Filamin C interacts with the muscular dystrophy KY protein and is abnormally distributed in mouse KY deficient muscle fibres

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The KY protein has been implicated in a neuromuscular dystrophy in the mouse, but its role in muscle function remains unclear. Here, we show that KY interacts with several sarcomeric cytoskeletal proteins including, amongst others, filamin C and the slow isoform of the myosin-binding protein C. These interactions were confirmed in vitro and because of its central role in skeletal muscle disease, characterized in more detail for filamin C. A role for KY in regulating filamin C function in vivo is supported by the expression analysis of filamin C in the null ky mouse mutant, where distinct irregular subcellular localization of filamin C was found in subsets of muscle fibres, which appears to be a specific outcome of KY deficiency. Furthermore, KY shows protease activity in in vitro assays, and specific degradation of filamin C by KY is shown in transfected cells. Given the enzymatic nature of the KY protein, it is likely that some of the identified partners are catalytic substrates. These results suggest that KY is an intrinsic part of the protein networks underlying the molecular mechanism of several limb-girdle muscular dystrophies, particularly those where interactions between filamin C and disease causing proteins have been shown.

INTRODUCTION

The ky-kyphoscoliosis mutant appeared spontaneously in an inbred mouse colony and was first identified by a prominent spinal deformity, a character of recessive inheritance (1). Subsequently, it was found that ky muscles are smaller, slower contracting and weaker than controls (2). However, only postural slow-contracting muscles such as the soleus, psoas or the erector spinae display typical muscle degeneration and regeneration hallmarks, for instance internal nuclei, fibre size variability or increased connective tissue, common to muscular dystrophies (3). The ensuing postural muscle weakness leads to muscle to body weight imbalance and secondary chronic thoraco-lumbar kyphoscoliosis, a feature of post-weaning onset. The ky mutation provokes a premature stop codon leading to a predicted total absence of the KY protein in the ky mouse mutant (4). Thus far, no molecular function has been assigned to the KY protein. The ky transcript has been detected at low levels only in skeletal muscle and heart and encodes a novel transglutaminase-like/protease protein (4).

Enzymatic activity in transglutaminases relies on the presence within the catalytic pocket of the active site triad, comprising a cysteine, a histidine and an aspartate. A similar structure is found in ancestral prokaryotic proteases from which modern transglutaminases are thought to have evolved (5). However, sequence alignments of KY with related transglutaminases are inconclusive regarding the functional conservation of the active site triad in the KY protein (4,6). On the other hand, this comparative analysis, together with the available expression data of the Caenorhabditis elegans and yeast homologues, clearly placed KY into a new highly conserved family of potential cytoskeletal proteins (6–8). In order to elucidate the role of KY in muscle function and disease, it is therefore imperative to analyze its enzymatic properties.

Here, we have uncovered functional features of the KY protein. Different tagged and untagged versions of the KY protein have been expressed in muscle and non-muscle cell lines and differentiated myotubes. Interactions have been identified using the yeast two-hybrid system and confirmed in vitro. To test the functionality of the conserved
transglutaminase/protease domain, protease in vitro assays using recombinant as well as wild-type KY proteins have been performed. Finally, this analysis has been extended to the null ky mouse mutant, a suitable genetic background from which to obtain insights into the role of the KY protein in vivo.

RESULTS

KY interacts with sarcomeric cytoskeletal proteins

A yeast two-hybrid screen of a human skeletal muscle cDNA library using the full length KY protein as a bait produced fragments containing open reading frames for the C-terminal end of filamin C (FLNC), the full length slow isoform of myosin binding protein C (MYBPC1), several fragments of giant titin protein (TTN) and several fragments of a novel protein ( provisionally named here as KYIP1). All known putative interacting partners are muscle-specific sarcomeric proteins made up of series of immunoglobulin and/or fibronectin-like domains (Ig-like and Fn-like, respectively). Interactions between KY and all the above proteins or protein fragments were confirmed within the yeast two hybrid system and corroborated in a pull down assay. For the latter, a fusion between the maltose-binding protein (MBP) and full length KY (MBP–KY) was used to test the specificity of the association between KY and the in vitro translated putative interacting partners (Fig. 1A and C). In addition, interactions were characterized in more detail for MYBPC1 and FLNC. MYBPC1 is made up of a series of 10 immunoglobulin and fibronectin-like domains, termed as C1–C10, in which interaction domains for myosin and titin as well as A band localization have been previously identified (reviewed in 9,10). A series of MYBPC1 derived constructs containing deletions for several of its domains was tested in the yeast two-hybrid system. The results showed that MYBPC1 contains at least two sites of interaction for KY, one at the N-terminus, within domains C2–C4, and another one at the C-terminus, within domains C9–C10 (Fig. 1B). Interactions with TTN were not mapped any further as the fragments recovered from the Y2H already suggested more than one well defined site of interaction. Thus, most fragments of the titin molecule (five out of a total of 50 clones sequenced in this screen; see Materials and Methods) contained only its last 50 aminoacids. However, one additional TTN fragment started at the 8th Ig-like domain from the C-terminal and did not include those aminoacids (data not shown). KYIP1 shows high homology to both TTN and MYBPC1, but other than being a predicted protein (Ensembl gene ID ENSG00000163395), the KYIP1 full length cDNA has not yet been cloned and therefore its exact domain composition is still unclear.

In this work, we have focused on the interaction with FLNC because of its central role in skeletal muscle function and disease. FLNC is a large actin-cross-linking protein containing an N-terminal actin-binding domain followed by 24 Ig-like domains, denoted here as d1–d24. All the recovered FLNC clones contained domains d19–d24. A deletion series analysis spanning the last six domains narrowed down the region of interaction to domains d20–d22 (Fig. 1C). A subset of these constructs were translated in vitro and tested in a pull down assay. Yeast two-hybrid interactions were readily reproduced by this independent biochemical assay (Fig. 1D). The reciprocal experiment was done by tagging FLNCd19/24 with MBP (MBP–FLNCd19/24) followed by incubation and pull down of in vitro translated KY. This experiment also showed high and specific affinity of binding of FLNC to KY compared to the negative control using MBP alone (Fig. 1D).

Interactions in COS-7 cells

To confirm that the interactions detected in vitro and in the yeast two-hybrid system also occur within cells, either untagged KY or KY tagged at its C-terminal with green fluorescent protein (GFP) (KY–GFP) and a His/V5-tagged FLNC clone containing domains d19–d24 (His/V5–FLNCd19/24) were co-expressed in COS-7 cells. Both KY–GFP and KY efficiently co-purified with FLNCd19/24 from co-transfected cells, whereas negligible co-purification was obtained with a His-tagged LacZ protein used as negative control (Fig. 2A).

FLNC interacts with endogenous KY

Rabbit polyclonal antibodies were raised against three KY fusion proteins encompassing residues 1–577, 1–270 and 207–630 (AbKyL1R4, AbKyL1R2 and AbKyL3R3, respectively) and eight peptides scattered along the KY sequence (see Materials and Methods for details of constructs and peptides). Out of the total, six KY antibodies recognized over-expressed heterologous KY fusion proteins on western blots from Escherichia coli or transfected COS-7 cell lysates (data not shown), but only AbKyL1R4 performed well on immunofluorescent labelling of transfected cells (Fig. 2C). Despite the variety of the immunogens used, none of the resulting antibodies obtained identified the native protein as the expected 73 kDa protein band on skeletal muscle western blots or gave a good signal to noise ratio on immunostainings of skeletal muscle sections. This apparent elusiveness of native KY suggests that it is expressed at very low levels, consistent with the low levels of transcript that we have observed (4) and/or it is a very short lived protein. More crucially, it precluded further confirmation of the KY–FLNC interaction by co-localization studies on adult muscle fibres. Hence, we resolved to test the binding of FLNC to endogenously produced KY by means of a pull down assay using MBP tagged FLNCd19/24 on whole skeletal muscle extracts. As negative controls, MBP alone and a muscle extract from the ky homozygous mutant were used. The ky mutation, a GC deletion at codon 24, predicts that no KY protein should be present in homozygous mutants (4). Using AbKyL1R4 antibodies on the resulting western blotted pull down samples, a single band of the expected 73 kDa was detected with wild-type but not ky mutant samples (Fig. 2B). This result substantiates the specificity of the interaction and confirms that such interaction also occurs with endogenous KY. In addition, it indirectly confirms the absence of KY protein in ky homozygous mice.
Figure 1. (A) Confirmation of the interactions between KY and MBPC1, TTN and KYIP1. Left, pull downs of radiolabelled proteins or protein fragments using MBP by itself or fused to KY (MBP–KY). Right, pictures of yeast two-hybrid assays showing that activation of the His and Ade reporters requires the presence of KY and the putative interacting partners as indicated. pGADT7 and pGBK7 are empty backbone control vectors. (B) Domain architecture at scale of skeletal muscle MBPC1. Known interactions are shown above as a grey solid bar that expands the domain(s) required for each interaction (MyosinS2 and LMM denote the myosin neck region S2 and the rod portion or light meromyosin, respectively). Below, black solid bars indicate the length of the different clones with the results on the yeast two-hybrid (Y2H) test next to each construct. (C) Domain architecture at scale of the C-terminal of skeletal muscle filamin C. Bars underneath denote the length of the different clones with the results on the yeast two-hybrid test (Y2H) and pull down (P.D.) assays indicated next to each construct (N.T., non tested). Right, picture of a yeast two-hybrid assay showing that reporters’ activation requires the presence of KY and FLNCd19/24 or FLNCd20/22. (D) In vitro interaction assays between KY and FLNC fragments. Left, pull-downs of radiolabelled FLNC fragments using MBP by itself or fused to KY (MBP–KY). Right, pull down of radiolabelled KY using MBP by itself or fused to FLNCd19/24 (MBP–FLNCd19/24) (see Materials and Methods for details).
Expression analysis

As co-localization studies could not be efficiently performed with any of the available anti-KY antibodies, we next attempted to study the sub-cellular localization of KY in vitro. Transfections of different muscle cell lines (mouse C2C12, rat L6.G8 and L8 and quail QM7) as well as non muscle cells (HeLa, COS-7 and NIH/3T3) with untagged KY or KY–GFP (pTRE2HygKY or pCTGFPKy, respectively) revealed the same smooth cytoplasmic pattern of expression (some examples of these are shown in Fig. 3A). Phalloidin staining on C2C12 cells showed no signs of KY being stably associated with the actin based cytoskeleton (Fig. 3B). In all cell lines tested, transient transfections with KY–GFP or KY invariably lead to cell death before 48 h (Fig. 3C). Transfected cells detached from the bottom of the plate but tested negative for active caspase-3 and in TUNEL assays, suggesting that apoptosis was not implicated in this process (data not shown). This toxic effect complicated the analysis of the KY expression within mature myotubes, as these will develop from confluent myoblasts only after several days in culture. Moreover, cell lines stably transfected with pCTGFPKy or pTriX1.1Ky (a bicistronic vector that encodes for KY and the neo selectable marker on a single transcript; see Materials and Methods) did not show any detectable KY expression either. To circumvent the toxicity effect, a new cell line derived from the rat L8 was selected for the inducible expression of KY in the presence of...
doxycycline (DOX; see Materials and Methods). This cell line was then differentiated into multinucleated myotubes, and KY expression was induced upon addition of DOX to the medium. A similar cytoplasmic pattern to that observed in undifferentiated myoblasts was displayed by these myotubes (Fig. 3D, see fully differentiated myotube and detail). This pattern clearly differs from reported sarcomeric proteins overexpressed under similar conditions, including FLNC (11).

KY has protease activity in vitro

A new database search of KY homologues revealed additional sequences from vertebrates and other eukaryotes (Fig. 4A). Alignment of the protease/transglutaminase-like core domain showed that, as noted before (4,6), replacement of the predicted active residues have occurred in many members of this family. However, the putative catalytic triad is conserved in all the available sequences of vertebrate KY homologues (Fig. 4A). This suggests that protease activity may be a preserved functional feature in the vertebrate subgroup, because the only functionally characterized member of this superfamily is the pseudomurein endopeptidase of the *Methanobacterium* phage psiM2 (12).

The KY protease activity was initially tested using two general substrates: fluorescently labelled but totally quenched casein and the conjugated peptide *N*-benzoyl-1-arginine-7-amido-4-methylcoumarin hydrochloride (*N*-Arg-MCA).
Casein releases green fluorescence only after protease-catalyzed hydrolysis, whereas fluorescent 7-amino-4-methylcoumarin is released upon cleavage of \(N_a\)-Arg-MCA. A qualitative protease activity test was performed by incubating the MBP–KY or MBP coated resin with casein or \(N_a\)-Arg-MCA in MES buffer (see Materials and Methods). Strong green and blue fluorescent signals were released from MBP–KY coated beads incubated with casein or \(N_a\)-Arg-MCA, respectively, whereas the MBP coated beads remained dim (Fig. 4B).

We then asked if FLNC is a catalytic substrate for KY. This was tested by comparing the expression levels of FLNC\(_{d19/24}\) in COS-7 cells co-transfected with KY or KY–GFP. The results showed a reduction in the amount FLNC\(_{d19/24}\) present in lysates from cells that had been co-transfected with either KY or KY–GFP (Fig. 4B), suggesting that KY directly or indirectly degrades FLNC.

**FLNC expression in the ky homozygous mutant**

FLNC is the only filamin isoform expressed in adult skeletal muscle (11). Given that KY enzymatically targets FLNC in vitro, the possibility exists that KY may play a role in regulatng FLNC expression in vivo. To explore the expression of FLNC in wild-type and ky mutant muscles, the monoclonal antibody RR90 was comprehensively used. RR90, although reactive with filamin A and filamin C (11), does exclusively stain filamin C in skeletal muscle fibres, as this is the only filamin isoform expressed in these cells (11). The immunostaining results are shown in Figure 5. Cross sections of the lower limb spanning the deeply affected soleus muscle as well as the unaffected gastrocnemius showed a much stronger fluorescent signal in ky muscles. This increment affected both type I and IIa fibres in dystrophic soleus and sporadic fibres in gastrocnemius (indicated by white arrows in Fig. 5A). This variation was consistent across muscle samples from young (30 days old) as well as very old (over 300 days) mice (data not shown). Patchy and irregular FLNC staining of homozygous ky soleus muscle was displayed by both regenerating and non-regenerating fibres (Fig. 5A, bottom), suggesting that it is not a hallmark of any particular phase of the dystrophic process. In contrast, western blots did not show any difference in the total amount of FLNC (Fig. 5B) suggesting that in ky muscles FLNC is irregularly distributed rather than upregulated. As FLNC has been shown to interact with myotilin at the Z-disc (13), we looked for evidence of FLNC and/or myotilin redistribution on longitudinal sections, on which the typical striations are exposed, by analyzing their co-localization...
Figure 5. Alterations of FLNC localization in ky muscles. (A) Confocal images of cross sections spanning soleus and adjacent gastrocnemius showing a higher green fluorescent signal with the anti-FLNC RR90 antibody in homozygous ky muscles. Fluorescent pictures were taken with identical settings. Two examples of homozygous ky are shown, the top one also including a section of gastrocnemius. White arrows point at highly reactive fibres within the ky gastrocnemius. Type I fibres were identified by MHC I staining with the monoclonal antibody A4.951 (red fluorescence). The overlay image of ky/ky sections reveals that some type I fibres express FLNC (yellow signal) whereas others do not (red signal), indicating that there is not direct correspondence between fibre type and FLNC expression. Note regenerating centrally nucleated fibres in the ky dystrophic samples in the H&E views on the right of the same muscle group (SOL, soleus; GM, gastrocnemius). Bottom, a detailed view of the 45 days old ky dystrophic soleus mentioned earlier. Continuous and discontinuous lines circle centrally nucleated regenerating fibres and normal non-regenerating fibres, respectively. Note that both type of fibres show irregular and inconsistent FLNC expression. (B) Loading control gel and a section of a western blot showing similar expression levels of FLNC (arrow) in mutant and control soleus muscles. The FLNC band was identified because of its upregulation upon differentiation of C2C12 cells (11) (C2 proliff. and C2 differ. denote C2C12 proliferating cells and C2C12 differentiated into myotubes, respectively). 3T3 cells do not express FLNC and are used as negative control (see Materials and Methods). (C) A longitudinal section of control and mutant soleus stained with RR90 (FLNC) and myotilin antibodies. Note in the ky mutant fibres, the increased accumulation of FLNC at the Z band, reactive dots and smears. (D) EM pictures at the same magnification of ky and control showing partial lack of register and increased thickness of the Z band in ky soleus. Right, a quantitative analysis of the Z band thickness.
pattern. As expected, FLNC and myotilin co-localize at the Z-disc in control sections. In kyd fibres, however, signs of FLNC mis-localization were evident. Thus, along with fibres showing apparently normal Z-band striation, there were fibres showing disproportionate accumulation of FLNC at the Z-band, very reactive dots and long smears (Fig. 5C and details). Distribution of myotilin, on the other hand, did not show any relevant difference between mutant and control samples.

As FLNC is a crucial component of the Z-band (11), we then asked if specific Z-band phenotypes could be unveiled in the kyd mutant at ultrastructural level. Electron microscopy revealed significant alterations of the sarcomere banding pattern and dimensions in dystrophic soleus (Fig. 5D). In dystrophic soleus, we observed clear myofibrillar alterations, including Z-line streaming and overlapping of thick filaments with loose A banding. Similar and other ultrastructural alterations have already been described in other kyd muscle tissues at all ages (3). Interestingly, the Z-band was much thicker in kyd soleus fibres than in controls. Measures of over 100 Z-discs from well preserved and non-degenerating fibres from three homozygous kyd mutants showed an average increase of 60% over age matched control samples (Fig. 5D). This result, together with the biochemical evidence of KY/FLNC binding, suggests that interactions between KY and FLNC are required for normal sarcomere formation and/or stabilization.

**DISCUSSION**

Several muscle specific sarcomeric cytoskeletal proteins, including TTN, MYBPC1, KYIP1 and FLNC have been identified as interacting partners of KY. These are modular
proteins containing repeats of Ig-like and/or Fb-like domains responsible, amongst other interacting functions, for correct sarcomeric targeting. We focused on the interaction with FLNC because of the pivotal role of this protein in the pathogenic mechanism of several limb-girdle muscular dystrophies (LGMD). Thus, FLNC interacts with myotilin (LGMD1A) (13,14), γ- and δ-sarcoglycan (LGMD2C and 2F, respectively) (15–17) and is an enzymatic target of calpain-3 (LGMD2A) (18–20). A serial deletion analysis showed that FLNC domains d20–d22 are necessary and sufficient for interaction in the yeast two-hybrid system. In addition, FLNC efficiently pulls down endogenous KY protein from wild-type but not ky mutant muscle extracts, indicating that such interaction is compatible with naturally occurring KY.

It has been previously suggested that members of the ky/cyk3 family are all cytoskeleton associated proteins (6). This was experimentally supported by the expression pattern of the KY C. elegans and yeast homologues (7,8). However, we found no evidence of KY stably associating with any cytoskeletal structure in non-differentiated and differentiated myotubes, a result consistent with the absence in the KY sequence of the LIM/SH3 protein–protein interaction domains that are found in the non-vertebrate homologues (4,6). On the other hand, a statistically significant sequence similarity between the animal transglutaminases and a distinct class of microbial proteins whose only functionally characterized representative is a protease has been previously found (5). As the KY protein belongs to this family, we looked for evidence of enzymatic activity. Here, we have shown that the mouse KY protein has endopeptidase activity in vitro over general fluorescent substrates and also specifically degrades FLNC when co-transfected in COS-7 cells.

We explored FLNC expression in the null ky spontaneous mutant. This revealed abnormal patterns of FLNC localization in adult muscle fibres. Thus, upregulation of membrane bound FLNC has been found in LGMD2C and DMD patients as well as within microsome preparations from the mdx and null γ-sarcoglycan and δ-sarcoglycan mice (16). However, in ky muscles we found no evidence of specific sarcolemmal-bound FLNC upregulation using the same polyclonal antibodies as in (16) (data not shown) or the RR90 monoclonal antibody (11) (Fig. 5). On the other hand, the non-specific accumulation of FLNC found in patients with central core disease, multi-minicore myopathy (21) and other myopathies (J. Beatham et al., unpublished data and R. Schröder, personal communication) also clearly differs from the expression pattern described here. Therefore, this abnormal distribution is a ky specific effect. It is not a direct consequence of myofibrillar degeneration, as it is found in both regenerating and non-regenerating fibres in ky soleus as well as in some fibres of the non dystrophic ky gastrocnemius. Surprisingly, total amount of FLNC, as revealed by western blots, is unchanged in mutant muscles. This suggests that redistribution of FLNC is the likely cause of its increased reactivity. We found evidence of this on longitudinal sections, where FLNC showed a variety of patterns from apparently normal sarcomeric localization to excessive accumulation at the Z-band and smeary stainings. In addition, the ultrastructural analysis of the sarcomere in the ky mutant revealed a considerable increment of the Z-disc thickness as well as previously reported myofibrillar alterations (3). Given that FLNC plays a role in myofibrillar formation (11), it is plausible that some of the morphological changes found in the KY deficient muscle fibres are contributed to by the lack of association and/or catalytic control of KY over FLNC.

FLNC has already been shown to be a catalytic target of Calpain3 (18,19), a cysteine protease that underlies the most prevalent form of LGMD (20). Although Calpain3 is a more complex enzyme than KY, in terms of its domain composition (22), it is worth noticing that both KY and Calpain3 are proteases (22,23) (this work) underlying muscular dystrophies, their overexpression in transfected cells provokes cell detachment and death by an apoptotic independent mechanism (19,23) (this work) and they both share FLNC and TTN as cytoskeletal partners. In addition, although the Calpain3 deficient mice showed variable alterations depending on genetic background and age, psoas and soleus, were amongst the most affected muscles (24). Pathological changes in ky muscles affect slow postural muscle only, with psoas and soleus prominent amongst them (3). It is therefore possible that these proteins play a complementary role in regulating muscle cytoskeleton homeostasis in response to changes in muscle activity, a question that may be addressed by test crosses between ky and Capn3 deficient mouse lines.

To date there are no reported mutations in FLNC associated with inherited muscle disorders in the human population (OMIM 102565). However, FLNC interacts with a number of proteins that underlie different forms of LGMDs. Given the profound muscular dystrophy that KY deficiency provokes in the mouse (3,4) and its association with FLNC, KY is a likely candidate for those existing forms of human LGMDs thus far excluded from any known chromosomal locus.

**MATERIALS AND METHODS**

**Immunohistochemistry**

Tissues from 30 to 45 days old mice were frozen in isopentane cooled in liquid nitrogen prior to cryosectioning and then stored at −70°C. After thawing and drying, sections were fixed with either cold acetone (pure or as a 1:1 mix with cold methanol) or 4% paraformaldehyde for 30 min and rinsed with PBS three times. Standard indirect immunohistochemistry was employed using primary rabbit and mouse monoclonal antibodies followed by FITC or TRITC-conjugated polyclonal anti-mouse, or anti-rabbit (Sigma) and visualized using a Zeiss Axiosope fluorescent microscope or a Bio-Rad MRC 500 confocal laser scanning system attached to a Nikon Diaphot inverted microscope.

Polycystolic anti-KY antibodies were raised using conjugated peptides from various commercial sources or in house using fusion proteins. The peptides and resultant serum were: ANHNFYSYLKKYKNDQ (KyAb1), GPTETEQRYYIFQLN (KyAb2), CLHGDGPIETEQTQRY (KyAb3), CGEF DHAWNAYLEGKR (KyAb4), CLKPPQSLROFENSYMKY (KyAb5), CLOQDKGNGNRPROPG (Ky136B), CIVHSE KRRAAQATHKL (Ky136A) and NAVAIMLIVHSEKCY (KySig1). Fusion proteins were constructed using the pMALC2 vector (New England Biolabs). ky gene fragments were amplified using the following primers to give the...
following vectors after cloning in frame with pMALC2 MBP, pL1R2 with forward: ACC ATG GAG TTT AAG AAG GAC AGC AAC and reverse: AGC GTT CCA GGA GTG GTC GAA; pL1R4 with forward: ACC ATG GAG TTT AAG AAG GAC AGC AAC and reverse: CCT GTC CCC AGT TGC CAA AGC; pL3R3 with forward: CAT GCT CAT CTG GCA AGC and reverse: CCT GTC CCC AGT TGC CAA AGC; KyAb2, KyAb136B and Ky136A specifically recognized This test showed that AbKyL1R2, AbKyL1R4, AbKyL3R3, as well as the anti-peptide antibodies was ascertained on western blots against bacterial extracts containing the fusion proteins. Fusion proteins were produced according to the instructions provided by the manufacturer (New England Biolabs). Purified fusion proteins were injected into rabbits. After the third boost serum was collected and characterized, the specificity of the obtained in vitro

**Cell culture and expression**

A full length ky cDNA was cloned into pCDNA3.1/ CTGfpTopo and pCDNA3.1/NTGfpTopo (Invitrogen) to give pCTGfpKy and pNTGfpKy, respectively. Fusion proteins were translated in vitro according to manufacturer’s instructions (kit STP3-T7, Novagen) to ascertain the integrity of the open reading frame. Cell lines were grown on DMEM medium supplemented with antibiotics and L-glutamine, and 15% bovine serum. At 70% of confluence, cells were transfected with pCTGfpKy, pNTGfpKy and the control vector pCDN3.1/NTGFP (Invitrogen) using Lipofectamin (Qiagen). Cells were visualized using a confocal microscope at 24, 48 and 72 h. When required, muscle cell lines (C2C12, L6 and L8) were allowed to differentiate after reaching confluency by switching to a low serum medium (2%). No expression was ever observed with pNTGfpKY. Alternative expression vectors were constructed to allow stable Ky expression. pTriX1.1Ky is full length Ky cloned into the bicistronic pTriX1.1.neo vector (Novagen), upstream of the second ribosome entry site that allows expression of the neo selection marker from the same mRNA. pTRE2HygKY is full length Ky cloned into the pTRE2Hyg vector (Clontech). Stable cell lines were selected by addition of G418 (500 µg/ml) to the growth medium 24 h post-transfection followed by clonal selection of resistant colonies after 15 days of selection. An L8 inducible cell line was selected by confluence transfections with pTri-On (the regulator plasmid) and pTRE2HygKY (the response plasmid containing full length KY) following the two step selection protocol recommended by the manufacturer (Tet-On expression system, Clontech). Induction of KY expression in the L8 derived cell line was obtained by adding 2 µg/ml of DOX to fully differentiated multinucleated myotubes 24 h before pictures were taken. Apoptosis on transiently transfected undifferentiated cells was assayed by TUNEL and anti-active caspase-3 immunostainings following the instructions provided by the manufacturer (Promega).

**Yeast two-hybrid screen**

A two-hybrid screen using a human muscle cDNA library was carried out using the Matchmaker GAL4 System 3 according to the instructions provided by the manufacturer (Clontech). The bait used was a full length mouse ky cDNA cloned in frame with the GAL4 DNA-binding (construct pGBK7KY). Fifty plasmids were recovered from positive colonies and sequenced. False interactions were detected at this point coming from artificial intronic frames or out frame peptides and discarded. Plasmids were then retransformed into the AH109 yeast strain together with the KY bait or the pGBK7 vector. The clones corresponding to sarcomeric cytoskeletal proteins (MYBPC1, TTN, FLNC and KYIP1) were the only confirmed interactions, activating all reporters only when the KY bait and any of those targets were present.

The FLNC clone pACT2FLNCd19/24 was used as template to generate smaller versions for interaction domain mapping. PCR products amplified with the indicated primer pairs were then subcloned into the same backbone vector (pACT2) for further Y2H tests. pACT2FLNCd20/24—FnClg20F: GCC CAT GGA GGC GTG GGA GTC CAC CC AGG TC plus FnClg24R: CTC GAG ACC GTA GGC TGA CAC CAA G. Fifty plasmids were recovered from positive colonies and sequenced. False interactions were detected at this point coming from artificial intronic frames or out frame peptides and discarded. Plasmids were then retransformed into the AH109 yeast strain together with the KY bait or the pGBK7 vector. The clones corresponding to sarcomeric cytoskeletal proteins (MYBPC1, TTN, FLNC and KYIP1) were the only confirmed interactions, activating all reporters only when the KY bait and any of those targets were present.

**Pull down assays**

Physical association between KY and putative interacting partners was confirmed in an in vitro pull down assay. KY was expressed as a MBP-fusion protein using the construct pL1R4 (see earlier). MBP was expressed from the control vector pMALC2X (New England Biolabs) and used as a negative control. Both proteins were purified according to the manufacturer’s instructions and kept bound to the manufacturer (Tet-On expression system, Clontech). Induction of KY expression in the L8 derived cell line was obtained by adding 2 µg/ml of DOX to fully differentiated multinucleated myotubes 24 h before pictures were taken. Apoptosis on transiently transfected undifferentiated cells was assayed by TUNEL and anti-active caspase-3 immunostainings following the instructions provided by the manufacturer (Promega).
amylose beads. Protein fragments of FLNC, TTN, KYIP1 and MBPC1 plus full length MBPC1 were PCR amplified and expressed radiolabelled from the yeast two-hybrid pACT2 plasmids using the in vitro translation system SPT37 (Novagen). MBP and MBP–KY beads were blocked in a buffer containing 250 mM HEPES, pH 7.6; 300 mM NaCl; 0.05% NP-40 and 3% BSA overnight at 4°C. An aliquot of 70 μl of each in vitro translation (IVT) reaction were pre-cleared by incubating with 1 μg MBP on beads in binding buffer (250 mM HEPES, pH 7.6; 300 mM NaCl; 0.05% NP-40 and 1% BSA) for 60 min. After spinning, the supernatant was split into two 35 μl samples, which were incubated with 4 μg of either pre-blocked MBP-FLNC beads or pre-blocked MBP beads in 1 ml 3% BSA in binding buffer. After 2 h incubation at room temperature on a rotating wheel, beads were washed three times for 10 min in 20 mM HEPES, pH 7.4; 400 mM NaCl; 10% glycerol and 0.2% NP40. Beads were mixed with sample buffer, boiled and run in a SDS–PAGE gel, and KY was detected using L1R4 antibodies following standard protocols.

Protease assays
The protease activity of KY was initially examined using the EnzChek protease assay kit (Molecular Probes). Wild-type KY was expressed as an MBP-fusion protein using the construct pL1R4. MBP was used as a negative control. Both proteins were purified according to the manufacturer’s instructions and kept bound to the amylose resin. Activity was assayed on the beads in MES buffer (see later). Tryptsin (10 ng/ml) was used as a positive control in the assays. The protease assay was carried out on microplates according to manufacturer’s instructions using either 200 mM Tris–HCl, pH 7.8 buffer or 10 mM 2(N-morpholino)ethanesulfonic acid (MES), pH 6.2 buffer incubated with 5 μg/ml casein or 200 μM Nε-Arg-MCA. Samples were incubated at room temperature for 2 h protected from light. The released fluorescence from the protease-catalyzed hydrolysis was visualised under a fluorescence microscope with a standard FITC or DAPI filters.

Preparation of tissue extracts and western blotting
Muscle specimens were frozen in liquid nitrogen directly upon dissection, and stored at −80°C until use. Total muscle protein extracts were prepared by grinding the muscle tissue under liquid nitrogen in a pre-cooled mortar. Ground tissue was gathered in a small amount of Laemmli buffer. Subsequently, the mixture was sonicated and heated for 10 min at 80°C. The insoluble fraction was sedimented by centrifugation (18 000 , 30 min, 4°C), and the supernatants were frozen in liquid nitrogen at −80°C until use. Total muscle tissue was gathered in a small amount of Laemmli buffer. Subsequently, the mixture was sonicated and heated for 10 min at 80°C. The insoluble fraction was sedimented by centrifugation (18 000 , 30 min, 4°C), and the supernatants were frozen in liquid nitrogen at −80°C until use. Western blots were carried out according to manufacturer’s instructions (BioRad). FLNC and LacZ proteins were detected using anti-V5 antibodies (Invitrogen), whereas KY and KY–GFP proteins were visualized using KyL1R4 antibodies. Protein concentration was estimated by the Bradford method (BioRad) and, additionally, anti-actin antibody was used as a loading control.

Interactions were also tested by using a pull down assay with whole skeletal muscle extracts using FLNC as bait. FLNC (d19–d24) was re-cloned from pACT2FLNCd19/20 into the pMALC2X vector by releasing a BamHI–SalI fragment from the donor vector and cloning it into the same sites into the recipient vector. The resulting construct, pMALFLNCd19/20, was expressed in E. coli as described earlier. MBP and MBP–FLNC beads were blocked in a binding buffer containing 20 mM HEPES, pH 7.4; 400 mM NaCl; 10% glycerol; 0.1% NP40 and 3% BSA overnight at 4°C. An aliquot of 1 g of whole skeletal muscle was added to 7.5 ml of binding buffer, homogenized and spun at 9000g for 15 min at 4°C. The supernatant was pre-cleared by incubating with 1 μg MBP on beads and 1% BSA in binding buffer for 60 min. After spinning, the supernatant was split into two samples, which were incubated with 4 μg of either

REFERENCES


