Transient association of titin and myosin with microtubules in nascent myofibrils directed by the MURF2 RING-finger protein

Véronique Pizon[1,2], Andrei Iakovenko[3], Peter F. M. van der Ven[4], Raymond Kelly[3], Cristina Fatu[3], Dieter O. Fürst[4], Eric Karsenti[1,2] and Mathias Gautel[5,*]

[1]European Molecular Biology Laboratory, Cell Biology Division, Heidelberg, Germany
[2]Institut Jacques Monod, Paris, France
[3]Max-Planck-Institut für molekulare Physiologie, Department of Physical Biochemistry, 44202 Dortmund, Germany
[4]Potsdam University, Department of Cell Biology, Potsdam, Germany
[5]King's College London, Muscle Cell Biology, The Randall Centre, New Hunt's House, London SE1 1UL, UK
*Author for correspondence (e-mail: mathias.gautel@kcl.ac.uk)

Accepted 24 August 2002
Journal of Cell Science 115, 4469-4482 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00131

Summary

Assembly of muscle sarcomeres is a complex dynamic process and involves a large number of proteins. A growing number of these have regulatory functions and are transiently present in the myofibril. We show here that the novel tubulin-associated RING/B-box protein MURF2 associates transiently with microtubules, myosin and titin during sarcomere assembly. During sarcomere assembly, MURF2 first associates with microtubules at the exclusion of tyrosinated tubulin. Then, MURF2-labelled microtubules associate transiently with sarcomeric myosin and later with A-band titin when non-striated myofibrils differentiate into mature sarcomeres. Finally, MURF2 labelled microtubules disappear from the sarcomere after the incorporation of myosin filaments and the elongation of titin. This suggests that the incorporation of myosin into nascent sarcomeres and the elongation of titin require an active, microtubule-dependent transport process and that MURF2-associated microtubules play a role in the alignment and extension of nascent sarcomeres. MURF2 is expressed in at least four isoforms, of which a 27 kDa isoform is cardiac specific. A C-terminal isoform is generated by alternative reading frame use, a novelty in muscle proteins. In mature cardiac sarcomeres, endogenous MURF2 can associate with the M-band, and is translocated to the nucleus. MURF2 can therefore act as a transient adaptor between microtubules, titin and nascent myosin filaments, as well as being involved in signalling from the sarcomere to the nucleus.

Key words: Myosin, Microtubules, Titin, MURF2, Myofibril assembly, Connectin

Introduction

The smallest contractile units in striated muscles, the sarcomeres, are highly regular arrays of the contractile proteins myosin and actin. The formation of sarcomeres in striated myofibrils requires that a large number of different proteins be assembled in polymeric structures of great precision (Gautel et al., 1999; Sanger et al., 2000; Trinick, 1994). Actin and myosin filaments of precise length have to be aligned in a regular fashion, together with a large number of structural and accessory proteins. The giant muscle protein titin has emerged as a major molecular blueprint for this function, assisted, possibly, by other giant signalling proteins, nebulin and obscurin (Sanger and Sanger, 2001; Trinick, 1996). Other cytoskeletal systems like intermediate filaments and microtubules undergo major rearrangements in parallel, but their function in muscle morphogenesis is still poorly understood. Microtubules are dynamic structures that fulfil many functions ranging from organelle transport to mitosis, including myogenesis (Goldstein and Entman, 1979; Guo et al., 1986; Rothen-Rutishauser et al., 1998; Saitoh et al., 1988; Toyama et al., 1982). During myogenesis, morphological analysis demonstrated that microtubules form an elongated network closely associated with sarcomeres in heart muscle (Goldstein and Entman, 1979), with individual microtubules integrated into the sarcomeric filament lattice. Induction of cardiac hypertrophy, and thereby formation of new sarcomeres, is associated with the rapid increase in the number of microtubules and their reorganisation parallel to the myofibrillar axis (Rappaport et al., 1984; Rappaport et al., 1985; Saitoh et al., 1988; Samuel et al., 1984). During the onset of myogenic differentiation, microtubule dynamics is reduced by the formation of stable arrays of glutaminated tubulin, with a concomitant reduction in the relative amount of the dynamic pool of tyrosinated tubulin (Gundersen et al., 1989). The ablation of microtubules by nocodazole results in defective myofibrillogenesis in re-differentiating adult cardiomyocytes, but does not affect sarcomeres in neonatal cardiomyocytes, presumably because their myofibrils are preformed (Rothen-Rutishauser et al., 1998). This is supported by pharmacological studies using the microtubule-depolymerising drug colcemid; muscle cells differentiate in the presence of colcemid and form all myofibrillar components, but these do not assemble with

precise lateral alignment (Toyama et al., 1982). A role of microtubules in the dynamic reorganisation of the cytoskeleton during myofibril assembly was also suggested by pharmacological experiments with the microtubule-stabilising drug taxol, which results in bizarre pseudo-sarcomeres with interdigitating assemblies of myosin and microtubules, lacking normal actin filaments (Antin et al., 1981; Toyama et al., 1982). Furthermore, morphological analysis in cultured cardiomyocytes suggested a direct role in the integration of myosin into nascent sarcomeres (Guo et al., 1986), as well as possibly for the localisation of myosin mRNA (Perhonen et al., 1998).

The intermediates of myofibrillogenesis are hard to study and still not fully characterised (Sanger et al., 2000). This is partly due to the increasing number of recently characterised components (Sanger and Sanger, 2001; Faulkner et al., 2000) and partly to difficulties in visualising the reorganization of these components with sufficient resolution. In cultured cells, stress-fibre like structures (SFLS), which contain some sarcomeric proteins but lack the regular periodicity of mature sarcomeres, assemble first and then undergo dramatic structural rearrangements, resulting finally in mature sarcomeres (Dlugosz et al., 1984; Rhee et al., 1994). In vivo, this process appears to be similar but in the heart, it seems to occur without some of the intermediates observed in culture (Ehler et al., 1999). During the early stages of myofibrillogenesis in cultured cells, titin colocalises with α-actinin in the Z-bodies of nascent myofibrils, and is thought to be responsible for the anchorage of α-actinin (Schultheiss et al., 1990; Sorimachi et al., 1997; Tokuyasu and Maher, 1987; Turnacioglu et al., 1996; Young et al., 1998). Titin, α-actinin and actin sequentially form a regularly arranged scaffold on SFLS, which progress on to form non-striated myofibrils or premyofibrils (NSMF) and nascent striated myofibrils (naSMF). As late components, myomesin, myosin-binding protein-C and finally myosin are incorporated. Nascent myofibrils align and fuse with adjacent nascent myofibrils to form the Z-discs of mature myofibrils (Dabiri et al., 1997). During these transitions, the spacing between α-actinin-containing Z-bodies increases from less than 1 μm in SFLS and nascent myofibrils/premyofibrils to more than 2 μm in mature myofibrils. Titin epitopes that initially colocalise at Z-bodies separate during this process (Mayans et al., 1998; Van der Loop et al., 1996), indicating that stretching of the titin molecule, and possibly exposure of binding sites for other myofibrillar proteins, is an essential process for the assembly of sarcomeres. Clearly, coordination and a series of regulatory processes are needed to organise the successive assembly states over time and space.

The recent discovery that a family of microtubule-binding proteins, the MURF proteins [first identified as putative interactors of the serum response factor by Spencer et al. (Spencer et al., 2000)], play a crucial role in myofibril assembly, points again to microtubules as important elements of myofibril morphogenesis. The MURFs are transcribed from three genes, (chromosomes 1p31.1-p33, 8q12-13, 2q16-21). They contain an N- terminal RING, followed by a B-box zinc-finger domain and a coiled-coil sequence, and thus belong to the RBCC subfamily of RING finger proteins (Centner et al., 2001; Spencer et al., 2000). MURFs can multimerise via the coiled-coil domain, resulting in homo- or heteromultimeres (Centner et al., 2001; Spencer et al., 2000). Intriguingly, the known cellular localisations of MURF1 and MURF3 are variable and include microtubules and Z-disks (MURF3), or both Z-disk and M-band (MURF1) (Centner et al., 2001; McElhinny et al., 2002; Spencer et al., 2000). Their exact functions are unclear, although roles in cytoskeletal dynamics, transcription regulation and cell signalling are conceivable. For MURF3, a stabilising effect on microtubules is established (Spencer et al., 2000). In addition, MURF3 seems to be required for the initiation of myogenesis in vitro: ablation of MURF3 in cell culture inhibits the expression of the early myogenic regulatory factors, MyoD and myogenin. Reduced MURF3 expression also diminishes myosin heavy chain (MHC) expression and concomitant myotube formation (Spencer et al., 2000). Interestingly, a knock-out mouse model of MURF1 however shows no primary defect in myofibrillogenesis (Bodine et al., 2001). The temporal dynamics of MURFs in myofibril assembly, crucial for the understanding of the mechanistic role in this process, is so far unknown.

We therefore investigated the spatio-temporal relocalisation of the least well-characterised member of the MURF protein family, MURF2, during myogenesis in vitro. Since primary myofibril assembly can not be unambiguously studied in neonatal cardiomyocytes due to their pre-existing myofibrils, we investigate differentiating skeletal myoblasts. We show that MURF2 is a microtubule-binding protein involved in early myofibrillogenesis as it interacts with both microtubules and M-bands in forming myofibrils. Moreover, we find MURF2 in the nucleus under certain conditions, suggesting that it could also play a role in the transcription programme during differentiation of myocytes.

Materials and Methods

Cloning of MURF and plasmid construction

The protein bait A164-169 comprises 6 titin A-band domains from A164 up to A169, and was expressed in *E. coli* using the pET system essentially as described previously (Freiburg and Gautel, 1996). The purified protein was immobilised to Sepharose using NHS-activated Sepharose (Amersham). We identified MURF by a proteomic search for titin ligands near the kinase domain, using a multidomain construct from the P-zone as protein bait in a pulldown experiment from cardiac muscle extracts. A protein mixture with major bands at around 50 and 60 kDa was retained, with minor bands at 97 kDa and 20 kDa. Peptides from the 60-kDa band were analysed by mass spectrometry and one peptide, HGVYGLQR NLLVGNIIDIYK, was mapped to a human EST, AA443443. The additional bands at 97 and 20 kDa were also found in experiments with control beads and represent phosphorylase and 14-3-3 epsilon, respectively. Starting from the EST AA443443, cDNA clones were isolated from a human cardiac cDNA library and analysed by sequencing. Subsequent sequencing of these cDNAs and RT-PCR analysis revealed that MURF2 is extensively differentially spliced between muscle types. These cDNA sequences, originally named tizian (Iakovenko and Gautel, 2000), were deposited in the EMBL data base, accession numbers AJ243488 (50 kDa isoform), AJ243489 (60 kDa isoform), AJ277493 (27 kDa isoform), AJ431704 (60 kDa alternative C-terminus). During the progress of this work, MURF3 was described (Spencer et al., 2000) and two additional MURF genes were identified (Centner et al., 2001). The peptides identified in our proteomic approach map to the nearly identical zinc-finger domains at the N-terminus of the MURF-family.

Two-hybrid analysis

Ig domains from the titin C-terminal region, or MURFs were cloned into a modified pLexA vector described previously (Young et al., 1998) and interactions with MURFs or titin in pGAD10 or MURFs in pLexA were monitored in L40 cells (Vojtek et al., 1993) as described (Young et al., 1998). Titin domain nomenclature followed the human cardiac titin sequence [EMBL X90568 (Labeit and Kolmerer, 1995)].

Antibodies

The polyclonal antibody α-HPC is directed against the unique, constitutive C-terminus of the 50, 60 and 27 kDa isoforms of MURF2. It was raised by immunisation of New Zealand rabbits with the keyhole-limpet hemocyanin-coupled peptide HP-C: DSEPARHIFSFSWLNSLNE following established procedures. The antibody was affinity-purified on matrix-coupled HP-C using standard methods.

The α-tubulin-specific rat monoclonal antibody YL 1/2, specific for the C-terminal EEY-epitope of tyrosinated tubulin (Wehland et al., 1984), was used to stain tyrosinated microtubules. The α-tubulin-specific monoclonal antibody ID5 (Rüdiger et al., 1999) was used to detect polyglutaminated tubulin and was a kind gift of J. Wehland. Antibodies against titin recognised the Z-disk [T12 (Fürst et al., 1988a)], I-band [N2A (Gautel et al., 1996)] and A-band [T31 (Fürst et al., 1989a)]. Monoclonal antibodies against tubulin (DM1A), skeletal myosin (MY-32) and α-skeletal and α-cardiac sarcomeric actins (5C5) were purchased from Sigma-Aldrich.

Cell culture and immunofluorescence

The C57 myogenic cell line was established by Pinset and Montarras as described previously (Pinset and Montarras, 1998). Cells were grown in a mixture (1/1volume) of Dulbecco's modified Eagle's and MCDB202 medium (Cryo Bio System) supplemented with 100 μg/ml penicillin, 100 units/ml streptomycin, 2 mM glutamine, 20% fetal calf serum (FCS) and 2% UltroserSF (Gibco BRL Life Science). For differentiation, cells were maintained in the previously described medium containing 2% FCS and 10 μg/ml insulin. In all conditions, cells were grown on gelatin-coated dishes or coverslips at 37°C in a 10% CO_2 atmosphere. Double immunofluorescence experiments were performed as follow: cells briefly rinsed with warm (37°C) BRB80 (8 mM K-Pipes pH 6.8, 1 mM EGTA and 1 mM MgCl2), permeabilised with 0.1% Triton X-100 in BRB80 for 45 seconds and then fixed in methanol at –20°C for 7 minutes. Mouse monoclonal anti α-tubulin (DM1A from Sigma), Titin or myosin antibodies and affinity purified rabbit anti-MURF2 antibodies, diluted in PBS containing 0.1% saponin and 5% FCS, were applied for 1 hour at room temperature. Following three washes with 0.1% saponin in PBS, primary antibodies were visualized by fluorescent conjugated secondary antibodies (Molecular Probes) for 30 minutes. Coverslips were mounted in AF1 solution (Citifluor) and observed with an LSM 510 confocal microscope (Zeiss).

BHK-21 cells were cultured and differentiated as described before (Van der Ven and Fürst, 1998). Transfection with MURF plasmids was carried out using Escort-III (Sigma) following the supplier's instructions. Neonatal rat cardiomyocytes were isolated from day 2-4 Wistar pups essentially as described (Auerbach et al., 1999; Young et al., 2001) and cultured in collagen-coated culture dishes at 37°C, 5% CO_2 either in M199, 10% fetal calf serum, 10 μM phenylephrine, 10 μM cytosine-arabinoside and penicilline/streptomycine or in low low-serum minimal medium consisting of M199, 1% Nutridoma-HU (Roche biochemicals), 10 μM cytosine-arabinoside and penicilline/streptomycine. Fixation followed the protocols developed for plakophilin (Mertens et al., 1996) by inclusion of $MgCl_2$ to the fixation medium.

In vitro assembly of microtubules

C57 myoblasts and myotubes differentiated for 2 and 5 days were scraped from dishes with a rubber policeman and centrifuged at 900 ***g*** for 5 minutes. Cell pellets resuspended with an equal volume of cold 2× extraction buffer (2% Triton X100, 50 mM Tris/HCl pH 7.5, 2 mM EGTA, 2 mM EDTA, 1% Nonidet P40, 200 mM NaCl, 2 mM sodium ortho-vanadate, 0.4 mM PMSF and protease inhibitors) were then incubated on a rotary device at 4°C for 90 minutes, before being centrifuged as previously described. The supernatants were then clarified by centrifugation at 50,000 ***g*** at 4°C for 15 minutes and protein quantification was performed by the Pierce Micro BCA assay according to the supplier's protocol. 400 μg of total protein, supplemented with 2 μg/ml of cytochalasine D or latrunculin A, were used for microtubules polymerisation or depolymerisation experiments. Microtubule polymerisation was performed by incubating the extracts with 2 mM MgGTP, 2 mM AMP-PNP and 20 μM taxol at 37°C for 30 minutes. Microtubule depolymerisation was obtained by incubating the extracts with 20 μM nocodazole on ice for 30 minutes. After centrifugation through a 40% sucrose cushion in BRB80 containing taxol or nocodazole (150,000 ***g***, 30 minutes, 25°C), pellets were resuspended with 100 μl of 1% SDS in PBS. In order to compare microtubule-associated proteins, western blot analyses were performed with 20 μg of proteins solubilized from the taxol pellet and identical volume of their corresponding pellet in presence of nocodazole. Samples were separated on a 10% SDS gel, transferred to nitrocellulose membranes, blocked and subsequently incubated with affinity purified anti-MURF2 and anti-tubulin antibodies following standard procedures.

Myosin and titin binding assays

MURF2 was transfected in HeLa cells and soluble cell supernatants collected as above. Myosin cosedimentation assays were carried out essentially as described (Gruen and Gautel, 1999) using 40 μg of rabbit skeletal muscle myosin [prepared by repeated high- and low-salt steps as described (Gruen and Gautel, 1999)] per assay, and 40 μg of total soluble HeLa proteins from MURF2 transfected cells. After incubation on ice for 20 minutes, myosin was pelleted (100,000 ***g***, 30 minutes, 25°C) and the pellets collected. Pellets were run on 12% SDS gels, blotted and bound MURF2 detected with the HPC antibody.

Titin binding was assayed in a column-based binding assay essentially as described for titin-α-actinin (Young et al., 1998) using GST-fused titin domains A164-169 and GST alone as control. Soluble proteins (40 μg) from MURF2 transfected HeLa cells were passed over 20 μl of glutathione beads loaded with 20 μg of GST or GST-titin fusion protein, washed in binding buffer (100 mM NaCl, 20 mM HEPES pH 7, 1 mM EDTA, 1 mM DTT, 0.05% Tween-20) and bound proteins were eluted with 10 mM glutathione in binding buffer. Eluted fractions were run on 12% SDS gels and MURF2 detected by western blot as above.

Results

MURF2 isoforms generated by two distinct differential splicing mechanisms

The MURF family of muscle ring finger proteins were isolated independently by yeast-two hybrid screens with the serum response transcription factor (Spencer et al., 2000) and C-terminal titin sequences (Centner et al., 2001), a human EST data base sequence similarity search (Dai and Liew, 2001) and proteomic ligand searches with recombinant P-zone titin (this study; see Materials and Methods, EMBL data base entries AJ243488, AJ243489, AJ277493). MURF family members are expressed in multiple isoforms (Centner et al., 2001). In this

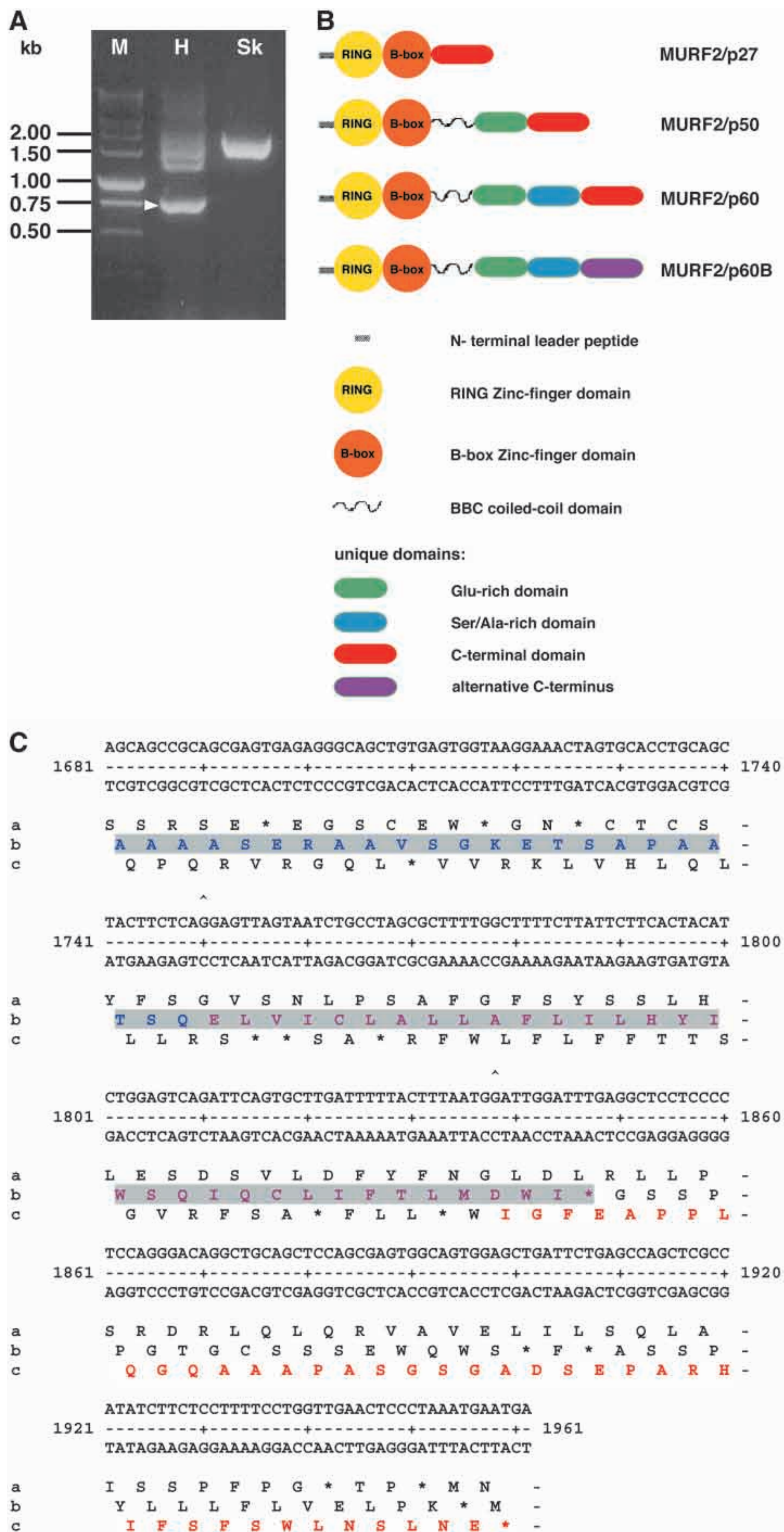

Fig. 1. Domain patterns and tissue-specific differential splicing of MURF2. (A) Tissue-specific differential splicing of MURF2. RT-PCR demonstrates tissue-specific co-expression of multiple MURF2 transcripts. The ~0.75 kb band of the 27 kDa isoform is detected only in cardiac muscle (arrowhead) but not in cDNA from adult skeletal muscle. Faint bands above 2 kb may represent further, yet unidentified isoforms. H, adult cardiac muscle; Sk, adult skeletal muscle; M, marker ladder, sizes given in kb. (B) MURF2 is expressed in four isoforms derived from differential splicing events. MURF2 isoforms of 27, 50 and 60 kDa are generated by sequential internal expansion of a core molecule containing the Ring, B-Box and C-terminal domain present in MURF2^{p27}. A third isoform, MURF2^{p60B}, is generated by the splicing of an additional exon before that of the C-terminal domain; a frameshift in the reading frame of this exon leads to the generation of an alternative C-terminus. (C) Generation of alternative C-termini in MURF2. Alternative splicing of the MURF2 gene that maintains the last exon (highlighted in red in B and C) while splicing in an additional 60 bp exon is observed in skeletal muscle. This leads to a frameshift in the last exon, omitting its reading frame and creating an alternative C-terminus (purple in B and C) which is fused directly to the Ser/Ala rich domain (blue in B and C). Note the alternative reading frame use in the last exon. The three C-terminal exons localise to a 20409 bp genomic region on chromosome 8q12, separated by introns of 1285 bp to 18762 bp. The large stretch of sequence between the last two exons may suggest the presence of further, as yet unidentified differentially spliced exons. In this figure, only the 3′ region of the p60B isoform was mapped. The start of a new exon is indicated (^). Reading frames are numbered a, b and c, and the MURF2^{p60B} reading frame is shadowed in grey. Nucleotide numbering is according to our data library entry AJ431704.

study, we focussed on the so far least characterised family member, MURF2. To complete the picture of MURF2 isoforms, we performed reverse transcription PCR from both human cardiac and skeletal cDNA using a primer pair flanking the open reading frame of MURF2 defined in our data library entry AJ243488. Amplifications from both tissues resulted reproducibly in strikingly different results: multiple bands ranging from 1.6 to ~2 kB were amplified from skeletal muscle and an additional ~0.75 kB band was specifically amplified from cardiac muscle (Fig. 1A). Sequence analysis of these possible isoforms demonstrated that they represent three different splice variants, which share the common N-terminal RING and B-box domains and end in a common C-terminal sequence. They encode peptides in the range of 27, 50 and 60 kDa. The smallest MURF2 isoform, MURF2^{p27}, is specific for cardiac muscle and contains only the N-terminal RING and B-box domains as well as the C-terminal MURF2-specific sequence (Fig. 1A,B). The proteomic approach (see Materials and Methods) as well as western blotting using MURF-specific antibodies (not shown) demonstrated that all three MURF family proteins could associate with P-zone titin, most likely by hetero-multimer formation via their coiled-coil domains. The coiled-coil domain, believed to be a hallmark for MURFs as members of the RBCC protein family, is spliced out in the MURF2^{p27} isoform. MURF2^{p27} can therefore not interact with other MURF isoforms, a prediction also verified in two-hybrid analysis (not shown). An additional MURF2 transcript was isolated in repeated RT-PCR experiments from skeletal muscle. Despite sharing the most C-terminal exon with the 27, 50 and 60 kDa isoforms, this transcript contains an additional exon spliced in before this last constitutive exon. This alternatively spliced exon leads to frameshift, resulting in the omission of the last coding exon peptide and creating an alternative C-terminus (Fig. 1B,C) by alternative reading frame use. Western

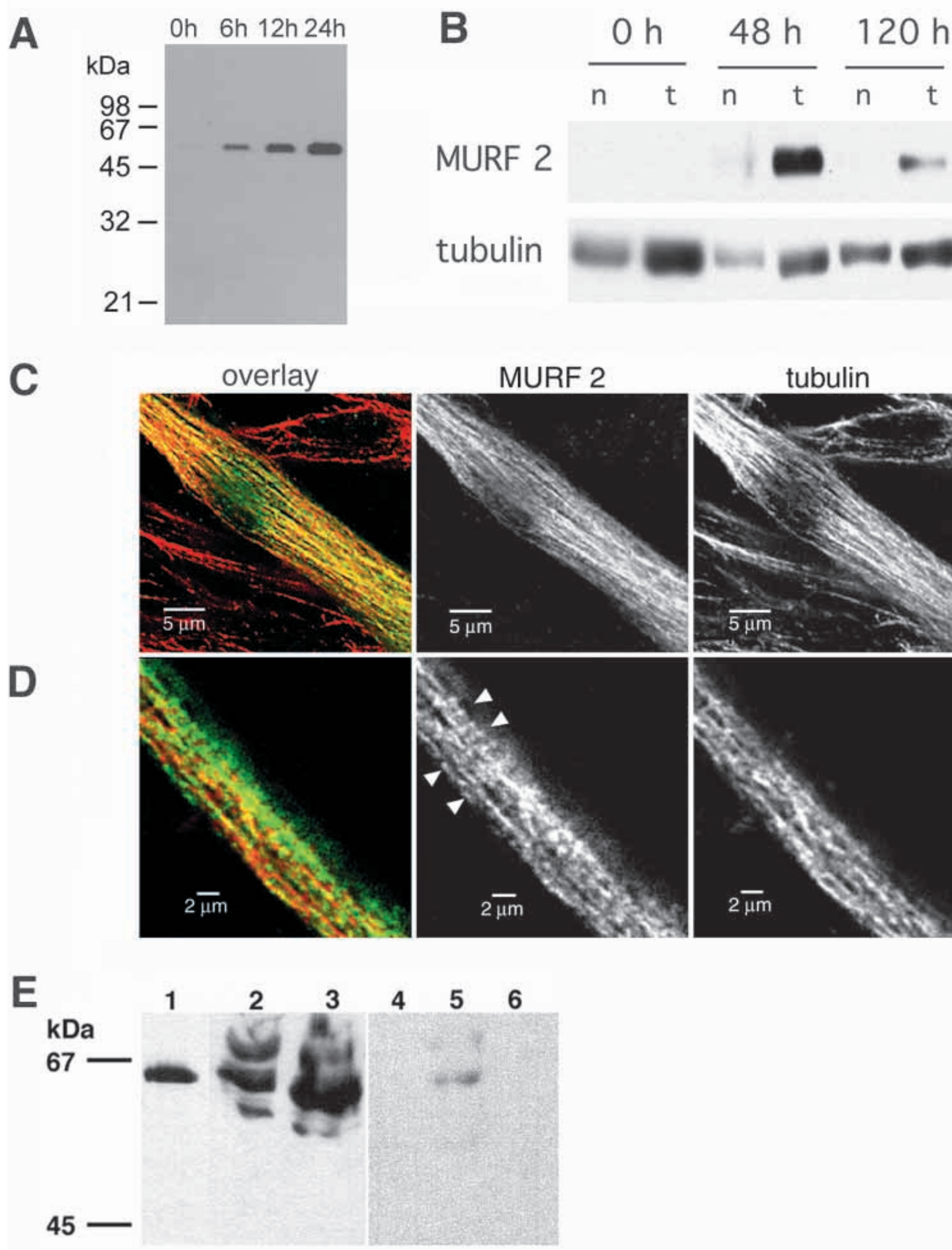

Fig. 2. Developmental expression of MURF2 and association with microtubules, myosin and titin. (A) Developmental time course of differentiating myoblasts probed with anti-MURF2 HPC. A single 60 kDa band is expressed upon myogenic differentiation. (B) Microtubule sedimentation assays were performed using protein extracts from undifferentiated (0 hours) and from differentiated C57 cells (48 hours, 120 hours) using nocodazole (n) or taxol (t). MURF2 was detected in the microtubule pellets of differentiated myotubes. (C-D) Localisation of endogenous MURF2 to microtubules in differentiated C57 cells. Double immunofluorescence was performed using anti-MURF2 and anti-tubulin antibodies. After 24 hours of differentiation (C), MURF2 directly colocalised with microtubules as seen in the overlay (yellow). In cells differentiated for 48 hours (D), some MURF2 protein (arrowhead) did not strictly associate with microtubules (green in overlay). (E) MURF2 interacts with sarcomeric myosin and P-zone titin. MURF2 was expressed in transfected HeLa cells and detected by the anti-MURF2-specific antibody HPC (lane 1). The myosin sedimentation assay demonstrates the presence of endogenous MURF2 in myosin preparations (lane 2); addition of transfected MURF2 increases the myosin-associated MURF2 fraction (lane 3). MURF2 associates with titin A164-169 (lane 5) but not with GST alone (lane 6). (Lane 4) Untransfected HeLa cell lysate control on titin A164-169 beads.

blot analysis of differentiating muscle cells using antibodies against the MURF2p60-specific Ser-Ala rich domain and the C-terminal domain demonstrated that differentiating skeletal muscle cells express the p60 isoform of MURF2. In summary, MURF2 isoforms can be generated by conventional alternative exon, as well as by alternative reading frame usage.

Endogenous MURF2 interacts with microtubules from skeletal muscle cell

MURF3 has been reported to be associated both with microtubules as well as with the mature Z-disk (Spencer et al., 2000). Given the very high amino acid sequence homology of MURF1, 2 and MURF3 (Centner et al., 2001), we tested the microtubule binding ability of MURF2. Microtubule sedimentation assays were performed using protein extracts from the mouse C57 skeletal muscle cell line (Pinset and Montarras, 1998), prepared at various times of differentiation, where MURF2^{p60} is expressed in a differentiation-dependent way (Fig. 2A). Cytoskeletal fractions were prepared in the presence of either taxol or nocodazole in order to stabilize, or to inhibit microtubule polymerization. However, as previously described, some microtubules remain resistant to cold and nocodazole treatment (Gundersen et al., 1989). Although some tubulin was present in the nocodazole pellets (Fig. 2B), higher amounts of tubulin were observed in the taxol pellets indicating efficient microtubule polymerisation and stabilization. MURF2^{p60} was not detected in proliferating myoblasts, demonstrating the specific expression in differentiating myogenic cells. Upon differentiation, MURF2^{p60} was specifically detected in cytoskeletal fractions obtained from taxol treated extracts (Fig. 2B). Interestingly, the highest amount of cytoskeleton-associated MURF2 was detected after 48 hours of differentiation, with a subsequent decline, suggesting that the physical association between MURF2 and microtubules could be a transient event occurring during the differentiation programme. To confirm the cellular localisation of MURF2, double immunofluorescence experiments were performed on differentiating C57 cells. Using specific affinity-purified anti-MURF2 and anti tubulin antibodies, we observed that at the beginning of differentiation, MURF2 colocalised with microtubules (Fig. 2C). Later, although most microtubules remained decorated with the anti-MURF2 antibody, some of the MURF2 staining appeared as small rod and dot structures scattered in a longitudinal and transverse orientation within the cytoplasm (Fig. 2D, arrowhead). In fully differentiated skeletal myofibrils, MURF2 labelling was not detected in mature sarcomeres (Fig. 6). In agreement with previous data (Spencer et al., 2000), overexpression of MURF1, 2 and 3 in C57 skeletal myoblasts was lethal. We found that the cytotoxic effect was least pronounced in myogenic BHK-21 cells (Van der Ven and Fürst, 1998). In these cells, MURF2 displayed a specific differential localisation on post-translationally modified microtubules. Using the antibody YL1/2 (Wehland et al., 1984) against tyrosinated tubulin, MURF2 was found to be specifically excluded from tyrosinated microtubules (Fig. 3A) but to localise with polyglutaminated MTs (Fig. 3B) labelled by the monoclonal antibody ID5 (Rüdiger et al., 1999). Altogether, these data demonstrate the association of MURF2 with microtubules at early stages of skeletal muscle differentiation, and suggest that this association is regulated by developmental post-translational tubulin modification.

MURF2 interaction with sarcomeric myosin and titin

Since endogenous MURF2 in muscle cells is largely associated with the cytoskeleton, and several MURFs are co-expressed, we expressed MURF2 selectively in non-myogenic HeLa cells to assay for a possible interaction with sarcomeric myosin and A-band titin. We observed that conventional preparations of sarcomeric myosin contain copurified MURF2 protein, and that exogenous MURF2 cosediments with myosin (Fig. 2E). This suggests that MURF2 can directly associate with myosin. Because of the high sequence homology between MURF1 and MURF2, we also investigated the possibility that MURF2 could interact with the MURF1-binding A-band titin region. Again, we used HeLa-expressed MURF2 free of MURF1 or MURF3. In a pulldown assay using GST-tagged titin A164-169, we observed binding of MURF2 to titin, but not to GST (Fig. 2E) or other protein constructs. These combined data suggest that MURF2 can interact with sarcomeric myosin and that muscle myosin preparations actually contain traces of MURF2, and furthermore, that MURF2 contains intrinsic titin-binding properties previously undiscovered by yeast two-hybrid analysis.

Dynamics of MURF2 during skeletal muscle differentiation

MURF2 and sarcomeric actin

The actin cytoskeleton undergoes major remodelling during myocyte differentiation, resulting ultimately in the highly ordered arrays of sarcomeric, parallel actin filaments cross-linked with the antiparallel filaments from the opposite sarcomere half at the Z-disk (Luther et al., 2002). The temporal and spatial distribution patterns of MURF2 were analysed by double immunofluorescence experiments with the anti-MURF2 antibody and anti-sarcomeric actin. In C57 cells, endogenous MURF2 and skeletal actin remain mostly segregated during myofibrillogenesis (Fig. 4). Although MURF2-decorated microtubules aligned parallel to actin filaments (arrowheads in 4B), strict colocalisation was rare and the bulk of both proteins were found in separate compartments at the beginning of differentiation (Fig. 4A). Later, in premyofibrils, regions displaying cross-striated actin filaments showed no MURF2 labelling (Fig. 4B). This suggests that MURF2 does not follow the integrative route of most classical sarcomeric proteins, which are found to colocalise with actin and α-actinin in premyofibrils or stress-fibre like structures.

MURF2 and the titin Z-disk and I-band regions

Titin follows a defined path of sarcomeric integration, starting from dot-like aggregates on SFLS, which localise to Z-bodies. Initially, the Z-disk portion becomes organised, followed by stretching-out of the molecule and the integration of the C-terminal M-band portion. The association of MURF2 with the A-band region of titin revealed by protein interaction analysis raises the question of the time course and the distribution pattern of both proteins. We therefore investigated the distribution of MURF2 in relation to the titin Z-disk, I- and

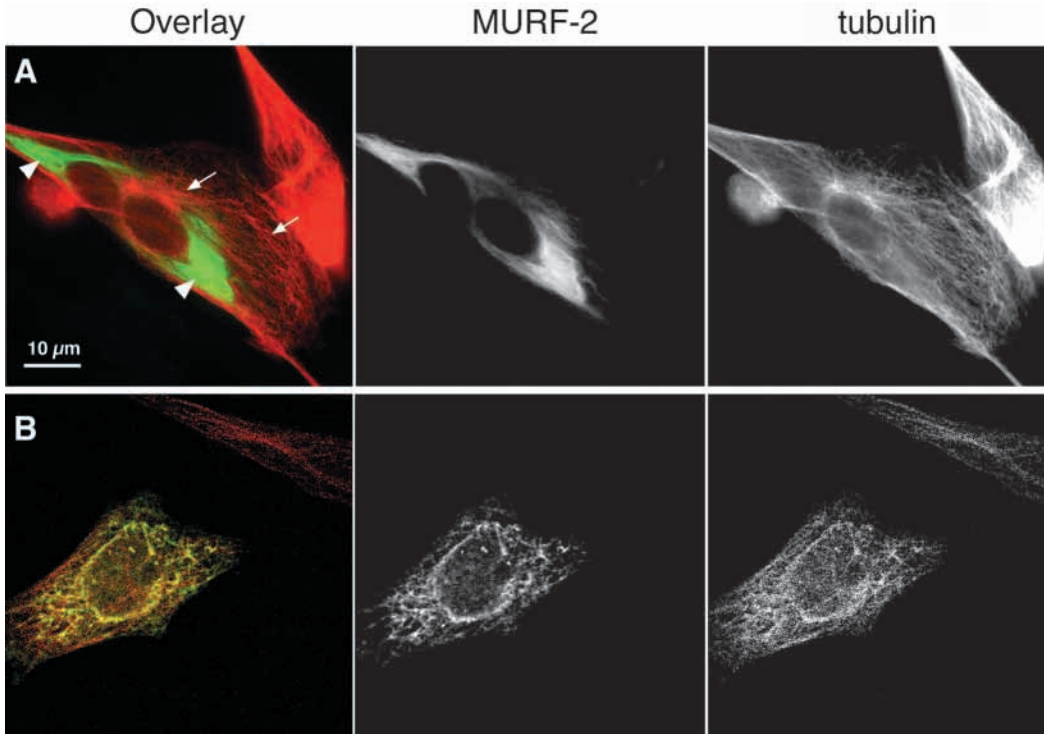

Fig. 3. Selective association of MURF2 with post-translationally modified microtubules. (A) Transfection of full-length T7-tagged MURF2 in the myogenic cell line BHK-21 shows that microtubule-associated MURF2 (green, arrows) is excluded specifically from tyrosinated microtubules, which are stained by the YL1/2 antibody (red, arrowheads). (B) Transfected MURF2 (green) is localised on polyglutaminated microtubules in HeLa labelled by the monoclonal antibody ID5 (red; overlay yellow).

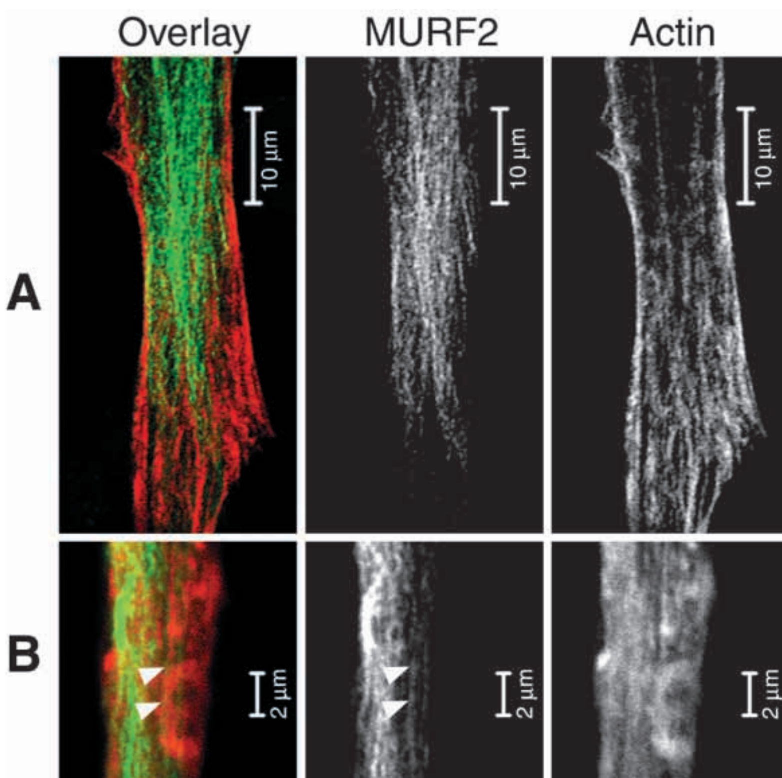

Fig. 4. MURF2 and sarcomeric actin are localized in different cell regions. Cells stained with anti-MURF2 (green) and with anti-sarcomeric actin (red) antibodies did not show significant colocalisation, neither after 48 hours (A) nor after 120 hours of differentiation (B). When cross-striated actin begins to be organised (B), MURF2 decorated microtubules align laterally to these structures (arrowheads in B).

A-band using the anti-MURF2 antibody in combination with the monoclonal anti-titin I-band N2A antibody (Gautel et al., 1996) or with the T12 anti-titin antibody [Z-band (Fürst et al., 1988b)]. At the beginning of differentiation, (Fig. 5A), stress fibre-like structures, stained with the titin N2A antibody, are completely excluded from the labelling revealed with the MURF2 antibody. Titin T12 Z-disk epitopes were identified as dots and patches aligned parallel to MURF2 positive filaments (Fig. 5C). In young myotubes, higher magnification showed that titin T12-positive spots can occasionally colocalise with long MURF2-decorated microtubules (Fig. 5D, arrowhead). Occasional colocalisation of both proteins was also observed even with Z-disk titin dots of a periodicity <1 μm (Fig. 5D). The absence of strict colocalisation at these early stages resembles the pattern of sarcomeric actin and reflects the early association of titin and actin. When sarcomeres start to form, MURF2 appears in small punctuate structures mostly arranged in a linear pattern (Fig. 5E, arrowhead). These structures seemed to be in register with more mature sarcomeric Z-disk titin displaying a 2 μm periodicity (Fig. 5E, arrow). During differentiation, colocalisation of MURF2 and titin I-band N2A could be observed in cell regions where I band cross striation starts to be organised (Fig. 5B). MURF2 and titin N2A antibodies decorated regions where non-striated myofibrils differentiate into striated myofibrils (Fig. 5B, arrowhead). It is noteworthy that MURF2 was barely detectable in mature sarcomeres (Fig. 5B, arrow). All these results indicate that even though

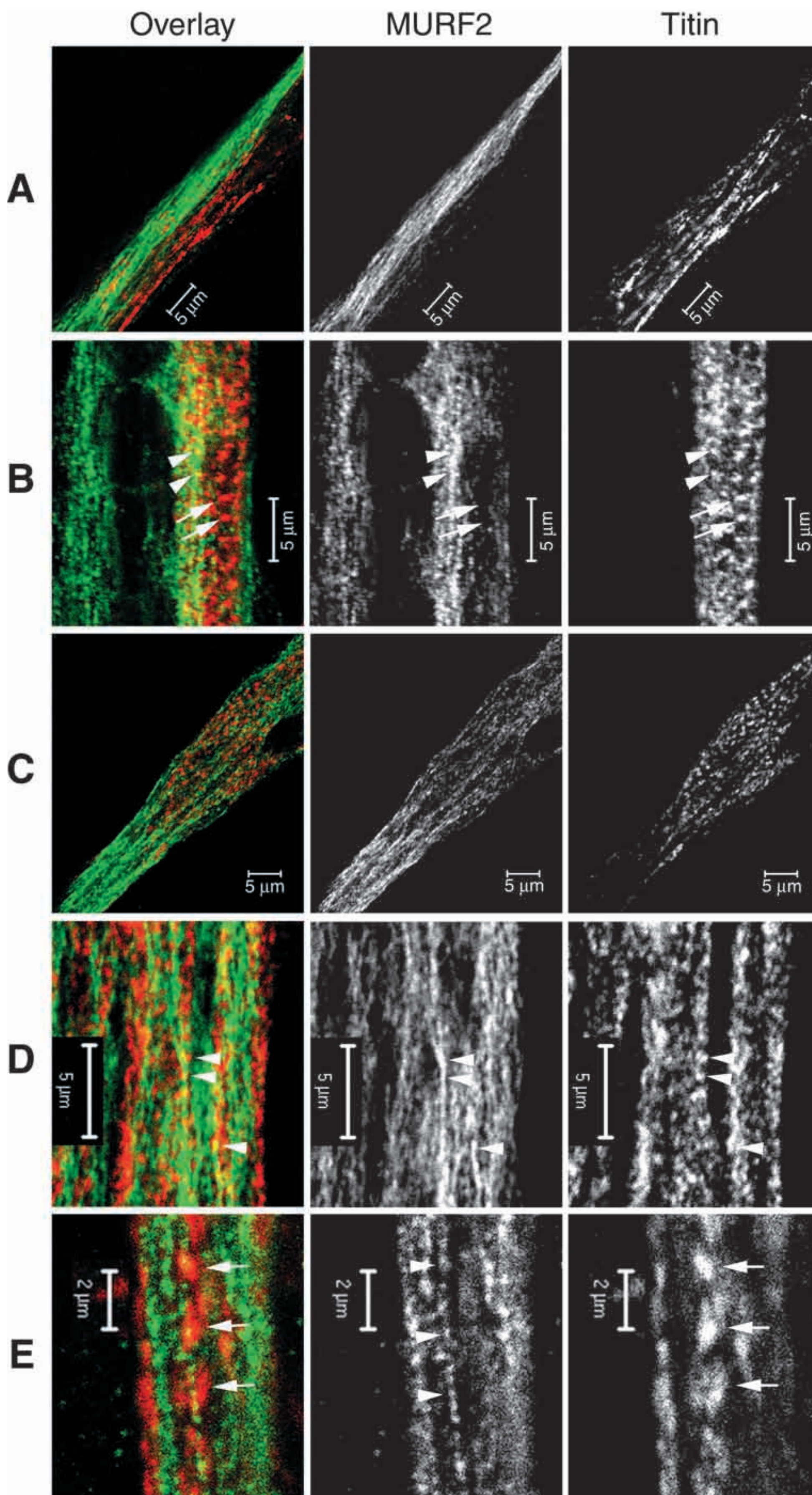

Fig. 5. Localisation of endogenous MURF2 relative to titin Z-disk and I-band epitopes. At different stages of differentiation, double immunofluorescence experiments were performed with anti-MURF2 (green), and either anti-titin I-band (A,B) or anti-titin Z-disk (C-E) antibodies (red). At 24 hours of differentiation (A) MURF2 did not decorate the stress fibre like structures revealed with titin I antibody. After 120 hours of differentiation (B), MURF2 and I-band titin epitopes showed occasional transversal colocalisation (arrowhead) near mature cross-striated regions of the myofibril (arrow). In young myotubes (24 hours of differentiation) Z-disk titin can align along MURF2 positive microtubules (C,D). When sarcomeres started to be organised (E), structures with MURF2 punctate staining (arrowhead) co-aligned with maturing Z-disk titin (arrow).

MURF2 can align with the Titin I and Titin Z regions, these associations take place only transiently and in different intracellular domains of differentiating cells.

MURF2 and the titin A-band region

A more complex picture was observed for the Titin A-band region (Fig. 6). C57 cells were double stained with the T31 antibody directed against a central A-band epitope of titin (Fürst et al., 1989a) in combination with the anti MURF2 antibody at different developmental stages. Early during differentiation (Fig. 6A), the MURF2 antibody stained MURF2-decorated microtubules whereas titin was visible as fluorescent spots dispersed longitudinally, apparently along SFLS and without obvious colocalisation with MURF2. This pattern is similar to that observed for the titin Z- and I-band epitopes. Titin A-band patches then accumulated and assembled along MURF2 positive structures (Fig. 6B). As shown by their overlap, MURF2 and A-band titin particularly colocalised in maturing non-striated myofibrils. In nascent striated myofibrils, MURF2 was seen mainly as

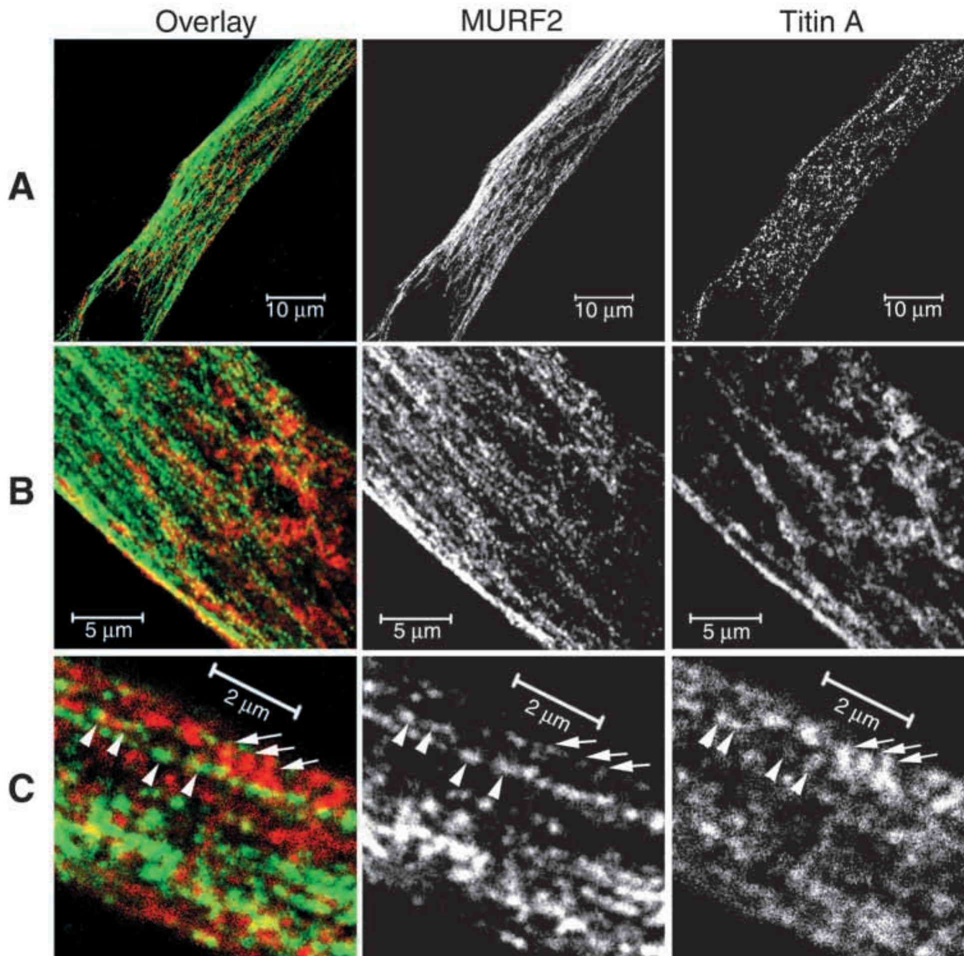

Fig. 6. MURF2 associates with A-band titin in nascent myofibrils. C57 cells were stained with anti-titin A-band (T31, red) and anti-MURF2 (green) antibodies. After 24 hours of differentiation (A), titin aligned with MURF2 positive microtubules in a dotty pattern. After 48 hours of differentiation (B), titin A-band epitopes were organised in patches along MURF2 positive structures. At 120 hours of differentiation (C), MURF2 colocalises with titin A-band epitopes as longitudinally oriented dots (arrowhead). Some dots were observed at both sides of nascent striated myofibrils (arrow).

dotted, rather than continuous, structures arranged in a linear fashion, sometimes on both sides of titin A-band labelled structures (Fig. 6C, arrowhead). When clear A-band doublets begun to form in nascent striated myofibrils, MURF2 showed lateral colocalisation with non-striated A-band titin on both sides of the transversal titin bands (Fig. 6C, arrow). This pattern suggests that initially, MURF2 and A-band titin were segregated, but transiently co-aligned during the progression of the non-striated myofibril to the striated myofibril. These results also confirm that MURF2 can associate with A-band titin, and suggest that this interaction occurs transiently in non-striated myofibrils.

MURF2 and the assembly of sarcomeric myosin filaments

Sarcomeric myosin is integrated into the nascent sarcomere at a late stage of myofibril formation, after the assembly of an initial titin/actin/M-band scaffold (Ehler et al., 1999; Van der Ven et al., 1999). Titin has been proposed to act as a molecular ruler that directs the assembly of thick filaments in the sarcomere (Trinick, 1992). An intact microtubule network dynamics is also required for the correct assembly of sarcomeres (Antin et al., 1981; Holtzer et al., 1985; Saitoh et al., 1988). Due to the interaction of MURF2 with microtubules and its transient association with A-band titin, we investigated the localisation of MURF2 in relation to sarcomeric myosin during differentiation. Double immunofluorescence was performed with anti-MURF2 and anti-myosin heavy chain antibodies. Myofibril assembly progresses from microscopically less ordered structures to the highly ordered contractile apparatus; as long as new sarcomeres and myofibrils develop, these distinct steps are observed simultaneously in a single cell (Fig. 7B).

Of all sarcomeric markers, myosin showed the earliest colocalisation with MURF2. Arrays of MURF2 labelled microtubules largely colocalised with parallel bundles of sarcomeric myosin in non-striated myofibrils (Fig. 7A). As differentiation proceeded, non-striated myofibrils co-aligned with more mature stages, nascent striated myofibrils and mature myofibrils. Clear colocalisation of sarcomeric myosin and MURF2 could then be observed in long filamentous structures (Fig. 7B). In these structures, MURF2 was found in strictest localisation with myosin, at the lateral boundaries of the nascent striated myofibrils where myosin lacked the exact alignment in-register found in fully mature sarcomeres (Fig. 7C, arrowhead). Interestingly, when myosin was organised in a typical periodic A-band pattern, MURF2 disappeared from the sarcomere (Fig. 7C, arrow). Altogether, these results reveal that MURF2 associates closely with sarcomeric myosin all along myogenic differentiation until mature sarcomeres are organized.

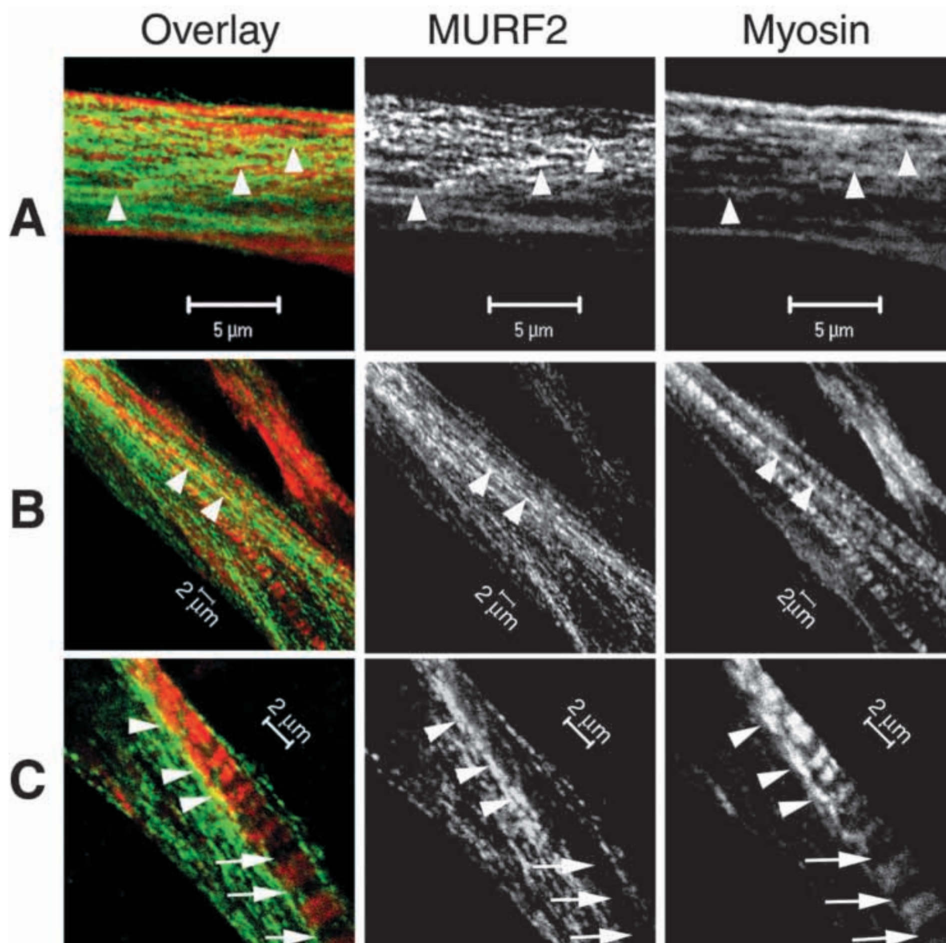

Fig. 7. MURF2 and sarcomeric myosin show colocalisation in premyofibrils. C57 cells were stained with anti-MURF2 (green) and with anti sarcomeric myosin heavy chain antibodies (red). Double immunofluorescence experiments revealed that MURF2 stained filamentous structures containing sarcomeric myosin at early stages of differentiation (A, arrowheads). Later, MURF2 and sarcomeric myosin colocalise in non-striated regions parallel to striated myofibrils (B, arrowheads). Upon sarcomere formation, MURF2 and sarcomeric myosin antibodies labelled identical rod-like structures (C, arrowheads). Mature skeletal sarcomeres (arrow) did not show colocalisation of MURF2 and sarcomeric myosin.

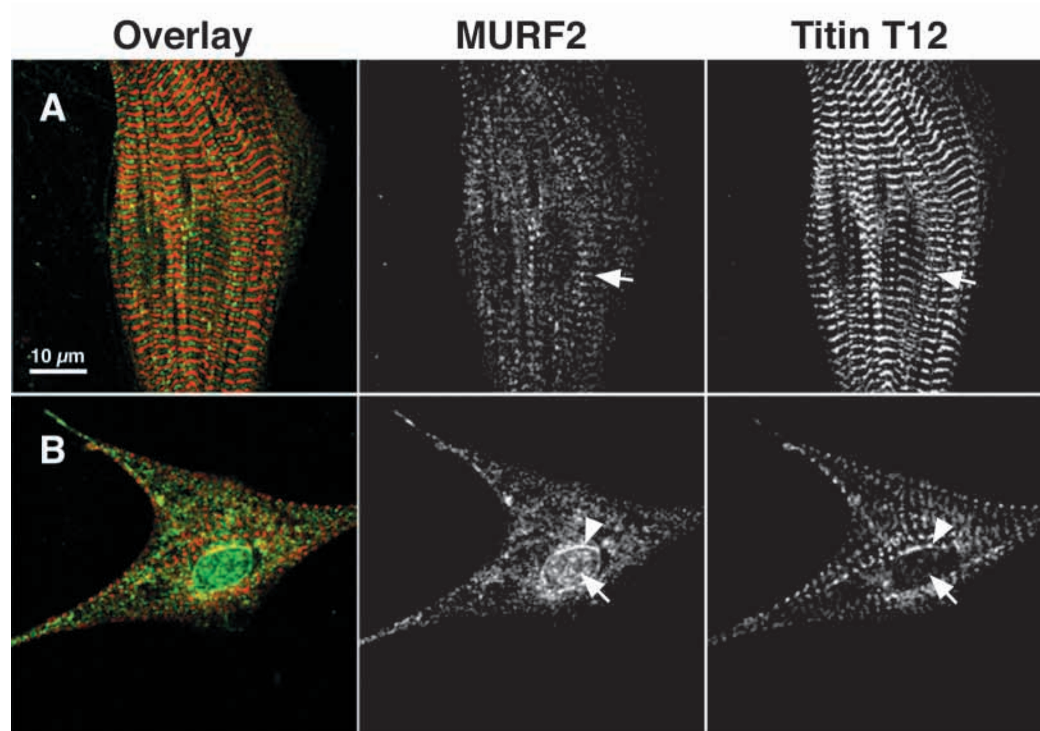

Fig. 8. MURF2 is translocated from the sarcomeric M-band to the nucleus in stressed cardiomyocytes. (A) In neonatal cardiomyocytes cultured for 72 hours in high-serum conditions, MURF2 (green) is found at the sarcomeric M-band (arrows) and excluded from the nucleus. These cells show a large myofibrillar area with mature, parallel alignment of sarcomeres visualised by the Z-disk titin antibody T12 (red). MURF2 is arranged centrally between Z-disks, at the M-band. (B) Cells cultured in minimal medium for 36 hours show few, poorly arranged and non-parallel myofibrils, stained by titin T12 (red). MURF2 (green) is diffusely distributed in the cytosol, and in a speckled pattern in the nucleus (arrow). MURF2 at the nuclear envelope is marked by an arrowhead. Bar, 20 µm.

Nuclear translocation of MURF2 in cardiomyocytes

MURF1 and -3 were reported to be localised at the sarcomeric Z- and M-bands of cardiomyocytes, with a surprising dual localisation of MURF1 at both sites in cardiomyocytes (Centner et al., 2001; Spencer et al., 2000). For the understanding of MURF functions, it is important to establish whether, and where MURF2, whose cellular localisation in both skeletal and cardiac myocytes was unknown to date, was present in the sarcomeres of cardiomyocytes. In neonatal rat cardiomyocytes cultivated for 72 hours under high-serum conditions and α-adrenergic stimulation, we detected MURF2 at the sarcomeric M-band as well as in a weak diffuse cytosolic pattern (Fig. 8A). In these cells, no nuclear localisation of MURF2 could be observed in agreement with (Spencer et al., 2000). In neonatal rat cardiomyocytes that had been serum-starved for 36 hours, MURF2 (green) is largely diffusely distributed in the cytosol but notably found in a speckled pattern in the nucleus (Fig. 8B, arrow). This pattern persists in cells re-exposed to serum for 3-12 hours. There is also significant accumulation of MURF2 at the nuclear envelope (Fig. 8B, arrowhead), the site of other proteins involved in SUMO-regulated nuclear transport like RanGAP. The diffuse cytosolic staining suggests a mobile pool of protein. These combined data identify MURF2 as a protein with triple cellular localisation: on microtubules, at M-bands, and in the nucleus. The latter localisation seems to correlate with the differentiation state of the cardiomyocyte and is induced by serum starvation.

Discussion

A growing number of proteins are being identified in the sarcomere, which do not fit to a more traditional view of the sarcomere as a static structure of great regularity (Sanger and Sanger, 2001). Some of these new proteins combine a structural role with signalling functions, like the differentially spliced giant G-protein regulator obscurin, which localises to the sarcomeric Z-disk in early developing hearts but is later found at the M-band (Young et al., 2001).

MURF2 belongs to the MURF family of muscle-specific RING/B-box zinc-finger proteins (Centner et al., 2001) first identified by Spencer et al. (Spencer et al., 2000) in a search for ligands of the serum response factor (SRF). A second MURF member, MURF1, was identified as a ligand of titin close to the M-band, and about 12 nm N-terminal to the kinase domain. It was hence speculated that MURFs might be regulators of the titin kinase domain (Centner et al., 2001). In adult cardiac muscle, MURF1 was found both at the Z-disk and the M-band; MURF3 was assigned to the Z-disk. MURF1 was recently proposed to play a role in thick filament assembly (McElhinny et al., 2002). However, a MURF1 knockout mouse shows no defects in primary myofibrillogenesis (Bodine et al., 2001), but rather a resistance to atrophy in agreement with the up-regulation of MURF1 under these conditions. These observations suggest that the functions of MURF1 in myofibril assembly are partly redundant or dispensable. To understand MURF functions, a detailed analysis of the role in myofibril assembly is clearly required. The previous studies on MURF1 and MURF3 have focussed on cardiac myocytes, where primary myofibril assembly cannot be studied due to the preformed myofibrils. In this study, we therefore present the first detailed temporal analysis of a MURF-protein, MURF2, during myogenic differentiation in skeletal muscle.

MURF2 was isolated on affinity beads of an A-band titin fragment (Iakovenko and Gautel, 2000) containing the binding site for MURF1, possibly due to its ability to form heterodimers with MURF1 and MURF3 (Centner et al., 2001; Spencer et al., 2000). However, we demonstrate here for the first time that MURF2 also displays intrinsic titin-binding properties which may contribute to the cellular localisation near the M-band of mature cardiac sarcomeres. Whereas MURF1 appears to remain expressed in all striated muscles at all stages of differentiation, we find that MURF2 is down-regulated in mature skeletal myotubes and is excluded from mature skeletal sarcomeres. These results are in good agreement with northern blot analysis which suggested that MURF2 is expressed at best weakly in adult cardiac and skeletal tissues (Centner et al., 2001), and that MURF1 is strongly expressed in fetal heart muscle (Dai and Liew, 2001). Our data together with those of Spencer et al. (Spencer et al., 2000) show that both MURF3 and MURF2 proteins are microtubule associated proteins but that expression and localisation remain distinct, indicating that MURF2 and MURF3 could fulfil different tasks during differentiation. Whereas MURF3 was detected in proliferating myoblasts and in adult skeletal and cardiac muscle (Spencer et al., 2000), MURF2 shows temporal dynamics in its expression in skeletal muscle cells with highest levels early after the onset of differentiation (Fig. 2A,B).

During differentiation, MURF2 distribution follows the morphological reorganisation undergone by the microtubule cytoskeleton (Fischman, 1970; Gundersen et al., 1989; Okazaki and Holtzer, 1965). At early stages of differentiation, the MURF2 antibody stains distinct microtubules all along the length of the nascent myotube, while at later stages, segmented rod-like and dotty structures were observed. These various morphologies reflect the reorganization of the microtubule network (Cartwright and Goldstein, 1982; Gundersen et al., 1989; Warren, 1974), and/or specific intracellular relocalisation of MURF2. This association is also evident in cells treated with nocodazole where MURF2 adopts a diffuse distribution whereas titin on SFLS is not impaired (data not shown).

The closest association between MURF2 and sarcomeric proteins is observed for sarcomeric myosin, well before the integration of myosin into nascent myofibrils. Although sarcomeric myosin appears as one of the first myofibrillar proteins, the striated A-band arrangement is observed only at a very late stage (Person et al., 2000; Rudy et al., 2001; Van der Ven et al., 1999). MURF2 is the first MURF protein known to show morphological myosin association. In agreement with this, we also detected MURF2 in preparations of sarcomeric myosin (Fig. 2E). According to our immunofluorescence data, MURF2 and myosin association take place at the beginning of differentiation, when the microtubule network is still preserved on large scale. Interestingly, myosin filament assembly is independent of actin filaments (Guo et al., 1986; Holtzer et al., 1997; LoRusso et al., 1997; Rhee et al., 1994; Sanger et al., 1986; Schultheiss et al., 1990; Wang and Wright, 1988), but requires the microtubule cytoskeleton for the formation and the organization of the thick filaments in A-bands (Antin et al., 1981; Toyama et al., 1982).

In contrast, actin and Z-disk titin co-assemble early on and initiate the formation of a Z-disk-titin-M-band scaffold into which myosin is finally integrated. Our observations suggest that the Z-disk/titin/M-band scaffold only transiently co-aligns with MURF2-containing microtubules during sarcomere formation. However, both myosin and A-band titin colocalise with MURF2 in non-striated and nascent striated myofibrils, parallel and closely apposed to striated myofibrils. Many observations showed that the organisation of the N-terminal titin domains precede those of the C-terminal regions, revealing the sequential and structural order of titin molecule unravelling (Ehler et al., 1999; Fürst et al., 1989b; Komiyama et al., 1993; Mayans et al., 1998; Schultheiss et al., 1990; Soeno et al., 1999; Van der Loop et al., 1996; Van der Ven et al., 1999). The temporal order of the spatial relationship of MURF2 with respect to Z-disk, I-band and A-band titin are in good agreement with an involvement in the straightening and stretching of the giant titin molecule during sarcomere formation. Co-alignment of MURF2 and Z-disk titin takes place at the beginning of differentiation, as indicated by the integrity of the microtubule network staining revealed with MURF2 antibody (Fig. 5D). Since overlap of MURF2 and I-band titin is only observed on irregular cross-striated sarcomeres (Fig. 5B), MURF2 seems to align with I-band titin only briefly. As expected due to the biochemical association with titin, MURF2 localises with A-band titin. Since definite striations of A-band titin epitopes are formed at a late stage of differentiation, one could hypothesise that MURF2 interacts with the folded-up titin molecule when their A-band portion starts to stretch out from the Z-disk portion.

The early and persistent association of MURF2 with both sarcomeric myosin and microtubules, and the parallel alignment of microtubules and their associated proteins with nascent striated myofibrils suggest that microtubules are indeed involved in translocating myosin filaments to the sites of final sarcomere assembly. MURFs appear to act as a transient adaptor between sarcomeric proteins, especially myosin and titin, and the microtubule network. At the beginning of differentiation, the MURF2/myosin complex interacts with microtubules, allowing the dispersal of myosin throughout the myotube along the microtubule network. During differentiation, MURF2 thus brings myosin filaments at the vicinity of maturing titin filaments. MURF2 homo- or hetero-multimeres could then coordinate binding sites on myosin and titin. MURF1, which interacts most strongly with A-band titin (McElhinny et al., 2002), may provide the link to the titin filament, although we show here that titin binding is not an exclusive property of MURF1. This may explain why a knockout of MURF1 is without consequences for primary sarcomere formation (Bodine et al., 2001). This proposed role of MURFs provides an explanation how A-band titin and myosin are finally brought in tight register, summarised synoptically in the sketch in Fig. 9. The processes of active transport of myosin, and the stretching out and aligning of nascent myofibrils, must require force and hence the activity of molecular motors. It will be interesting to see whether these are microtubule-based and whether such motors associate with MURFs.

We found that MURF2 is expressed in multiple isoforms, some of which are tissue-specific. MURF2^{p60B} is generated by alternative reading frame use, the first description of this novel

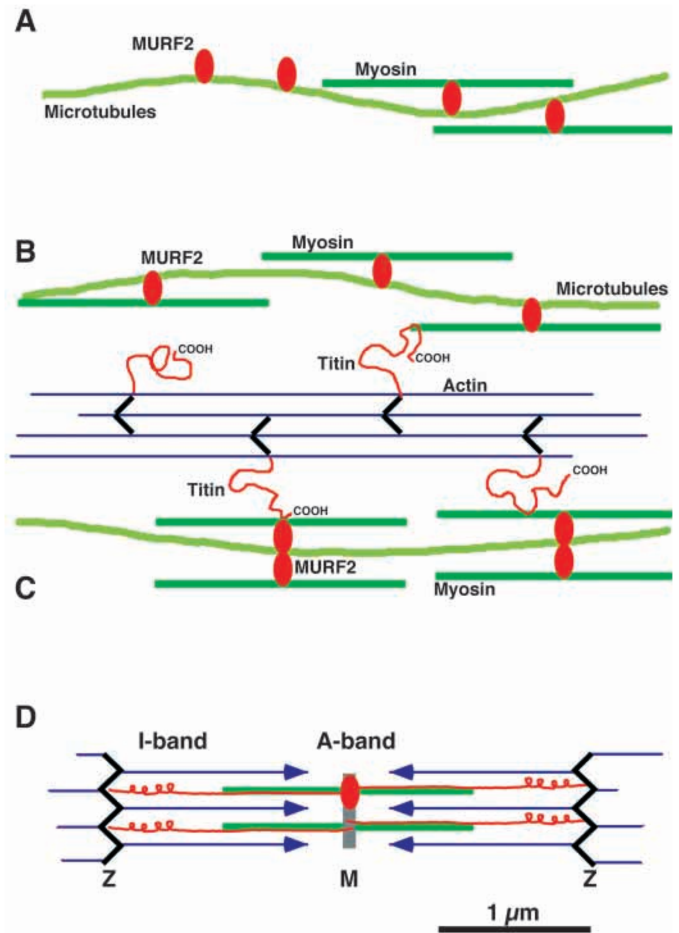

Fig. 9. MURF2 in relation to titin, myosin and the filamentous systems of actin and microtubules during sarcomere formation. (A) At initial stages of myofibril assembly, MURF2 (red ovals) is microtubule associated. Myosin (dark green rods) colocalises early with MURF-decorated microtubules (light-green). (B) Titin (red chains) localises in dot-like aggregates on actin SFLS (blue) with a spacing of around 1 μm. Black links, α-actinin crosslinks. These structures co-align with MURF-decorated microtubules. (C) MURF2 and myosin colocalise strictly in nascent striated myofibrils, when the cross-striated pattern of myosin begins to form and titin Z-Z staining increases to the mature approximately 2 μm pattern. Transient colocalisation with A-band titin is observed at this stage. MURF-MURF heteromultimeres could link different sarcomeric components at this stage and result in the exact alignment of titin and myosin. (D) In mature myofibrils, myosin and actin are arranged in highly ordered cross-striated patterns with ordered polarity (actin pointed ends marked by arrows), and the titin molecule is extended, with the N-terminal portion remaining in the Z-disk and the C-terminus integrated into the M-band. Depending on differentiation state and/or muscle type, MURF2 can be present in the M-band or the nucleus. Bar, 1 μm.

splice mechanism (Klemke et al., 2001) in a muscle protein. Apart from creating the potential for innumerable permutations in complex formation during sarcomere assembly, the differentially expressed MURFs may have other, muscle-type-specific functions. The ablation of MURF3 by antisense RNA dramatically suppresses myogenic differentiation on the transcriptional level (Spencer et al.,

2000) apart from impairing myofibril formation. This may suggest a role in the control muscle differentiation apart from that of a transient structural adaptor during myofibril formation. A role for MURF3 in muscle gene transcription could be inferred from the putative interaction with SRF (Spencer et al., 2000). MURF1, also known as SMRZ (Dai and Liew, 2001), was found to localise to the nucleus when transfected into C2C12 myoblasts, as well as to translocate in cardiac myocytes (McElhinny et al., 2002). MURF1 can also interact via the highly conserved RING domain with the ubiquitin-like SUMO-2/SMT3b (Dai and Liew, 2001), linking MURFs to potential roles in nuclear transport, transcription regulation and signal transduction (Müller et al., 2001). Our data provide the first evidence that endogenous MURF2 is translocated to the nucleus and the nuclear lamina in response to stimulation of serum-starved cardiac myocytes. MURF2 can thus shuttle between three cellular compartments: microtubules, M-bands and the nucleus. However, MURF2 is detectable in the nucleus of neonatal rat cardiomyocytes only briefly after shifting serum-starved cells from low to high-serum conditions, indicating an involvement in nuclear signalling in a narrow time window and related to the stress of serum-withdrawal. Although we could not observe MURF2 in skeletal myotube nuclei, we are currently investigating the localisation in skeletal muscle tissues. Whether MURFs act as transcriptional co-activators or co-repressors in the nucleus in addition to a more structural involvement in sarcomere assembly will now need to be elucidated. Our observations suggest that the sarcomere not only receives input from many signal transduction pathways, but may also relay information to the transcriptional machinery and could thus regulate muscle-specific gene expression as first proposed by Iakovenko and Gautel (Iakovenko and Gautel, 2000). MURFs emerge as novel components of this crosstalk, and the various signals resulting in stress-induced MURF translocation now need to be identified.

We are greatly indebted to Gitte Neubauer at Cellzome Heidelberg for mass-spectrometrical protein identification. We are most grateful to Nathalie Bleimling for excellent technical assistance and to Roger Goody for continuous support and encouragement and critical reading of this manuscript. This work was supported by the MRC (M.G.) and the DFG (D.F., M.G.).

References

Antin, P. B., Forry-Schaudies, S., Friedman, T. M., Tapscott, S. J. and Holtzer, H. (1981). Taxol induces postmitotic myoblasts to assemble interdigitating microtubule-myosin arrays that exclude actin filaments. *J. Cell Biol.* **90**, 300-308.

Auerbach, D., Bantle, S., Keller, S., Hinderling, V., Leu, M., Ehler, E. and Perriard, J. C. (1999). Different domains of the M-band protein myomesin are involved in myosin binding and M-band targeting. *Mol. Biol. Cell* **10**, 1297-1308.

Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K., Nunez, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E., Dharmarajan, K. et al. (2001). Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294**, 1704-1708.

Cartwright, J. and Goldstein, M. (1982). Microtubules in soleus muscles of the postnatal and adult rat. *J. Ultrastruct. Res.* **79**, 74-84.

Centner, T., Yano, J., Kimura, E., McElhinny, A. S., Pelin, K., Witt, C. C., Bang, M. L., Trombitas, K., Granzier, H., Gregorio, C. C. et al. (2001). Identification of muscle specific ring finger proteins as potential regulators of the titin kinase domain. *J. Mol. Biol.* **306**, 717-726.

Dabiri, G., Turnacioglu, K., Sanger, J. M. and Sanger, J. W. (1997). Myofibrillogenesis visualized in living embryonic cardiomyocytes. *Proc. Natl. Acad. Sci. USA* **94**, 9493-9498.

Dai, K. S. and Liew, C. C. (2001). A novel human striated muscle RING zinc finger protein, SMRZ, interacts with SMT3b via its RING domain. *J. Biol. Chem.* **276**, 23992-23999.

Dlugosz, A. A., Antin, P. B., Nachmias, V. T. and Holtzer, H. (1984). The relationship between stress fiber-like structures and nascent myofibrils in cultured cardiac myocytes. *J. Cell Biol.* **99**, 2268-2278.

Ehler, E., Rothen, B. M., Hammerle, S. P., Komiyama, M. and Perriard, J. C. (1999). Myofibrillogenesis in the developing chicken heart: assembly of Z-disk, M-line and the thick filaments. *J. Cell Sci*. **112**, 1529-1539.

Faulkner, G., Pallavicini, A., Comelli, A., Salamon, M., Bortoletto, G., Ievolella, C., Trevisan, S., Kojic, S., Dalla Vecchia, F., Laveder, P. et al. (2000). FATZ, a filamin-, actinin-, and telethonin-binding protein of the Z-disc of skeletal muscle. *J. Biol. Chem.* **275**, 41234-41242.

Fischman, D. A. (1970). The synthesis and assembly of myofibrils in embryonic muscle. *Curr. Top. Dev. Biol.* **5**, 235-280.

Freiburg, A. and Gautel, M. (1996). A molecular map of the interactions of titin and myosin-binding protein C: implications for sarcomeric assembly in familial hypertrophic cardiomyopathy. *Eur. J. Biochem.* **235**, 317-323.

Fürst, D., Osborn, M., Nave, R. and Weber, K. (1988a). The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z line extends close to the M line. *J. Cell Biol.* **106**, 1563-1572.

Fürst, D. O., Osborn, M., Nave, R. and Weber, K. (1988b). The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy; a map of ten non-repetitive epitopes starting at the Z line extends close to the M line. *J. Cell Biol.* **106**, 1563-1572.

Fürst, D. O., Nave, R., Osborn, M. and Weber, K. (1989a). Repetitive titin epitopes with a 42 nm spacing coincide in relative position with known A band striations also identified by major myosin-associated proteins; an immunoelectron microscopical study on myofibrils. *J. Cell Biol.* **94**, 119-125.

Fürst, D. O., Osborn, M. and Weber, K. (1989b). Myogenesis in the mouse embryo: differential onset of expression of myogenic proteins and the involvement of titin in myofibril assembly. *J. Cell Biol.* **109**, 517-527.

Gautel, M., Lehtonen, E. and Pietruschka, F. (1996). Assembly of the cardiac I-band region of titin/connectin: expression of the cardiac-specific regions and their relation to the elastic segments. *J. Muscle Res. Cell Motil.* **17**, 449-461.

Gautel, M., Mues, A. and Young, P. (1999). Control of sarcomeric assembly: the flow of information on titin. *Rev. Physiol. Biochem. Pharmacol*. **138**, 98-137.

Goldstein, M. A. and Entman, M. L. (1979). Microtubules in mammalian heart muscle. *J. Cell Biol.* **80**, 183-195.

Gruen, M. and Gautel, M. (1999). Mutations in beta-myosin S2 that cause familial hypertrophic cardiomyopathy (FHC) abolish the interaction with the regulatory domain of myosin binding protein-C. *J. Mol. Biol.* **286**, 933-949.

Gundersen, G., Khawaja, S. and Bulinski, J. C. (1989). Generation of stable, posttranslationally modified microtubule array is an early event in myogenic differentiation. *J. Cell Biol.* **109**, 2275-2288.

Guo, J. X., Jacobson, S. L. and Brown, D. L. (1986). Rearrangement of tubulin, actin, and myosin in cultured ventricular cardiomyocytes of the adult rat. *Cell Motil. Cytoskeleton* **6**, 291-304.

Holtzer, H., Forry-Schaudies, S., Dlugosz, A., Antin, P. and Dubyak, G. (1985). Interactions between IFs, microtubules, and myofibrils in fibrogenic and myogenic cells. *Ann. NY Acad. Sci.* **455**, 106-125.

Holtzer, H., Hijikata, T., Lin, Z. X., Zhang, Z. Q., Holtzer, S., Protasi, F., Franzini-Armstrong, C. and Sweeney, H. L. (1997). Independent assembly of 1.6 microns long bipolar MHC filaments and I-Z- I bodies. *Cell Struct. Funct.* **22**, 83-93.

Iakovenko, A. and Gautel, M. (2000). Titin-associated zinc-finger proteins link titin kinase to transcriptional control. *J. Muscle Res. Cell Motil.* **21**, 833.

Klemke, M., Kehlenbach, R. H. and Huttner, W. B. (2001). Two overlapping reading frames in a single exon encode interacting proteins – a novel way of gene usage. *EMBO J.* **20**, 3849-3860.

Komiyama, M., Kouchi, K., Maruyama, K. and Shimada, Y. (1993). Dynamics of actin and assembly of connectin (titin) during myofibrillogenesis in embryonic chick cardiac muscle cells in vitro. *Dev. Dyn.* **196**, 291-299.

Labeit, S. and Kolmerer, B. (1995). Titins: giant proteins in charge of muscle ultrastructure and elasticity. *Science* **270**, 293-296.

LoRusso, S. M., Rhee, D., Sanger, J. M. and Sanger, J. W. (1997). Premyofibrils in spreading adult cardiomyocytes in tissue culture: evidence

for reexpression of the embryonic program for myofibrillogenesis in adult cells. *Cell Motil. Cytoskeleton* **37**, 183-198.

Luther, P. K., Barry, J. S. and Squire, J. M. (2002). The three-dimensional structure of a vertebrate wide (slow muscle) Z-band: lessons on Z-band assembly. *J. Mol. Biol.* **315**, 9-20.

Mayans, O., van der Ven, P., Wilm, M., Mues, A., Young, P., Fürst, D. O., Wilmanns, M. and Gautel, M. (1998). Structural basis of the activation of the titin kinase domain during myofibrillogenesis. *Nature* **395**, 863-869.

McElhinny, M. S., Kakinuma, K., Sorimachi, H., Labeit, S. and Gregorio, C. C. (2002). Muscle-specific RING finger-1 interacts with titin to regulate sarcomeric M-line and thick filament structure and may have nuclear functions via its interaction with glucocorticoid modulatory element binding protein-1. *J. Cell Biol.* **157**, 125-136.

Mertens, C., Kuhn, C. and Franke, W. W. (1996). Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. *J. Cell Biol.* **135**, 1009-1025.

Müller, S., Hoege, C., Pyrowolakis, G. and Jentsch, S. (2001). SUMO, ubiquitin's mysterious cousin. *Nat Rev Mol Cell Biol* **2**, 202-210.

Okazaki, K. and Holtzer, H. (1965). An analysis of myogenesis in vitro using fluorescein-labeled antimyosin. *J. Histochem. Cytochem.* **13**, 726-739.

Perhonen, M., Sharp, W. W. and Russell, B. (1998). Microtubules are needed for dispersal of alpha-myosin heavy chain mRNA in rat neonatal cardiac myocytes. *J. Mol. Cell Cardiol.* **30**, 1713-1722.

Person, V., Kostin, S., Suzuki, K., Labeit, S. and Schaper, J. (2000). Antisense oligonucleotide experiments elucidate the essential role of titin in sarcomerogenesis in adult rat cardiomyocytes in long-term culture. *J. Cell Sci.* **113**, 3851-3859.

Pinset, C. and Montarras, D. (1998). *Cell Biology: A Laboratory Handbook*. Sheffield: Acadamic Press.

Rappaport, L., Samuel, J. L., Bertier, B., Bugaisky, L., Marotte, F., Mercadier, A. and Schwartz, K. (1984). Isomyosins, microtubules and desmin during the onset of cardiac hypertrophy in the rat. *Eur. Heart J.* **5 Suppl. F**, 243-250.

Rappaport, L., Samuel, J. L., Bertier-Savalle, B., Marotte, F. and Schwartz, K. (1985). Microtubules and desmin filaments during the onset of heart growth in the rat. *Basic Res. Cardiol.* **80**, 129-132.

Rhee, D., Sanger, J. M. and Sanger, J. W. (1994). The premyofibril: evidence for its role in myofibrillogenesis. *Cell Motil. Cytoskeleton* **28**, 1-24.

Rothen-Rutishauser, B. M., Ehler, E., Perriard, E., Messerli, J. M. and Perriard, J. C. (1998). Different behaviour of the non-sarcomeric cytoskeleton in neonatal and adult rat cardiomyocytes. *J. Mol. Cell Cardiol.* **30**, 19-31.

Rüdiger, A. H., Rüdiger, M., Wehland, J. and Weber, K. (1999). Monoclonal antibody ID5: epitope characterization and minimal requirements for the recognition of polyglutamylated alpha- and beta-tubulin. *Eur. J. Cell Biol.* **78**, 15-20.

Rudy, D. E., Yatskievych, T. A., Antin, P. B. and Gregorio, C. C. (2001). Assembly of thick, thin, and titin filaments in chick precardiac explants. *Dev. Dyn.* **221**, 61-71.

Saitoh, O., Arai, T. and Obinata, T. (1988). Distribution of microtubules and other cytoskeletal filaments during myotube elongation as revealed by fluorescence microscopy. *Cell Tissue Res.* **252**, 263-273.

Salmikangas, P., Mykkanen, O. M., Gronholm, M., Heiska, L., Kere, J. and Carpén, O. (1999). Myotilin, a novel sarcomeric protein with two Ig-like domains, is encoded by a candidate gene for limb-girdle muscular dystrophy. *Hum. Mol. Genet.* **8**, 1329-1336.

Samuel, J. L., Bertier, B., Bugaisky, L., Marotte, F., Swynghedauw, B., Schwartz, K. and Rappaport, L. (1984). Different distributions of microtubules, desmin filaments and isomyosins during the onset of cardiac hypertrophy in the rat. *Eur. J. Cell Biol.* **34**, 300-306.

Sanger, J. W. and Sanger, J. M. (2001). Fishing out proteins that bind to titin. *J. Cell Biol.* **154**, 21-24.

Sanger, J. M., Mittal, B., Pochapin, M. B. and Sanger, J. W. (1986). Myofibrillogenesis in living cells microinjected with fluorescently labeled alpha-actinin. *J. Cell Biol.* **102**, 2053-2066.

Sanger, J. W., Ayoob, J. C., Chowrashi, P., Zurawski, D. and Sanger, J. M. (2000). Assembly of myofibrils in cardiac muscle cells. *Adv. Exp. Med. Biol.* **481**, 89-102.

Schultheiss, T., Lin, Z., Lu, M., Murray, J., Fischman, D., Weber, K., Masaki, T., Imamura, M. and Holtzer, H. (1990). Differential distribution of subsets of myofibrillar proteins in cardiac nonstriated and striated myofibrils. *J. Cell Biol.* **110**, 1159-1172.

Soeno, Y., Yajima, H., Kawamura, Y., Kimura, S., Maruyama, K. and Obinata, T. (1999). Organization of connectin/titin filaments in sarcomeres of differentiating chicken skeletal muscle cells. *Mol. Cell Biochem.* **190**, 125-131.

Sorimachi, H., Freiburg, A., Kolmerer, B., Ishiura, S., Stier, G., Gregorio, C. C., Labeit, D., Linke, W. A., Suzuki, K. and Labeit, S. (1997). Tissue-specific expression and alpha-actinin binding properties of the Z-disc titin: implications for the nature of vertebrate Z-discs. *J. Mol. Biol.* **270**, 688-695.

Spencer, J. A., Eliazer, S., Ilaria, R. L., Jr, Richardson, J. A. and Olson, E. N. (2000). Regulation of microtubule dynamics and myogenic differentiation by MURF, a striated muscle RING-finger protein. *J. Cell Biol.* **150**, 771-784.

Tokuyasu, K. T. and Maher, P. A. (1987). Immunocytochemical studies of cardiac myofibrillogenesis in early chick embryos. I. Presence of immunofluorescent titin spots in premyofibril stages. *J. Cell Biol.* **105**, 2781-2793.

Toyama, Y., Forry-Schaudies, S., Hoffman, B. and Holtzer, H. (1982). Effects of taxol and Colcemid on myofibrillogenesis. *Proc. Natl. Acad. Sci. USA* **79**, 6556-6560.

Trinick, J. (1992). Molecular rulers in muscle? *Curr. Biol.* **2**, 75-77.

Trinick, J. (1994). Titin and nebulin: protein rulers in muscle? *Trends Biochem. Sci.* **19**, 405-409.

Trinick, J. (1996). Titin as a scaffold and spring. *Curr. Biol.* **6**, 258-260.

Turnacioglu, K. K., Mittal, B., Sanger, J. M. and Sanger, J. W. (1996). Partial characterization of zeugmatin indicates that it is part of the Z-band region of titin. *Cell Motil. Cytoskeleton* **34**, 108-121.

Van der Loop, F. T. L., van der Ven, P. F. M., Fürst, D. O., Gautel, M., van Eys, G. J. J. M. and Ramaekers, F. C. S. (1996). Integration of titin into the sarcomeres of cultured differentiating human skeletal muscle cells. *Eur. J. Cell Biol.* **69**, 301-307.

Van der Ven, P. F. M. and Fürst, D. O. (1998). Expression of sarcomeric proteins and assembly of myofibrils in the putative myofibroblast cell line BHK-21/C13. *J. Muscle Res. Cell Motil.* **19**, 767-775.

Van der Ven, P. F. M., Ehler, E., Perriard, J. C. and Fürst, D. O. (1999). Thick filament assembly occurs after the formation of a cytoskeletal scaffold. *J. Muscle Res. Cell Motil.* **20**, 569-579.

Van der Ven, P. F. M., Wiesner, S., Salmikangas, P., Auerbach, D., Himmel, M., Kempa, S., Hayess, K., Pacholsky, D., Taivainen, A., Schröder, R. et al. (2000). Indications for a novel muscular dystrophy pathway. gamma-filamin, the muscle-specific filamin isoform, interacts with myotilin. *J. Cell Biol.* **151**, 235-248.

Vojtek, A. B., Hollenberg, S. M. and Cooper, J. A. (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74**, 205-214.

Wang, K. and Wright, J. (1988). Architecture of the sarcomere matrix of skeletal muscle: immunoelectron microscopic evidence that suggests a set of parallel inextensible nebulin filaments anchored at the Z line. *J. Cell Biol.* **107**, 2199-2212.

Warren, R. (1974). Microtubular organization in elongating myogenic cells. *J. Cell Biol.* **63**, 550-566.

Wehland, J., Schröder, H. C. and Weber, K. (1984). Amino sequence requirements in the epitope recognized by the α-tubulin-specific rat monoclonal antibody YL 1/2. *EMBO J.* **3**, 1295-1300.

Young, P., Ferguson, C., Bañuelos, S. and Gautel, M. (1998). Molecular structure of the sarcomeric Z-disk: two types of titin interactions lead to an asymmetrical sorting of alpha-actinin. *EMBO J.* **17**, 1614-1624.

Young, P., Ehler, E. and Gautel, M. (2001). Obscurin, a giant sarcomeric Rho guanine nucleotide exchange factor protein involved in sarcomere assembly. *J. Cell Biol.* **154**, 123-136.