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## INVOLVEMENT OF BTBD1 IN MESENCHYMAL DIFFERENTIATION

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Running title: Myogenic versus adipogenic regulation of BTBD1.

**ABSTRACT**

BTBD1 is a recently cloned BTB-domain containing protein particularly expressed in skeletal muscle and interacting with DNA topoisomerase 1 (Topo1), a key enzyme of cell survival. We have previously demonstrated that stable overexpression of a N-terminal truncated BTBD1 inhibited *ex vivo* myogenesis but not adipogenesis of pluripotent C2C12 cells. Here, BTBD1 expression was studied in three models of cellular differentiation: myogenesis (C2C12 cells), adipogenesis (3T3-L1 cells) and osteogenesis (hMADS cells). BTBD1 mRNA was found to be upregulated during myogenesis. At the opposite, we have not observed BTBD1 upregulation in an altered myogenesis cellular model and we observed a downregulation of BTBD1 mRNA expression in adipogenesis. Interestingly, amounts of Topo1 protein, but not Topo1 mRNA, were found to be modulated at the opposite of BTBD1 mRNA. No variation of BTBD1 expression was measured during osteogenesis. Taken together, these results indicate that BTBD1 mRNA is specifically regulated during myogenic and adipogenic differentiation, in relation with Topo1 expression. Moreover, they corroborate observations made previously with truncated BTBD1 and show that BTBD1 is a key protein of balance between adipogenesis and myogenesis. Finally, a transcriptome analysis gave molecular clues to decipher BTBD1 role, with an emphasis on the involvement in ubiquitin/proteasome degradation pathway.

Keywords: BTB domain, DNA topoisomerase 1, myogenesis, adipogenesis, osteogenesis.

## **INTRODUCTION**

BTBD1 is a recently characterized protein expressed in the whole animal kingdom from *C. elegans* to human, with an unknown function. This protein was detected first by Carrim-Todd *et al.* in 2001 along with its homologue BTBD2 [1]. BTBD1 gene encodes a 482 amino acid protein with a N-terminal end containing a proline-rich region and a BTB domain, which are both involved in protein-protein interactions [2]. In 2002, Xu *et al.* demonstrated that BTBD1 and BTBD2 interact with DNA topoisomerase 1 (Topo1) by their C-terminal region (317-482 positions for BTBD1) [3]. BTBD1 function has not been characterized, but its colocalization in cytoplasmic bodies with TRIM5 $\delta$  [4], an E3 ubiquitin ligase, suggests its involvement in protein degradation. Moreover, BTBD1 can be classed in a novel functional family of BTB-domain containing proteins: the family of “Cullin-3 (Cul3) associated proteins” [5-7]. Cul3 is a subclass of Cullin-dependent ubiquitin ligases, a multi-subunit enzyme class of E3s involved in specific degradation of various proteins by the ubiquitin/proteasome pathway [8]. In this model, BTB proteins, like BTBD1, would determine selectivity of the degradation complex by linking specific substrates to Cul3 [7, 9, 10]. For instance, since Topo1 degradation can be performed by the ubiquitin/proteasome pathway [11, 12], BTBD1 could link Cul3 by its N-terminal region and Topo1 by its C-terminal region, to promote Topo1 degradation. Topo1 is the key enzyme of DNA molecule relaxation, a paramount event in the cell life for all species. It is a determining factor for DNA replication, recombination and repair, chromatin condensation and remodelling, and finally RNA transcription [13-15]. The crucial activity of Topo1 for cell survival and differentiation requests a strict regulation of its expression, which is principally determined by the balance between its synthesis and degradation [16-18].

Our studies are focused on BTBD1 function in skeletal muscle. We have previously demonstrated that BTBD1 is essential for myoblast growth and differentiation [19]. Analysis of stable altered BTBD1 protein expression (BTBD1 truncated of its BTB and proline-rich N-terminal domains, designated as  $\Delta$ -BTBD1) in C2C12 cells (a murine cell line of myoblasts) has shown a defect of these cells to accomplish myogenesis. The mechanism of BTBD1 implication in myogenic differentiation is unknown, but several characteristics of these modified cells are in favour of Topo1 deregulation. Thus,  $\Delta$ -BTBD1 overexpression was found to be associated with deregulation of the nuclear Topo1 level and localization [19].

In this study, we assess the involvement of BTBD1 in mesenchymal differentiation and we attempt to clarify its specificity of action. First, by  $\Delta$ -BTBD1 C2C12 cell differentiation assays based on C2C12 pluripotentiality, we confirmed the involvement of BTBD1 in myogenesis and adipogenesis, but not in osteogenesis. Then, we analyzed BTBD1 basal expression along myoblast, adipocyte and osteoblast differentiations, as well as in a myoblast altered differentiation model. We found close correlations between BTBD1 mRNA expression and progression of adipocytic and myogenic differentiations, and we correlated those with Topo1 expression. A transcriptome analysis of gene deregulations induced by the overexpression of  $\Delta$ -BTBD1 in C2C12 cells submitted to myogenic differentiation conditions gave some molecular basis to decipher BTBD1 function. Finally, our study demonstrates that BTBD1 is a key protein involved in adipogenic/myogenic differentiation balance, potentially *via* Topo1 regulation through its degradation by the ubiquitin/proteasome pathway.

## **MATERIALS AND METHODS**

**Cell culture.** The C2C12 mouse skeletal muscle cell line (CRL 1772) was obtained from the American Type Culture Collection. Wild type (WT) and transfected cell lines were grown at 37°C in a 5% CO<sub>2</sub> atmosphere in a growth medium (GM) composed of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Myotube C2C12 differentiation was induced by withdrawing fetal calf serum from confluent cells and adding 10 µg/mL insulin, 5 µg/mL transferrin and 2% horse serum; this medium was named differentiation medium (DM). C2C12 adipogenic differentiation was induced by adding rosiglitazone (5 µM final concentration) in GM to confluent cells. Rosiglitazone is a member of the glitazone family known to induce adipogenesis in various cell types [20]. C2C12 osteogenic differentiation was induced by adding 0.1 µM dexamethasone, 10 mM β-glycerophosphate and 50 µg/mL ascorbic acid 2-phosphate in GM to confluent cells [21]. The C2C12 DN MKK3 cell line has been previously described [22].

For adipogenesis experiments, 3T3-L1 cells (ATCC-CL 173) were grown in GM until confluence and stimulated to differentiate by addition of 0.5µM dexamethasone, 0.5 mM 1-methyl-3-isobutylmethyl-xanthine, and 170 nM insulin [23]. Two days later, cells were replaced in GM complemented with 170 nM insulin. For osteogenesis analysis, hMADS cells were grown in GM supplemented with 2,5 ng/mL FGF2 and differentiated in osteoblasts at confluence by adding 0.1 µM dexamethasone, 10 mM β-glycerophosphate, and 50 µg/ml ascorbic acid 2-phosphate [24].

For WT C2C12, DN MKK3 C2C12, 3T3-L1 and hMADS cells, experiments were performed with a pool of clones routinely used. For Δ-BTBD1 C2C12 cells, only experiments

reproducible on three independent modified cell lines were considered because of the clonal heterogeneity of derived C2C12 cells.

**Cells staining and visualization.** Oil red O and Alizarin red staining were performed as previously described [20, 21]. Cells were mounted and visualized on a Leitz Wetzlar light microscope. Photographs were taken with a Nikon Coolpix 990 digital camera. Mineralization areas were quantified after numerization with Multi Gauge V 2.3 (Fuji) software.

**Total RNA extraction and quantitative RT-PCR analysis.** Total RNAs were extracted using TRI-REAGENT (Euromedex) according to the manufacturer's instructions. RNAs were treated with DNase I for 30 min (Promega) before reverse transcription. First strand cDNA was generated with Superscript II reverse transcriptase (Invitrogen) in presence of 12.5 ng/l random primers (Promega), following the manufacturer's instructions. Real-time PCRs were performed on an ABI Prism 7000 real-time PCR machine (Applied Biosystem). For each PCR run, a master mix was prepared and composed of "qPCR MasterMix Plus for SYBR green I" (Eurogentec) and 300 nM of each primer. Primers were designed using Primer Express software (Applied Biosystem) and validated by testing PCR efficiency using standard curves (efficiency >85%). Gene expression was quantified using the comparative Ct (threshold cycle) method, mouse 36B4 and human TATA box binding protein (TBP) RNAs were used as reference. Forward and reverse primers are as follows : mouse 36B4 5'-TCCAGGCTTTGGGCATCA-3' and 5'-CTTTATCAGCTGCACATCACTCAGA-3' ; mouse aP2 5'-CTTCAAACCTGGGCGTGGAA-3' and 5'-CTAGGGTTATGATGCTCTTCACCTT-3'; mouse BTBD1 5'-CCTAAACCCCGAGTAGAGTA<sup>2</sup>ATTG-3' and 5'-TTGATGCAGCACTCCTTTCCT-3'; mouse myogenin 5'-GAAGAGGAGCACCCCTGCTCA-3' and 5'-ATCTCCACTTTAGGCAGCCG-3'; mouse Topo1 5'-TCGAGCTGTTGCAATTCTTTGTA-3' and 5'-TGCAAGTTCATCATTGACTTCTCA-3' ; human alkaline phosphatase (ALP) 5'-

GCGCAAGAGACACTGAAATATGC-3' and 5'-TGGTGGAGCTGACCCTTGAG-3' ; human BTBD1 5'-GACCAAGGCGTCGCTGAA-3' and 5'-CCCAGTACGAAGCGCACAT-3' ; human TBP 5'-CACGAACCACGGCACTGATT-3' and 5'-TTTTCTTGCTGCCAGTCTGGAC-3'. The data presented are means  $\pm$  sem of triplicates from two independent experiments. Statistically significant changes (\*,  $p < 0.01$ ) were detected using unpaired Student's t-test.

**Western blot analysis.** Cell protein extracts and Western blots analysis were performed as previously described [19]. Anti-Topo1 rabbit polyclonal (H-300) and anti- $\beta$ -actin (AC-15) mouse monoclonal antibodies were purchased respectively from Santa Cruz and Sigma-Aldrich. The anti-aP2 rabbit polyclonal antibody was a gift of Dr. David Bernlohr (University of Minnesota, St. Paul, MN, USA).

**Microarrays analysis.** 50mers oligonucleotide-microarrays manufacturing, labelling, hybridizations and imaging, array data analysis and expression statistical analysis are described previously [25]. Briefly, total RNAs for each conditions under comparison were prepared, using the RNeasy Midi Kit (Qiagen) with DNase treatment, from three independent culture of WT C2C12 cells pool or one culture of three independent  $\Delta$ -BTBD1 C2C12 cells clones. 15  $\mu$ g of RNA were reverse transcribed using the CyScribe cDNA Post labelling kit (Amersham Biosciences) from oligo dT and random nonamers with dUTP-aminoallyl incorporation. cDNAs were purified on GFX columns (Amersham Biosciences) then coupled to Cy3 and Cy5. The microarrays contained 16254 spots representing 5418 genes in triplicates. Hybridization signals were read on ScanArray 3000 (Perkin Elmer) and analysed with Genepix Pro software. Data were filtered and normalized with Madscan software available at <http://cardioserve.nantes.inserm.fr/mad/madscan/login.php>. Statistical analyses were made with the Significance Analysis of Microarrays software.



## **RESULTS**

**Multiple differentiation assays with  $\Delta$ -BTBD1 C2C12 cells.** In a previous work [19], we have constructed a plasmid expressing  $\Delta$ -BTBD1 protein, a partially truncated form of BTBD1 that lacks the 108 N-terminal amino acid residues, which correspond to the complete proline-rich domain and a large part of the BTB domain, basis of the N-terminal protein/protein interaction domain.  $\Delta$ -BTBD1 stable expression in pluripotent C2C12 cells, *i.e.* a murine skeletal muscle cell line that is able to differentiate *ex vivo*, prevented their myogenesis capacity, but not their adipogenesis capacity. To precise these observations, and to analyze the general effect of  $\Delta$ -BTBD1 expression on C2C12 cell pluripotency, we performed in parallel myogenesis, osteogenesis and adipogenesis assays with  $\Delta$ -BTBD1 C2C12 (3 clones) and wild-type cells (WT) as control (Fig. 1). Confluent cells were cultivated with the adequate differentiation medium. Then, cells differentiation potential was evaluated on the basis of specific terminal morphologic modification. Myogenesis was assessed by myotube apparition within 2-4 days; adipogenesis was assessed by lipid droplets Oil Red O specific staining after 1 week; osteogenesis was assessed by calcification Alizarin red specific staining after 2 weeks. As expected, WT C2C12 cells were able to differentiate into myotubes, osteoblasts and adipocytes (Fig. 1 A-F), while  $\Delta$ -BTBD1 C2C12 cells were able to differentiate into osteoblasts and adipocytes, but not in myotubes (Fig. 1 G-L). Moreover, their capacity to differentiate in adipocytes was found to be increased in comparison with WT C2C12 cells (Fig. 1: compare E-F with K-L). This was confirmed by real-time PCR quantification of an adipocyte marker, the fatty acid binding protein (aP2) (Fig 1M). Conversely,  $\Delta$ -BTBD1 C2C12 cells capacity to differentiate in osteoblasts was not found different than WT C2C12 cells capacity, as assessed by quantification of mineralization area (Fig.1N). These results

establish that  $\Delta$ -BTBD1 expression affects C2C12 cell pluripotency: myogenesis capacity is inhibited, adipogenesis is enhanced and osteogenesis seems unaffected.

Moreover, confluent  $\Delta$ -BTBD1 C2C12 cells are able to differentiate spontaneously in adipocytes, but not in osteoblasts, without adequate differentiation medium (data not shown). This result is in favour of a specific implication of BTBD1 in adipocyte differentiation.

**BTBD1 expression is positively correlated with C2C12 myogenic differentiation.** In order to investigate BTBD1 function in skeletal muscle and to clarify the relation between  $\Delta$ -BTBD1 expression and defect in *ex vivo* myogenic differentiation, we studied BTBD1 basal expression in differentiation of C2C12 cells. In growth medium (GM), C2C12 cells are mononucleated and undifferentiated (myoblasts). At confluence, if these cells are switched to differentiation medium (DM), they differentiate into myocytes which fuse after 2-3 days to form syncytial structures (myotubes) (Fig. 1B). The kinetic of BTBD1 mRNA variations was studied by quantitative RT-PCR during C2C12 cell differentiation, from confluence (day 0) to day 5 in DM, in comparison with mRNA variations of myogenin, a key myogenic regulator crucial for myocyte fusion. Quantification of RNA content indicated a continuous increase of BTBD1 mRNA amounts throughout the differentiation process with an approximate seven-fold maximum reached 4 days after differentiation induction (Fig. 2A). This pattern was very well correlated with the important up-regulation of myogenin.

To further assess BTBD1 involvement in C2C12 cell myogenic differentiation, we investigated BTBD1 mRNA expression in a C2C12 cell model in which differentiation is altered. This model is a stable C2C12 cell line expressing a dominant-negative form of the p38 activator MAP kinase kinase 3 and designated DN MKK3 C2C12 cells [22]. The cells are characterized by their inability to complete differentiation due to inhibition of p38 activity. They are blocked at the

myocyte step and are unable to fuse into myotubes (similar morphology than Fig. 1G). BTBD1 and myogenin expressions were compared by quantitative RT-PCR throughout 5 days in DM, the treatment that induces myogenic differentiation in WT C2C12 cells (Fig. 2B). A basal and constant expression, without any upregulation was found for both genes. Thus, in our model, alteration of C2C12 differentiation aborts BTBD1 and myogenin expression regulation.

Taken together, these results demonstrate a strong positive correlation between BTBD1 expression and C2C12 cell myogenic differentiation.

**BTBD1 gene is differently modulated during *ex vivo* mesenchymal differentiations.** Firstly, to elucidate further the previous observation that C2C12 adipogenic differentiation was increased by  $\Delta$ -BTBD1 stable expression (Fig. 1), we studied BTBD1 mRNA basal expression in a widely used adipogenesis model, the mouse pre-adipocyte 3T3-L1 cell line which is able to differentiate into mature adipocytes with more than 90% efficiency after a 6-day exposure to adipogenic signals [23]. Analysis by quantitative RT-PCR of 3T3-L1 cells mRNAs at different time-points during differentiation showed a significant gradual decrease of BTBD1 mRNA expression from day 0 to day 9 (Fig. 3A). More than 80% of decrease was observed at day 9, which is a time-point corresponding to 90% of 3T3-L1 cells adipogenic differentiation. Induction of the fatty acid binding protein aP2, a specific adipocyte marker, was quantified in parallel as a positive control of adipogenic differentiation.

Secondly, in the same manner, we studied BTBD1 expression during osteogenic differentiation. We used hMADS cells described to differentiate in osteoblasts under specific culture conditions [24]. We detected no significant variation of BTBD1 mRNA level from day 0 to day 24 of differentiation (Fig. 3B), despite a clear expected extracellular matrix mineralization.

At the opposite, the mRNA level of phosphatase alkaline, an osteogenic differentiation marker, was found to be gradually increased between days 8 and 24.

Together, these data are consistent with the results obtained with  $\Delta$ -BTBD1 and suggest that BTBD1 is differently implicated in mesenchymal differentiations.

**Different correlations between BTBD1 and Topo1 expressions.** We have previously demonstrated that BTBD1 — which binds to Topo1 — is essential for normal Topo1 activity and speculated that BTBD1 could negatively contribute to Topo1 regulation, via its ubiquitination and degradation by the ubiquitin/proteasome pathway [19]. The upregulation of BTBD1 in C2C12 myogenic differentiation and its downregulation in C2C12 adipogenic differentiation prompted us to analyse Topo1 expression in the same cellular differentiations. Results are shown in Fig. 4. Topo1 protein levels were assessed on Western blots, but no information could be obtained on BTBD1 protein since no anti-BTBD1 antibodies are available. Topo1 mRNA were quantified with quantitative RT-PCR performed on the same C2C12 RNAs than mentioned above for BTBD1 expressions. Very interestingly, C2C12 myogenic differentiation, which is associated to an upregulation of BTBD1 mRNA (Fig. 2A), was found to be characterized by a significant approximately two-fold downregulation of Topo1 protein between days 0 and 4 (Fig. 4A). However, no significant modification of Topo1 mRNA was seen during myogenic differentiation. A converse situation was observed with C2C12 adipogenic differentiation. The downregulation of BTBD1 mRNA (Fig. 3A) contrasted with an approximately twofold upregulation of Topo1 protein between days 0 and 9 (Fig. 4B). As for myogenic differentiation, no variation in Topo1 mRNA amounts was detected during adipogenic differentiation. These data indicate opposite post-translational regulations of Topo1 during C2C12 myogenic and adipogenic differentiations and corroborate our hypothesis of a negative regulation of Topo1 by BTBD1.

**Transcriptome analysis of  $\Delta$ -BTBD1 C2C12 cells myogenic differentiation.** To start to decipher BTBD1 function we have analyzed transcriptome modifications induced by  $\Delta$ -BTBD1 overexpression in C2C12 cells at the onset of myogenic differentiation. Transcriptomes of  $\Delta$ -BTBD1 and WT C2C12 cells were compared along a three time-points kinetic, i.e. day -2 (proliferating stage), day 0 (confluence) and day +1 (differentiating stage: myogenic differentiation is induced at day 0). The comparison was stopped at this latter stage before myotube apparition in WT C2C12 cells since  $\Delta$ -BTBD1 C2C12 cells do not fuse into myotubes [19]. mRNAs were hybridized to murine cDNA microarrays developed in a previous work [25].

Figure 5 presents meaningful variations which were found in four gene families. A large defect of expression for many myogenic markers (troponin, tropomyosin, myosin...) including key muscle regulatory factors (MEF2C and MEF2D) have been found during  $\Delta$ -BTBD1 C2C12 cells differentiation compared to WT cells. This is in accordance with the failure of  $\Delta$ -BTBD1 C2C12 cells to differentiate into myotubes. Some of these genes are not upregulated at all, but some of them are induced in a much weaker manner than in WT C2C12 (*i.e.* troponin T3). The second striking result was the almost opposite expression profiles found for extracellular matrix genes, such as many collagen subtypes, between  $\Delta$ -BTBD1 and WT C2C12 cells. In most of the cases, the genes were specifically induced in  $\Delta$ -BTBD1 C2C12 cells. In fact only one gene clearly related to the extracellular matrix, *i.e.* fibronectin 1, was found to share a common expression profile between the two types of C2C12 cells (not shown). These opposite expression profiles were also observed for the four integrin genes found differentially expressed on the microarrays. This is important to note since integrins are involved in muscle differentiation, especially at the membrane fusion stage [26]. All these results sustain important differences

between  $\Delta$ -BTBD1 and WT C2C12 cells and support myogenesis defect in  $\Delta$ -BTBD1 C2C12 cells.

Finally, the last gene group of interest concerns genes involved in the ubiquitin/proteasome degradation pathway. This pathway is clearly affected in the  $\Delta$ -BTBD1 C2C12 cells since 13 out of 14 genes with differential microarray signals harboured opposite expression profiles (essentially down-regulated in  $\Delta$ -BTBD1 cells) between  $\Delta$ -BTBD1 and WT C2C12 cells (see Fig. 5 Proteasome panel).

## **DISCUSSION**

BTBD1 is a new protein that contains several protein-protein interaction motifs. It was discovered at the same time as its homologue BTBD2 by Carrim-Todd *et al.* in 2001. Xu *et al.* showed that BTBD1 and BTBD2 were able to bind Topo1 and that BTBD2 has a weak Topo1 inhibitor activity *in vitro*. BTBD1 is preferentially expressed in skeletal muscle and heart, which suggests that BTBD1 has a more specific function in striated muscles [1, 3]. In a previous study, we have investigated BTBD1 function in modified murine C2C12 skeletal muscle cells expressing a truncated form of BTBD1 ( $\Delta$ -BTBD1) which is expected to act as a dominant-negative form. The most important findings were the incapacity of  $\Delta$ -BTBD1 C2C12 cells to differentiate in myotubes and alterations in Topo1 protein level and localization. These results suggested that BTBD1 may be involved in myogenic differentiation *ex vivo* [19].

In the present study, we investigated the effect of  $\Delta$ -BTBD1 expression on C2C12 cells pluripotency by performing myogenesis, adipogenesis and osteogenesis assays. Then, to clarify BTBD1 implication in different lineage differentiations, we studied BTBD1 mRNA expression in cellular models for *ex vivo* myogenesis, adipogenesis and osteogenesis. In parallel, Topo1 expression was assessed at the RNA and protein levels. Finally, to get insights into the molecular mechanism involving BTBD1, we analyzed by cDNA microarrays gene expression modifications induced by overexpression of  $\Delta$ -BTBD1 in C2C12 cells cultured under myogenic differentiation conditions.

**Opposite involvement of BTBD1 in myogenic and adipogenic differentiations.** To precise the general effect of  $\Delta$ -BTBD1 expression on C2C12 cell pluripotency,  $\Delta$ -BTBD1 C2C12 cells were treated to induce myogenesis, adipogenesis and osteogenesis. Our results indicated that expression of  $\Delta$ -BTBD1 in C2C12 cells inhibits myogenesis, induces adipogenesis and does not

affect osteogenesis. Two hypotheses can be raised to explain the opposite effect of  $\Delta$ -BTBD1 expression on myogenic and adipogenic differentiations. (i) Inhibition of the process of myogenesis facilitates the differentiation of C2C12 cells into adipocytes as previously described with DN MKK3 C2C12 cells [27]. (ii) BTBD1 has opposite functions in myogenic and adipogenic differentiations. To discriminate between these two hypotheses, we analyzed endogenous BTBD1 mRNA expression in models for *ex vivo* myogenesis and adipogenesis.

*In vitro* myogenic C2C12 differentiation is considered to mimic most of the *in vivo* differentiation steps. Under low serum culture conditions, confluent myoblasts complete a last cell cycle and then differentiate into myocytes, which align contiguously to each other and finally fuse into myotubes. This is the last structure before *in vivo* terminal myofiber formation. We found a close correlation between BTBD1 expression and differentiation from myoblasts to myotubes in C2C12 cells. A continuous increase in BTBD1 expression matched the progression of differentiation in WT C2C12 cells. Conversely, a low and constant BTBD1 expression was observed for several days under differentiation culture conditions in the DN MKK3 C2C12 cell line which stayed blocked at the myocyte stage for several days [22]. In conclusion, a link between BTBD1 and myogenesis is underlined by our previous and present observations: (i) overexpression of altered BTBD1 protein blocks C2C12 cell myogenic differentiation, (ii) myogenic differentiation and progressive increase of BTBD1 expression are positively correlated and (iii) inhibition of myotube formation, leaded by inhibition of p38 activity in C2C12 cells, prevents normal upregulation of BTBD1 expression. An obvious hypothesis to explain these results is that upregulation of functional BTBD1 protein is necessary for myogenesis progress.

Adipogenesis was simulated *ex vivo* using 3T3-L1 cells able to form mature adipocytes after 6 days in appropriate differentiation medium. Using this model, we have demonstrated a continuous decrease of BTBD1 mRNA expression during adipogenesis. While BTBD1



expression is upregulated during myogenesis, it is downregulated during adipogenesis. Since overexpression of  $\Delta$ -BTBD1 increases C2C12 cell adipogenic differentiation [ref], we can speculate that BTBD1 is directly involved in adipogenesis by preventing this process. Thus, a defect in BTBD1 function, as caused by overexpression of  $\Delta$ -BTBD1, could explain in the same time an inhibition in myogenic differentiation and an induction of adipogenic differentiation. Therefore we propose that BTBD1 supports opposite functions in myogenic and adipogenic differentiations.

To precise a specific or a wide involvement of BTBD1 in mesenchymal differentiation, we also studied BTBD1 expression during osteoblastic differentiation. No significant variation in BTBD1 expression was detected during osteoblastic differentiation, in accordance with the lack of effect of  $\Delta$ -BTBD1 expression in C2C12 cell osteoblastic differentiation. BTBD1 function in mesenchymal differentiation seems thus restricted to myogenic and adipogenic lineages.

Taken together, these results indicate that BTBD1 could be involved in myogenic/adipogenic differentiation balance. Recent experimental works have shown that satellite cells, the main myogenic precursors, can differentiate in several lineages and in particular give rise to adipocytes. [Asakura et al., *Differentiation*, 2001, 68, 245-253; De Coppi et al., *Diabetologia*, 2006]. Environmental cellular interactions are important cues for pluripotent-cell differentiation fates. Thus, Shefer *et al.* have shown that satellite cells, maintain in fact their myogenic potential when they are close to myofibers, but they can adopt an adipogenic potential when they are dissociated from myofibers [28]. Underlying molecular mechanisms need still to be clarify. An essential role has been found for Wnt signalling [Vertino et al., *Mol Cell Biol*, 2005, 16, 2039-2048].

Maybe, BTBD1 could be another important protein of this adoptive potential of satellite cells and more generally of the differentiation engagement regulation of mesenchymal committed progenitors (myo-adipogenic or myo-adipo-chondro-osteogenic lineages).

**Molecular events correlated to BTBD1 mRNA expression during myogenesis.** Transcriptome modifications induced by  $\Delta$ -BTBD1 in C2C12 cells grown under myogenic differentiation conditions, clearly indicate that the lack of differentiation in myotubes is associated with a wide and drastic impairment of myogenic markers RNA accumulation. This underlines the crucial role of BTBD1 in myogenic differentiation. Modifications in expression of extracellular matrix and integrin genes are likely related to the faint myogenic differentiation. Integrins are important for myoblasts membrane fusion in connection with extracellular matrix proteins. However it cannot be ruled out that expression modification of extracellular matrix genes can also be related to other cellular mechanisms since these genes are involved in many aspects of cell behaviour, as chondrogenic differentiation for collagen IIa1. The clearest hypothesis on BTBD1 role suggested by the microarray study is the implication in the ubiquitin/proteasome degradation pathway. It seems that  $\Delta$ -BTBD1 expression profoundly modifies composition of multiple ubiquitin/proteasome complexes. This is based on the observation that 13 genes of this pathway showed opposite expression profiles between  $\Delta$ -BTBD1 and WT C2C12 cells. They consist of ubiquitin activating enzymes, ubiquitin conjugating enzymes or ubiquitin ligases (Siah2), as well as proteasome subunits. The extent of these changes at mRNA level is surprising but of special interest, and could be a consequence of a large disruption in ubiquitin/proteasome system for protein degradation due to  $\Delta$ -BTBD1 expression. These data reinforce our hypothesis for an implication of BTBD1 in this pathway, but with a more important involvement that we previously hypothesized.

Thus BTBD1 involvement in differentiation process is likely to consist in targeting key proteins to the ubiquitin/proteasome pathway. More precisely, BTBD1 could link specific substrates to E3 ubiquitin ligases, such as Cul3. In this context, expression of the truncated N-terminal form of BTBD1 which disrupts myogenesis and promotes adipogenesis, could prevent degradation of key substrates linked to its C-terminal region and not recruited to the E3 complex normally linked to the N-terminal containing proline-rich and BTB domains of BTBD1. Topo1 is likely to be one of the proteins targeted to degradation by BTBD1 during myogenesis and adipogenesis since i) BTBD1 is a Topo1 interacting protein [3], ii) its expression is negatively correlated with Topo1 protein expression and activity — without variations in Topo1 mRNA quantities —during myogenesis [19] as well as adipogenesis (Fig. 4), iii) although no clear molecular evidence demonstrate the role of Topo1 in mesenchymal differentiation, links between Topo1 and myogenic differentiation are enlightened by the following point. A strategy to treat rhabdomyosarcoma, a malignant pathology characterized by uncontrolled skeletal muscle cell differentiation, is the use of Topo1 inhibitors such as topotecan or irinotecan [29]. Thus, an abnormal upregulation of Topo1 activity can be linked to anarchic muscle cell differentiation, which suggests a direct link between Topo1 activity and myogenic differentiation. Taken together, these and our previous results clearly suggest an original function of Topo1 in mesenchymal differentiation with a main regulation at the protein level .

Finally, it also should be mentioned that Topo1 is a key enzyme of cell cycle progress in regard to its function in DNA replication. Another point to consider is that BTBD1 can be involved in cell cycle regulation, through Topo1 degradation *via* BTBD1 activity in the Cul3 complex. In this sense, mice invalidated for Cul3 die at day 7.5 post-coïtum with aberrations in several cyclin levels, which control cell cycle checkpoint [30]. This lethality could be explained

by the potential involvement of Cul3 complex in degradation of a multiplicity of substrates [31]. More precisely, Cul3 disruption could involve Topo1 level aberrations leading to cell cycle perturbations. Because of the multiple functions of Topo1, it is difficult at this point to link or not the roles of Topo1 in cellular differentiation and cell cycle.

In conclusion, we clearly describe a primordial regulation of BTBD1 expression in myogenic differentiation, which could be related to its high expression in this tissue. Indeed, alteration of normal BTBD1 expression impairs myogenesis. Moreover, our results indicate a potential key function of BTBD1 in mesenchymal differentiation, regarding adipogenesis/myogenesis balance. The current and previous findings suggest that the dual and opposite involvement of BTBD1 in this two different differentiation pathways could be explained by its capacity to regulate Topo1 content and/or activity. We propose that BTBD1 is important for Topo1 regulation through its ubiquitin/proteasome pathway degradation during mesenchymal differentiation.

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## **FIGURE LEGENDS**

**Figure 1.** Analysis of  $\Delta$ -BTBD1 C2C12 cells pluripotency potential. Control (A-F) and  $\Delta$ -BTBD1 (G-L) C2C12 cells were grown either in DM for myogenesis or in GM containing 5  $\mu$ M rosiglitazone to study adipogenesis, or containing 0.1  $\mu$ M dexamethasone, 10 mM  $\beta$ -glycerophosphate and 50  $\mu$ g/mL ascorbic acid 2-phosphate to study osteogenesis. Morphological analysis was performed after 4 days of treatment for myogenesis (A, B, G, H), 7 days for adipogenesis (C, D, I, J) and 14 days for osteogenesis (E, F, K, L). Adipogenesis was confirmed by Oil red O staining and osteogenesis by Alizarin red staining. These dyes are specific of lipid droplets containing neutral lipids and calcification, respectively (arrows). Adipogenesis variations between control and  $\Delta$ -BTBD1 C2C12 cells were checked on aP2 mRNA expression kinetics by quantitative RT-PCR from day 0 to day 8 after differentiation induction (M). Osteogenesis was analyzed at day 14 by mineralization area quantification (N). Scale bar = 20  $\mu$ m.

**Figure 2.** Analysis of BTBD1 mRNA expression during *ex vivo* myogenesis of C2C12 cells. Quantitative RT-PCR analysis were performed with total RNAs extracted from WT C2C12 cells (A) or DN MKK3 C2C12 cells (B) at days 0, 1, 2, 3, 4 and 5 in myogenic differentiation medium. Myogenin was used as myogenesis control. Histograms represent  $\Delta$ Ct mean  $\pm$  sem (normalized by 36B4 expression) of three PCR assays for two independent kinetics.

**Figure 3.** Analysis of BTBD1 mRNA expression level during *ex vivo* adipogenesis of 3T3-L1 cells and adipogenesis of hMADS cells. Total RNAs were extracted (A) from 3T3-L1 cells at days 0, 2, 6 and 9 in adipogenic differentiation medium and (B) from hMADS cells at days 0, 2, 8, 15 and 24 after osteogenic stimulation. BTBD1, aP2 (adipogenesis control) and alkaline

phosphatase (osteogenesis control) mRNA expressions were studied by quantitative RT-PCR. Histograms represent  $\Delta\text{Ct}$  mean  $\pm$  sem (normalized by 36B4 expression for 3T3-L1 cells and TBP expression for hMADS cells) of three PCR assays for two independent kinetics.

**Figure 4.** Analysis of Topo1 expression level along myogenesis and adipogenesis. Total proteins and RNAs from (A) C2C12 cells in myogenic medium and (B) 3T3-L1 cells in adipogenic medium were extracted at indicated days after differentiation inductions. Topo1 protein level were analyzed by Western blots and Topo1 mRNA levels by quantitative RT-PCR. Myogenesis was checked with myogenin mRNA expression presented in Fig.2 and adipogenesis with aP2 protein expression. For protein level analysis, bands were quantified and standardized with  $\beta$ -actin protein amounts. Histograms represent mean  $\pm$  sem of three independent Western blot experiments. For quantitative RT-PCR, histograms represented  $\Delta\text{Ct}$  mean  $\pm$  sem (normalized by 36B4 expression) of three PCR assays for two independent kinetics.

**Figure 5.** Microarrays analysis of genes expression alteration during  $\Delta$ -BTBD1 C2C12 cells myogenic differentiation. All differential gene expressions are represented as normalized hybridization signal ratios with a commune reference and with a log<sub>2</sub> scale. The commune reference is the normalized hybridization signal obtained at day 0 for WT C2C12 cells. Myogenic differentiation was induced at day 0. For each gene, the slide grey bars represent ratios at day -2, day 0 and day +1 for WT C2C12 cells and the dark grey bars represent respectively the same ratio for  $\Delta$ -BTBD1 C2C12 cells. Abbreviated gene names are (i) for muscle markers: myosin heavy polypeptide 3 (MYH3) and 7 (MYH7), phosphofructokinase (PFKM), myocyte enhancer factor 2C (MEF2C) and 2D (MEF2D), ATPase, Ca<sup>++</sup> transporting, slow twitch 2 (ATP2A2),



tropomyosin 1 (TPM1) and 2 (TPM2), troponin C (TNNC1) and T3 (TNNT3); (ii) for extracellular matrix: chondroitin sulfate proteoglycan 2 (Cspg2), epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (Efemp1); (iii) for ubiquitin/proteasome pathway: ubiquitin carboxyl-terminal esterase L3 (Uchl3), seven in absentia 2 (Siah2).

Figure 1.

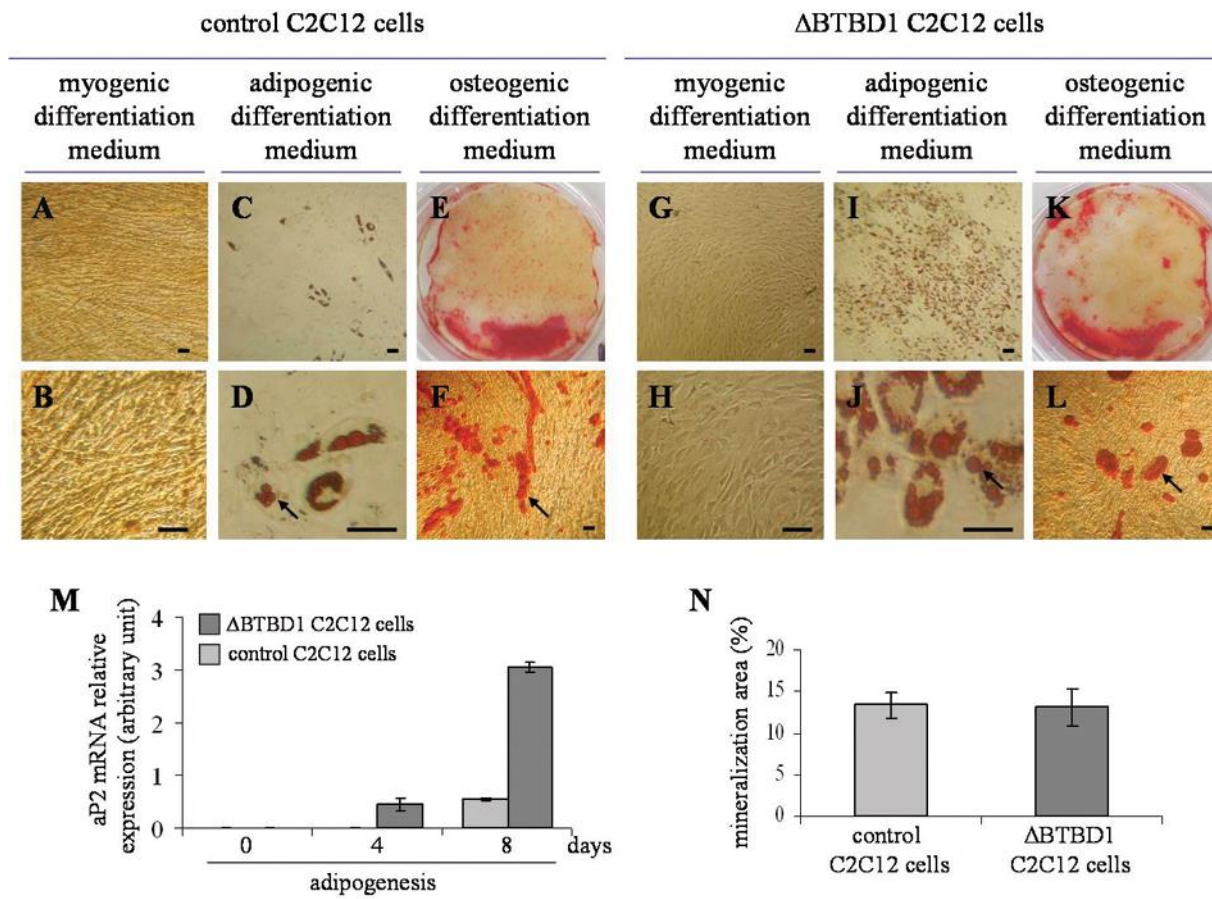
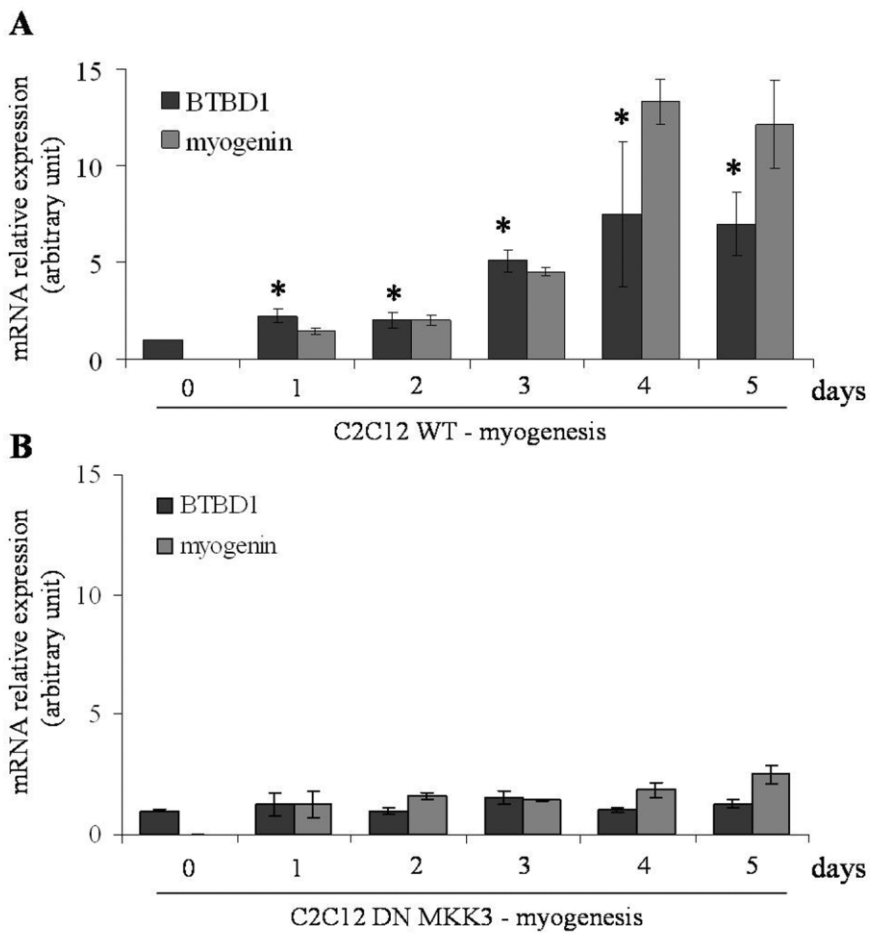


Figure 2.



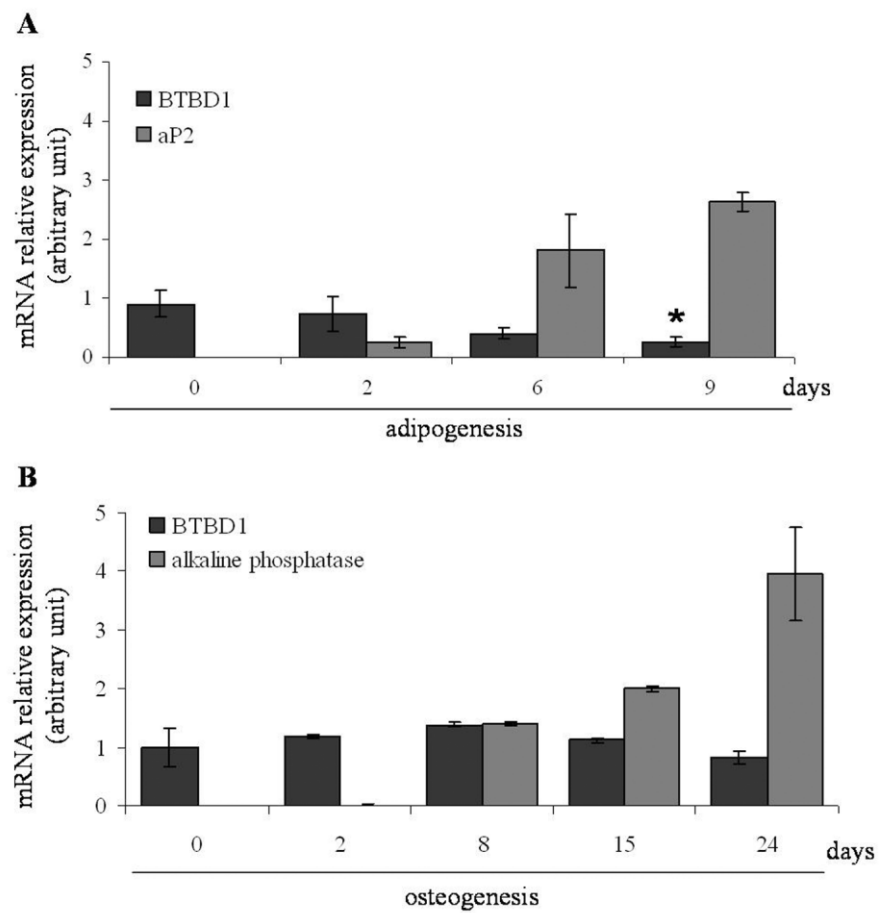
**Figure 3.**

Figure 4.

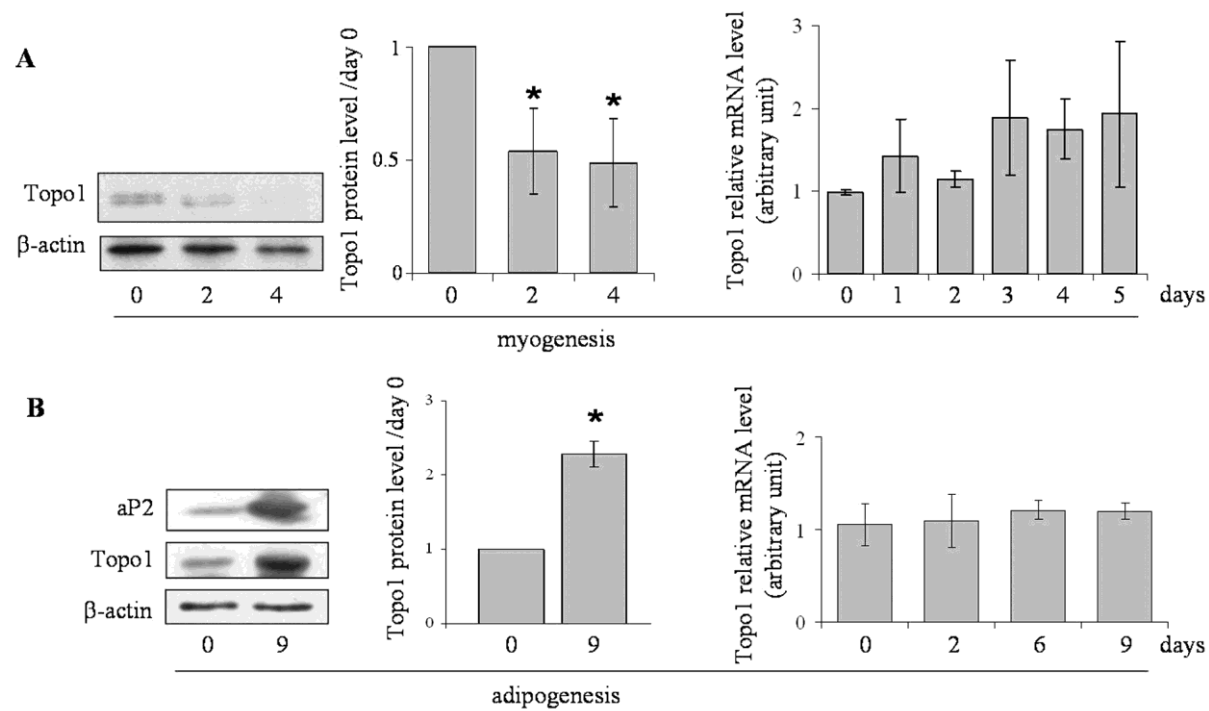


Figure 5.

