous clones as detailed in Fig. 1. (A) Table showing the sizes of the expected *Eco*RI restriction fragment sizes for each type of clone as detected by the 5′ probe. (B) Southern blot analysis of *Eco*RI digested genomic DNAs from clones heterozygous and homozygous for the deletion using the 5′ probe. The result for 1-D9 appears to show a single band, but there are in fact two restriction fragments running very close together (8.5 and 8.4 kb).

disk localisation of obscurin (data not shown). Both M-band and Z-disk obscurin staining has been previously reported (Bang et al., 2001; Borisov et al., 2004; Young et al., 2001).

We further investigated whether deletion of the kinase and downstream sequence in a single titin allele affected the localisation of the first-described substrate for YK, telethonin. We found that in both control and heterozygous targeted cells, telethonin was colocalised with Z-disk titin epitopes (Fig. 4) with a significant pool of diffusely localised protein. This localisation was previously described for 6-day human myotube cultures (Mues et al., 1998). Therefore, telethonin localisation appears unaffected in the heterozygous targeted cells.

Finally, we investigated whether MURF2 localisation was affected in heterozygous targeted cells, and found that there appeared to be some disruption to the M-band localisation of MURF2 in heterozygous targeted cells (Fig. 4). In WT cells,

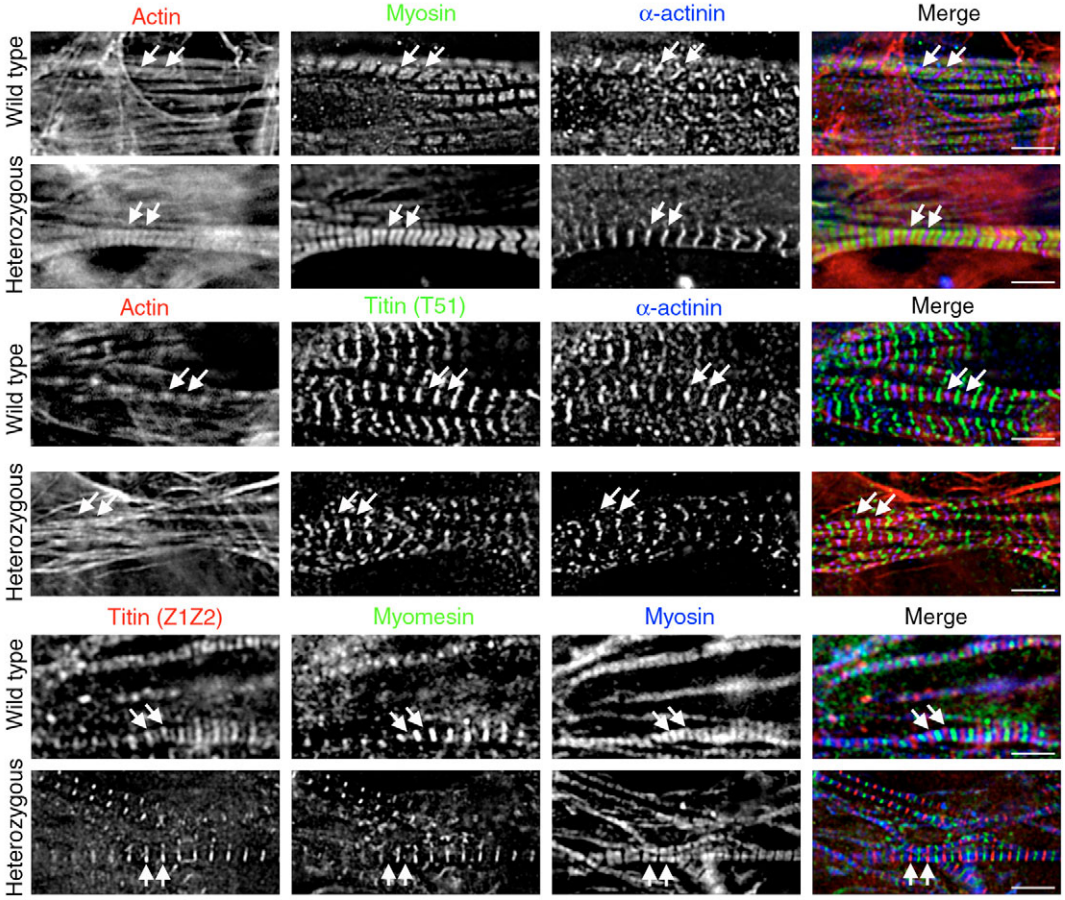

Fig. 3. Immunostaining of WT and heterozygous targeted cells shows myofibrillogenesis is normal. WT and heterozygous targeted cells (clone 5-A8) were coimmunostained for α-actinin and skeletal myosin or α-actinin and M-band titin, and for actin using Alexa-546 phalloidin, or stained for titin (Z1-Z2 antibody) myomesin and myosin, and imaged using a deconvolution microscope. Arrows show the positions of the Z-disks in each of the images. In the top four panels, the Z-disks stain positively for α-actinin and actin (thus, purple in the merged image). Myosin staining is clearly defined by a doublet between the Z-disks stained for α-actinin. The M-band region of titin is clearly seen as a stripe at the middle of the sarcomere between the two Z-disks stained for α-actinin. In the bottom two panels, the Z-disks stain immunopositively for titin (Z1-Z2 antibody), and myomesin is found midway between the Z-disks at the M-band. Occasional weak staining with the Z1-Z2 antibody at the M-band, which we assume is an anomalous cross-reaction, is sometimes observed. Bars, 5 μm.

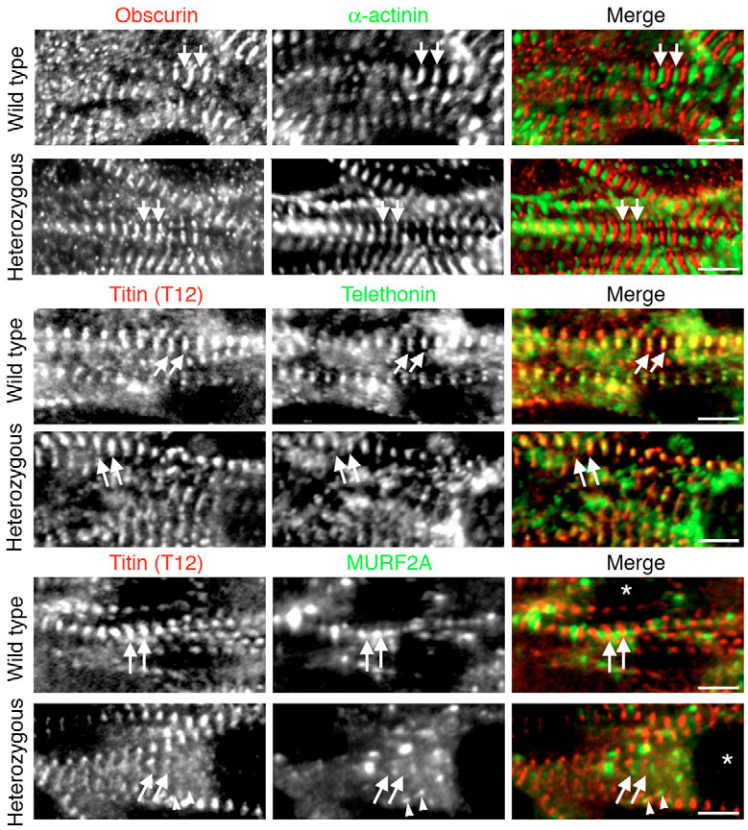

Fig. 4. Immunostaining of WT and heterozygous targeted cells shows normal localisation of obscurin and telethonin, but some mislocalisation of MURF2. WT and heterozygous cells were coimmunostained for obscurin and α-actinin, Z-disk titin (T12 antibody) and telethonin, or for Z-disk titin (T12 antibody) and MURF2 and imaged using a confocal microscope. The arrows show the alternate localisation of obscurin at the M-band and α-actinin at the Z-disk in the top pair of panels for WT and heterozygous cells (clone 5-A8). Titin and telethonin are both localised to the Z-disk (arrows in the middle panels) for WT and heterozygous cells (clone 1-F9). There is also a diffuse cytoplasmic pool of telethonin. In the bottom pair of panels, the arrows show the alternate localisation of titin at the Z-disk and MURF2 at the M-band in WT cells, but this alternate localisation is less clear in the heterozygous cells (clone 5-A8). Arrowheads indicate the M-band localisation, arrowheads show spots and/or streaks in the heterozygous cells. Bars, 5 μm.

we found that MURF2 localised to the M-band, and was also found in spots, occasional streak-like structures that are likely to arise from its association with a subset of microtubules (Fig. 4). We found a similar pattern in heterozygous cells, except that the M-band localisation was less clear and MURF2 appeared in spots and aggregates more frequently.

Mutation of both titin alleles arrests myofibrillogenesis at an early stage

In contrast to the heterozygous targeted cells, differentiated cultures of cells homozygous for the titin mutant allele failed to beat at all, or rarely only beat very weakly, even when allowed to differentiate for up to 4 weeks. We observed the same results for all the homozygous targeted ES lines generated. However, the number of cells that stained positively for sarcomeric myosin was similar to that in WT and heterozygous targeted cells, suggesting that a similar number of cells in the culture became committed to differentiating into the cardiac lineage. The lack of beating was explained by a severe disruption of myofibrillogenesis, which was clearly shown by immunostaining for a wide range of sarcomeric proteins.

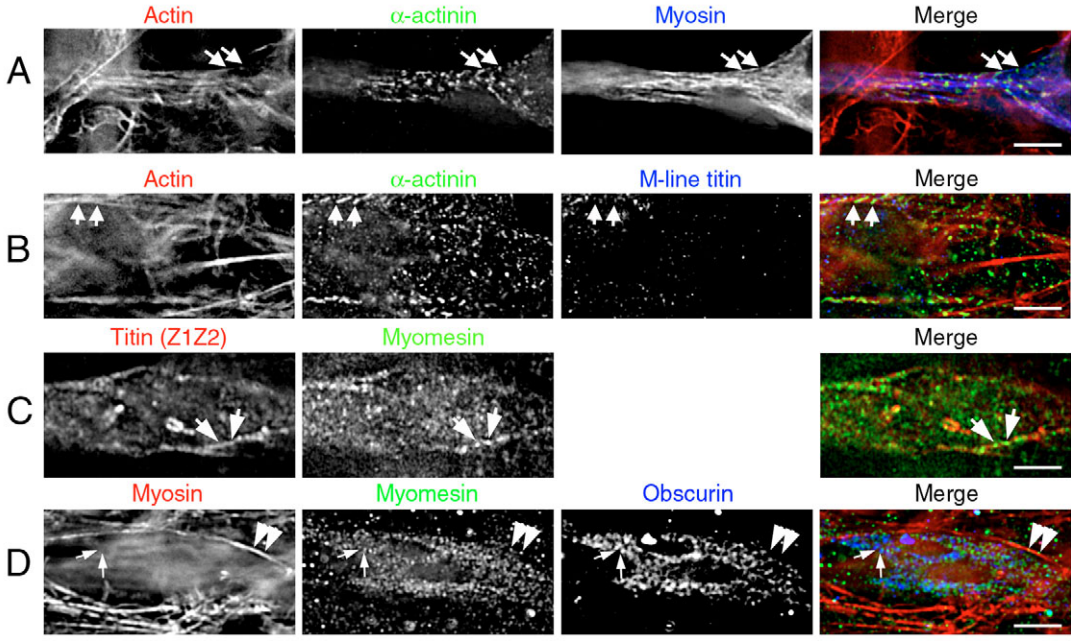

Fig. 5. Myosin, myomesin, obscurin and titin lack ordered organization in homozygous targeted cells. Confocal images of triple-stained homozygous targeted cells. Cells were co-stained for (A) actin (using Alexa-546 phalloidin), the Z-disk protein α-actinin, and myosin; (B) actin (using Alexa-546 phalloidin) α-actinin, and the M-band region of titin; (C) the Z-disk epitope of titin, myomesin and DAPI; or (D) myosin, myomesin and obscurin. Arrows in A and B show stripes of α-actinin staining that might be immature Z-disks and which have a spacing of approximately 1 μm. Arrows in C show close association of titin spots with myomesin. Arrows in D show occasional colocalisation of myomesin and obscurin. Arrowheads show spots of the rare association of myomesin spots with myosin. Bars, 5 μm.

Although many cells could be identified that stained positively for a variety of sarcomeric proteins, myofibrillar organisation was completely lacking (Figs 5, 6). Triple coimmunostaining for actin, myosin and α-actinin in homozygous targeted cells showed a primitive pattern of localisation, which resembles that of nascent myofibrils despite the long time allowed for differentiation (Fig. 5). Myosin is not organised into the characteristic double stripes found in WT and heterozygous targeted cells, but found in a disorganised filamentous pattern, similar to that found in very early myofibrillogenesis, before myosin is organised into muscle sarcomeres (Fig. 5A). These filamentous myosin structures colocalise with actin filaments, and α-actinin staining along these actin filaments is punctate or striped. However, the spacing of the α-actinin stripes is shorter – only approximately 1 μm compared with over 2 μm in mature myofibrils (Fig. 5). Triple staining for actin, α-actinin and M-band titin, using an antibody (Ti51) raised against MEx9 (Van der Ven et al., 1999), showed that the staining for Ti51 was absent as expected, as this region of the titin is missing (Fig. 5B). However, puncta of α-actinin on actin filaments can be found in the same cells, indicating that these cells are myogenic (Fig. 5B).

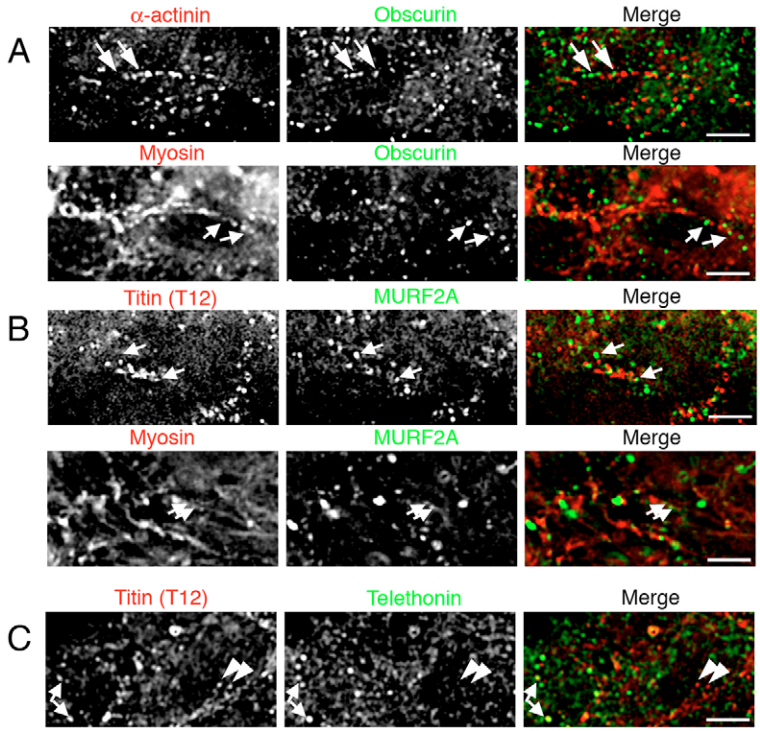

Fig. 6. Mislocalisation of obscurin, telethonin and MURF2 in homozygous targeted cells. Cells were coimmunostained for a range of sarcomeric proteins as indicated. Obscurin is shown for cells co-stained with either α-actinin or myosin. (A) In the top two panels, arrows indicate puncta that stain positively for obscurin. (B) In the middle two panels, MURF2 is shown for cells co-stained with either the Z-disk epitope of titin (T12) or myosin. Arrows indicate puncta that stain positively for MURF2. (C) In the bottom set of panels, cells are co-stained for Z-disk titin and telethonin, and there is some colocalisation of these two proteins (arrows). Many titin dots do not localise with telethonin, however (arrowheads). In all cases, sarcomeric organisation is lacking, and only sporadic colocalisation with titin is observed even for obscurin and telethonin, both of which contain Z-disk-binding sites. Bars, 5 μm.

Myomesin, obscurin and MURF2, which were localised at the M-band in WT cells, were all mislocalised in homozygous cells. In the homozygous targeted cells, staining for myomesin was weak, but showed that myomesin did not associate with the Z-disk epitope of titin or myosin (Fig. 5C,D). However, there was a better correlation of myomesin along filamentous structures stained for titin, than for myosin. Myomesin is known to bind to the light meromyosin region of myosin as well as to the M-band region of titin (Obermann et al., 1997). Thus both of these interactions are ablated by deletion of the M-band region of titin.

Obscurin was found in the same filamentous structures as puncta of myomesin (Fig. 5D), but neither obscurin nor myomesin showed clear colocalisation with filamentous myosin structures (Fig. 5D). Further examples of co-staining for obscurin with either titin or myosin showed a better correlation of obscurin along filamentous structures stained for titin, than for myosin (Fig. 6A), as observed for myomesin. However, no colocalisation with Z-disk titin was observed. By contrast, co-staining for MURF2 and either myosin or titin showed that MURF2 puncta could be found along the same filamentous structures co-stained for myosin, and to a lesser extent along filamentous structures marked by puncta positive for the Z-disk epitope of titin, but there is only occasional co-staining of both antibodies (Fig. 6B).

Deletion of TK might be also expected to affect localisation of telethonin. Coimmunostaining for Z-disk titin and telethonin showed small dots for Z-disk titin that only rarely colocalised with telethonin (Fig. 6C). Thus, telethonin does not consistently bind to the nascent Z-disks in the absence of the kinase and the remainder of the M-band region of titin (Fig. 6C). Moreover, the staining for both α-actinin and Z-disk epitopes of titin (Z1Z2 or T12 antibodies) all show that deletion of the kinase and M-band region of titin affects Z-disk as well as M-band organisation (Figs 5, 6). Staining for Z-disk titin with two different antibodies (Z1Z2, Fig. 5B and T12, Fig. 6) showed random punctate staining and primitive, dot-shaped Z-disk precursors aligned along actin-containing bundles, resembling stress-fibre-like structures (Fig. 5C; Fig. 6B,C). This also demonstrated that the targeted deletion of the M-band region of titin did not result in the complete loss of titin, because the Z-disk epitopes were still present. No ordered Z-disks with the expected mature spacing of approximately 2 μm could be observed, either for the Z-disk titin epitopes or for α-actinin staining (Fig. 5A,B; Fig. 6A).

Finally, we further investigated MURF2 staining to determine whether this protein re-localised to the nucleus, as found for mature muscle when mechanically inactivated or the kinase is mutated (Lange et al., 2006). Normally, MURF2 interacts at the M-band with the TK-associated zinc-finger proteins Nbr1 and p62, but TK has been deleted. We found that the ubiquitously expressed proteins Nbr1 and p62 were localised in a cytoplasmic dot-like pattern, which was very similar to that in the surrounding non-myocytes (Fig. 7). Although Nbr1 can also associate with Z-disks (Lange et al., 2005), no colocalisation with the stress-fibre-like structures stained for Z-disk titin by T12

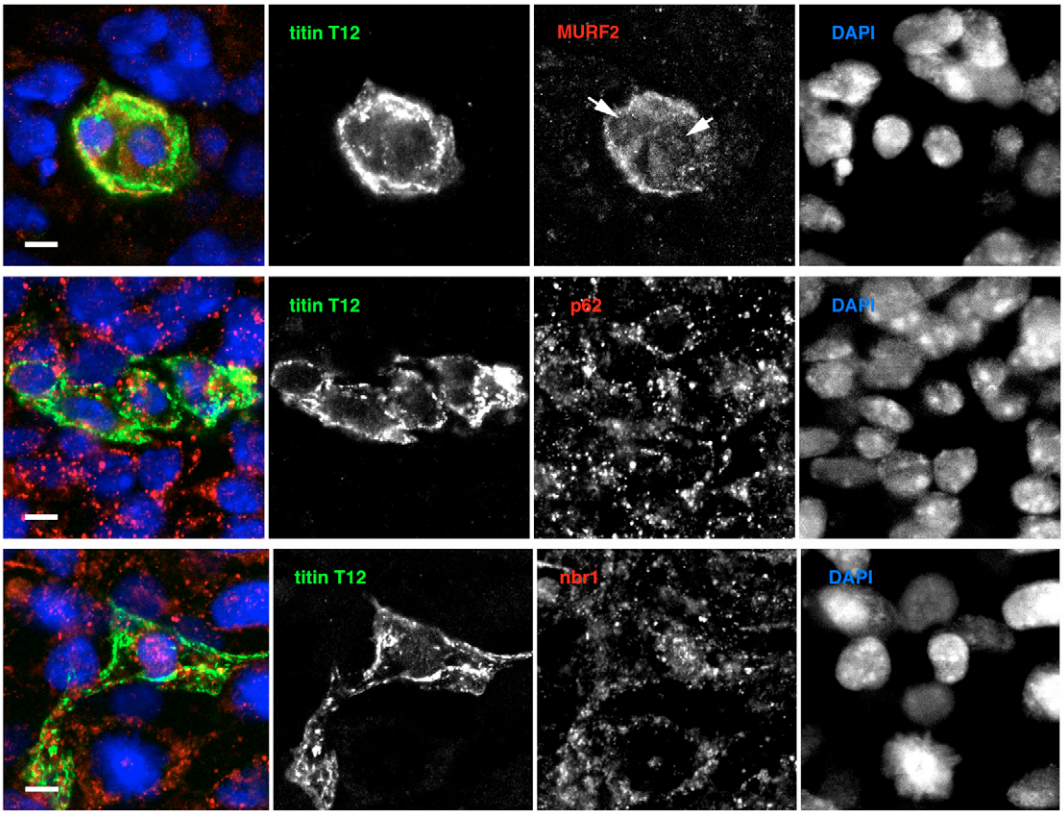

Fig. 7. MURF2, Nbr1 and p62 fail to assemble with titin in homozygous M-band-deficient cells. WT and homozygous targeted cells stained for the titin Z-disk epitope T12 (red) and for Nbr1 p62, and MURF2 (green) under conditions optimal for any nuclear localisation for MURF2, are shown. Association with sarcomeric structures is completely lost for both proteins, which adopt a punctate and diffuse staining pattern. MURF2 and Nbr1 can sometimes be found weakly in the nuclei of some ES cells (arrows), which are stained by DAPI (blue). Bars, 5 μm.

could be detected; a similar picture was observed for p62. Surprisingly, both the muscle-specific MURF2 as well as Nbr1 were only rarely and weakly detected in the nuclei of differentiated homozygous targeted cells (arrows in Fig. 7, and see Fig. 6). This might be because myofibrillogenesis is interrupted at an early stage in the homozygous targeted cells, before levels of MURF2 have become significant, and thus it would be difficult to detect its re-localisation to the nucleus.

Discussion

Our results demonstrate that a targeted deletion of the TK including the C-terminal M-band region of titin severely disrupts myofibrillogenesis in ES-cell-derived cardiomyocytes when homozygous. Myofibrillogenesis is arrested at an early stage in that Z-disc assembly is abnormal, M-bands are not formed and myosin is not organised into muscle sarcomeres. However, the presence of a single WT allele is sufficient in heterozygous targeted cells to ensure almost normal primary myofibrillogenesis with very little detrimental effect on either Z-disk or M-band organisation. The severe effects of the complete deletion of the TK domain and the entire M-band region of titin shows for the first time that the C-terminus of titin must be intact for organisation and assembly of muscle sarcomeres.

The disruption of the M-band is not very surprising, because the entire M-band region of titin is missing in homozygous targeted cells. Deletion of this region prevents M-band formation by blocking the formation of antiparallel dimers of titin in the M-band and by the loss of other proteins important for the formation of the M-band structure. One of these proteins is myomesin (Obermann et al., 1996), which crosslinks myosin and titin in the M-band (reviewed by Agarkova and Perriard, 2005). The localisation of myomesin was disrupted in the homozygous targeted cells. A second protein is obscurin, suggested to form an elastic link between M-bands, or M-bands and the plasma membrane (reviewed by Agarkova and Perriard, 2005). The majority of obscurin localises to the M-band in WT and heterozygous targeted cells, although this protein has been shown in other studies to localise to both the Z-disk and M-band (Bang et al., 2001; Borisov et al., 2004; Young et al., 2001). This M-band localisation is disrupted in homozygous targeted cells and we did not find any obscurin in the primitive Z-disks either, even though binding sites in titin (Z9-Z10) in the Z-disk persist in the homozygous targeted cells. Obscurin is the only known vertebrate analogue of the *C. elegans* giant muscle protein Unc-89, and Unc-89 mutants result in severe disruption of A-bands (Benian et al., 1996). Thus, the failure to recruit obscurin to the M-band in homozygous targeted cells might be expected to have similar effects.

Loss of the M-band region of titin will also affect other proteins that bind in this region, which are involved in downstream signalling pathways important for myofibrillogenesis. The signalling protein MURF2 is mislocalised in homozygous targeted cells, and we expect that other signalling proteins, such as MURF1 and DRAL/FHL2, will also be mislocalised with resultant effects on downstream signalling. The lack of obscurin binding at the Z-disk, mentioned above, could depend on its initial failure to localise to the M-band. Furthermore, until titin is incorporated into the M-band, it is unlikely that mechanical tension will be transmitted to the Z-disk, potentially affecting activation of the

TK. This might explain why we observed very little colocalisation of telethonin with Z-disk titin in homozygous targeted cells, if this localisation is dependent on its phosphorylation by the TK. Although a muscle-specific calpain (calpain-3, or p94), a Ca^{2+}-activated protease, implicated in limb girdle muscular dystrophy type 2A (Kramerova et al., 2004) has also been reported to interact with M-band titin (Sorimachi et al., 1995), this protein is not expressed in the heart, and thus effects on calpain 3 will not contribute to the phenotype we observe here.

As well as the loss of the M-band, we found that myosin failed to incorporate normally into muscle sarcomeres, and was either found in random dot-like structures or in stress-fibre-like structures, similar to those seen at an early stage of myofibrillogenesis (Rhee et al., 1994). This result supports the idea of a hierarchical order of sarcomere formation, where the M-band must form before myosin is incorporated (Ehler et al., 1999; Van der Ven et al., 1999; Rhee et al., 1994). Since M-bands do not form in the homozygous targeted cells, it is unlikely that myosin will become organised into muscle sarcomeres, even though the A-band region of titin – which contains over 170 domains that can bind to myosin (Labeit et al., 1992) – remains intact. In addition, the incorporation of myosin into nascent sarcomeres might require the microtubule network (Pizon et al., 2002 and references therein), with which MURF2 and MURF3 also interact. Myofibrils in neonatal rat cardiomyocytes are relatively stable when treated with nocodazole, which depolymerises microtubules (Rothen-Rutishauer et al., 1998). However, MURF2 preferentially interacts with modified (detyrosinated) microtubules (Pizon et al., 2002), which are relatively resistant to depolymerisation by nocodazole (Gundersen et al., 1994). The deletion of the MURF2-binding site on titin in the targeted cells might contribute to the disrupted assembly of sarcomeric myosin if this interaction is required to regulate microtubule-directed myosin integration with the titin filament.

More surprising is the disruption of Z-disk assembly in the homozygous targeted cells, and this suggests that functions of the titin M-band are required for maturation of the Z-disk. Z-disk formation is an early process believed to occur before mature sarcomeres form (Van der Ven et al., 1999), and thus we might have expected Z-disk formation to be normal. However, as already discussed, many proteins bind at both the M- and Z-disks, and are possibly involved in signalling between these two regions. The loss of the M-band-binding domains for these proteins could result in aberrant, or loss of signalling at the Z-disk that prevents its maturation. Furthermore, the loss of ordered myosin integration could abolish the contractile rearrangement of nascent sarcomeres (Ferrari et al., 1998) that would align nascent Z-disks laterally.

The phenotype described here for homozygous deleted cells is different to that for the previously reported mouse model (Gotthardt et al., 2003; Peng et al., 2006; Weinert et al., 2006). In these mouse models, the exon containing the kinase has been deleted, but the titin domains M8 to M10 remain intact, and apparently M-bands and muscle sarcomeres can initially form even when deleted early in embryogenesis. However, the instability of these sarcomeres, and their failure to persist might be explained by as yet unidentified stabilising interactions in the most C-terminal domains with either myomesin or titin itself.

Interestingly, the heterozygous deletion mutation in ES cells had very little effect on myofibrillogenesis, whereas a similar heterozygous deletion mutation in cultured muscle cells did (Miller et al., 2003; Peng et al., 2006). It is possible that, in cardiac muscle, a single WT titin allele is sufficient to drive myofibrillogenesis. One possible explanation for this is that cardiac cells spontaneously beat, whereas skeletal muscle myotubes only beat sporadically. As mentioned above, the TK interacts in a mechanically sensitive complex with Nbr1, p62 and MURF2 (Lange et al., 2005). In inactive cells, MURF2 relocalises to the nucleus, and this contributes to downregulation of muscle gene expression. Since beating in heterozygous targeted ES cells is similar to that in WT cells, this might be sufficient for recruitment of MURF2 to the M-band, rather than relocalisation to the nucleus, such that control of myofibril turnover is normal. In the homozygous targeted cells, the lack of mechanical activity and the absence of the TK domain does not result in strong nuclear translocation of MURF2, or in intercalated disk localisation of Nbr1 or p62. However, in this case, myofibrillogenesis is arrested at a very early stage and these signalling pathways may not yet be switched on. It appears, therefore, that for the functioning of Nbr1/p62/MURF2 signalling, an intact M-band is required that might recruit further components involved in this process.

In summary, this study underscores the importance of the M-band for myofibrillogenesis. Deletion of this region abrogates binding of M-band proteins and surprisingly also disrupts Z-disk organisation. Such a dramatic effect on myofibrillogenesis conveys the importance of this region of titin. To further study the exact role played by the TK domain, and elucidate how the TK domain is linked to feedback-signalling pathways that control sarcomeric organisation and muscle turnover, more subtle genetic interventions are now required.

Materials and Methods

Targeted modification of the titin gene in ES cells

Two versions of a replacement targeting vector, based in pBluescript plasmid, were constructed, which were identical other than containing different drug-resistance selection markers. Each consisted of approximately 8.6 kb cloned genomic DNA sequence (derived from a 129/Ola strain genomic library) corresponding to the region of the titin gene spanning all the M-line exons. The cloned genomic sequence had been modified with an internal 1.98 kb nucleotide deletion created by removal of an *Ava*I restriction fragment in MEx1 encompassing the coding sequence for the entire kinase domain. The deleted interval was replaced in one of these versions with a cassette consisting of both positive, G418 (*neo*) resistance, and negative, HSV thymidine kinase (*tk*), genetic selection markers flanked by directly repeated *loxP* sites and *I-Sce*I sites (the *I-Sce*I/*loxP*/PGK*neo*/MC1*tk*/*loxP*/*I-Sce*I cassette, henceforth referred to as the *neo/tk* cassette). In the other version a similar cassette was used but containing a puromycin (*puro*) instead of a *neo* resistance marker (the *I-Sce*I/*loxP*/PGK*puro*/MC1*tk*/*loxP*/*I-Sce*I cassette, henceforth referred to as the *puro/tk* cassette), in order to permit a sequential round of transfection and selection in G418-resistant targeted clones (see below). The *puro/tk* cassette was inserted in the reverse orientation to facilitate discrimination from the *neo/tk* targeted allele in the subsequent Southern blot analysis. The vectors were both linearised at a unique *Not*I site prior to transfection into ES cells by electroporation (5×10^7 cells mixed with 75 μg DNA in 600 μl PBS in a 0.4-cm-gap electroporation cuvette, using a Bio-Rad gene pulser set at 0.8 kV, 3 μF). The starting ES cell line was E14-TG2a.IV cultured in standard feeder-independent conditions using medium supplemented with leukaemia inhibitory factor (LIF) (Smith, 1991). Cells were selected for approximately eight to 10 days (in 200 μg/ml G418 or 1.75 μg/ml puromycin, as appropriate), drug-resistant colonies were then picked into 96-well plates, grown for three to four days, and replica plates made for freezing and DNA analysis. To identify correctly targeted drug-resistant clones, their DNAs from 96-well plates were analysed by Southern blot analysis after *Eco*RI and *Eco*RV digestion and hybridisation with probes flanking and external to the vector 5′ and 3′ homology arm sequences, respectively (Fig. 1A-C).

The vector with the *neo/tk* cassette was used for the initial round of targeting to generate G418-resistant ES cell clones heterozygous for the targeted deletion. ES

cells homozygous for the targeted deletion were then generated from one of the heterozygous targeted clones by two alternative methods. In one, a sequential round of transfection was performed using the second version of the targeting vector with the *puro/tk* cassette. Puromycin-resistant clones were analysed as above to identify those in which the remaining WT allele had been targeted. In the second method the cassette in the G418-resistant heterozygous targeted clone was first deleted by Cre recombination between its flanking *loxP* sites. Cells were electroporated with a Cre recombinase expressing plasmid pCAGGS-Cre-IRES*puro*, passaged for four days without selection and then plated at clonal density (10^3 cells per 10-cm plate). Two days after plating the cells were selected in 2.5 μM ganciclovir for four days to recover clones that had lost the cassette. Ganciclovir-resistant clones were screened by Southern blot analysis to confirm these clones contained a deletion with only a single *loxP* site remaining. A second round of homologous recombination was performed on one of these ganciclovir-resistant targeted clones using the targeting vector containing the *neo/tk* cassette, and G418-resistant clones were then screened as above to identify those in which the remaining WT allele was targeted.

Targeted clones at each of the above stages were analysed to determine whether they had a normal karyotype.

In vitro differentiation of ES cells

Non-transfected E14-TG2a.IV cells, and cells of heterozygous and homozygous targeted clones were expanded for in vitro differentiation studies. For differentiation, 20 μl aliquots of cell suspension in LIF-supplemented medium (containing approximately 800 cells) were placed on the upturned lids of Petri dishes containing PBS. Cell were cultured in hanging drops for 2 days and then transferred to Petri dishes for a further 3 days. During this 3-day period, LIF was removed form the media. The 5-day embryoid bodies were separately plated onto Matrigel®-coated cover slips and left to differentiate for 10 days in growth medium without LIF. Cardiac myocyte differentiation was evaluated and these cells fixed with methanol, 4% paraformaldehyde in PBS or 4% paraformaldehyde, 5 mM Mg^{2+}, 5 mM EGTA in cytoskeletal buffer (optimal conditions for observing MURF2 in the nucleus).

Immunofluorescence

After differentiation, cells were fixed as above and permeabilised using 0.1% Triton X-100 prior to staining. The antibodies used in the study were: anti-Z-disk Titin [T12, a gift from D. Fürst (Institute of Cell Biology, University of Bonn, Germany), or Z1/Z2, a gift from S. Labeit (Institute for Anaesthesiology and Intensive Care, University Clinic Mannheim, Germany)]; anti-M-band titin T51 (Van der Ven et al., 1999) a gift from D. Fürst; anti-myomesin My190Nrt (Obermann et al., 1996); A1025, which recognises all skeletal myosin (Cho et al., 1994); anti-sarcomeric α-actinin (Sigma; A7811); anti-obscurin Ob48 (Young et al., 2001); and the proteins of the TK signalosome anti-Nbr1, anti-p62 and anti-MURF2 (Lange et al., 2005). The anti-MURF2 antibody used was first described by Pizon et al. (Pizon et al., 2002) and recognises the A-splice variants p60A, p50A and p27A. Secondary antibodies conjugated to Alexa Fluor-488, Alexa Fluor-564 or Alexa Fluor-647 were purchased from Molecular Probes. Actin was visualised using Alexa Fluor-546-conjugated phalloidin (Molecular Probes). Stained cells were mounted in Pro-Long Antifade (Molecular Probes), and imaged using a Zeiss LSM 510 Meta confocal microscope, using a 64× objective, or a DeltaVision deconvolution microscope (Applied Precision) using 60× or 100× objectives. The resultant images were imported into Adobe Photoshop to generate the figures and to add labelling.

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