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# The E3 ubiquitin ligase specificity subunit ASB2β is a novel regulator of muscle differentiation that targets filamin B to proteasomal degradation

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## Abstract

Ubiquitin-mediated protein degradation is the main mechanism for controlled proteolysis, which is crucial for muscle development and maintenance. The ankyrin repeat-containing protein with a suppressor of cytokine signalling box 2 gene (*ASB2*) encodes the specificity subunit of an E3 ubiquitin ligase complex involved in differentiation of hematopoietic cells. Here, we provide the first evidence that a novel ASB2 isoform, ASB2β, is important for muscle differentiation. ASB2β is expressed in muscle cells during embryogenesis and in adult tissues. ASB2β is part of an active E3 ubiquitin ligase complex and targets the actin-binding protein filamin B (FLNb) for proteasomal degradation. Thus, ASB2β regulates FLNb functions by controlling its degradation. Knockdown of endogenous ASB2β by shRNAs during induced-differentiation of C2C12 cells delayed FLNb degradation as well as myoblast fusion and expression of muscle contractile proteins. Finally, knockdown of FLNb in ASB2β knockdown cells restores myogenic differentiation. Altogether, our results suggest that ASB2β is involved in muscle differentiation through the targeting of FLNb to destruction by the proteasome.

### Keywords

ubiquitin-proteasome system; ASB2; muscle differentiation; filamin

## Introduction

The ubiquitin-proteasome system (UPS) is one of the major mechanisms for controlled proteolysis which is a crucial determinant of many cellular events in eukaryotes. Degradation

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of a protein by the ubiquitin-proteasome pathway entails two successive events: the covalent attachment of ubiquitin chains to lysine residues in a substrate protein leading to its recognition and ATP-dependent proteolysis by the proteasome. Ubiquitylation of protein substrates occurs through the sequential action of distinct enzymes: a ubiquitin-activating enzyme E1, a ubiquitin-conjugating enzyme E2 and a ubiquitin ligase E3 responsible for the specific recognition of substrates through dedicated interaction domains (1). ASB2 is one of 18 members of the ankyrin repeat-containing SOCS box protein family (ASB) that are characterized by variable numbers of N-terminal ankyrin repeats (2). The *ASB2* gene was originally identified as an retinoic acid-inducible gene involved in induced-differentiation of myeloid leukemia cells (3,4). We have previously demonstrated that, by interacting with the Elongin BC complex, ASB2 can assemble with a Cullin5/Rbx module to form an E3 ubiquitin ligase complex that stimulates polyubiquitylation by the E2 ubiquitin-conjugating enzyme UbcH5a (5,6). This strongly suggests that ASB2 targets specific proteins to destruction by the proteasome during differentiation of hematopoietic cells. We have recently shown that ASB2 ubiquitin ligase activity drives proteasome-mediated degradation of the ubiquitously expressed actin-binding protein FLNs, FLNa and b, and can regulate integrin-mediated cell spreading (6).

During muscle development, dramatic changes in protein expression and cell morphology rely on the turnover of regulatory and structural components. Indeed, myogenic transcription factors such as MyoD and its E2A partner or negative Id regulator as well as myofibrillar proteins were shown to be degraded by the UPS (7–11). While some E3 ubiquitin ligases active during myogenesis have been identified (12–23), a precise understanding of the role of ubiquitylation in muscle development and the identities of specific ubiquitin-ligases and their potential substrates is lacking.

Here we show that *ASB2* expression is not restricted to hematopoietic cells but is also expressed and regulated in muscle cells during mouse and chick embryogenesis. Furthermore, *ASB2* transcripts expressed in muscle cells encode for a novel ASB2 isoform that we have named ASB2β. Its expression is induced during myogenic differentiation of C2C12 and primary myoblasts. By interacting with the elongin BC complex, ASB2β can assemble with the Cul5/Rbx2 module to reconstitute an active E3 ubiquitin ligase complex and we show that ASB2β triggers ubiquitylation and drives proteasomal degradation of FLNb but not of FLNa. Knockdown of ASB2β expression markedly delayed FLNb degradation and decreased C2C12 differentiation. Thus, our study provides the first evidence that FLNb regulation, via proteosomal degradation pathways, may regulate muscle differentiation.

## Results

### ASB2 is expressed in developing and mature muscle cells

ASB2 is known to be expressed in hematopoietic cells, where it is important for cell differentiation (3,6). To examine whether ASB2 may have roles outside the hematopoietic system we examined expression of *ASB2* mRNA in a variety of other tissues. Human ASB2 mRNA was detected in bone marrow, skeletal muscle, heart, foetal heart, small intestine, appendix, bladder, aorta, stomach, uterus, prostate, colon and thyroid gland (Figure 1a). Human ASB2 transcripts were relatively less abundant in tissues containing non-striated muscle than in skeletal and cardiac muscle. *ASB2* expression was induced during mouse embryonic development (Figure 1b) and its expression was maintained in skeletal muscle and heart in the adult mice (Figure 1c). To examine the expression of *ASB2* during chick embryogenesis, we performed *in situ* hybridization to whole-mount embryos and to tissue sections at various stages of development (Figure 2). *In situ* hybridization to whole-mount embryos showed an expression of *ASB2* in the somites (Figure 2a–c, e), and in the heart (Figure 2c and g) from E3 and in the limb (Figure 2d*)* from E6. *In situ* hybridization to tissue sections showed that the

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somitic expression of *ASB2* corresponds to the myotome (Figure 2e). *ASB2* transcripts are detected in all the heart (Figure 2g). An additional site of *ASB2* expression was observed in smooth muscle cells of the intestine at E6 (Figure 2f). Finally, the limb *ASB2* expression corresponds to skeletal muscle expression (Figure 2d and h). Altogether these results demonstrated that *ASB2* is developmentally regulated and that its expression previously described in hematopoiesis is also associated with the formation of all types of muscles, including skeletal, smooth and cardiac muscles.

## ASB2 is induced during myogenic differentiation

Since myogenic differentiation can be recapitulated *in vitro*, wherein myoblasts can be converted to myotubes, we examined the expression of ASB2 mRNA throughout the well-established differentiation model of the C2C12 mouse cell line. Differentiation of C2C12 cells was confirmed by their morphological changes such as alignment, elongation and fusion of mononucleated cells to multinucleated myotubes after switching cells from growth medium (GM) to differentiation medium (DM) (Figure 3a). Accompanying these morphological changes, the expression of muscle-specific proteins, myogenin, myosin heavy chain (MHC) and troponin T was up-regulated (Figure 3b and c). The ASB2 transcripts were barely detectable in undifferentiated C2C12 cells, increased in cells cultured in DM for 8 hours and were continuously expressed until day 8 (Figure 3d). By this time, myogenin, an early marker for the entry of myoblast into the differentiation, was induced (Figure 3b) suggesting that *ASB2* up-regulation may coincide with the differentiation commitment of myoblasts. Altogether, our results show that *ASB2* is induced during myogenic differentiation.

## ASB2β a novel ASB2 isoform

The cDNA sequences encoding mouse ASB2 proteins were first analysed in EST databases. Two different isoforms, a hematopoietic- and a muscle -type of ASB2 were identified and named ASB2α and ASB2β, respectively (Figure 4a). The human ASB2α has been previously published (3, 5). The mouse ASB2α was recently described in UniProtKB/Swiss-Prot database (release 12.0/54.0) as isoform 2 of Q8K0L0 whereas mouse ASB2β corresponds to isoform 1 of Q8K0L0. To confirm the expression of two ASB2 mRNAs, quantitative RT-PCR experiments were performed by amplification of cDNAs from skeletal muscle, heart, smooth muscle and hematopoietic cells with primers specific to ASB2α, ASB2β or with primers common to both cDNAs. As shown in Figure 4b, ASB2β mRNAs were mainly expressed in muscle cells while ASB2α mRNAs were expressed in hematopoietic cells. The new β isoform of ASB2 retains ankyrin repeats and the SOCS box (Figure 4a). In addition, ASB2β harbours a ubiquitin-interacting motif (UIM) at its N-terminus (Figure 4a and b). The ASB2 SOCS box can be further divided into a BC-box that defines a binding site for the elongin BC complex and a Cul5 box that determines the binding specificity for Cullin5 (5, 24) (Figure 4c). ASB2α and ASB2β are predicted to have molecular weights of 64 and 70 kDa, respectively (Figure 4a). To extend and further validate the finding that the ASB2β isoform is expressed in muscle cells, anti-peptide polyclonal antibodies were raised against an epitope within the N-terminal extension of ASB2β (2PNAB1 serum) or against an epitope within the C-terminus common to both ASB2 isoforms (1PLA serum) (Figure 4a). Flag-tagged ASB2 isoforms were expressed in HeLa cells to test the specificity of the antibodies by western blot analysis. As expected, anti-ASB2 antibodies from the 1PLA serum recognized both ASB2α and ASB2β isoforms whereas the anti-ASB2β antibodies from the 2PNAB1 serum recognized only the ASB2β isoform (Figure 4d). In protein lysates from differentiating C2C12 cells, a 70-kDa band was detected by the 2PNAB1 serum (Figure 4e) indicating that ASB2β protein is induced during skeletal muscle differentiation.

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### ASB2β is the specificity subunit of an E3 ubiquitin ligase complex

Given that ASB2α is the specificity subunit of an E3 ubiquitin ligase complex and that ASB2β contains both BC and Cul5 boxes, we determined whether ASB2β can also assemble with elongin B, elongin C and a Cullin5/Rbx module to reconstitute an E3 ubiquitin ligase complex. Therefore, anti-Flag immunoprecipitations were carried out on lysates of Sf21 cells co-infected with baculoviruses encoding Flag-ASB2β, Elongin B, HSV-Elongin C, Rbx2 and HA-Cul5, as indicated (Figure 4f). Like ASB2α, ASB2β can interact with Elongin B, Elongin C, Cul5 and Rbx2 whereas an ASB2β BC-box mutant (ASB2βLA) did not (Figure 4f). To determine whether the ASB2β/Elongin BC/Cul5/Rbx2 complex possesses ubiquitin ligase activity, the complex was immunoaffinity-purified and assayed for its ability to activate formation of polyubiquitin chains by the E2 ubiquitin-conjugating enzyme UbcH5a in the presence of ATP, E1 ubiquitin-activating enzyme Uba1, and ubiquitin. As shown in Figure 4g, the ASB2β complex stimulated formation of ubiquitin conjugates by E2. In contrast, anti-Flag immunoprecipitation from lysates of insect cells not expressing ASB2β did not support formation of polyubiquitin conjugates (Figure 4g). Furthermore, the ASB2β BC-box mutant that cannot assemble with Elongin B, elongin C, Cul5 and Rbx2 did not stimulate the polyubiquitylation activity of UbcH5a (Figure 4g). Among the proteins that were polyubiquitylated in this *in vitro* ubiquitylation reaction, ASB2β and Cul5 were found to be polyubiquitylated (Figure 4h). Altogether, our results indicated that ASB2β can assemble with elongin B, elongin C, Cullin 5 and Rbx2 to reconstitute an active E3 ubiquitin ligase complex.

### ASB2β triggers ubiquitylation and proteasome degradation of FLNb

We previously reported that ASB2α targets FLNa and b to proteasome degradation (6). We therefore assessed the expression of the ubiquitously expressed FLNa and FLNb as well as the muscle-specific FLNc during differentiation of C2C12 cells. Interestingly, ASB2β up-regulation correlated with loss of FLNb (Figure 5a). In contrast, expression of FLNa was not regulated and expression of FLNc was induced (Figure 5a). As shown in Figure 5b, accelerated degradation of FLNb was observed in differentiating C2C12 cells compared to proliferating cells. Furthermore, loss of FLNb was mediated via the proteasome as treatment of differentiating C2C12 cells with the proteasome inhibitor MG132 reduced FLNb degradation (Figure 5c). Altogether, these suggest that FLNb may be a substrate of ASB2β. To determine whether ASB2β can promote FLNb ubiquitylation, *in vitro* substrate ubiquitylation assays were performed using purified GFP-tagged FLNb. When FLNb-GFP was used as a substrate, ubiquitylation of FLNb by UbcH5a in the presence of the ASB2β/Elongin BC/Cullin5/Rbx2 or the ASB2α/Elongin BC/Cullin5/Rbx2 complexes but not in the presence of ASB2 E3 ligase defective mutants (ASB2βLA or ASB2αLA) was observed (Figure 5d). To confirm these results, NIH3T3 cells were co-transfected with vectors expressing GFP, GFP-ASB2β, GFP-ASB2βLA, GFP-ASB2α or GFP-ASB2αLA together with a FLNb-GFP or a FLNa-GFP expression vector. Twenty-four hours post transfection western blotting revealed that GFP-ASB2β expression resulted in a loss of FLNb-GFP (Figure 5e) but not of FLNa-GFP (Figure 5f). However, loss of both FLNa-GFP and FLNb-GFP was observed in cells transfected with a GFP-ASB2α expression vector as previously reported (6). Furthermore, FLNb-GFP levels were not altered in cells expressing GFP-ASB2βLA (Figure 5e). As expected, proteasome inhibitors blocked FLNb-GFP degradation induced by GFP-ASB2β (Figure 5e). To determine whether ASB2β induces degradation of endogenous FLNb, C2C12 myoblasts were transfected with vectors expressing GFP-ASB2β or GFP-ASB2βLA. Twenty hours after transfection, FLNb could not be detected in cells expressing GFP-ASB2βwt while FLNb expression was unaffected by GFP-ASB2βLA (Figure 5g). To extend and further validate the finding that ASB2β induced FLNb degradation during myogenic differentiation, we investigated ASB2β and FLNb expression during differentiation of human primary myoblasts. Differentiation was confirmed by their morphological changes (Figure 6a) and the expression of myogenin, MHC and troponin T (Figure 6b) after switching cells from growth medium to differentiation

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medium. In these cells, ASB2β up-regulation correlated with decrease of FLNb (Figure 6b). Altogether, our results indicate that ASB2β ubiquitin ligase activity drives ubiquitin-mediated proteasomal degradation of FLNb.

### Inhibition of ASB2β expression blocks myoblast fusion and myotube formation

To determine whether ASB2β is required for myoblast differentiation, ASB2β knockdown stable cell populations were generated by transfection of vectors encoding short hairpin RNAs (shRNAs) directed against ASB2β. Knockdown of ASB2β expression in C2C12 cells cultured in DM was demonstrated by Northern blot (data not shown) and Western blot (Figure 7a and b) analyses. In these cells, FLNb degradation was delayed (Figure 7a and b). Knockdown of ASB2β expression delayed myotube formation as evaluated by morphological observations (Figure 7c) and confirmed by the reduction in the level of both MHC and troponin T expression (Figure 7f). Furthermore, quantification of the fusion index demonstrated that ASB2β is required for myotube formation (Figure 7d and e). Conversely, the cell population transfected with an empty vector formed myotubes and expressed markers of muscle differentiation upon a shift to DM as expected (Figure 7c–f). To demonstrate the involvement of FLNb degradation in ASB2β-mediated effects on cell differentiation, we have investigated whether FLNb knockdown in ASB2β knockdown C2C12 cells can rescue the differentiation defects of these cells. Therefore, we have generated stable FLNb knockdown in ASB2β knockdown C2C12 cells. In these cells, FLNb expression was reduced to 50% compared to ASB2β knockdown cells expressing constructs that generate a shRNA targeting luciferase as controls (Figure 7g). The low level of FLNc present in undifferentiated C2C12 cells was not increased in ASB2β knockdown cells indicating that there is no functional compensation between FLNb and FLNc in these cells (Figure 7g). When cultured in DM, ASB2β/FLNb knockdown cells differentiate more rapidly than ASB2β knockdown cells transfected with a vector expressing the control shRNA as demonstrated by the expression of differentiation markers (Figure 7h). Altogether, our results indicated that ASB2β is required for the differentiation of C2C12 myoblasts into myotubes and regulates cell differentiation through FLNb degradation.

## Discussion

The *ASB2* gene was originally identified as a retinoic acid-inducible gene whose expression recapitulates early differentiation events critical to induced-differentiation of myeloid leukemia cells (3). EST database searches identified two different ASB2 protein isoforms, a hematopoietic- and a muscle -type that we named ASB2α and ASB2β, respectively. Our results show that ASB2β mRNAs are expressed in muscle cells while ASB2α mRNAs are mainly expressed in hematopoietic cells. Whether the tissue-specific control of ASB2 transcription is achieved through two different promoters resulting in the synthesis of two cell-specific ASB2 isoforms is the subject of ongoing experiments. We further demonstrate that ASB2β-specific antibodies recognize a 70-kDa protein in differentiated muscle cells. The two ASB2 isoforms differ in their $NH_2$-terminal region but share ankyrin repeats and a SOCS box. As demonstrated for ASB2α, ASB2β, by interacting with the Elongin BC complex, can assemble with Cul5 and Rbx2 to form a *bona fide* multimeric RING-type E3 ubiquitin ligase complex that stimulates polyubiquitylation by the E2 ubiquitin-conjugating enzyme UbcH5a. The ASB2β isoform harbours an UIM at its N-terminus. The UIM was initially identified in the proteasomal S5a subunit as involved in recognition of ubiquitylated substrates (25). UIMs form a single α-helix that binds polyubiquitin chains as well as monoubiquitin and promotes ubiquitylation of proteins that contain them (26). Recently, the UIM motif of Met4 was shown to protect polyubiquitylated Met4 from proteolysis by the proteasome (27). Whether ASB2 UIM is involved in the regulation of ASB2 stability and/or activity remains to be determined.

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While it is known that ASB proteins are implicated in diverse biological functions such as hematopoiesis, the substrates that are targeted for polyubiquitylation by ASB proteins are largely undefined. Although ASB2α induces proteasomal degradation of both FLNa and b ((6); this report), we showed here that ASB2β induces FLNb ubiquitylation and subsequently FLNb degradation, indicating that ASB2β is specific for FLNb over FLNa. Furthermore, knockdown of endogenous ASB2β in C2C12 cells delays myogenic differentiation and FLNb degradation. These results are in line with the previously reported FLNb down-regulation during C2C12 myoblast differentiation (28). Since loss of FLNb is markedly delayed in ASB2β KD2 cells, we can not exclude that a threshold of ASB2β is necessary to target FLNb to proteasomal degradation. Furthermore, we cannot exclude the possibility that other ASB2β targets are also important for differentiation of C2C12 cells.

Cell migration is a crucial step in skeletal muscle development during which myogenic progenitors migrate from the somites to the limb musculature. To differentiate and fuse to form syncytial skeletal muscle fibers, myoblasts must become less motile and establish cell-cell and cell-extracellular matrix contacts leading to cytoskeletal rearrangements (29). In this regard, it is noteworthy that *ASB2* is expressed in the myotome of the somites and in the limb during chick embryogenesis. This together with the fact that ASB2β can regulate the degradation of FLNb, a protein involved in actin remodelling, suggest that ASB2β can contribute to cytoskeletal reorganization during myogenesis. The appearance of ASB2β marks a very early event in differentiation of C2C12 cells; ASB2β was up-regulated at the same time as myogenin, the earliest known marker of myoblasts committed to the differentiation pathway expressed before the establishment of the postmitotic state (30). Inactivation of ASB2β by shRNAs interfered with the normal induction of muscle-specific proteins in C2C12 cells and delayed myotube formation. This suggests that ASB2β is important for myogenic differentiation. Furthermore, a partial knockdown of FLNb in ASB2β knockdown C2C12 cells accelerated the induction of differentiation markers demonstrating that the cell differentiation defect of ASB2 knockdown cells is due, at least in part, to its effect on FLNb degradation.

Interestingly, ASB2β induction and subsequent FLNb down-regulation correlates with the switch of the β1A to the β1D splice variant of the integrin β1 subunit associated with the commitment to differentiation of C2C12 myoblasts. A critical role of integrins during myogenesis has been proposed since antibody ligation of β1 integrins perturbed myotube formation *in vitro* (31) and inactivation of the mouse β1 integrin gene in developing myoblasts inhibited myoblast fusion and sarcomere assembly (32). Previous reports have indicated that FLNb binds strongly to β1A integrin but poorly to β1D whereas talin binds strongly to β1D and with intermediate affinity to β1A (28,33). Thus, differential binding of FLNb or talin to β1A integrin may modulate integrin-dependent functions such as cytoskeleton remodelling and signalling. It is therefore tempting to speculate that ASB2β may impact integrin-dependent functions. Our results provide a mechanism through which expression of FLNb and integrins are coordinately regulated, allowing myogenic differentiation. Alternatively, since FLNs act as scaffolds for signalling molecules involved in actin remodelling and/or transcriptional regulation, ASB2β may regulate pathways downstream of FLNb that have to be activated during muscle differentiation. Which signal transduction pathways are regulated, are the subject of ongoing investigation.

Expression of *ASB2* in axial and limb skeletal muscles during chick embryogenesis is consistent with ASB2β expression in myotubes. ASB2β is expressed in adult muscles suggesting that ASB2β may also play a role in muscle remodelling. Interestingly, ASB2β is up-regulated during mouse embryonic development at 17 dpc, a period associated with synaptic connections. Hence, it will be important to determine whether ASB2β expression correlates with muscle innervation. Furthermore, in a recent work aimed to the identification of transcripts with a circadian pattern of expression in adult skeletal muscle, *atrogin-1*, *MURF1* as well as *ASB2*

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were found to be circadian genes (34), suggesting that these E3 ubiquitin ligases play a role in maintaining cellular homeostasis in skeletal muscle cells. An interesting possibility is that ASB2β regulate independent mechanisms in myoblasts and skeletal myotubes. In this regard, it will be important to identify ASB2β substrate(s) in fully differentiated muscle cells. Future studies will also be necessary to further our understanding of FLNb function in myoblasts.

## Materials and methods

### Cell Lines, culture conditions, induction and differentiation

The mouse myoblasts of the C2C12 cell line were grown in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/l glucose, 10 % fetal bovine serum (PAA laboratories), 1% sodium pyruvate, 1% non essential amino acids and penicillin-streptomycin (Invitrogen). For differentiation studies, C2C12 cells were plated at 7,500 cells/$cm^2$, grown to 80 % confluence in two days and then cultured in differentiation media containing DMEM supplemented with 2% horse serum (PAA laboratories). Differentiation media was changed every 48 h. The fusion index, i.e., the number of nuclei in troponin T positive multinucleated myotubes divided by the total number of nuclei, calculated for C2C12 parental cells at day 6 was 65 %. Human Primary myoblasts isolated from a quadriceps muscle biopsy of a new-born infant as described (35) were obtained from V. Mouly (Institut de Myologie, Paris, France). Human myoblasts were grown in F10 medium (Invitrogen) supplemented with 20% fetal bovine serum and penicillin-streptomycin. For differentiation studies, human myoblasts were plated at 3,500 cells/$cm^2$, grown to 80 % confluence in six days and then cultured in differentiation media containing DMEM supplemented with 10 μg/ml insulin (Sigma) and 100 μg/ml transferin (Sigma). Differentiation media was changed every 24 h. HeLa cells were grown on Petri-dishes in DMEM containing 4.5 g/l glucose, 10 % fetal bovine serum (PAA laboratories), glutamax, pyruvate and penicillin-streptomycin (Invitrogen). NIH3T3 cells were grown in DMEM containing 4.5 g/l glucose (Invitrogen), 1% sodium pyruvate, 10 % new born calf serum (PAA laboratories) and penicillin-streptomycin. NB4 cells were used as described (3). Cells were maintained in a 5% $CO_2$ incubator at 37°C. For proteasome inhibition, NIH3T3 and C2C12 cells were incubated with 1 and 5 μM MG132 (Euromedex), respectively. To inhibit *de novo* protein synthesis, C2C12 cells were treated with 5 μg/ml cycloheximide (Sigma).

### Plasmid constructs

The pCMVSport6-mASB2β vector was obtained from RZPD (Deutsches Ressourcenzentrum für Genomforshung GmbH). The mASB2β open reading frame was subcloned into a pBacPAK9 (Clontech) -derived vector to direct the expression of mASB2β fused to the FLAG epitope at its N terminus (pBacPAK9FN-mASB2β), into a pCMV-derived vector to direct the expression of mASB2β tagged with two FLAG epitopes at its N terminus (pCMV-FLAG2-mASB2β) and into the pEGFP-C3 expression vector (Clontech). Mutation L595A was introduced into mASB2β using the QuikChange site-directed mutagenesis kit (Stratagene) and the mutated oligonucleotide sequence, as indicated in boldface, 5′-CTCCGAGACCT**GCG**GCTCACCTCTGCCG-3′. The pcDNA3-cASB2 plasmid was obtained from the University of Delaware and contains the 3′ end of chicken ASB2 cDNA (accession number AI982288). The hASB2α open reading frame (3,4) was subcloned into the pCMV-FLAG2 generating the pCMV-FLAG2-ASB2α vector. The pcDNA3-FLNa-GFP (36), pCl-puro-FLNb-GFP (28) and pEGFP-C3-ASB2α (6)expression constructs have been used previously.

Specific silencing of mASB2β was achieved by using a shRNA-expressing vector. Nucleotides 96–114 (sh#1) and 1370–1388 (sh#2) of the mASB2β coding sequence were chosen as target for shRNA. The shRNA sequences were used to construct 60-mer short hairpin (sh)RNA oligonucleotides, which were then synthesized (MWG), and ligated into the pSUPER.neo.gfp

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expression vector (Oligoengine) under the control of the H1 promoter. The following oligonucleotides were used (underlined, sense and antisense sequences; boldface, restriction enzyme sites; lightface italics, polIII termination signals; boldface italics, loop with linker): sh#1: 5′-
**GATCC***CC*GAGTCATAACGTCTTATAG***TTCAAGAGA***CTATAAGAACGTTATGACT C*TTT TTGGAA***A**-3′, sh# 2: 5′-
**GATCC***CC*CGCCGATGCTAACAAAGCC***TTCAAGAGA***GGCTTTGTTAGCATCGGCG *TTT TTGGAA***A**-3′. All constructs were verified by DNA sequencing.

## Northern blotting

Total RNA was isolated from mouse tissues following the method of Chomczynski and Sacchi (37) and from C2C12 cells using a nucleospin RNA II kit (Macherey-Nagel). Hybridization was as described (38). The ASB2 probe corresponded to the mouse ASB2β open reading frame. The human RNA Master blot and the mouse embryo MTN blot were obtained from Clontech. Poly A+ RNA samples on Master blot have been normalized to the mRNA levels of eight different “housekeeping” genes. The β-actin probe was from Clontech and the Arbp probe was previously described as 36B4 probe.

## Quantitative RT-PCR

Total mRNAs from human skeletal muscle, heart and smooth muscle were from Clontech. Total mRNA was extracted and purified from NB4 acute promyelocytic leukemia cells treated for two days with $10^{-6}$ M *all-trans* retinoic acid as described (3). cDNA was synthesized using superscript III first-strand synthesis kit as recommended by the manufacturer (Invitrogen). cDNA synthesis experiments were repeated three times. Real-time PCR was carried out with the 7300 real-time PCR system using the SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer’s instructions. The specificity of the PCR primers was confirmed by melting curve analyses. Primers for detection of human ASB2 mRNAs were designed based on chromosome 14 sequence according to the requirements for real-time RT-PCR using the Perl Primer software. Oligonucleotide primer sequences corresponding to distinct exons were: forward 5′-ATTCCTGCCTGAAGCC -3′ and reverse 5′-TGCAGTGGACCTGGA -3′ for ASB2α, forward 5′-GAATTGTACCCTTGTTTCAGAG -3′ and reverse 5′-CTCCAGAACAGACACCC -3′ for ASB2β, forward 5′-GCCCAGAGTGGACAGTTGGA -3′ and reverse 5′-TGGCCTGCGTGTTGATGT -3′ common to both ASB2isoforms. Efficiency of amplification wa s determined using the standard curves method. Fold changes were quantified as $2^{-(\text{Ct isoform}-\text{Ct common})}$.

## *In situ* hybridization

*In situ* hybridization to whole-mount embryos and to tissue sections was performed at various developmental stages, ranging from E2 to E10 as previously described (39). The fragment corresponding to part of the coding sequence of chicken ASB2 was isolated from pcDNA3-cASB2 and used to generate a single-stranded anti-sense digoxygenin-labeled RNA probe.

## Transfections

Exponentially growing HeLa and NIH3T3 cells were transfected using the Jet PEI reagent (Polyplus transfection) as recommended by the manufacturer. For transient expression, C2C12 cells were transfected using LipofectAMINE as per manufacturer’s instructions. To establish stable transfectants, C2C12 cells were transfected using the nucleofector V solution and the B32 program, as recommended by the manufacturer (Amaxa). ASB2β knockdown was obtained transfecting C2C12 cells with shRNA against mouse ASB2β. Cells were then cultured for 48 hours prior to selection with 0.5 mg/ml G418 (Invitrogen). FLNb knockdown was obtained transfecting C2C12 cells that have been previously transfected with sh#2 directed

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against ASB2β with an shRNA against mouse FLNb in pGIPZ vector (OpenBiosystem). A vector expressing an shRNA in pGIPZ vector targeted to luciferase (OpenBiosystem) was used as a control. After two days the transfected cells were selected using 1 μg/ml puromycin together with 0.5 mg/ml G418.

### Whole cell extracts

C2C12 cells were washed twice in PBS and resuspended in whole cell extract buffer containing 50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.1 % NP40, 10 % glycerol, 1 mM dithiothreitol (DTT), 1 mM $Na_3VO_4$, 50 mM NaF and 1 % protease inhibitor cocktail (P8340; Sigma). After three freeze-thaw cycle in liquid nitrogen, the resulting cell lysates were cleared by a 10 min 20,000 g centrifugation at 4°C.

### Analysis of detergent soluble, detergent insoluble and urea soluble fractions

$10^6$ cells were collected and washed twice in ice-cold PBS. Cell pellets were lysed in 100 μl detergent-soluble fraction (DSF) buffer containing 10 mM Tris-HCl pH 7.5, 1 % Triton X100, 5 mM EDTA and supplemented with 1 mM $Na_3VO_4$, 50 mM NaF and 1 % protease inhibitor cocktail. Insoluble material was recovered by centrifugation at 16 000 g for 15 min at 4°C. Pellets were then washed with supplemented DSF buffer, resuspended in 20 μl detergent-insoluble fraction (DIF) buffer containing 10 mM Tris-HCl pH 7.5, 1 % SDS and supplemented with 1 mM $Na_3VO_4$, 50 mM NaF and 1 % protease inhibitor cocktail, incubated for 15 min at room temperature and for 2 min on ice, and sonicated following the addition of 50 μl DSF buffer. After centrifugation at 16,000 g for 5 min at 4°C, the remaining insoluble material was resuspended in 10 μl 50 mM Tris-HCl pH 7 containing 8 M urea and sonicated. Equal amounts of each fraction were heated for 15 min at 37°C in SDS-PAGE sample buffer and analysed by SDS-PAGE.

### Antibodies

Two peptides, an amino-terminal specific to the mouse ASB2β isoform (ISTRGRQRAIGHEE) and a C-terminal common to ASB2α and β proteins (LAPERARLYEDRRS) were synthesized and coupled to keyhole limpet hemocyanin through a cysteine residue added to the carboxy- or amino-terminal amino acid of the peptides, respectively (Millegen). Rabbit sera were collected 6 months after the initial injection (Millegen). The serum raised against human ASB2 (1PNA) has been described previously (3). Primary antibodies were: anti-myosin heavy chain (F59), anti-elongin B (FL-118), anti-Rbx2 (N15), anti-Erk2 (C-14), anti-myogenin (F5D) (Santa Cruz Biotechnology, Inc), anti-troponin T (JLT-12) (Sigma-Aldrich), anti-HA (1D1) (Euromedex), anti-polyubiquitinylated proteins (FK1) (Biomol), anti-Elongin C (SIIIp15) (Transduction Laboratories), anti-FLNc (Kinasource), anti-Flag (F7425) (Sigma) and anti-GFP (Rockland). The anti-human FLNa antiserum which cross-reacts with mouse FLNa has been described (40). Rabbit anti-FLNb and goat anti-FLNb (N-16) were purchased from Chemicon and Santa Cruz, respectively. Secondary antibody anti-mouse, anti-rabbit and anti-goat conjugates with HRP were from Jackson Laboratories.

### Immunofluorescence

Cells were fixed in 4 % paraformaldehyde in PBS supplemented with 15 mM sucrose and permeabilized with 0.1 % Triton X-100. After blocking with 3 % BSA in PBS, immunostaining of cells was performed using antibodies to FLNb from Chemicon in 1:1000 and to troponin-T in 1:1000. Secondary antibodies used were Alexa Fluor 546 or 488 coupled to goat anti-rabbit or goat anti-mouse (Invitrogen). For nuclear staining, fixed cells were incubated with 0.4 μM DAPI for 5 min after secondary antibody incubation. Preparations were mounted in mowiol (Calbiochem).

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### Ubiquitylation assays

Recombinant baculoviruses encoding mASB2β, mASB2βL595A and Rbx2 were generated with the BacPAK baculovirus expression system (Clontech). *In vitro* ubiquitylation assays were carried out as described (5). FLNb ubiquitylation assays were performed using immunopurified FLNb-GFP as substrate in the presence of the NEDD8 machinery as described (6). Briefly, NIH3T3 cells were transfected for 24 h with FLNb-GFP expression vector. Anti-GFP antibodies immobilized onto protein A sepharose were added to the cell protein extract in a binding buffer adjusted to 20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% NP40. After 2 h of incubation on ice and after 3 washes with binding buffer, proteins were eluted with 100 mM phosphate buffer, pH 12.5 and buffered to pH 8.5 for *in vitro* ubiquitylation assays. Reaction products were fractionated by SDS-PAGE and analyzed by immunoblotting with anti-GFP antibodies.

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## Abbreviations

**ASB2**
: ankyrin repeat-containing protein with a suppressor of cytokine signalling box 2 gene

**CRL**
: Cullin RING ligase

**FLN**
: filamin

**HECT**
: homologous to the E6-associated protein carboxyl terminus

**MAFbx**
: Muscle Atrophy F-box

**MHC**
: myosin heavy chain

**MURF**
: muscle RING finger

**RING**
: really interesting new gene

**SOCS**
: suppressor of cytokine signalling

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**UIM**

ubiquitin-interacting motif

**UPS**

ubiquitin-proteasome system

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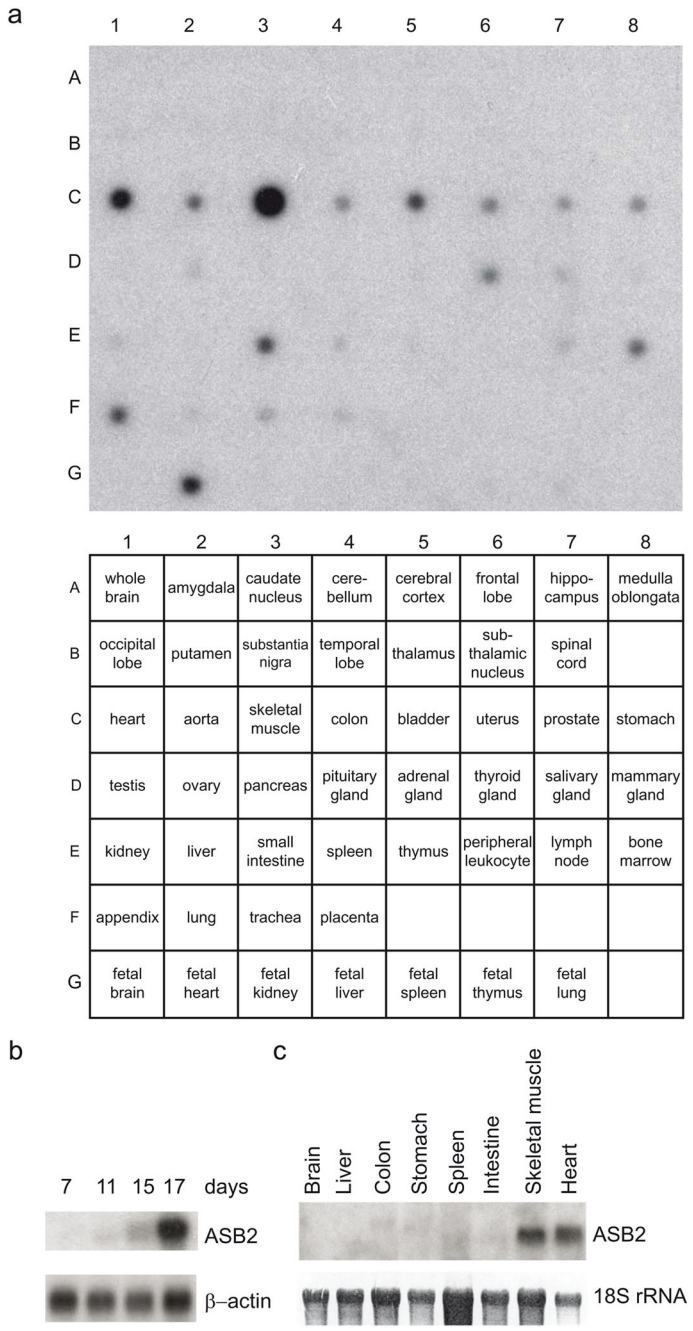


**Figure 1.**

Expression of *ASB2* mRNA in tissues. (**a**) Autoradiogram of *ASB2* mRNA expression in human primary tissues (*upper*) and diagram showing the type and position of polyA+ RNAs on the membrane (*lower*). Autoradiograms of ASB2 mRNAs expression in mouse embryos (**b**) in mouse adult tissues (**c**). Northern blot was performed using 2 μg of polyA+ RNA (b) or 5 μg of total RNA (c). To confirm RNA loading and integrity, β-actin was used as a probe (b) or 18S rRNA was methylene blue-stained on membrane after transfer (c).

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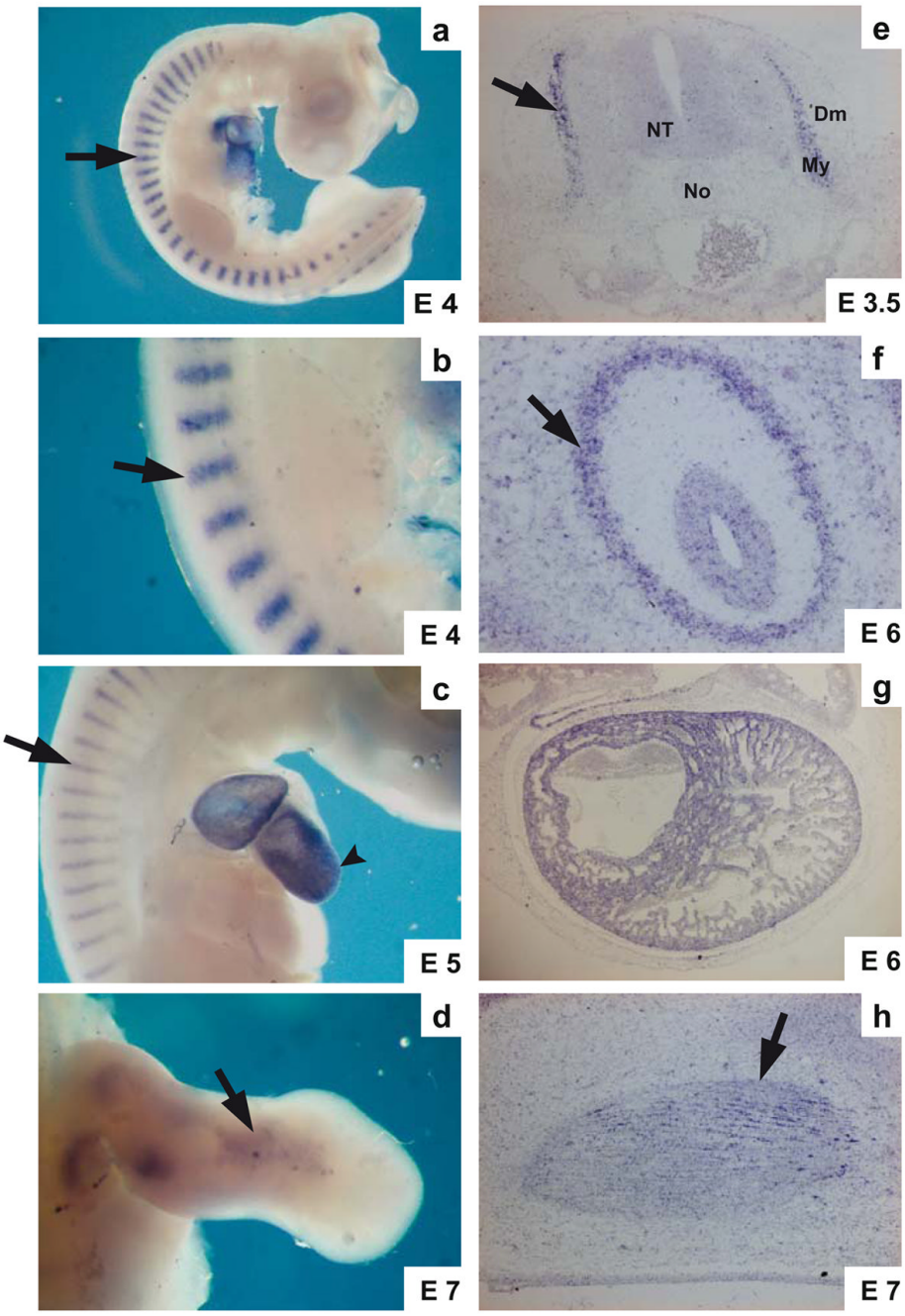


**Figure 2.**

Expression pattern of *ASB2* during chick embryogenesis. ASB2 mRNAs were detected in whole mount preparations (**a** to **d**) and sections through the trunk region (**e**), the intestine (**f**), the heart (**g**) or the developing limb skeletal muscle (**g**) of chick embryos by *in situ* hybridization. In each panel, the developmental stage is indicated. NT, neural tube; No, notochord; Dm, dermomyotome; My, myotome. Arrows indicate somites in a–c and e, limb skeletal muscle in d and h, and smooth muscle cells of the intestine in f. Arrowhead points to the heart.

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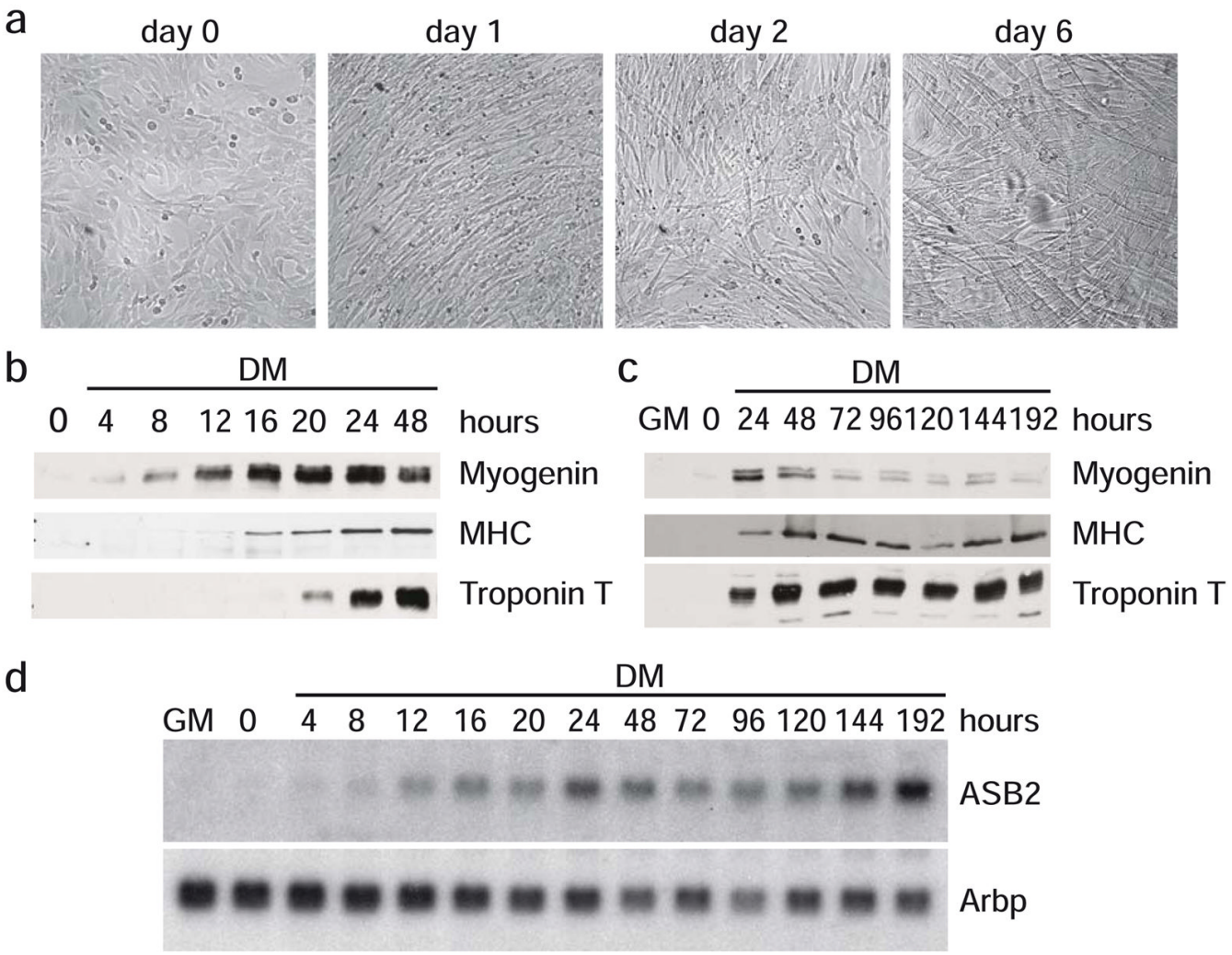


**Figure 3.**

Induction of *ASB2* mRNA during differentiation of C2C12 myoblasts. C2C12 cells were cultured in growth medium (GM) and shifted to differentiation medium (DM) for 6 days. (**a**) Morphological changes of C2C12 cells for assessment of alignment, elongation and fusion were observed under a phase-contrast microscope. (**b** and **c**) Expression of myogenin, myosin heavy chain (MHC) and troponin T during differentiation of C2C12 cells. 10 μg-aliquots of each whole cell extract were analyzed by western blot using indicated antibodies. (**d**) Detection of ASB2 mRNA in C2C12 cells cultured in GM or in DM for 1 to 6 days. Northern blot analysis was performed using 5 μg of total RNA. Upper and lower panels are autoradiograms of mRNA of ASB2 and Arbp as assessment of RNA quantities in each lane, respectively.

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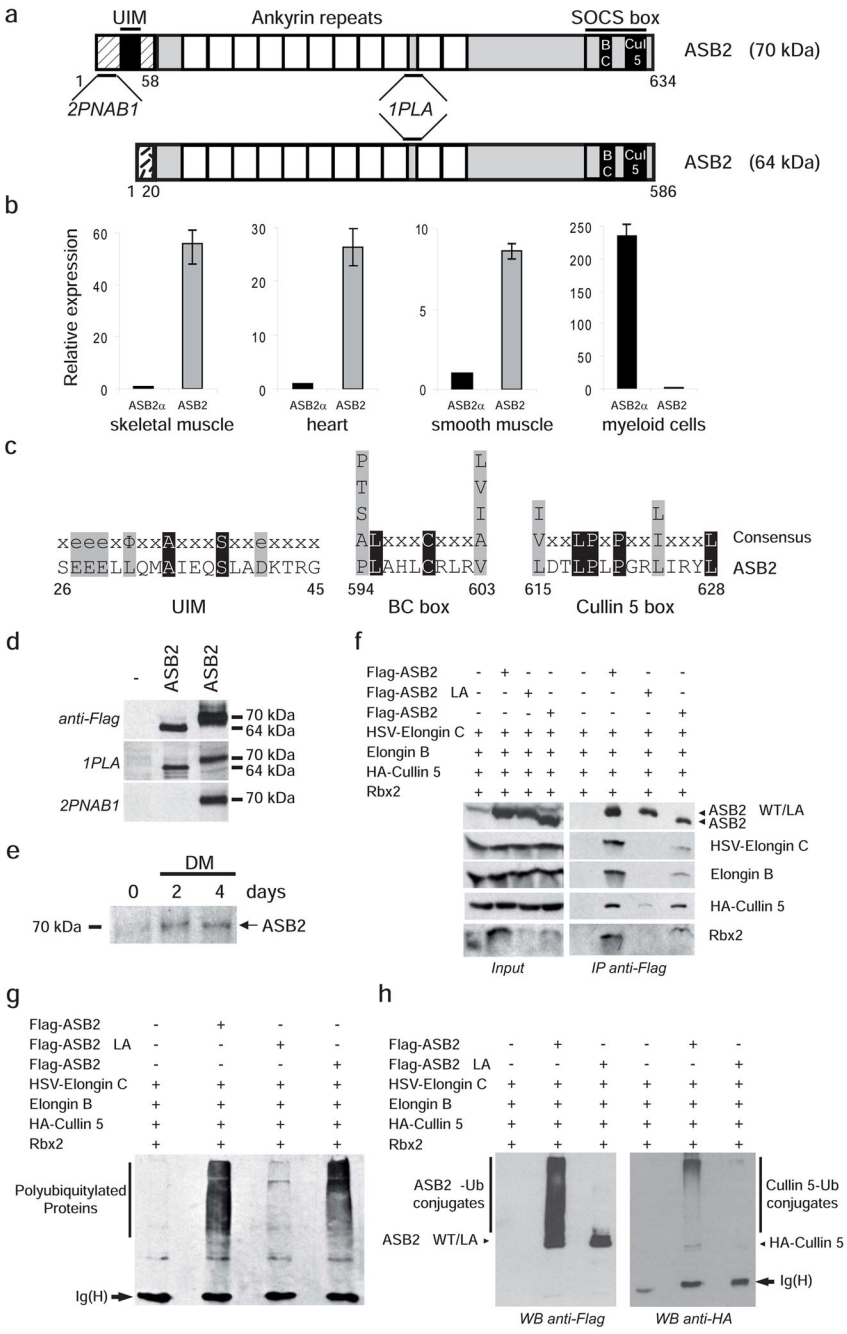


**Figure 4.**

Characterization of ASB2 isoforms. (**a**) Schematic representation of the domains of mouse ASB2 isoforms. Positions of the peptide sequences used for production of the 2PNAB1 and 1PLA sera are shown (**b**) Relative expression of ASB2 mRNAs in human skeletal muscle, heart, smooth muscle and myeloid cells. Quantitative real-time RT-PCR were carried out as described in Materials and Methods. Results are plotted as relative expression for ASB2α and ASB2β. Data corresponding to one out of three independent experiments are shown as mean ± standard deviation. (**c**) Alignments of the ubiquitin interacting motif (UIM), the BC-box and the Cul5 box of mouse ASB2β with the consensus sequences. Shaded regions represent residues identical (black) or similar (grey) to the domain class consensus sequences. e is a

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negatively charged residue, Φ represents hydrophobic residue and x is any amino acid. In a and B, amino acid numbering is indicated. (**d**) Isoform specificity of ASB2-specific antibodies. HeLa cells were transfected with FLAG-tagged ASB2β and FLAG-tagged ASB2α expression vectors or the corresponding empty vector (−). Urea soluble fractions (5 μg) were separated by SDS-PAGE and subjected to immunoblotting with anti-FLAG antibodies and 1PLA or 2PNAB1 sera, as indicated. (**e**) ASB2β protein is induced during differentiation of C2C12 myoblasts. C2C12 cells were cultured in GM and were shifted to DM for 2 and 4 days. 30 μg-aliquots of each urea soluble fraction were analyzed by western blot using the 2PNAB1 serum. (**f**) ASB2β associated with Elongins B and C, Cullin 5 and Rbx2. Sf21 cells were infected with baculoviruses expressing the proteins indicated. The lysates were immunoprecipitated (*IP*) with anti-FLAG antibodies. Crude extracts (input) and immune complexes were separated by SDS-PAGE and immunoblotted with the indicated antibodies. ASB2 isoforms were detected using the 1PLA serum. (**g–h**) The ASB2β/ElonginBC/Cul5/Rbx2 complex had ubiquitin ligase activity. The cell lysates of (f) were subjected to anti-Flag immunoaffinity purification. The purified ASB2 complexes were incubated with Uba1, UbcH5a, ubiquitin and ATP to assess their ability to stimulate ubiquitylation by UbcH5a by Western blot using anti-polyubiquitin (**g**), anti-Flag (**h**, left panel) and anti-HA (**h**, right panel) antibodies. Arrow indicates the heavy chain of immunoglobulins (Ig(H)).

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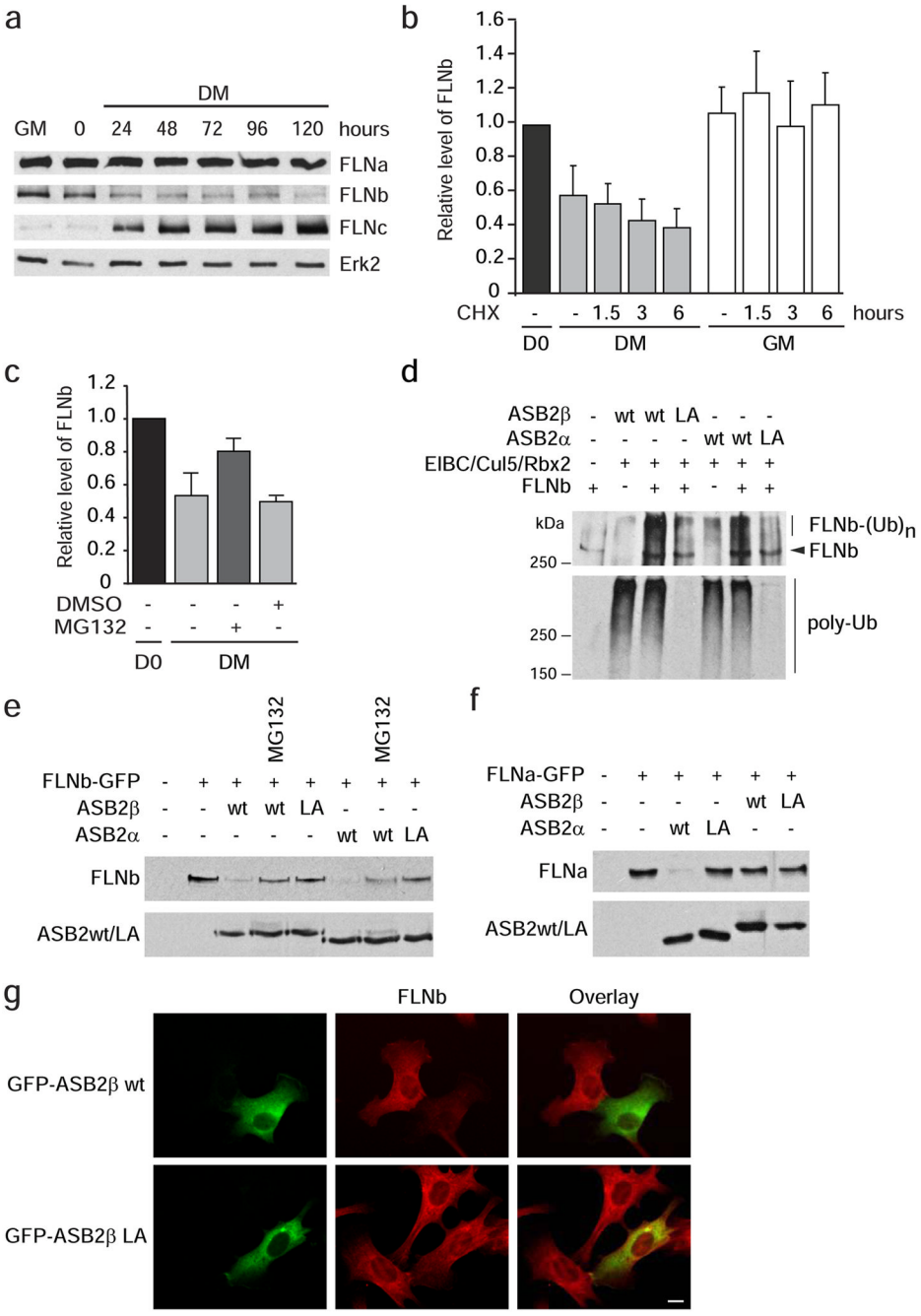


**Figure 5.**

ASB2β induced ubiquitin-mediated FLNb degradation. (**a**) Expression of FLNa, FLNb and FLNc in C2C12 cells induced to differentiate. C2C12 cells were cultured in growth medium (GM) and shifted to differentiation medium (DM) for 6 days. Expression of FLNa, FLNb, FLNc and Erk2 was analyzed by Western blot using 20 μg-aliquots of whole cell extracts. (**b**) Quantification of FLNb-turnover following CHX-treatment. C2C12 cells were cultured in GM or shifted to DM for 18 hours and subsequently treated with cycloheximide (CHX) to block protein synthesis for various time (1.5, 3 and 6 hours). (**c**) FLNb degradation in differentiating C2C12 cells is dependent on the proteasome. C2C12 cells were cultured in DM for 16 hours and subsequently treated with MG132 or DMSO for 8 hours. In b and c, 10-μg

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aliquots of each whole cell extract was immunoblotted with antibodies to FLNb and Erk2 and quantification of FLNb level relative to Erk2 by densitometric scanning of three independent experiments is shown. (**d**) ASB2β induces polyubiquitylation of FLNb. Recombinant ASB2/elongin BC/cullin5/Rbx2 complexes were purified as in Figure 3f. All samples contained purified Uba1, UbcH5a, ubiquitin, APP-BP1/Uba3, Ubc12 and NEDD8. Purified FLNb-GFP was also provided as indicated and subjected to ubiquitylation. Aliquots of the reaction mixture were analyzed by western blotting using anti-GFP (upper panel) and antibodies to polyubiquitylated proteins (lower panel). (**e**) ASB2β-induced FLNb degradation is dependent on ASB2 ubiquitin ligase activity and the proteasome. NIH3T3 cells were mock-transfected or transfected for 24 h with FLNb-GFP together with GFP (−), or GFP-ASB2αwt, GFP-ASB2αLA, GFP-ASB2βwt, GFP-ASB2βLA expression vectors in the absence or presence of 1 μM MG132 for 18 h, as indicated. (**f**) ASB2β does not induce FLNa degradation. NIH3T3 cells were mock-transfected or transfected for 24 h with FLNa-GFP together with GFP (−), or GFP-ASB2 expression vectors, as indicated. In (e) and (f), 20 μg aliquots of whole cell extracts were immunoblotted with antibodies to GFP. (**g**) ASB2β induced degradation of endogenous FLNb. C2C12 myoblasts were transfected with GFP-ASB2βwt or GFP-ASB2βLA expression vectors, plated on fibronectin-coated coverslips 5 h after transfection, fixed 15 h later and analyzed using an antibody directed against FLNb. Scale bar, 10 μm.

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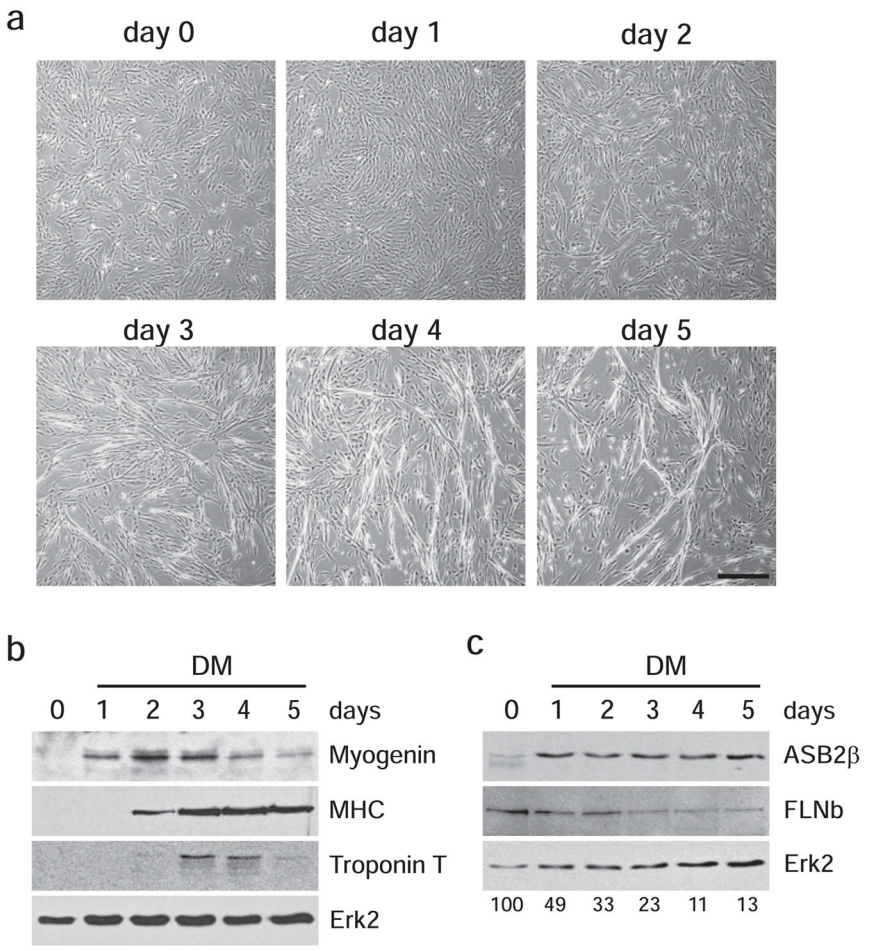


**Figure 6.**

Down-regulation of FLNb in primary myoblasts induced to differentiate correlates with ASB2β induction. Human myoblasts were cultured in growth medium (GM) and shifted to differentiation medium (DM) for 5 days. (**a**) Morphological changes of primary cells for assessment of alignment, elongation and fusion were observed under a phase-contrast microscope. Scale bar, 500 μm. (**b**) Expression of Myogenin, MHC, Troponin T and Erk2 during differentiation of primary cells. (**c**) Expression of ASB2β, FLNb and Erk2 during differentiation of primary cells. The numbers under the blot represent the percentages of FLNb relatively to day 0, calculated after normalization to Erk2. In b and c, 5 μg-aliquots of each whole cell extract were analyzed by western blot using indicated antibodies.

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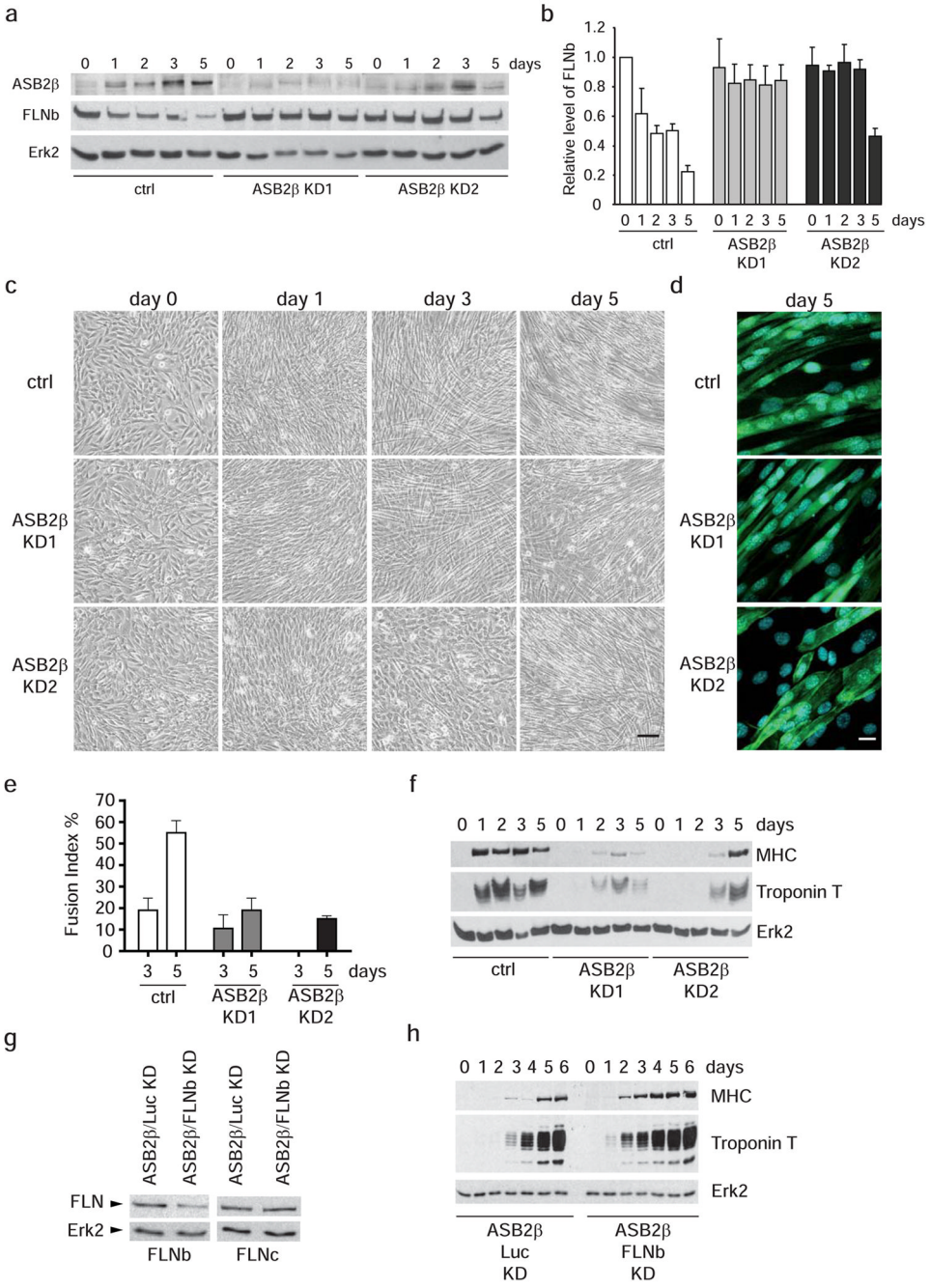


**Figure 7.**

ASB2β knockdown delayed differentiation of C2C12 myoblasts. (**a** to **f**) Stable C2C12 cell populations expressing shRNAs directed against ASB2β (ASB2β KD1 and ASB2β KD2) or transfected with the empty vector (ctrl) were shifted to DM for 5 days. (**a**) 20 μg-aliquots of urea soluble fractions were analyzed by western blot using 2PNAB1 serum. 5 μg-aliquots of whole cell extracts were analyzed by western blot using anti-FLNb (N-16) and anti-Erk2 antibodies. (**b**) Expression of FLNb relative to Erk2 based on densitometric scanning. Results are mean ± standard deviation of three independent experiments. (**c**) Images of differentiating C2C12 cell populations (1, 3 and 5 days after initiation of differentiation) observed with a phase-contrast microscope are shown for each stable cell populations. Scale bar, 100 μm. (**d**)

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Fluorescence images of C2C12 cell populations, 5 days after induction of differentiation. Troponin T (green) and nuclei (blue) were stained to identify troponin T positive cells and facilitate myotube identification. Scale bar, 20 μm. (**e**) Histograms represent the fusion index calculated for C2C12 cell populations at day 3 and 5. Results are mean ± standard deviation from 2 independent experiments, where at least 200 nuclei/experiment were counted. (**f**) Expression of myosin heavy chain (MHC) and troponin T during differentiation of C2C12 cell populations. Ten μg-aliquots of each whole cell extract were analyzed by western blot, using anti-MHC and anti-troponin T antibodies. (**g**) FLNb knockdown in ASB2β knockdown C2C12 cells. ASB2β KD2 cells were stably transfected with constructs that generate shRNAs targeting ASB2β (ASB2β/FLNb KD) or luciferase (ASB2β/Luc KD). Ten μg-aliquots of each whole cell extract were immunoblotted with antibodies to FLNb, FLNc and Erk2. (**h**) Expression of myosin heavy chain (MHC) and troponin T during differentiation of ASB2β and FLNb double knockdown C2C12 cells. ASB2β/FLNb KD and ASB2β/Luc KD cells were shifted to DM for 6 days. Ten μg-aliquots of each whole cell extract were analyzed by western blotting using indicated antibodies.