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Research Article

The ubiquitin-specific protease USP25 interacts with three sarcomeric proteins

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Abstract. The biological functions of the more than one hundred genes coding for deubiquitinating enzymes in the human genome remain mostly unknown. The USP25 gene, located at 21q11.2, encodes three protein isoforms produced by alternative splicing. While two of the isoforms are expressed nearly ubiquituously, the expression of the longer USP25 isoform (USP25m) is restricted to muscular tissues and is upregulated during myogenesis. USP25m interacts with three sarcomeric proteins: actin alpha-1 (ACTA1), filamin C (FLNC), and myosin binding protein C1 (MyBPC1), which are critically involved in muscle differentiation and maintenance, and have been implicated in the pathogenesis of severe myopathies. Biochemical analyses demonstrated that MyBPC1 is a short-lived proteasomal substrate, and its degradation is prevented by over-expression of USP25m but not by other USP25 isoforms. In contrast, ACTA1 and FLNC appear to be stable proteins, indicating that their interaction with USP25m is not related to their turnover rate.

Keywords. Ubiquitin-specific protease, ACTA1, MyBPC1, FLNC, sarcomere, alternative splicing.

Ubiquitin (Ub)-dependent proteasomal degradation, which was previously assumed to be a non-specific means for disposal of misfolded, damaged, or non-functional proteins, is now considered a tightly regulated process that controls the intracellular protein pools, and hence, the metabolism and fate of the cell (reviewed in [1–3] and references therein). In addition to proteolysis, new regulatory functions for Ub and Ub-like (Ubl) proteins have been described, thus providing the grounds for comparing protein ubiquitination to phosphorylation [4]. Moreover, similar to the better-studied Ub conjugation, deubiquitination is a regulation checkpoint of the Ub-proteasome system (UPS), as the attachment of a polyubiquitin chain to a specific substrate is not a unidirectional process.

The deubiquitinating enzymes (DUBs) are proteases that hydrolyse Ub from Ub-conjugated protein substrates, process Ub from Ub precursors, and recycle Ub from Ub adducts. DUBs constitute one of the largest, albeit relatively unknown, protein family involved in the UPS (reviewed in [5–7]). The DUB superfamily was initially divided into Ub-specific proteases (UBPs, also named USPs in humans) and Ub C-terminal hydrolases (UCHs), the latter being smaller in size and fewer in number. However, new DUB families are emerging, such as the OTU-related, Josephin domain and the JAMM/MPN+ proteases (reviewed in [7]).

The UBP family contains highly divergent members, whose sequence homology is restricted to the catalytic and Ub-binding domains. Indeed, the large number of putative UBPs encoded in the human genome indicates a high degree of functional specificity in either regulation and/or substrate recognition. However, the study of UBPs has been hampered by their partial functional redundancy, with mild or absent phenotype in most yeast knockouts [8], and by their broad-range specificity, as they are able to cleave Ub – with varying degrees of affin-

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ity – from recombinant synthetic Ub-protein substrates *in vitro* (reviewed in [6]). Hence, one of the major challenges in the field is to unravel the specific physiological roles of the UBPs.

It is becoming increasingly clear that UBPs are involved in human pathological disorders. The first DUB associated to an inheritable disorder was cylindromatosis tumour suppressor (CYLD), a negative regulator of the NF-κB pathway, which when mutated causes cylindromatosis [9, 10]. Other examples worth mentioning are UCHL-1, whose mutations were reported to be involved in familial Parkinson's disease [11]; USP7 (also named HAUSP), which regulates the p53 pathway by interacting with both p53 and its cellular Ub ligase Mdm2 [12]; USP2a, which rescues prostate cancer cells from apoptosis by stabilising fatty acid synthase [13]; USP6 (also named tre-2), with a putative role in growth control [14]; USP4 (also named UNP), which binds members of the Rb family and has been suggested to be involved in lung carcinogenesis [15] and USP14, involved in synaptic defects in an ataxia murine model [16].

While searching for genes on chromosome 21, we identified a new Ub-specific protease gene, *USP25*, located at 21q11.2 [17], which is over-expressed in Down's syndrome (DS) foetal brains. *USP25* is highly expressed in skeletal muscle, heart, and testis. Three alternatively spliced isoforms were identified in human [18]. The most abundant transcript contained the in-frame fusion of exons 18 and 20, while two additional isoforms involved exons 18–19b-20, and 18–19a-19b-20. The latter isoform was expressed only in skeletal muscle and heart and was therefore called USP25m (USP25 muscle isoform). Alternatively spliced protein isoforms may show differences in tissue specificity, subcellular localisation, regulation, and substrate recognition. In this context, it is remarkable that, in addition to *USP25* and the closely related *USP28* [18], other USP genes have been reported to code for different protein isoforms by alternative splicing, i.e. *USP4* (or *UnpEL-UnpES* [15]), *USP5* (or *ISOT-1/2* [19]), *USP9X* (previously named *DFFRX* [20]), and *Ubp45/69* [21].

Myogenic differentiation requires cell cycle exit, muscle-specific gene expression, and myotube formation, and in all these processes the UPS plays a regulatory role [22]. A role for DUBs in myogenesis was earlier suggested by the finding that UBP45 and UBP69, two protein isoforms encoded by alternatively spliced transcripts of the same gene, act antagonistically in myogenic differentiation [21]. The identification of a muscle-specific isoform of USP25 prompted us to investigate its involvement in muscle cell physiology. Here we report on the identification of three muscle-restricted cytoskeletal and sarcomeric proteins that specifically interact with USP25m, hence suggesting an important role for this enzyme in the regulation of muscular differentiation and function.

Material and methods

Human and murine tissue panel RT-PCR. Multiple tissue cDNA panels were obtained from Clontech (BD Biosciences). Of the first-strand cDNA (1 ng/ µl), 2 ng were used in two-step PCRs with USP25-specific primers from exons 18 and 20, respectively (Hs E18: 5′-TTGTTGAGGAAGACAACCAACGA-3′ and Hs E20: 5′-GCACAGATGAGGAACAGCAAGG-3′; Mm E18: 5′-GAAGAATGGGACACGCAGCTT-3′ and Mm E20: 5′-TCTGCATGCCACTCCTCATATTC-3′).

cDNA purification and real-time quantitative PCR. C2C12 cells were seeded in a 24-well plate and total cDNA was extracted at different time points using the 'Cells-to-cDNA II' kit (Ambion) following the manufacturer's instructions. For each transcript tested, a primer pair and a probe designed by PE Applied Biosystems were used. Real-time PCR reactions were performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Amplification plots and predicted threshold cycle (Ct) values (fractional cycle number at which the amount of amplified target reaches a fixed threshold) were obtained with the Sequence Detector Software (SDS version 1.9.1, PE Applied Biosystems). Data were obtained from two separate experiments, each with three replicates and normalised according to the expression of the housekeeping gene PGK1.

Generation of rabbit polyclonal antibodies against human USP25. The full-length USP25 coding sequence was cloned in-frame into the pGEX-4T-1 vector to produce the glutathione S-transferase (GST)-USP25 fusion protein. After transforming on BL21 *Escherichia coli* cells, the recombinant protein was purified using glutathione-agarose beads by standard procedures. The immunisation of rabbits was performed following conventional protocols using 100 µg of the purified fusion protein in Freund's adjuvant. Specificity and sensitivity of the serum were determined after each bleeding by Western blots.

Immunodetection on Western blots. Cell lysates were obtained by resuspension in Laemmli loading buffer. Samples were then boiled for 5 min and centrifuged to sediment cellular debris. Protein preparations were loaded either onto 7.5% or 10% SDS-PAGE gels. Primary antibodies used were as follows: anti-USP25 polyclonal antibody (pAb) (1:4000), anti-filamin C (FLNC) pAb (kindly provided by Dr. L. M. Kunkel) (1:2000), anti-actin alpha-1 (ACTA1) monoclonal antibody (mAb) (Sigma; 1:2000), anti-HA mAb (BaBCO; 1:1000) anti-Ub mAb (Zymed; 1:1000), anti-p53 pAb (Santa Cruz; 1:200), and anti-α-tubulin mAb (Sigma; 1:8000). Secondary antibodies were conjugated to horseradish perox-

idase (1:3000). Immunodetection was performed using the chemiluminescent ECL reagents (AP Biosciences).

Yeast two-hybrid screening. A Matchmaker library of *Saccharomyces cerevisiae* (Y187, Matα), pretransformed with a human skeletal muscle cDNA collection cloned in pACT2 (Clontech), was used as prey in a yeast two-hybrid (Y2H) screening. Two bait proteins were used: (i) the full-length cDNA (from ATG to the 'stop' codon) of USP25m, and (ii) a PCR-generated fragment encompassing the muscle-specific alternative exon of USP25m (exons 18, 19a, 19b, and 20). The two coding fragments were cloned in-frame with the GAL4-binding domain (amino acids 1–147) into the pGBKT7 vector. AH109 *S. cerevisiae* cells (Mata) were transformed with each bait and, subsequently, mated with the pretransformed *S. cerevisiae* Y187 prey library. More than 2×10^8 colonies were screened in each mating. The screening and selection of positive clones were performed according to the manufacturer's instructions (Matchmaker two hybrid system 3, Clontech). Clones growing on selective medium (-Trp, -Leu, -His, -Ade) were also assessed for β-galactosidase reporter activity with the qualitative X-Gal lift test and/or the quantitative assay using the *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate. The enzymatic generation of *o*-nitrophenol was measured at 420 nm and normalised to cell density for each sample. All positive clones were confirmed by retransformation. The library inserts were sequenced for all the confirmed positive clones using vector primers (5′-GATACCCCACCAAACCC-3′ and 5′-GTGAACTTGCGGGGTTT-3′) and Blast searches were performed against the nr database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

Expression constructs and cell culture. Full-length USP25 and USP25m and the 18-19a-19b-20 exon fragment were cloned in-frame with the cMyc epitope into the eukaryotic expression vector pcDNA3 (Invitrogen). The cDNAs encoding myosin binding protein C1 (MyBPC1) (RZPD clone DKFZp451B236Q2) and ACTA1 (obtained by PCR amplification from a human skeletal muscle cDNA collection, BD-Biosciences) were cloned in pcDNA3, downstream of an HA epitope tag. COS-7 and HEK293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin, in 5% CO_2 at 37 °C. Mouse C2C12 myoblasts were maintained in DMEM (ATCC) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, in 5% CO_2 at 37 °C. For differentiation, preconfluent myoblasts were shifted to differentiation medium (DMEM containing 5% horse serum) and allowed to differentiate for 7–10 days.

Transient transfections, proteasome inhibitors and cycloheximide treatment. COS-7 and HEK293T cells were seeded on 100-mm dishes at 3×10^6 cells/dish or in 6-well plates at 5×10^5 cells/well. After 12 h, cells were transiently co-transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For treatment with proteasome inhibitors, epoxomicin (0.5 µM) or MG132 (10 µM) were added to the medium during the last 16 h of culture. Both reagents were purchased from Biomol. For protein synthesis inhibition, cycloheximide (Sigma) was added to the culture (30 h post transfection) at a final concentration of 100 µg/ml for the specified times before cell harvesting. α-Tubulin was used as an internal control. Western blots were scanned and quantified by densitometry using the Scion Image software.

Co-immunoprecipitation. COS-7 and C2C12 cells were seeded on 100-mm tissue culture dishes at 3×10^6 cells and 9×10^5 cells/dish, respectively. After 12 h, transient co-transfection was performed using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. At 24 h post transfection, COS-7 cells were harvested, whereas C2C12 cells were shifted to differentiation medium and allowed to differentiate for 6 days. All cells were collected and lysed in a buffer containing 0.5% Nonidet P-40, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl and a protease inhibitor cocktail (Roche) by 15-min incubation at 4 °C. Following removal of cellular debris by centrifugation, protein concentration was determined by the Bradford assay. After adjusting the NaCl concentration to 250 mM, cell lysates corresponding to 500 µg protein were incubated on ice for 90 min with 1–5 µg anti-cMyc mAb (Santa Cruz Biotechnology). The protein-antibody complexes were removed by a 16-h incubation with protein G-Sepharose beads (Amersham-Pharmacia Biotech) at 4 °C with end-over-end mixing. The bound proteins were washed five times with ice-cold lysis buffer, eluted from the beads by boiling with protein loading buffer, resolved by electrophoresis on 10% SDS-PAGE and analysed by Western blot analysis. We used an anti-HA mAb (BAbCO; 1:1000) to immunodetect the fusion protein HA-MyBPC1.

Production of GST-fusion proteins and GST pull-down assays. Full-length USP25 and USP25m as well as the 18-19a-19b-20 exon fragment were cloned into pGEX-4T-1 (Stratagene) and expressed as GST-fusion proteins in *E. coli* BL21 cells. Protein production was induced for 2 h at 30 °C by adding 1 mM isopropyl-β-D-thiogalactopyranoside to the bacterial culture. Cells were harvested, resuspended in ice-cold PBS with protein inhibitors and lysed by sonication. Protein extracts were recovered after removal of cellular debris by centrifugation. The fusion proteins were bound to glutathione-Sepharose

beads (Amersham-Pharmacia Biotech) and posteriorly cleaved from GST following standard procedures. The purified proteins were boiled in protein sample buffer and then directly loaded onto 10% SDS-PAGE gels for Western blot analysis. Immunodetection of ACTA1 was carried out as stated above.

Immunofluorescence. COS-7 cells grown on glass coverslips placed in 12-well plates were transiently transfected with pcDNA3-cMyc-USP25m or co-transfected with pcDNA3-cMyc-USP25m and pcDNA3-HA-MyBPC1. C2C12 myoblasts were grown on glass coverslips to near confluence, shifted to differentiation medium and allowed to differentiate for the specified times before proceeding with fixation. At 30 h post transfection for COS-7, or at the specified times for C2C12, the cells were washed in ice-cold PBS, fixed at 4 °C with freshly prepared 3% paraformaldehyde and 2% sucrose in PBS for 30 min, permeabilised with 0.1% Triton X-100 and 20 mM glycine in PBS for 10 min, and blocked with 1% BSA in 20 mM glycine and PBS for 1 h. Samples were immunodetected with anti-USP25 rabbit pAb and anti-HA mAb (all diluted 1:200) in blocking solution for 1 h at 37 °C. The cells were then rinsed three times with 20 mM glycine in PBS and incubated with AlexaFluor 660-conjugated anti-rabbit IgG and AlexaFluor 488-conjugated anti-mouse IgG (Molecular Probes) secondary antibodies (all diluted 1:300) for 45 min at 37 °C. Actin filaments were stained by TRITC-conjugated phalloidin (Sigma; 1:50). The cells were rinsed two times in PBS and the coverslips were mounted using Vectashield (Vector Laboratories, Inc.) to prevent bleaching. Confocal fluorescent microscopy was performed with an Olympus Fluoview 500, and Leica TSC NT and TSC SP2 laser scanning microscopes.

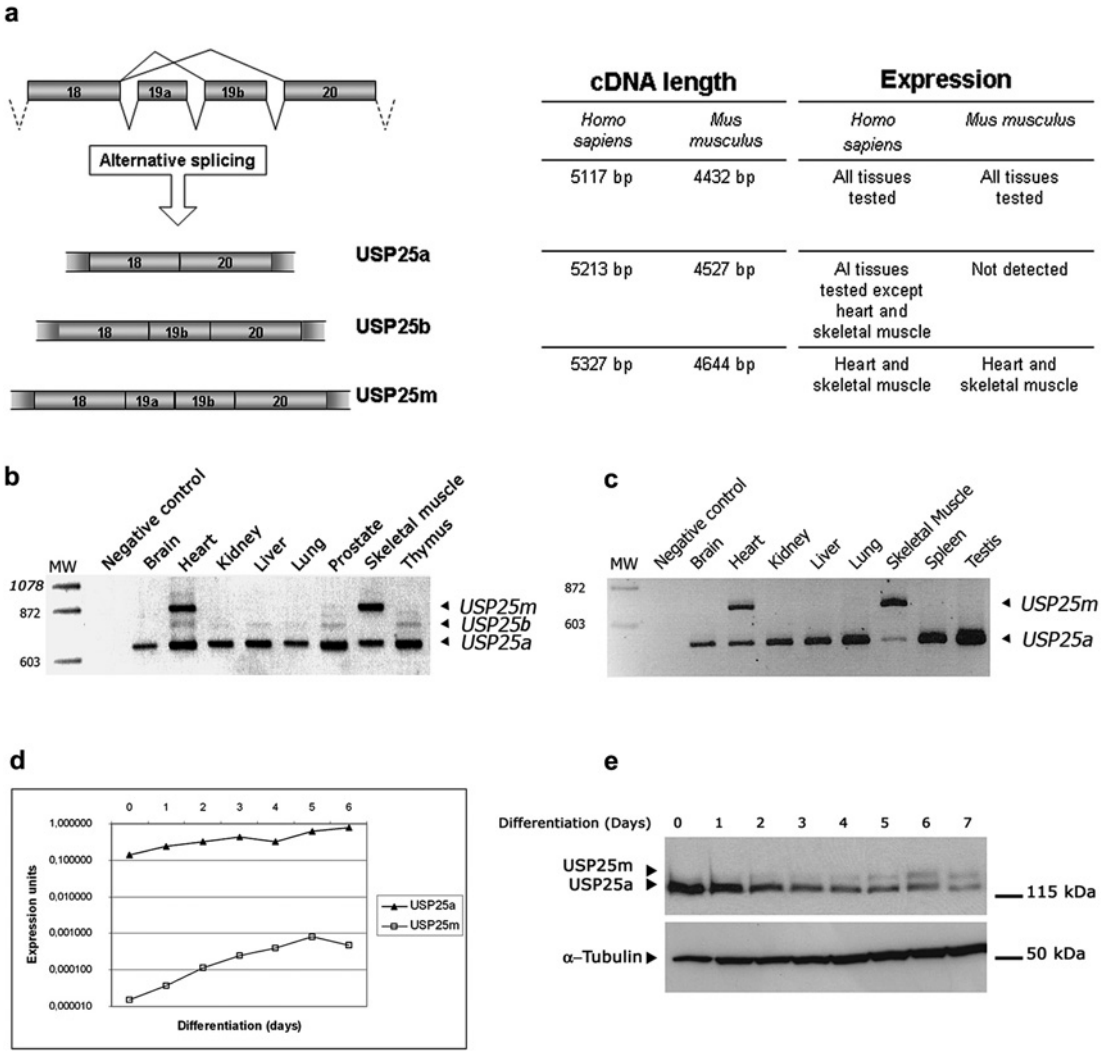
Mouse skeletal muscle fluorescence staining. Adult C57BL/6 mice were killed in a CO_2 environment. Quadriceps were excised and fixed by immersion in 4% paraformaldehyde for 24 h at 4 °C, cryoprotected by immersion in a graded series of sucrose solutions (3 h in 7% sucrose, 3 h in 15% sucrose, and 17 h in 30% sucrose), and embedded in Tissue-Tek OCT compound (Sakura Finetek, USA). Cryosections, 10 µm thick, were used for subsequent immunohistochemistry. Slides were initially fixed with 4% paraformaldehyde and blocked with goat serum (Sigma, 1:50 in 0.2% Triton X-100/PBS). After rinsing three times with PBS, sections were incubated with the primary antibody diluted in blocking solution (1:200 anti-USP25 rabbit pAb and anti-ACTA1 mAb) overnight at 4 °C, rinsed three times with PBS and incubated for 45 min at room temperature with the secondary antibodies (Molecular Probes) diluted 1:1000 in blocking solution. Slides were washed three times in PBS, incubated in Hoechst solution (2 µg/ml in PBS) for 15 min and mounted in DABCO mounting solution (75% glycerol, 25 mM Tris-HCl pH 8.0, 2.5% 1,4-diazabicyclo-(2,2,2)octane).

Results

***USP25* expression in human and murine adult tissues and during muscular differentiation.** The organisation of *USP25* and its three mRNA isoforms referred to as USP25a (ubiquitous isoform fusing exons 18–20), USP25b (containing exons 18-19b-20), and USP25m (containing exons 18-19a-19b-20), are shown in Figure 1a. We and others had already shown that the three corresponding encoded proteins were able to deubiquitinate a recombinant Ub-β-galactosidase substrate, thus proving that all such isoforms retained a Ub-specific protease activity [18, 23].

To validate USP25 expression in mouse, we performed PCR amplification with specific oligonucleotides from exons 18 and 20 on a panel of cDNAs. A similar experiment was performed on a panel of human cDNAs to compare the expression patterns. In human samples, USP25a mRNA was ubiquitously expressed in all the tested tissues; USP25b was expressed, although at very low levels, in all tested tissues except skeletal muscle, whereas USP25m was only detected in muscle lineage tissues, such as heart and skeletal muscle (Fig. 1b). In contrast, only two mRNA isoforms, corresponding to USP25a and USP25m, were detected in mouse tissues (Fig. 1c). No evidence for USP25b mRNA could be found under our conditions. Again, USP25m expression was restricted to skeletal muscle and heart.

To assess whether *USP25* expression and splicing pattern shifted during myogenesis, we performed real-time quantitative PCR analyses to detect the USP25 mRNA levels in cultured C2C12 cells, a well-established cellular model for muscle differentiation [24]. (Fig. 1d). To detect the corresponding encoded proteins, we generated a rabbit pAb against a USP25 peptide contained in all USP25 isoforms for immunodetection on C2C12 cell protein extracts obtained at different times during differentiation (Fig. 1e). High levels of the ubiquitous USP25a mRNA and the encoded protein were detected throughout differentiation. In contrast, the USP25m-specific mRNA showed low levels at day 2 and showed an approximately 100-fold increase towards the completion of differentiation, around day 6, concurrently to the highest levels of the encoded protein. These experiments revealed that myoblasts only expressed the USP25a isoform, while the USP25m isoform correlates with myotube formation. Therefore, the regulation of the USP25m splicing is dependent on muscle myogenesis, suggesting a physiological role for this isoform in muscle cells.



cDNA length		Expression	
Homo sapiens	*Mus musculus*	*Homo sapiens*	*Mus musculus*
5117 bp	4432 bp	All tissues tested	All tissues tested
5213 bp	4527 bp	Al tissues tested except heart and skeletal muscle	Not detected
5327 bp	4644 bp	Heart and skeletal muscle	Heart and skeletal muscle

Figure 1. Analysis of the alternatively spliced USP25 isoforms in human and murine tissues. (*a*) Diagram depicting the exon structure of the three USP25 isoforms. (*b*, *c*) Simultaneous detection of the three alternatively spliced mRNA products in human and murine tissues, respectively. Note that *USP25b* is not detected in murine tissues, although it is weakly expressed in human tissues. (*d*, *e*) Endogenous levels of USP25m expression increases along muscular differentiation, as assessed by real-time PCR (*d*) and Western blot (*e*) in cultured C2C12 cells. The levels of USP25 mRNA in (*d*) are normalised against PGK1 expression, a highly constantly expressed gene throughout all differentiation stages. Each point represents the average value obtained from two separate experiments, each with three replicates. Error bars are not shown for the sake of clarity. The USP25m protein is detected only in differentiated cells. Time in days indicate the time elapsed since the shift to the differentiation medium. α-Tubulin is used as a loading control in (*e*).

***In vitro* and *in vivo* expression of *USP25* and *USP25m*.** The endogenous expression of USP25 isoforms was analysed in the murine C2C12 cell line: in myoblasts (before differentiation), and throughout differentiation into myotubes at different times (from day 0 to day 10 after shifting to the differentiation medium; Fig. 2a). The subcellular localisation was mostly cytosolic except for a transient nuclear localisation of USP25 in some myotubes (between days 6 and 8, white arrowheads in Figure 2a). This pattern was consistently obtained in three replicates of two independent experiments.

We aimed then to investigate whether these different USP25 subcellular localisations were specific to the different isoforms. Given that the polyclonal USP25 antibody does not discriminate between different USP25 isoforms and that, at least two of them, were concurrently present in differentiating muscle cells, we performed independent transient transfection on COS-7 cells of the muscle and non-muscle isoforms. In addition, phalloidin co-staining was performed to visualise the organisation of the cytoskeleton as well as the cell shape, revealing no abnormalities after the over-expression of any USP25 isoform (Fig. 2b). The two isoforms showed similar cytosolic localisation by immunostaining (Fig. 2b), and no nuclear staining was found, indicating that the nuclear localisation observed was dependent on myotube differentiation.

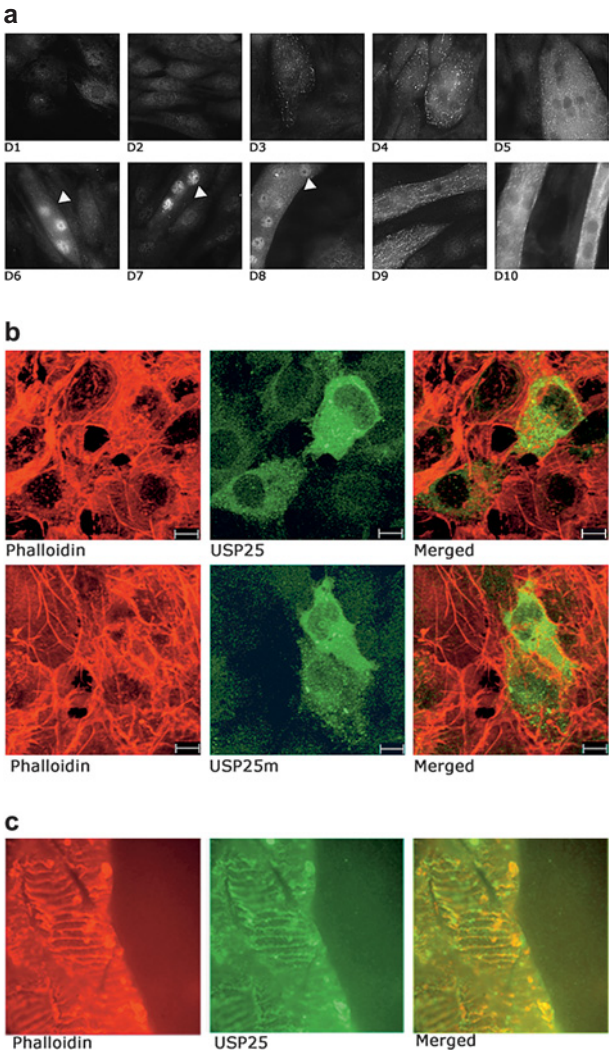


Figure 2. Detection of USP25 isoforms by immunofluorescence analysis. (*a*) Subcellular localisation pattern of endogenous USP25 in C2C12 myoblasts and throughout differentiation into myotubes (days after shift to the differentiating medium are indicated). Note that this polyclonal anti-USP25 antibody does not discriminate between isoforms. Although the subcellular localisation is mainly cytosolic, the white arrowheads point to transient nuclear localisation of some USP25 in cells at days 6–8 of differentiation. (*b*) USP25 and USP25m show a similar cytoplasmic localisation when transiently expressed in COS-7 cells (1000× magnification). TRITC-phalloidin counterstaining signals the actin cytoskeleton. Bar 10 µm. (*c*) USP25 is endogenously expressed in mouse muscle. TRITC-phalloidin counterstaining points to colocalisation with actin.

Immunostaining of endogenous USP25 in adult mouse muscle showed the striated pattern typical of skeletal muscle proteins, as shown by counterstaining with phalloidin (Fig. 2c).

USP25m specifically interacts with MyBPC1, ACTA1, and FLNC. The identification of USP25m as a differentiation-associated protein in muscular cells prompted us to attempt to identify its interacting partners and putative substrates. To this end, a human skeletal muscle cDNA library was screened by a Y2H strategy using two different baits: a PCR fragment encompassing USP25 exons 18-19a-19b-20 (muscle-specific exon is underlined) and the full-length USP25m cDNA. The two coding sequences were cloned into the bait vector to screen a pretransformed library of human skeletal muscle cDNAs by mating (>2×10^8 clones). The positive clones transactivating the selector genes HIS3, ADE2, and the reporter LacZ were fully sequenced (Tables 1 and 2) and, when isolated repeatedly, were confirmed by retransformation and growth on selective media (Fig. 3a). A quantitative approach to the interactions was attained by monitoring the β-Gal activity on the ONPG substrate (data not shown). According to these criteria, the methalothionein 2A (MT2A) protein was discarded as it turned out to be a false positive, whereas glyceraldehyde-3-phosphate dehydrogenase (GAPD), MyBPC1, ACTA1, and FLNC were identified as *bona fide* interacting proteins. MyBPC1, ACTA1, and FLNC are muscle-specific proteins involved in structural, sarcomeric, and cytoskeletal functions [25–28], and all of them are involved in severe muscular disorders. Therefore, we focused on these three proteins. Real-time quantitative PCR and immunoblot analyses of these three proteins and USP25m in C2C12 cells at various differentiation times showed that their expression is activated in parallel. Interestingly, USP25m expression correlates with the expression of these interacting partners during differentiation (data not shown).

Table 1. Results obtained in the Y2H screening.

	Bait construct	
	pGBKT7-USP25m	pGBKT7-19a10b
Total colonies[a]	300	30
False positives[b]	37	24
Positives[c]	263	6

[a] Total number of colonies that grew on selective medium SD-Leu-Trp-His-Ade
[b] False positives identified by X-gal lift, sequencing or Southern blot
[c] Remaining clones

Table 2. Summary of the positive clones that appeared more than once.

	Bait construct	
	pGBKT7-USP25m	pGBKT7-19a19b
MT2A	1	2
GAPD	3	0
MyBPC1	13	2
ACTA1	7	0
FLNC	2	2

MT2A: methalothionein 2A, GAPD: glyceraldehyde-3-phosphate dehydrogenase, MyBPC1: myosin binding protein C1, ACTA1: actin alpha-1, FLNC: filamin C.

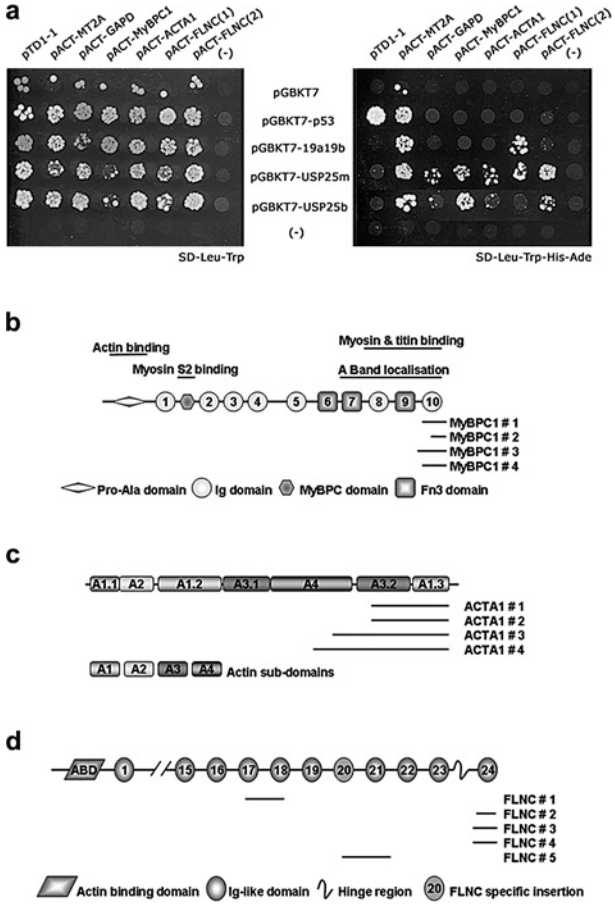


Figure 3. USP25m interaction with ACTA1, MyBPC1, and FLNC by the Y2H assay. (*a*) Growth of retransformed yeast clones on selective medium SD lacking leucine, tryptophan, histidine and adenine show that GAPD, MyBPC1, ACTA1, and FLNC interact specifically with USP25, whereas the interaction detected with MT2A appears to be non-specific, revealed by the growth of yeast colonies in the absence of USP25 or in the presence of the non-specific protein p53 (panel to the right). Two different clones of FLNC were retransformed [FLNC(#1) and FLNC(#2)], because they corresponded to different domains of the protein. Growth control of the retransformed yeast clones on SD lacking leucine and tryptophan (panel to the left). pTD1-1 is a positive control plasmid that encodes the SV40 large T antigen, which interacts strongly with p53. (*b–d*) Domain organisation of MyBPC1, ACTA1, and FLNC proteins, respectively. Solid bars below diagram indicate the length of the independently isolated clones on the Y2H screen. All clones represented here were isolated with the full-length USP25m, except for FLNC#1 and #2, which were isolated using the muscle specific region 19a-19b.

The domain architecture of these proteins supports their ability to form protein complexes, *e.g*. the Fn3 and Ig domains present in MyBPC1 and FLNC are well-known protein-protein interaction motifs. The isolation of several positive clones corresponding to the same interacting proteins allowed us to map their USP25 binding motifs (Fig. 3b–d). The USP25m interacting region of MyBPC1 comprised the last Ig-like C-terminal domain (C10) (Fig. 3b), which was previously shown to be responsible for A-band localisation and for myosin and titin binding. Sequence analysis of the ACTA1-positive clones revealed that the USP25m interacting region also maps to the C terminus, comprising the A3.2 and the A1.3 subdomains of the actin domain (Fig. 3c). The full-length USP25m recognised three protein segments of FLNC: Ig17-18, Ig20-21, and Ig24 (Fig. 3d). Remarkably, the Ig17-18 and Ig24 domains were also recognised by the short bait, containing only the 19a-19b muscle-specific exons of USP25m (clones FLNC#1 and FLNC#2 in Figure 3d), thus suggesting that the muscle-specific region of USP25m, encompassing just 38 amino acids, is sufficient for protein-protein interactions and may, therefore, play a major role in substrate recognition.

***In vitro* and *in vivo* interactions.** The interactions of MyBPC1 and ACTA1 with USP25m were verified by different complementary strategies, depending on the availability of a suitable antibody (e.g. there is not any available antibody against MyBPC1) and thus the requirement for epitope tagging and cell transfection to detect the proteins. The lack of a full-length FLNC cDNA, due to the large size of the gene, hampered further analysis.

Co-immunoprecipitation studies in COS-7 cells transiently co-transfected with epitope-tagged HA-MyBPC1 and either cMyc-USP25b or cMyc-USP25m showed that the interaction was specific for USP25m, as no MyBPC1 was immunoprecipitated in cells co-expressing USP25b and MyBPC1 (Fig. 4a).

The same approach could not be used for ACTA1 because high over-expression of ACTA1 and USP25 in COS-7 cells proved to be toxic. As a specific antibody against ACTA1 was available, we resorted to a GST-pull down strategy using differentiated muscle cells. Various GST-USP25 fusion proteins produced in *E. coli* were incubated with lysates from differentiated C2C12 myotubes, where endogenous ACTA1 is highly expressed. As shown in Figure 4b, ACTA1 interacted with USP25m much more efficiently than with the non-muscular USP25b isoform. Interestingly, the specific-muscular peptide encoded by exons 19a-19b was also able to bind to ACTA1.

In agreement with these findings, immunostaining studies either in co-transfected COS-7 cells with HA-MyBPC1 and cMyc-USP25m or in murine muscle tissues showed that MyBPC1 and ACTA1 co-localise with USP25 (Fig. 4c, d).

USP25m regulates the turnover of MyBPC1. One of the assumed functions of USPs is the editing of poly-ubiquitinated substrates, which may rescue them from proteasome-dependent destruction. We explored, therefore, whether these three USP25m interacting proteins identified in our study were degraded by the proteasome and, if so, whether USP25m regulated their turnover.

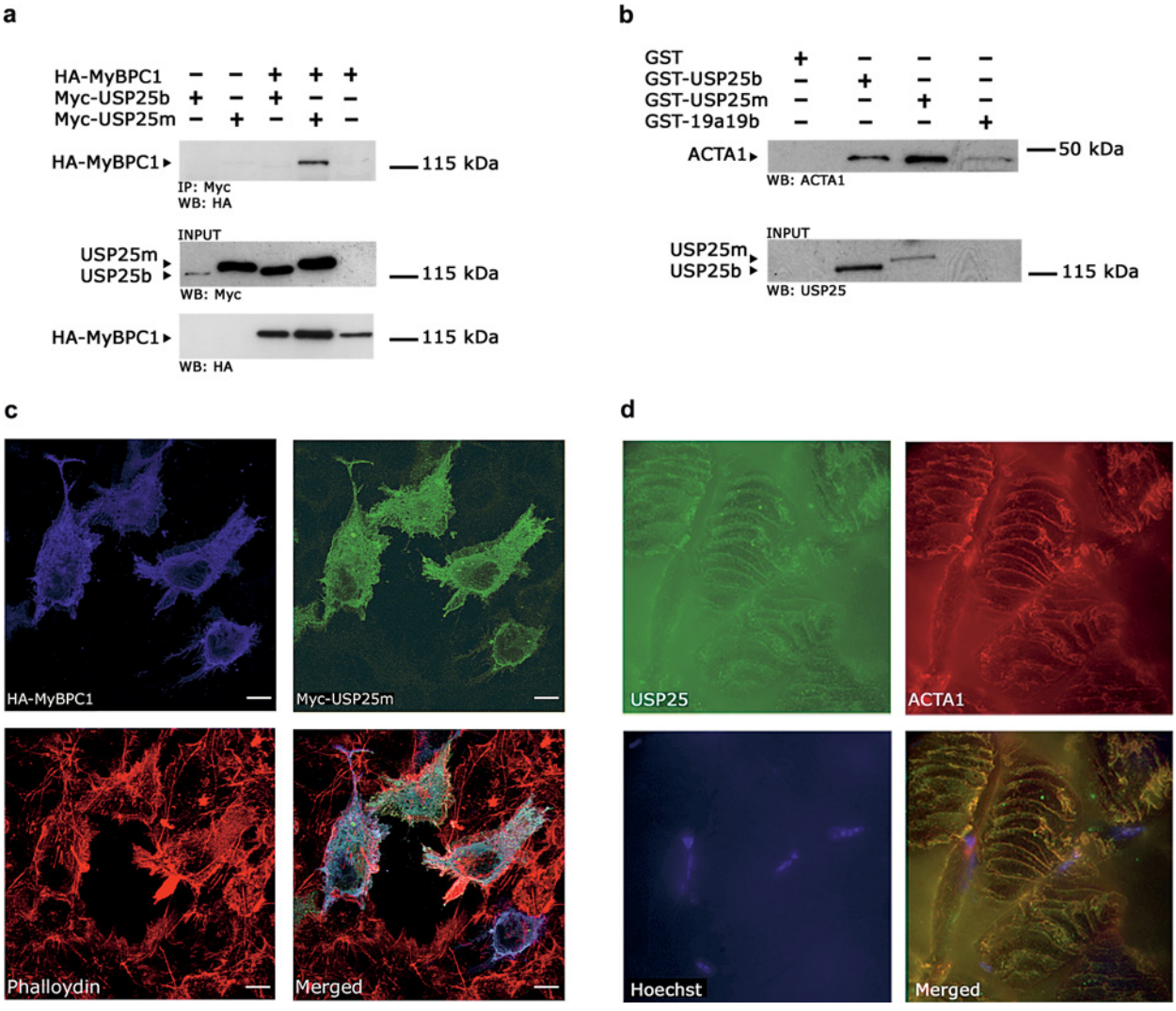


Figure 4. Confirmation of the interactions between USP25 and MyBPC1 and ACTA1. (*a*) Co-immunoprecipitation of HA-MyBPC1 with cMyc-USP25m but not with the cMyc-USP25b isoform. Immunoprecipitation and immunodetection were performed, respectively, with an anti-cMyc antibody and an anti-HA antibody. The two bottom panels correspond to the protein expression controls of the input samples. (*b*) GST-pull down assay, where the two GST-fused USP25 isoforms bind to endogenous ACTA1 from C2C12 muscle cells. Note that the interaction is much stronger with USP25m than with the ubiquitous USP25b. A weak interaction is also detected with the peptide encoded by the alternatively spliced exons. The bottom panel shows the protein expression control of the GST-fused USP25 isoforms. (*c*) Co-localisation (merged figure, in grey) of HA-MyBPC1 (blue) and cMyc-USP25m (green) in transiently transfected COS-7 cells. Cells are counterstained with TRITC-phalloidin (red), which stains the actin filaments (1000× magnification). Bar 10 µm. (*d*) *In vivo* co-localisation (merged figure, in yellow-orange) of endogenous ACTA1 (red) and USP25 (green) in adult mouse muscles. Hoechst staining (blue) shows the nuclei localisation.

We assessed the contribution of the proteasome to the regulation of the intracellular pools of MyBPC1, ACTA1, FLNC, and USP25 itself in cells treated with proteasome inhibitors. High levels of the exogenous MyBPC1 protein were detected in HEK293T transfected with the HA-MyBPC1 construct following treatment with the proteasome inhibitors epoxomicin and MG132 (Fig. 5a), confirming that this protein is a probable proteasome substrate. Untreated cells showed low levels of exogenous MyBPC1 (Fig. 5a, first lane). HA-ACTA1, cMyc-USP25b, and cMyc-USP25m did not accumulate in transfected cells treated with proteasome inhibitors (not shown). Furthermore, the endogenous intracellular pools of FLNC, ACTA1, USP25a, and USP25m were not altered by treatment with proteasome inhibitors in C2C12 cells, thus confirming that the turnover of these proteins is not regulated by the proteasome (Fig. 5b). Proteasome inhibition treatment was validated by detecting the accumulation of the unrelated protein p53 (a well-known proteasome substrate) on the same samples (Fig. 5b). The same experiment could not be performed for endogenous MyBPC1, due to the lack of a suitable antibody.

In the final set of experiments we explored whether USP25 over-expression could rescue MyBPC1 from de-

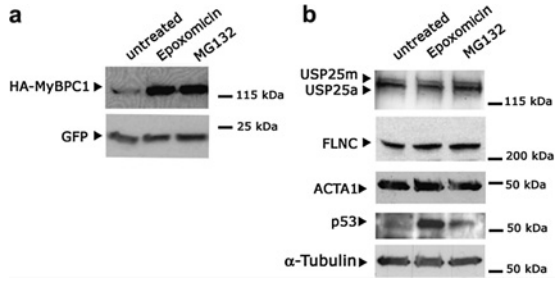


Figure 5. MyBPC1 is a proteasome substrate. (*a*) HA-MyBPC1 is degraded by the 26S proteasome in transiently transfected HEK293T cells, as the addition of proteasomal inhibitors epoxomicin (0.5 µM) and MG132 (10 µM) increase its intracellular levels after 16-h treatment. A co-transfected GFP expressing vector was used as both transfection and loading control. (*b*) Endogenous protein levels of USP25, ACTA1, and FLNC in differentiated C2C12 myotubes are not altered after 16-h treatment with the proteasome inhibitor epoxomicin (0.5 µM) or MG132 (10 µM), indicating that they are relatively insensitive to proteasomal degradation, whereas p53 (a well-known proteasome substrate) accumulates. Immunodetection of α-tubulin was used as a loading control.

gradation. To avoid undesired interferences with the endogenous USP25 isoforms, we over-expressed the muscle isoform of USP25 with HA-tagged MyBPC1 in transiently transfected HEK293T cells. As shown in Figure 6a, over-expression of USP25m was indeed able to protect MyBPC1 from proteasomal degradation, as it induced accumulation of MyBPC1 to levels comparable with those achieved after proteasome inhibitor treatment. Contrary to what had been shown for other DUBs with a broader substrate specificity [29–31], this rescue was not due to a general effect on protein deubiquitination, as the overall ubiquitination pattern was not altered in USP25m over-expressing cells (Fig. 6b). Furthermore, MyBPC1 rescue was highly specific for the USP25m isoform, since the degradation of MyBPC1 was not affected by over-expression of USP25b (which has already been proven to be a catalytically active isoform [17]) nor by over-expression of the unrelated DUB USP15 (Fig. 6a).

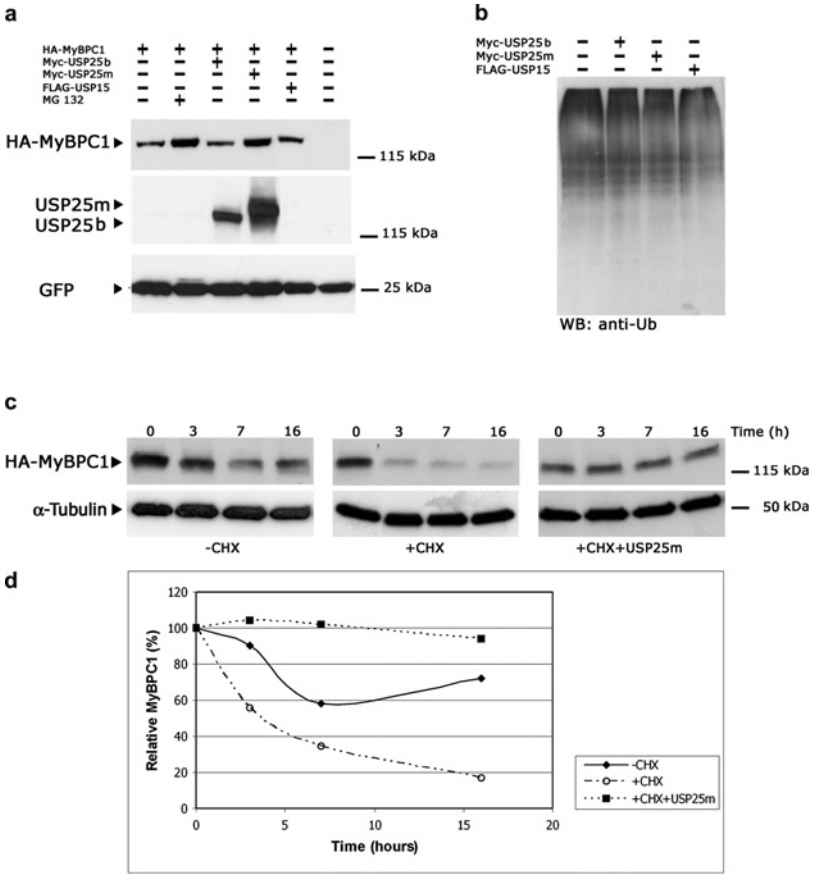


Figure 6. MyBPC1 is a short-lived protein, and it is stabilised by the over-expression of USP25m in transiently transfected HEK293T cells. (*a*) HA-MyBPC1 is rescued from proteasomal degradation by over-expression of USP25m, but not by over-expresion of the ubiquitous USP25b isoform or by an unrelated USP (USP15). These results have been consistently obtained in two different experiments, with two replicates each. (*b*) Transfections with either USP25, USP25m, or USP15 (negative control) do not alter the general ubiquitination pattern of the cells. (*c*) Inhibition of new protein synthesis by treatment with cycloheximide (CHX, 100 µg/ml final concentration) in a time-course experiment shows that MyBPC1 is a short-lived protein. Concurrent co-expression of USP25m stabilises the protein. Cycloheximide treatment was started 30 h post transfection, and cells were harvested at different time points, in hours, as indicated. (*d*) Immunoblots were quantified by densitometric analysis and HA-MyBPC1 levels (normalised with α-tubulin expression) were expressed relatively to that observed at 30 h post transfection before cycloheximide treatment (considered as 100%).

To assess the stabilisation of MyBPC1 protein by the expression of USP25m, we determined its half-life in transiently transfected HEK293T cells following cycloheximide treatment without or with concurrent over-expression of USP25m (Fig. 6c, d). Following the corresponding treatments, protein lysates were electrophoresed, immunoblotted, quantified by densitometric scanning and normalised against α-tubulin, a stable protein whose concentration is not modified by cycloheximide addition after a 16-h treatment (as shown in Figure 6c). MyBPC1 was a short-lived protein, with a half-life of around 3 h (Fig. 6d). Over-expression of USP25m clearly stabilises MyBPC1 in absence of new protein synthesis (Fig. 6c, d), maintaining its levels unaltered (at least up to 16 h of treatment with cycloheximide), thus providing strong support for the role of USP25m in deubiquitinating MyBPC1 and rescuing it from proteasome degradation.

Discussion

Despite growing evidence suggesting a pivotal role of DUBs in the regulation of cellular function and their implication in a variety of diseases, few UBP substrates are known. Here we show that an isoform of USP25, a human DUB located on chromosome 21 that is over-expressed in DS, is selectively expressed in differentiated muscle cells and specifically interacts with three proteins associated to muscle differentiation: ACTA1, MyBPC1, and FLNC. Furthermore, we show that MyBPC1 is a substrate for Ub-dependent proteolysis and that over-expression of USP25m rescues this protein from proteasomal destruction.

We screened a muscle cDNA library by the Y2H strategy to seek for USP25 partners and/or substrates. We focused on the specific muscular isoform of USP25, instead of the more ubiquitous isoforms, assuming that the additional exons would confer new specificities in substrate binding, irrespective of other cellular functions shared by all the isoforms. The use of two baits, one consisting of a short peptide encompassing the alternatively spliced exons (19a-19b) and the other comprising the full-length muscle isoform, allowed us to assess the relevance of the additional amino acids in substrate recognition. In fact, the detection of positive interactions with the short peptide as a bait (such as the ones found with MyBPC1 and FLNC) indicates that the alternatively spliced exons of USP25m directly contribute to the recognition and binding of substrates.

Several clones of the 300 initially isolated using as a bait the full-length isoform were false positives as they were not in-frame or encoded previously reported 'sticky' proteins, whereas others were isolated only once and were classified under our stringent criteria as feeble-interacting USP25 partners or weakly expressed proteins in muscle tissue. Among the consistently isolated clones, we focused on the muscle-specific proteins MyBPC1, ACTA1, and FLNC, given the relevance of the UPS in the maintenance of tightly regulated intracellular pools of sarcomeric proteins and the fact that alterations of these proteins have been associated with severe muscle disorders. In this respect, mutations of the cardiac-MyBPC gene are the major cause of hypertrophic cardiomyopathy [32], and more than 60 different mutations of the ACTA1 gene are responsible for actin and nemaline myopathies [33], while FLNC is involved in limb-girdle muscular dystrophies [27]. Several complementary approaches further refined the initially described interactions. Thus, although MyBPC1 appeared to interact with both USP25m and USP25b in the Y2H assay, only the interaction with the USP25m isoform was confirmed in the more stringent co-immunoprecipitation assays. Similar results were obtained in GST-pull downs, where ACTA1 interacted with USP25m much more strongly than with USP25b.

The three muscle proteins that interact with USP25m have a pivotal function in the regulation of sarcomere structure, where they act as scaffold proteins, as well as in muscle cell contraction capabilities. MyBPC1 has been proposed to stabilise the structure of thick filaments by acting as a crossbridge protein in the A-band and to contribute to contractile regulation. Its interaction domains with relevant muscle proteins, such as myosin, actin, and titin (for review see [34]) have been mapped at the most C-terminal region, which overlaps with the USP25m interacting segment. FLNC is a large actin-cross-linking protein (more than 290 kDa), with both structural and signalling roles and, consistently, it is localised in Z-discs and in the subsarcolemma. Filamins are reported to bind to actin by the N terminus and to many other sarcomeric proteins including myotilin, myozenin, γ and δ-sarcoglycan, actin, and KY protein [27, 35–37] by a series of Ig-like domains that span most of the protein length (Ig1-Ig24). FLNC is restricted to skeletal muscle and heart and includes a specific insertion in the Ig20, absent in the other filamin genes, responsible for anchoring to the Z-discs and for specific protein-protein interactions. Notably, this Ig20 filamin domain is responsible for some of the interactions with USP25m. Concerning actins, the most abundant proteins in striated muscle, mammals have up to six actin isoforms, encoded by different genes. Although four of these actin genes are co-expressed in muscle at varying levels depending on the developmental and differentiation stage, ACTA1 is the most prominently expressed in differentiated adult muscle. As actins are a highly conserved protein family, we cannot exclude that USP25m and the more ubiquitous isoforms of USP25 also interact with other actins, the expression of the three isoforms allowing for differences in the recognition and binding to the different actin isoforms. In this context, our

results with the GST-pull down assays, where ACTA1 interacted with the USP25m and USP25b with different affinity, would support this hypothesis.

Overall, the USP25m partners appear to be relevant components of large multiprotein complexes where they act bridging thin and thick filaments, and are probably also involved in signal regulation, establishing links between extracellular stimuli and signal integration and transduction at the sarcolemma. It is worth noting that both MyBPC1 and FLNC bind the KY protein, a protease for which no function has been yet identified, that is responsible for kyphoscoliosis in mice [35]. The fact that MyBPC1 and FLNC share at least two interacting proteins, USP25m and KY, suggests a shared regulatory network. The co-expression of all these proteins along muscle differentiation further supports this notion.

It is well known that the regulated balance of sarcomeric and sarcomeric-associated proteins in the functional muscle is crucial to maintain contractile activity (for a comprehensive review, see [34]). In this context, the interaction of some of these proteins with components of the UPS is particularly interesting: MURF-1, a cytoskeletal E3-ligase localised to the M-line, seems to be involved in many muscle processes, from differentiation to disease (reviewed in [34]), and additional E3 ligases involved in muscle atrophy, including atrogin-1, MURF-2, and MURF-3, have been identified [38, 39]. Similarly to Ub ligases, DUBs will undoubtedly be shown to play important regulatory roles in these same processes.

Protein tagging by Ub, as with phosphorylation, is a tightly regulated and versatile signal that has been shown to be involved in many cellular processes other than proteasomal degradation, including regulation of protein trafficking, subcellular localisation, partner recruitment and enzymatic activity (reviewed in [3]). According to our results, FLNC and ACTA1 levels were relatively insensitive to treatments with proteasome inhibitors, indicating that their interaction with USP25m is not related to their protein turnover rate, but probably to these other regulatory roles of ubiquitination. In contrast, we have clearly showed that (at least in transiently transfected cells) MyBPC1 is a short-lived protein with a half-life of 3 h, whose intracellular pools are regulated by proteasome degradation. Interestingly, the over-expression of the muscle USP25 isoform, but not that of the ubiquitous isoform, is able to specifically rescue this protein from degradation, maintaining high intracellular levels of MyBPC1 even after 16 h of protein synthesis inhibition. MyBPC1 function is already known to be regulated by phosphorylation, hence, its ubiquitination (and deubiquitination) adds a novel regulatory step. The distribution of MyBPC1 in transverse stripes within the thick filament and the presence of myosin, titin, and actin binding sites indicate a crossbridging function for MyBPC1 between the thick and thin filaments, linking and/or aligning them. On the basis of our finding, it may be speculated that hypomorphic or inactivating mutations of USP25m would increase the MyBPC1 degradation rate, resulting in shorter and less uniform myosin filaments, whereas hypermorphic mutations or over-expression of USP25m would lead to MyBPC1 accumulation, possibly resulting in longer and thicker myosin filaments, and disarrayed myofibrils. Both MyBPC1 and FLNC are known to bind actin filaments and, therefore, the effect of subtle variations in their ubiquitination state upon the sarcomere might be additive in either structure maintenance or regulation of muscle contraction. In addition, some of the muscular dystrophies with severe myofibrillar alterations involve insoluble actin aggregates and deregulation of the UPS. Hence, subtle differences in USP25 expression levels and/or substrate recognition may synergistically alter the pools of monomeric actin, the dynamics of actin polymerisation, or the rates of aggregate formation and deposition.

Given that USP25 is over-expressed in DS patients, and that muscular abnormalities are invariably associated to this disease, it is tempting to speculate on its contribution to some of the DS phenotypic traits.

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