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Downregulation of Protein tyrosine phosphatase

PTP-BL represses adipogenesis

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Abstract

The insulin / insulin-like growth factor 1 (IGF-1) signaling pathway is a major regulator of adipose tissue growth and differentiation. We recently demonstrated that human protein tyrosine phosphatase (PTP) L1, a large cytoplasmic phosphatase also known as PTP-BAS/PTPN13/PTP-1E, is a negative regulator of IGF-1R/IRS-1/Akt pathway in breast cancer cells. This triggered us to investigate the potential role of PTP1L in adipogenesis. To evaluate the implication of PTP-BL, the mouse orthologue of PTP1L, in adipose tissue biology, we analyzed PTP-BL mRNA expression in adipose tissue in vivo and during proliferation and differentiation of 3T3-L1 pre-adipocytes. To elucidate the role of PTP-BL and of its catalytic activity during adipogenesis we use siRNA techniques in 3T3-L1 pre-adipocytes, and Mouse embryonic fibroblasts that lack wildtype PTP-BL and instead express a variant without the PTP domain (ΔP/ΔP MEFs). Here we show that PTP-BL is strongly expressed in white adipose tissue and that PTP-BL transcript and protein levels increase during proliferation and differentiation of 3T3-L1 pre-adipocytes. Strikingly, knockdown of PTP-BL expression in 3T3-L1 adipocytes caused a dramatic decrease in adipogenic gene expression levels (PPARγ, aP2) and lipid accumulation but did not interfere with the insulin/Akt pathway. ΔP/ΔP MEFs differentiate into the adipogenic lineage as efficiently as wildtype MEFs. However, when expression of either PTP-BL or PTP-BLΔP was inhibited a dramatic reduction in the number of MEF-derived adipocytes was observed. These findings demonstrate a key role for PTP-BL in 3T3-L1 and MEF-derived adipocyte differentiation that is independent of its enzymatic activity.

Key words: Protein Tyrosine Phosphatase; adipocyte differentiation; PTP-BL
Introduction

Adipose tissue plays critical roles in the regulation of energy homeostasis; as a reservoir, by storing and releasing fuel, and as an endocrine organ, by secreting a number of hormones and cytokines (Spiegelman and Flier, 2001). Excess body fat, or obesity, is a major public health problem, particularly in industrialized countries, increasing the risk of diabetes, cardiovascular diseases and several types of cancers (Aviva Must, 1999). Conversely, lipoatrophy, the lack of adipose tissue, is also associated with diabetes and a number of other metabolic abnormalities (Spiegelman and Flier, 2001). Hence, understanding the signaling pathways that govern adipocyte differentiation is necessary to develop comprehensive therapeutic strategies for the prevention and treatment of these disorders.

Adipogenesis involves the formation of pre-adipocytes from mesenchymal progenitor cells and their differentiation into adipocytes (Rosen and Spiegelman, 2000). The cellular and molecular mechanisms of adipocyte differentiation have been extensively studied using pre-adipocyte culture systems, such as 3T3-L1 and 3T3-F442A cell lines (Rosen and Spiegelman, 2000) (Li et al., 2007). Differentiation of these cells occurs in two discrete steps. The cells first undergo several rounds of mitosis, known as clonal expansion, after which they become quiescent again and gradually acquire biochemical and morphological characteristics of mature adipocytes (Hwang et al., 1997) (Rosen and Spiegelman, 2000). Both steps are characterized by marked changes in the pattern of gene expression that are achieved by the sequential induction of transcription factors. Exposure of pre-adipocytes to appropriate hormonal inducers of adipogenesis thus results in early and transient expression of the β and γ isoforms of the CCAAT/enhancer-binding protein (C/EBP) (Evans et al., 2004), which contribute to the proliferation of these cells as well as to the subsequent increase in the expression of C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ) (Lane et al., 1999). The latter two proteins then mediate the activation of a variety of adipocyte-specific genes that lead to further differentiation (Hwang et al., 1997) (Lane et al., 1999).

Adipocyte differentiation is tightly regulated by insulin and IGF-1 signaling (Tseng et al., 2005). Insulin is a major hormone controlling critical energy functions, such as glucose and lipid metabolism. IGF-1 has been suggested to be a major regulator of adipose tissue growth and differentiation of pre-adipocytes into adipocytes. Both insulin and IGF-1 mediate adipocyte differentiation through their binding to the IGF-1 receptor (IGF-1R) (Tseng et al., 2005), which results in the induction of its
intrinsic tyrosine kinase activity and recruitment and phosphorylation of multiple proteins including
the insulin receptor substrates (IRSs). These enable the formation of macromolecular complexes close
to the receptor that elicit two main signaling cascades; the phosphatidylinositol 3-kinase (PI3K)
pathway and the MAPK pathway (Sanchez-Margalet et al., 1994). The MAPK pathway is considered
to be involved in proliferation and differentiation, whereas the PI3K pathway plays a major role in
metabolic functions, mainly via the activation of the Akt cascade. Activation of Akt stimulates glucose
uptake, glycogen synthesis, protein synthesis and cell survival, and inhibits lipolysis. This pathway is
also considered to be important for adipogenesis (Fasshauer et al., 2001; Sakaue et al., 1998; Xia and
Serrero, 1999; Yoshiga et al., 2007).

We recently demonstrated, in human breast cancer cells, that the Protein Tyrosine Phosphatase (PTP)
PTPL1 (PTPN13/PTP-BAS/PTP-1E) inhibits the IGF1-R/IRS-1/Akt pathway through
dephosphorylation of IRS-1 (Dromard et al., 2007). This suggests a potential role for PTPL1 in
adipogenesis. We here demonstrate that PTP-BL, the mouse ortholog of PTPL1, is strongly expressed
in white adipose tissue and that PTP-BL expression levels are increased during proliferation and
differentiation of mouse 3T3-L1 pre-adipocytes. Depletion of PTP-BL expression by RNAi profoundly
suppressed adipocyte differentiation of 3T3-L1 and mouse embryonic fibroblast (MEF) cells. Because
MEFs that lacked wildtype PTP-BL and instead expressed a truncated protein without the phosphatase
domain (PTP-BLΔP) differentiate into the adipogenic lineage as efficiently as wildtype MEFs, we
conclude that PTP-BL represents a novel adipogenic factor that is required for adipocyte
differentiation but that this is independent of its catalytic activity.
**Materials and methods**

**Materials.**

All chemicals, except if stated otherwise, were purchased from Sigma (St. Louis, Missouri). Pioglitazone was a kind gift of Takeda Pharmaceuticals (Osaka, Japan). Oligofectamine reagent was purchased from Invitrogen, and Interferin from Polyplus. Anti-Akt and anti-Akt phospho-Ser473 antibodies were from Cell Signaling Technology. The antibodies against α-tubulin and β-actin were purchased from Sigma. Anti-PTPL1 antibody (H300) was from Santa Cruz Biotechnology. Purified porcine insulin was a gift from Eli Lilly (Indianapolis, Indiana).

**RNA isolation; quantitative real-time PCR.**

RNA isolation was carried out using Trizol reagent according to the manufacturer instruction. Reverse transcription of total RNA was performed at 37°C using the M-MLV reverse transcriptase (Invitrogen SARL, France) and random hexanucleotide primers, followed by a 5 min inactivation at 95°C. Quantitative real-time PCR was carried out using a LightCycler and the DNA double-strand-specific SYBR Green I dye for detection (Roche, Basel, Switzerland). Results were normalized to RS9 mRNA levels.

**Cell culture and transient transfection.**

MEFs were isolated from PTP-BLΔP/ΔP (Wansink et al., 2004) or wild type mice and retrovirally transduced with a TBX2 expression construct to bypass senescence (Jacobs et al., 2000). MEFs and 3T3-L1 cells were grown in DMEM, 10% fetal bovine serum (FBS). 2 days after reaching confluence (day 0), cells were placed in differentiation media consisting of DMEM, 10% serum, 0.5 mM 3-Isobutyl-1methylxanthine (IBMX), 10 μg/ml insulin, 1 μM dexamethasone, and 100nM pioglitazone for 2 days. From day 3 onwards, cells were incubated with DMEM, 10% serum, 10 μg/ml insulin, and 100 nM pioglitazone. Medium was renewed every 2 days until the end of the experiment. SiRNA transfections were carried out using the Oligofectamine reagent or Interferin methods according to the manufacturer's instructions. The PTP-BL-specific siRNA 1 (5’-G G A C C G A A U U C G A G A G A G A G A G A G A G A U U U-3’), the PTPL1-specific siRNA 2 (5’-C A A A G A C G A U U C C A C U C U A C U U-
3’), and the control non-targeting siRNA from Dharmacon were used at a final concentration of 0.1µM and 2.5nM for Oligofectamine and Interferin methods, respectively.

**Immunoblotting analysis.**

Equal amounts of lysate were separated on an SDS/polyacrylamide gel and electrotransferred onto PVDF membrane. Blots were stained with Coomassie blue (R250, Sigma) to ensure that protein amounts were comparable. Membranes were blocked with TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk and immunoblotted overnight at 4°C with the indicated primary antibodies. Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG, along with the Western Lighting ECL detection kit (PerkinElmer Life Sciences).

Before reprobing with different primary antibodies, blots were stripped by incubation in 50 mM Tris pH 7.5, 150 mM NaCl, 2% SDS and 100 mM β-mercaptoethanol, for 30 min at 50 °C.

**Oil Red-O staining.**

Detection of neutral lipids with Oil Red-O was performed as described previously (Ramirez-Zacarias et al., 1992). Briefly, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 30 min, and then stained for 2 h at room temperature with Oil Red-O solution (0.5% Oil Red-O in isopropanol). The cells were washed twice with PBS and visualized with an inverted microscope.

**BrdU incorporation.**

Cells grown on coverslips were incubated 4 hr in the presence of BrdU (100 µM), and subsequently treated with 1.5N HCl for 10 min at 21°C. After fixation and permeabilization with 100% methanol, cells were incubated with antibodies directed against BrdU (Dako A/S, Glostrup, Denmark). Preparations were then incubated with an FITC-conjugated anti-mouse IgG.

**Statistical analysis.**

Results are presented as mean +/- SD and the number of experiments is indicated in the figure legends. In each experiment, all determinations were performed at least in triplicate. Statistical significance was assessed using student’s t-test.
Results

Expression pattern of PTP-BL during adipogenesis.

To determine the relative expression of PTP-BL mRNA in vivo, we performed quantitative real-time PCR, comparing PTP-BL mRNA expression in mouse white adipose tissue with different adult mouse tissues. As previously described (Hendriks et al., 1995; Thomas et al., 1998; Wansink et al., 2004), PTP-BL gene (Ptpn13) is expressed in many epithelia and in brain (Fig. 1A). Interestingly, PTP-BL is strongly expressed in white adipose tissue from visceral and epididymal depots as compared to other tissues (Fig. 1A). Mouse 3T3-L1 pre-adipocytes are a well-characterized in vitro model of adipocyte differentiation that can reliably differentiate into mature fat cells upon exposure to differentiation media (Kehinde, 1974) (Green and Meuth, 1974). We analyzed PTP-BL mRNA and protein expression levels during proliferation of 3T3-L1 pre-adipocytes and their differentiation into mature adipocytes (Fig. 1B). Interestingly, PTP-BL expression was increased during pre-adipocyte proliferation and differentiation and peaked at confluency (day 0). This elevated expression was also observed during the differentiation process. Differentiation of the cells was verified by measuring the mRNA expression of the adipogenic markers PPARγ and ap2 (Fig. 1C-D). Altogether, these results are in line with a role for PTP-BL in adipose tissue biology and/or differentiation.

PTP-BL knockdown inhibits 3T3-L1 pre-adipocyte differentiation.

To elucidate the role of PTP-BL during adipogenesis, we silenced PTP-BL expression using siRNA techniques. 2 days before optimal confluence, 3T3-L1 cells were transfected with two different siRNA oligonucleotides complementary to the mouse PTP-BL transcript or with an irrelevant siRNA and were compared for their ability to differentiate into adipocytes. Specific siRNA transfection resulted in a 50% inhibition of PTP-BL transcription and expression, quantified by RT-PCR (Fig.2A) and immunoblot analysis (Fig. 2B), respectively, corresponding at confluency in an expression level equal to this of proliferating pre-adipocyte. After 6 days in differentiation medium, normal lipid accumulation was observed in control cells, whereas a dramatic decrease in lipid accumulation was observed in PTP-BL knockdown cells as assessed by Oil Red O staining (Fig. 2C). By quantitative RT/PCR we detected a reduced PPARγ gene induction after 3 days (30%) or 6 days (50%) of differentiation in PTP-BL knockdown cells as compared to control cells (Fig. 2 D-E). We also
observed a dramatically reduced induction (80%) of the adipogenic gene marker \textit{aP2} at these time points following PTP-BL knockdown (Fig. 2F-G), in line with an important role for PTP-BL in adipogenesis. We next monitored the transcript levels of the second master regulator gene, C/EBP\textit{α}, and two upstream regulator genes, C/EBP\textit{β} and \textit{δ}. We detected reduced C/EBP\textit{α} gene induction after 4 days (66%) or 6 days (64%) of differentiation in PTP-BL knockdown cells as compared to control cells (Fig. 2 H-I). However, C/EBP\textit{β} and \textit{δ} transcript levels, and that of the clonal expansion associated gene c-myc, were identical in control and PTP-BL knockdown cells after 3 hours of differentiation (Fig. 2 H-I). Together, these data suggest that PTP-BL expression affects PPAR\textit{γ} and C/EBP\textit{α} regulation but not through regulation of C/EBP\textit{β} or \textit{δ} expression.

**Inhibition of PTP-BL impairs 3T3-L1 pre-adipocyte differentiation in the early phases without interfering with clonal expansion phase.**

Having verified that PTP-BL knock-down did not affect 3T3-L1 pre-adipocyte cell growth arrest at confluency (data not shown), we next investigated whether PTP-BL exerts its effect at the proliferative, clonal expansion phase (days 1 and 2) or rather at the differentiation stage (days 3-6) following hormonal induction of 3T3-L1 pre-adipocytes. To this end, we silenced PTP-BL expression using siRNA transfection two days before hormonal stimulation and performed BrdU incorporation studies. Consistent with our findings on the expression profiles of c-myc and the early adipogenic regulators C/EBP\textit{β} and \textit{δ}, PTP-BL-silenced 3T3-L1 cells proliferated as well as control-siRNA cells (Fig. 3A), indicating that PTP-BL rather participates in stages of the 3T3-L1 differentiation process that are beyond the clonal expansion phase.

We next silenced PTP-BL gene expression at day 2 or 4 after induction of differentiation, (Fig. 3B-F), which is coincident with the completion of clonal expansion. In both cases, siRNA transfection resulted in a 50 % inhibition of PTP-BL transcription as quantified by RT-PCR (Fig.3B-F). Adipocyte differentiation was also indeed inhibited upon PTP-BL knockdown 2 days after hormonal stimulation as assessed by Oil Red O staining (Fig. 3C) and \textit{aP2} expression levels (Fig. 3D-E). Surprisingly, no effect was observed on the differentiation process when PTP-BL gene expression was silenced 4 days after hormonal induction (Fig. 3G-H) suggesting that PTP-BL levels are not critical for 3T3-L1 pre-adipocytes that have already started the differentiation process. Together, these data reveal that PTP-
BL is important for the early steps of adipocyte differentiation, but not proliferation program. The up-regulation of PTP-BL gene expression that occurs during the proliferation and differentiation of 3T3-L1 pre-adipocytes thus appears to be essential for the initiation of the differentiation process.

**PTP-BL regulates adipogenesis independently of its phosphatase activity.**

Insulin/IGF-1/IRS pathway has an important role in the regulation of genes that are involved in multiple early adipogenic events in brown pre-adipocytes (Tseng et al., 2005). Activation of Akt is considered to be important for adipogenesis (Fasshauer et al., 2001; Sakaue et al., 1998; Xia and Serrero, 1999; Xu and Liao, 2004; Yoshiga et al., 2007). We have recently shown that PTPL1 expression was sufficient to modulate Akt activation in the IGF1-R/IRS-1/Akt pathway through IRS-1 dephosphorylation in breast cancer cells (Dromard et al., 2007). PTP-BL gene expression was silenced before the clonal expansion and we tested whether PTP-BL’s critical contribution to the early phase of 3T3-L1 differentiation into adipocytes involves IRS-1/Akt pathway deactivation. In confluent 3T3-L1 pre-adipocytes (day 0), the level of IRS-1 tyrosine phosphorylation in response to insulin is very low compared to mature adipocyte and, in contrast to our findings in human breast cancer cells, PTP-BL knock-down has no effect on IRS-1 activation (data not shown) or insulin-induced Akt phosphorylation in 3T3-L1 cells (Fig. 4A).

Finally, we wanted to assess the participation of PTP-BL’s tyrosine phosphatase activity in adipocyte differentiation. Poor transfection efficiency in 3T3-L1 pre-adipocytes prevented experiments using overexpression of PTP-BL phosphatase-dead mutant. We therefore turned to mouse embryonic fibroblast (MEF) cells that were derived from mice that lack the PTP-BL phosphatase moiety (PTP-BLΔP/ΔP mice) (Wansink et al., 2004). The capacity of ΔP/ΔP MEFs, that express an enzymatically inactive truncated version of the protein (PTP-BLΔP), to differentiate into adipocytes in vitro in response to hormone stimulation was compared to that of wild-type (wt) MEFs. Adipocytes were scored using Oil Red O staining to detect lipid droplets and mRNA expression of adipogenic markers was quantified. Hormonally stimulated ΔP/ΔP MEFs differentiated into adipocytes in vitro as efficiently as wt MEFs (Fig. 4B-C), indicating that PTP-BL catalytic activity is not required for adipocyte differentiation of MEFs. However, like in 3T3-L1 cells, PTP-BL is required for MEF
adipocyte differentiation since PTP-BL inhibition by siRNA technique in wt (data not shown) as well as ΔP/ΔP MEFs caused a dramatic decrease in lipid droplets accumulation as assessed by Oil Red O staining (Fig. 4E). Gene expression analysis of the adipose tissue-specific genes $PPAR_\gamma$ and $aP2$ (Fig. 4F-G) further corroborated this finding. Taken together, these data imply that not the catalytic activity but PTP-BL’s anchoring and scaffolding functions are required for adipocyte differentiation.
Discussion

Time-dependent modulation of intracellular signaling molecules and sequential induction of transcriptional regulators that generate marked changes in gene expression are essential for the differentiation of pre-adipocytes into adipocytes (Rangwala and Lazar, 2000) (Koutnikova and Auwerx, 2001). The transcriptional regulation of adipogenesis is relatively well characterized, with PPARγ and C/EBPα as two master regulators that control adipogenic genes (Nedergaard et al., 2005). However, the factors that influence pre-adipocyte determination remain poorly understood. Here we demonstrate that the mouse protein tyrosine phosphatase PTP-BL is strongly expressed in white adipose tissue and its expression levels are upregulated during proliferation and differentiation of 3T3-L1 pre-adipocytes, suggesting a role for PTP-BL in adipose tissue biology. Indeed, we found that PTP-BL plays a key role in 3T3-L1 adipocyte differentiation. We show that blocking the up-regulation of PTP-BL expression in the early phases of adipogenesis was associated with a dramatic decrease in adipogenic gene expression and lipid accumulation in 3T3-L1 adipocytes.

A number of diverse functions have been described for PTP-BL, given the varied proteins that it has been shown to interact with. For example, PTP-BL has been suggested to act as a scaffolding protein in the regulation of the cytoskeleton (Erdmann, 2003), to dephosphorylate EphrinB and thus regulate various developmental processes (Palmer et al., 2002), and to be involved in the regulation of cytokinesis (Herrmann et al., 2003). Reports based on the characterization of mice that lack PTP-BL phosphatase activity revealed a phenotype of impairment in motor nerve repair (Wansink et al., 2004) and the involvement of PTP-BL in both retinal ganglion cell neurite initiation and survival of activated retinal glia (Lorber et al., 2005). Recently, a published study on mutant mice that are completely devoid of PTP-BL did not report phenotypic consequences or an effect on adipogenesis and solely focalized on haematopoietic cell lineages (Nakahira et al., 2007) which were previously shown to express the phosphatase (Gjorloff-Wingren et al., 2000). For the human homologue of PTP-BL, PTPL1, a role in apoptosis has been proposed, but this is still a matter of debate and depending on Fas pathway effectiveness and PTPL1 expression level (Sato et al., 1995) (Abaan et al., 2005; Bompard et al., 2002; Cuppen et al., 2000; Miyazaki et al., 2006; Toretsky et al., 1997; Wieckowski et al., 2007).
Our own studies have shown that PTPL1 is necessary (Bompard et al., 2002) and sufficient (Dromard et al., 2007) for the early inhibition of the IRS-1/PI3K/Akt pathway through IRS-1 dephosphorylation.

The insulin/IGF-1/IRS signaling pathway has an important role in the regulation of genes that are involved in multiple early adipogenic events in brown pre-adipocytes (Tseng et al., 2005). Activation of Akt is considered to be important for adipogenesis (Fasshauer et al., 2001; Sakaue et al., 1998; Xia and Serrero, 1999; Xu and Liao, 2004; Yoshiga et al., 2007). The Akt signal cascade appears to induce or activate PPARγ and C/EBP during the induction of 3T3-L1 adipocyte differentiation (Kim and Chen, 2004; Kortum et al., 2005; Lazar, 2005; Nedergaard et al., 2005). Recently, PPARγ2 expression was shown to be negatively regulated by Foxo-1, through binding to the PPARγ promoter and inhibition of transcription. (Armoni et al., 2006). Insulin induces phosphorylation and nuclear export of Foxo-1 through Akt. Therefore, the IR/IGF-1R-IRS-PI3-kinase-Akt pathway promotes PPARγ expression. Surprisingly, we found that PTP-BL knock-down had no apparent effect on IRS-1 tyrosine phosphorylation and Akt activation in 3T3-L1 pre-adipocytes. This may be indicative for compensatory actions from other PTPs present in these cells but may also point to a regulatory mechanism controlling PTP-BL activity as encountered during oocyte meiotic maturation (Nedachi and Conti, 2004). Still, PTP-BL was found to be required during the early phase of adipocyte differentiation that directly follows the clonal expansion phase. Interestingly, we could demonstrate that PTP-BL phosphatase activity is not required for this effect on adipocyte differentiation, in line with the absence of an effect of PTP-BL on the IRS-1/Akt pathway. Indeed, MEFs (C57BL/6) expressing a PTP-BL protein lacking its PTP domain differentiated into the adipogenic lineage as efficiently as wild type cells. This is in accordance with the fact that PTP-BLΔP/ΔP mice, that lack the PTP-BL catalytic domain, do not display any abnormality in adipose tissue development when bred onto a C57BL/6 genetic background (Wansink et al., 2004). In that light, it will be interesting to learn whether any adipose tissue abnormalities become apparent in the full PTP-BL knock-out mice that were recently generated (Nakahira et al., 2007).

PTP-BL and PTPL1 represent the largest mammalian intracellular PTP in mice and human, respectively, and possess at least seven potential protein-protein interaction domains; a kinase noncatalytic C-lobe (KIND) domain, a Four-point-one-Ezrin-Radixin-Moesin (FERM) domain, and
five PSD-95-\textit{Drosophila} discs large-Zonula occludens (PDZ) domains. Many potential interacting proteins have been identified (Erdmann, 2003). The contribution of PTP-BL to the adipocyte differentiation process seems to be reflected by its potency to orchestrate the composition and dynamics of large protein machines through these many protein interaction modules rather than by its enzymatic activity. The precise molecular mechanism by which PTP-BL participates in adipogenesis, however, remains to be uncovered.

Elucidation of PTP-BL’s working mechanism as an adipogenic factor might result in new therapeutic strategies for the treatment of obesity that will involve the abrogation of PTP-BL protein complexes rather than inhibition of its enzymatic activity.

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Figure legends

Figure 1: Expression pattern of PTP-BL during adipogenesis. (A) Relative expression of PTP-BL mRNA as determined by quantitative real-time PCR in different mouse tissues. BAT, brown adipose tissue; WAT, white adipose tissue from epididymal fat pad. Visceral, but not subcutaneous, WAT also displays high PTP-BL expression levels (data not shown). Results were normalized to the expression of RS9 mRNA and are expressed as the mean +/- SD from four independent experiments. (B) Quantitative real-time PCR showing PTP-BL gene expression during proliferation of 3T3-L1 cells and at the indicated days of adipocyte differentiation. Results are expressed as a mean from two independent experiments. Insert: Western blot, representative of two independent experiments, showing the increased expression of PTP-BL protein (upper panel) during 3T3L1 proliferation and differentiation. Immunodetection of β-actin served as loading control (lower panel). (C-D) Analysis of the expression of adipocyte markers αP2 and PPARγ just before (d0) and 6 days after initiation of 3T3-L1 adipocytes. Results are representative of two independent experiments.

Figure 2: PTP-BL knockdown inhibits 3T3-L1 adipogenesis. Cells were transfected with siRNA (control or PTP-BL specific) two days before hormonal stimulation. (A) Quantitative real-time PCR showing PTP-BL gene expression. (B) Western blot showing knockdown of PTP-BL expression. (C) Micrographs of Oil Red O staining of PTP-BL-silenced 3T3-L1 cells (PTP-BL-siRNA1 and 2) and control (control-siRNA) after 6 days of differentiation. (D-G) Quantitative real-time PCR showing expression of the adipogenic markers PPARγ (D-E) and αP2 (F-G), in knockdown versus control differentiation experiments, at the indicated days of 3T3-L1 adipocyte differentiation. Results were normalized to the expression of RS9 mRNA and are expressed as a mean +/- SD from four independent experiments. *P<0.001; ** P<0.0001. (H-I) Quantitative real-time PCR showing expression of the adipogenic marker C/EBPα, the upstream regulator genes C/EBPβ and C/EBP δ and the clonal expansion associated gene c-myc, in PTP-BL-siRNA- versus control-siRNA-treated 3T3-L1 adipocytes, at the indicated time of differentiation. Results were normalized to RS9 mRNA levels and are expressed as a mean +/- SD from four (C/EBPα) or two (C/EBPβ and δ, c-myc) independent experiments. *P<0.04.
**Figure 3:** Inhibition of PTP-BL impairs 3T3-L1 pre-adipocyte early phase differentiation but not clonal expansion. (A) 3T3-L1 cells were transfected with siRNA (control or PTP-BL-specific) two days before stimulation and BrdU incorporation was quantified at the indicated days of differentiation by immunofluorescence. Results are the mean of at least 500 cells in two independent experiments. (B-E) Cells were transfected with siRNA (control or PTP-BL-specific) two days after stimulation. (B) Knockdown of PTP-BL gene expression in 3T3-L1 cells transfected with siRNA was assessed by quantitative real-time PCR. (C) Micrographs of Oil Red O staining of PTP-BL-silenced 3T3-L1 cells (PTP-BL-siRNA) and control (control-siRNA) after 6 days of differentiation. (D-E) Quantitative real-time PCR showing gene expression of the adipogenic markers aP2 in knockdown versus control cells at the indicated days of 3T3-L1 adipocyte differentiation. Results were normalized to the expression of RS9 mRNA and are expressed as the mean +/- SD from four independent experiments. *P<0.001. (F-H) pre-adipocytes were transfected with siRNA (control or PTP-BL-specific) four days after stimulation. (G) Micrographs of Oil Red O staining of PTP-BL-silenced 3T3-L1 cells (PTP-BL-siRNA) and control (control-siRNA) after 6 days of differentiation. (H) Quantitative real-time PCR showing aP2 gene expression levels in knockdown versus control cells after 6 days of differentiation. Results are expressed as the mean +/- SD from three independent experiments. *P<0.001.

**Figure 4:** PTP-BL regulation of adipogenesis does not require its phosphatase activity. (A) 3T3-L1 cells were serum-starved for 8h and were stimulated (+) or not (-) for 10 min with insulin at the indicated days of 3T3-L1 cells differentiation. Equal amounts of cell lysates were resolved by two separate 10 % SDS-PAGE gels. One membrane was probed with anti-phosphorylated Akt (pAkt; upper panels) antibody and the other with anti-Akt antibody (middle panels) before reprobing with anti-β-actin antibody (lower panels). (B) Micrographs of Oil Red O staining of ΔP/ΔP MEFs or wt MEFs 10 days after differentiation. (C) Quantitative real-time PCR showing gene expression of the adipogenic markers aP2 and PPARγ in ΔP/ΔP MEFs or wt MEFs after 10 days of differentiation. (D-E) PTP-BLΔP expression and lipid storage in ΔP/ΔP MEFs cells transfected with a siRNA sequence directed against mouse PTP-BL two days before optimal confluence. (D) Cells were lysed at confluency and PTP-BLΔP expression was evaluated by Western blot. (E) Micrographs of Oil Red O
staining of PTP-BL-silenced ΔP/ΔP MEFs (PTP-BL-siRNA) and control (control-siRNA) after 10 days of differentiation. (F-G) Quantitative real-time PCR showing gene expression of the adipogenic markers \(PPAR_\gamma\) (F) and \(aP2\) (G) in knockdown versus control ΔP/ΔP MEFs following 10 days of differentiation. Results were normalized to the expression of RS9 mRNA and are expressed as the mean +/- SD from three independent experiments. *\(P<0.001\); ** \(P<0.0001\).