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Akt- and Foxo1-interacting WD-repeat-FYVE protein promotes adipogenesis

Thorsten Fritzius and Karin Moelling*

Institute of Medical Virology, University of Zurich, Zurich, Switzerland

We have previously identified a protein, consisting of seven WD-repeats, forming a putative β-propeller, and an FYVE domain, ProF, which is highly expressed in 3T3-L1 cells, a cell line that can be differentiated into adipocytes. We recently found ProF to interact with the kinases Akt and protein kinase Cζ. Here we demonstrate that ProF is a positive regulator of adipogenesis. Knockdown of ProF by RNA interference leads to decreased adipocyte differentiation. This is shown by reduced lipid accumulation, decreased expression of the differentiation markers PPARγ and C/EBPα, and reduced glucose uptake in differentiated cells. Furthermore, ProF overexpression leads to increased adipogenesis. ProF binds to the transcription factor Foxo1 (Forkhead box O1), a negative regulator of insulin action and adipogenesis, and facilitates the phosphorylation and thus inactivation of Foxo1 by Akt. Additionally, dominant-negative Foxo1 restores adipogenesis in ProF knockdown cells. Thus, ProF modulates Foxo1 phosphorylation by Akt, promoting adipocyte differentiation. Furthermore, ProF might be involved in metabolic disorders such as diabetes.

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Introduction

We recently described the WD-repeat propeller-FYVE protein (ProF) as a binding partner of the serine/threonine protein kinases Akt and protein kinase Cζ/λ (PKCζ/λ) (Fritzius *et al*, 2006). ProF was found to be strongly expressed in 3T3-L1 cells, a well-established cell line for the study of adipocyte differentiation or adipogenesis (Green and Kehinde, 1975).

Adipocyte differentiation is controlled by complex signal transduction events. The importance of Akt for adipogenesis is demonstrated by various knockout mice studies (Peng *et al*, 2003; Baudry *et al*, 2006). Numerous targets of the Akt kinase have been identified. One of them is the family of Forkhead box O (Foxo) transcription factors (summarized in Greer and Brunet, 2005). The Foxo isoform Foxo1 has been shown to be highly expressed in adipose tissue and is a negative regulator of insulin action and differentiation in adipocytes (Nakae *et al*, 2003). Foxo1 inhibits adipogenesis at least partially by repressing PPARγ gene transcription (Armoni *et al*, 2006).

Ser253, located in the DNA binding domain (Zhang *et al*, 2002), is the most important Akt target site on Foxo1 (Van Der Heide *et al*, 2004). Its phosphorylation by Akt decreases the DNA binding capacity of Foxo1 (Zhang *et al*, 2002) in numerous tissues and organs, including adipose tissue (Armoni *et al*, 2006). A mutant of Foxo1, which carries mutations in Ser253 and two other Akt phosphorylation sites, inhibits adipocyte differentiation (Nakae *et al*, 2003). Additionally, embryonic fibroblasts derived from Akt1/Akt2 double knockout mice show impaired adipogenesis and also decreased Foxo1 phosphorylation and inactivation (Peng *et al*, 2003). However, the mechanisms regulating the Akt-dependent phosphorylation of Foxo1 and thus adipogenesis remain largely unknown to date.

In the present study, we demonstrate that ProF regulates adipocyte differentiation as a mediator between Akt and Foxo1. ProF and Foxo1 expression is transiently upregulated during differentiation. ProF binds Foxo1 and Akt and facilitates Akt phosphorylation of Foxo1 at Ser253 in its DNA binding domain. This leads to reduced transcriptional activity of Foxo1 and thus to increased adipogenesis and glucose uptake in differentiated cells. By this mechanism, ProF has a substantial role in the regulation of adipocyte differentiation.

Results

ProF expression is transiently upregulated during adipogenesis

ProF consists of seven WD-repeats, acting as a protein–protein binding platform, whereas the FYVE domain interacts with phosphatidylinositol-3-phosphate on vesicular membranes (Figure 1A, top). ProF preferentially binds the activated kinases Akt and PKCζ/λ upon stimulation of the cell with insulin-like growth factor 1. Akt is a serine/threonine kinase with an N-terminal Pleckstrin homology domain for binding to phosphoinositides, a central kinase domain, and a C-terminal regulatory domain (Figure 1A, middle). Targets of Akt are the Foxo transcription factors that comprise Foxo1, Foxo3, Foxo4, and Foxo6 in mice (Greer and Brunet, 2005). Foxo1, which is highly expressed in adipose tissue, contains a central DNA binding domain and a C-terminal transactivation domain (Figure 1A, bottom).

Owing to the high expression levels of ProF in 3T3-L1 cells compared to other tissues (Fritzius *et al*, 2006), we assumed that ProF might have a role in this model system for adipocyte differentiation or adipogenesis. Adipocyte differentiation is a multi-step process that requires growth inhibition by cell–cell contact, hormonal induction for mitotic clonal expansion (designated as day 0; Figure 1B), followed by growth arrest (corresponding to early differentiation at days 1–3), accumulation of triacylglycerol storage vacuoles or lipid droplets

*Corresponding author. Institute of Medical Virology, University of Zurich, Gloriastrasse 30, Zurich 8006, Switzerland.
Tel.: +41 44 634 26 52; Fax: +41 44 634 49 67;
E-mail: moelling@immv.uzh.ch

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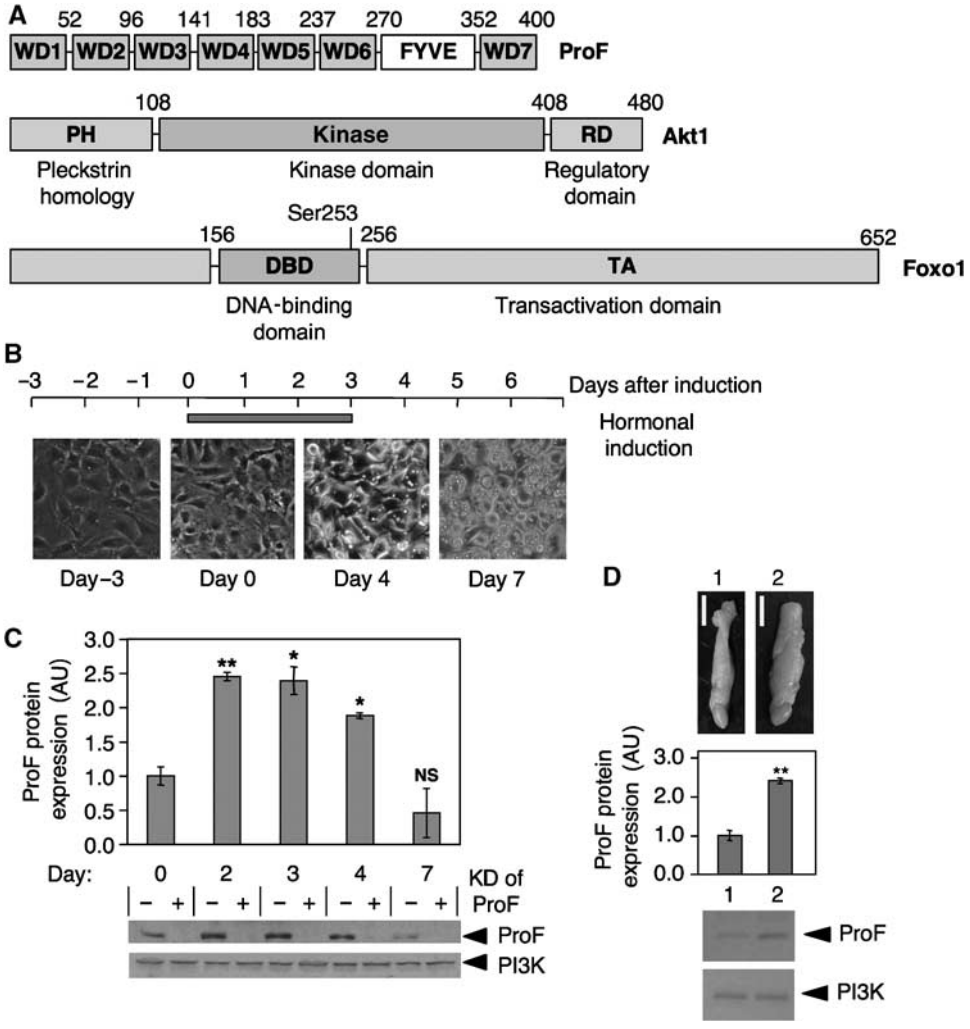


Figure 1 ProF is transiently upregulated during adipogenesis. (**A**) Domain structure of mouse ProF, Akt1, and Foxo1. ProF consists of seven WD-repeat motifs (WD1–7) for protein–protein interaction and an FYVE domain for binding to phosphatidylinositol-3-phosphate on vesicular membranes. Akt1 possesses an N-terminal Pleckstrin homology (PH) domain for binding to phosphoinositides, a central serine/threonine protein kinase domain (KINASE), and a C-terminal regulatory domain (RD). Foxo1 contains a central DNA binding domain (DBD) and a C-terminal transactivation domain (TA). The serine residue 253 (Ser253), located in the C-terminus of the DNA binding domain, is an Akt target site. (**B**) Time line of adipocyte differentiation. Addition of the hormonal induction medium is indicated as a grey bar (days 0–3). Shown below is the morphology of 3T3-L1 cells during cell cycle arrest (day −3), start of hormonal induction (day 0), late differentiation (days 4–6), and terminal differentiation (day 7 and later) as observed by light microscopy. (**C**) Protein expression levels of ProF during adipogenesis. The expression of ProF protein at the indicated days of adipocyte differentiation was measured by densitometric scanning of ProF protein bands from immunoblots. Data are mean values ± s.d. of three separate differentiations (NS = nonsignificant, $*P<0.05$, $**P<0.01$, relative to day 0). One representative blot shows the expression of ProF during adipocyte differentiation in control cells (−) and cells with knockdown of ProF (KD of ProF, +). Expression of PI3-kinase (PI3K) was assessed as a loading control. (**D**) ProF expression in adipose tissue. Epididymal white adipose tissue (eWAT) of mice, fed standard chow (1) or HFD (2) for 12 weeks. Scale bar is 1 cm (top). ProF protein expression in eWAT from mice, fed standard chow (1) or HFD (2), was measured by densitometric scanning. Data are mean values ± s.d. of eWAT from three mice ($**P<0.01$). One representative blot shows the expression of ProF in mice, fed standard chow (1) or HFD (2). A full-colour version of this figure is available at *The EMBO Journal* Online.

(late differentiation, at days 4–6), and terminal differentiation (day 7 and later).

To characterize ProF in 3T3-L1 cells, we first measured its expression during adipogenesis by densitometric scanning of ProF protein bands from immunoblots (Figure 1C). ProF protein expression is detected by an antiserum, described earlier (Fritzius *et al*, 2006). ProF expression levels increased significantly during early differentiation (days 1–2 after induction) and declined during terminal differentiation, shown up to day 7. This suggests that ProF expression is regulated during adipogenesis.

To investigate whether ProF expression is also regulated during adipogenesis *in vivo*, ten 4-week-old male C57BL/6J mice were fed for 12 weeks with either the standard chow (10% energy as fat) or high-fat diet (HFD, 60% energy as fat). After 12 weeks, ProF protein expression was measured in epididymal white adipose tissue and found to be significantly (270%, $P<0.01$) increased in mice fed HFD relative to those fed standard diet (Figure 1D). These results demonstrate that ProF expression is regulated during adipocyte and adipose tissue differentiation.

Knockdown of ProF impairs adipocyte differentiation

To analyse a possible role of ProF during adipocyte differentiation, we performed knockdown of ProF by stable transduction of 3T3-L1 cells with a lentiviral vector expressing either a small hairpin RNA (shRNA) targeted against nucleotides 1154–1172 of the ProF mRNA open reading frame (ProF KD1) or against nucleotides 131–149 of the 3′-untranslated region (ProF KD2). shRNA against firefly luciferase (shGL2) was used as control. Introduction of ProF KD2 led to 75% downregulation of endogenous ProF protein, whereas ProF KD1 introduction caused 90% downregulation (see Figure 2C). The cells were subjected to differentiation using a hormonal induction medium. Oil Red O staining showed a decreased lipid accumulation in differentiated ProF KD1 and ProF KD2 cells during late differentiation when compared to controls (Figure 2A). Quantification of the stained triglyceride content revealed that lipid accumulation was impaired by ~55% in ProF KD1 cells and ~40% in ProF KD2 cells compared to controls at day 7 (Figure 2B). This reduced lipid accumulation correlated with the decrease in ProF expression in ProF KD1 and ProF KD2 cells. Thus, knockdown of ProF inhibits adipogenesis.

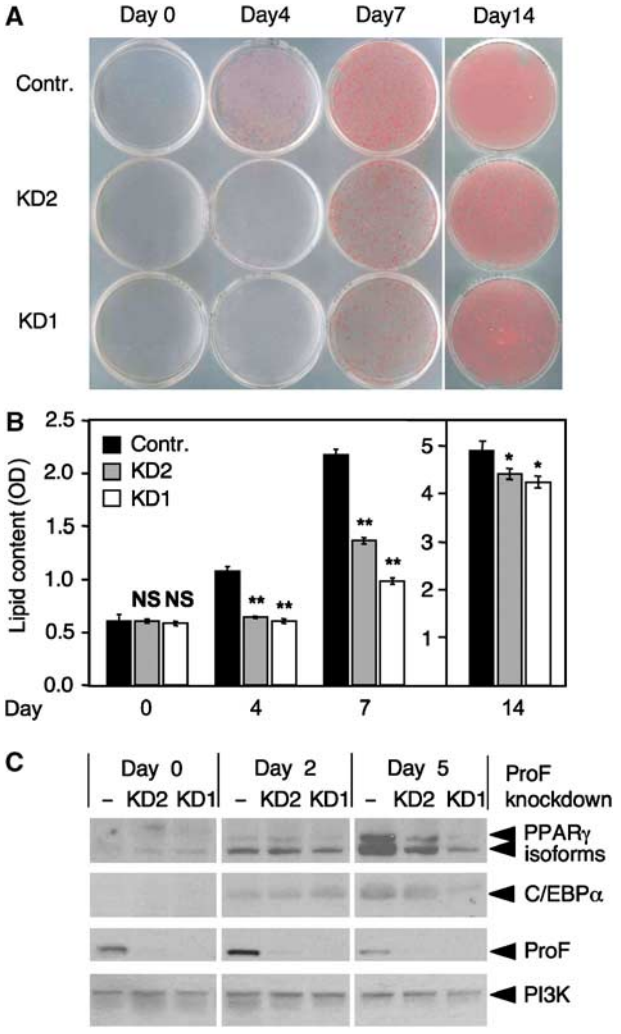


Figure 2 Knockdown of ProF impairs adipocyte differentiation. (**A**) Oil Red O staining of control-vector-transduced cells (Contr.) and cells with two different shRNAs for knockdown of ProF (KD2 and KD1) at the indicated days of differentiation. Cells from day 14 are derived from a separate differentiation experiment. (**B**) Triglyceride content of control (Contr., black bars) and ProF knockdown cells (KD2, grey bars; KD1, white bars) was measured spectrophotometrically at the indicated days of differentiation. Data are mean values ± s.d. of three individual points (NS = nonsignificant, $*P<0.05$, $**P<0.01$). Cells from day 14 are derived from a separate differentiation experiment. (**C**) Control cells (−) or cells with two different shRNAs for ProF knockdown (KD2 and KD1) were lysed at the indicated days of differentiation. Time-course analysis of expression of adipocyte differentiation markers was performed by immunoblotting with antibodies for the indicated proteins.

To find out if the inhibitory effect of ProF on adipogenesis persists at later time points, control cells (Contr.) and cells with ProF knockdown (ProF KD1 and KD2) were subjected to differentiation until day 14. Oil Red O staining revealed that the effect of ProF knockdown was partly but not fully abrogated at day 14 (Figure 2A and B).

Next, we investigated whether ProF affected the expression of the transcription factors PPARγ and C/EBPα, two important regulators of terminal adipogenesis (summarized in Rosen and MacDougald, 2006). For this, cells were lysed at different stages of adipogenesis, followed by SDS–PAGE for immunoblot analysis (Figure 2C). Knockdown of ProF impaired the expression of PPARγ and C/EBPα. PPARγ is known to regulate the expression of C/EBPα (Rosen *et al*, 2002), and the deletion of PPARγ in ProF knockdown cells may be responsible for the reduced expression of C/EBPα.

As the expression of PPARγ and C/EBPα is dependent on the early differentiation markers C/EBPβ and C/EBPδ (Tanaka *et al*, 1997), we analysed their expression in ProF KD1 knockdown cells (Figure 3A). The rapid upregulation and downregulation of C/EBPβ and C/EBPδ was unaffected by ProF knockdown, indicating that ProF is dispensable for C/EBPβ and C/EBPδ expression during early adipogenesis.

Knockdown of ProF also caused a decreased PPARγ and C/EBPα mRNA expression during adipogenesis as evidenced by reverse transcriptase–PCR (RT–PCR) (Figure 3B). Furthermore, it led to a decreased expression of glucose transporter 4 (GLUT4) mRNA (Figure 3B) and GLUT4 protein expression (Figure 3C). GLUT4 is a marker for terminally differentiated adipocytes and its expression is dependent on the transcriptional activity of PPARγ (Wu *et al*, 1998). Consistent with these results, 3T3-L1 adipocytes with ProF knockdown displayed a decreased glucose uptake compared to controls (Figure 3D). This is in agreement with the finding that GLUT4 is the major glucose transporter isoform in terminally differentiated adipocytes (Holman *et al*, 1990).

The role of ProF as a regulator of PPARγ expression was further investigated by using troglitazone, a PPARγ ligand, which stimulates the transcriptional activity of PPARγ and thus promotes adipogenesis (summarized in Rangwala and Lazar, 2000). Troglitazone did not restore the reduced lipid accumulation in ProF knockdown cells at day 4, but increased lipid accumulation in control cells (Figure 3E, left). At day 7 however, troglitazone-treated ProF knockdown cells displayed higher triglyceride levels than untreated control cells (Figure 3E, right). Thus, troglitazone can fully compensate for the inhibitory effect of ProF knockdown on adipogenesis at day 7, when PPARγ expression is detectable in ProF knockdown cells, but not at earlier time points (Figure 3F). These data show that ProF has a role in adipogenesis mainly by affecting the expression of PPARγ.

To confirm that the role of ProF in adipocyte differentiation is not confined to immortalized cell lines, primary human

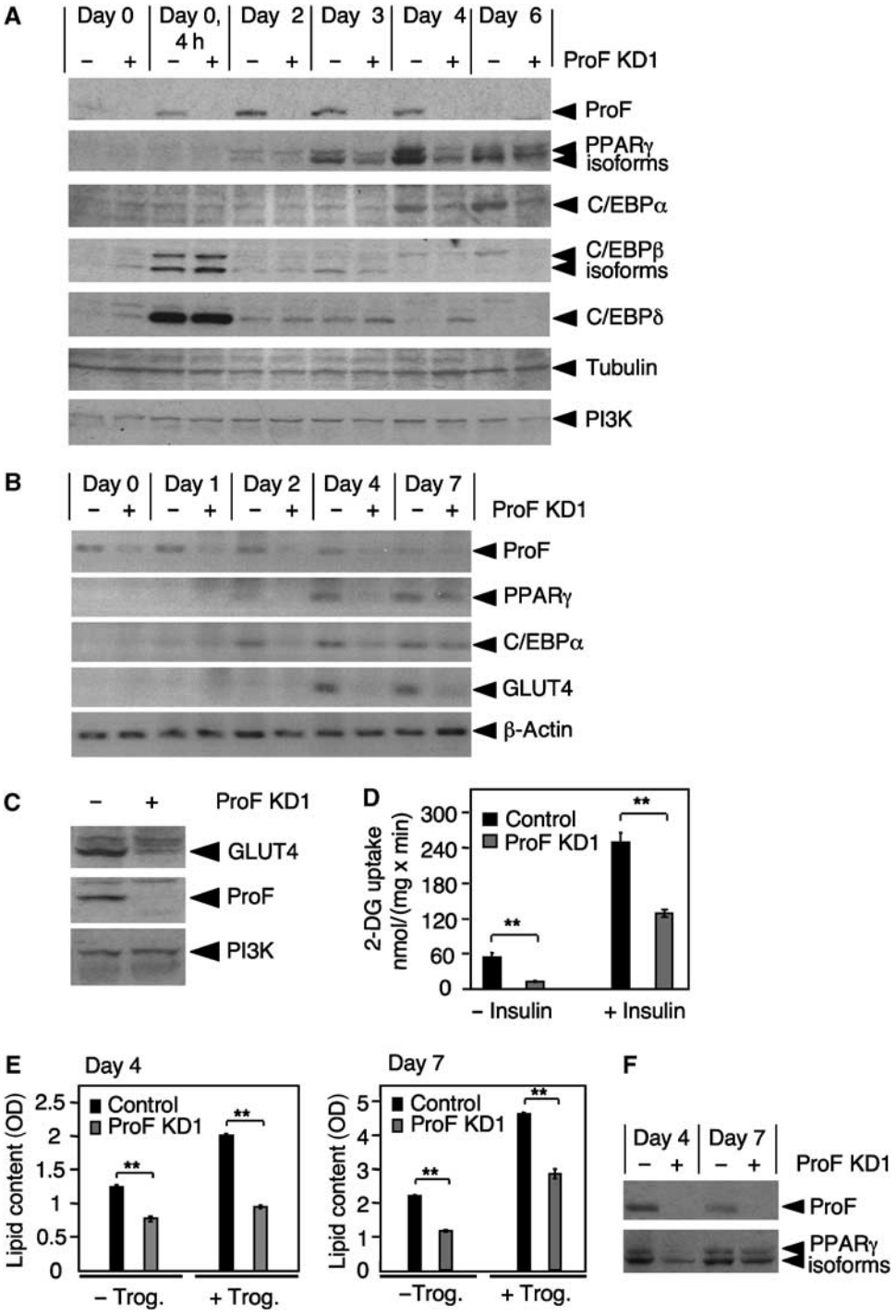


Figure 3 ProF knockdown affects the expression of key proteins of adipogenesis. (**A**) 3T3-L1 control cells (−) or cells with ProF knockdown (+) were lysed at the indicated days of differentiation. Time-course analysis of expression of adipocyte differentiation markers was performed by immunoblotting with antibodies for the indicated proteins. Expression of tubulin and PI3K was used as loading control. (**B**) 3T3-L1 control-vector-transduced cells (−) or cells with ProF knockdown (+) were lysed at the indicated days of differentiation. Time-course analysis of mRNA expression of adipocyte differentiation markers was performed by RT–PCR from total RNA with primers as indicated. β-Actin was used as control. (**C**) Expression of GLUT4, ProF, and PI3K at day 7 of differentiation was detected in control cells (−) or cells with ProF knockdown (+) by immunoblotting using antibodies as described above. (**D**) Control cells (black bars) or cells with ProF knockdown (ProF KD1, grey bars) were starved at day 7 and stimulated for 30 min with insulin or left unstimulated before incubation with 2-deoxy-D-[1-^{3}H]-glucose (2-DG) for 10 min. Data are mean values ± s.d. of four individual points (**$P<0.01$). (**E**) Control cells (Control, black bars) or cells with ProF knockdown (ProF KD1, grey bars) were subjected to differentiation in the absence (−Trog.) or presence (+Trog.) of troglitazone, a PPARγ ligand. At day 4 (left) and day 7 (right) of differentiation, triglyceride content was measured spectrometrically. Data are mean values ± s.d. of three independent points (**$P<0.01$). (**F**) Expression of ProF and PPARγ at days 4 and 7 in lysates of control cells (−) and ProF knockdown cells (+) is shown by immunoblotting. Lysates were derived from the same differentiation assay as in (E).

white preadipocytes (HWP-c) were transduced with a lentiviral vector expressing shRNA against human ProF (ProF KDh) or shGL2 as control. Afterwards, cells were subjected to differentiation using a hormonal induction medium. Oil Red O staining showed a decreased lipid accumulation in terminally differentiated ProF KDh cells when compared to controls (Figure 4A and B). Counting of Oil Red O-positive cells in randomly chosen sections revealed that the number of Oil Red O-positive cells is ~10 times higher in control cells than in ProF KDh cells (Figure 4C). Furthermore, ProF knockdown led to decreased expression of PPARγ in terminally differentiated adipocytes (Figure 4D). This finding shows that ProF has a role in primary adipocyte differentiation and furthermore suggests that the role of ProF in adipogenesis is conserved in all mammals.

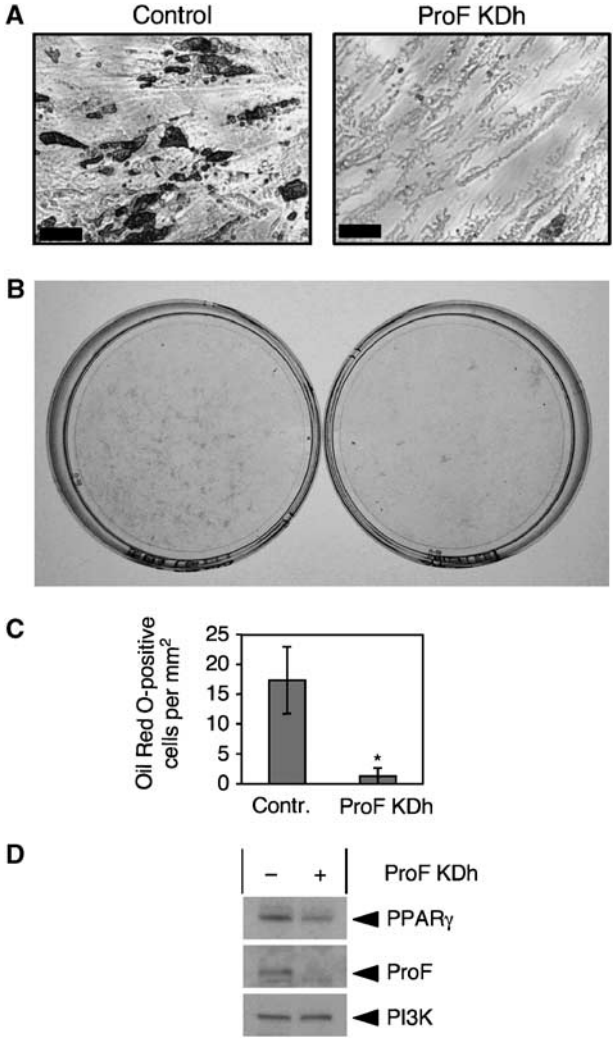


Figure 4 Knockdown of ProF impairs differentiation of primary adipocytes. (**A, B**) Oil Red O staining of control-vector-transduced cells (Control) and cells with shRNAs for knockdown of human ProF (ProF KDh) in terminally differentiated adipocytes (day 17). Scale is 50 μm (top). (**C**) Measurement of Oil Red O-positive cells per mm^2 in control cells (Contr.) and cells with ProF knockdown (ProF KDh), chosen by random selection. Data are mean values ± s.d. of three separate differentiations (NS = nonsignificant, $^*P<0.05$). (**D**) Expression of PPARγ, ProF, and PI3K in terminally differentiated adipocytes (day 17) was detected in control cells (−) or cells with ProF knockdown (+) by immunoblotting using antibodies for the indicated proteins. A full-colour version of this figure is available at *The EMBO Journal* Online.

Overexpression of ProF increases adipocyte differentiation

To further understand to role of ProF in adipogenesis, we overexpressed Myc-tagged ProF (Myc–ProF) or an empty vector control (Control) by stable transduction of 3T3-L1 cells with a retroviral vector. Cells were differentiated as described above. Indeed, lipid accumulation was increased in Myc–ProF- overexpressing cells compared to controls (Figure 5A). This difference was more striking during differentiation and less in terminally differentiated cells. Furthermore, ProF overexpression led to increased levels of expression of PPARγ and C/EBPα at days 2–4 after induction (Figure 5B), whereas C/EBPβ and C/EBPδ were unaffected (data not shown). Myc–ProF appeared to decrease the levels of endogenous ProF during terminal differentiation, possibly because overexpressed ProF accelerated the differentiation. In summary, overexpression of ProF increases adipocyte differentiation.

ProF specifically regulates Akt-mediated Foxo1 phosphorylation

The Akt- and PKCζ-interacting protein ProF (Fritzius *et al*, 2006) has recently been shown to simultaneously bind the kinase PKCζ and the kinase substrate VAMP2 in an adaptor protein-like manner to increase VAMP2 phosphorylation by PKCζ (Fritzius *et al*, 2007). Therefore, it seemed possible that ProF acts as an adaptor for facilitated phosphorylation of an Akt substrate to regulate adipogenesis.

Previously, several Akt targets, which have a role in adipogenesis, have been uncovered. These include the Foxo transcription factor Foxo1 (Nakae *et al*, 2003), which is negatively regulated by Akt phosphorylation (Kops and Burgering, 1999). Furthermore, the serine/threonine kinase mammalian target of rapamycin (mTOR), an indirect target of Akt (Lawrence *et al*, 2004), and the glycogen synthase kinase 3 (GSK-3), an important Akt substrate (Cross *et al*, 1995), are also implicated in adipogenesis (Ross *et al*, 1999; Cho *et al*, 2004).

To test whether ProF affected the phosphorylation of Akt substrates, 3T3-L1 cells with ProF knockdown as well as cells overexpressing ProF (Figure 6A and B) were subjected to differentiation. The phosphorylation of the Akt substrates Foxo1, mTOR, and GSK-3β at Ser253, Ser2448, and Ser9, respectively, was assessed on immunoblots by the use of phospho-specific antibodies. Knockdown and overexpression of ProF only affected phosphorylation of Foxo1 (Figure 6A and B, top lane), suggesting that the ProF-mediated effect on Foxo1 phosphorylation is specific. Knockdown of ProF by ProF KD1 caused an ~55% decrease in Foxo1 substrate phosphorylation (Figure 6C), whereas it only weakly affected its expression (~20%).

Knockdown of ProF does not affect Foxo1 phosphorylation and myotube differentiation of C_2C_{12} cells

Akt phosphorylation of Foxo1 has recently been shown to regulate the differentiation of C_2C_{12} myoblast cells into myotubes (Hribal *et al*, 2003), similar to its role in adipogenesis. To find out if ProF also modulates Foxo1 phosphoryla-

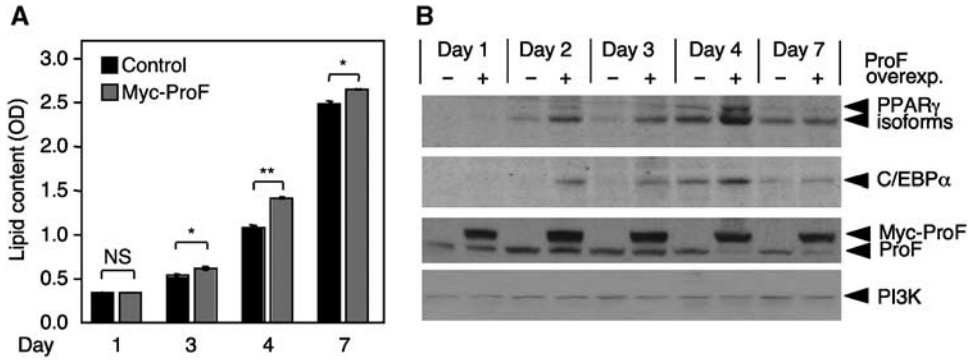


Figure 5 Overexpression of ProF increases adipocyte differentiation. (**A**) Triglyceride content of empty control-vector-transduced cells (Control, black bars) or Myc–ProF-expressing cells (Myc–ProF, grey bars) was measured spectrophotometrically at the indicated days of differentiation. Data are mean values ± s.d. of three individual points (NS = nonsignificant, $*P<0.05$, $**P<0.01$). (**B**) 3T3-L1 cells, transduced with an empty control vector (−) or a vector expressing Myc-tagged ProF (+), were lysed at the indicated days of differentiation. Time-course analysis of expression of adipocyte differentiation markers was performed by immunoblotting with antibodies for the indicated proteins.

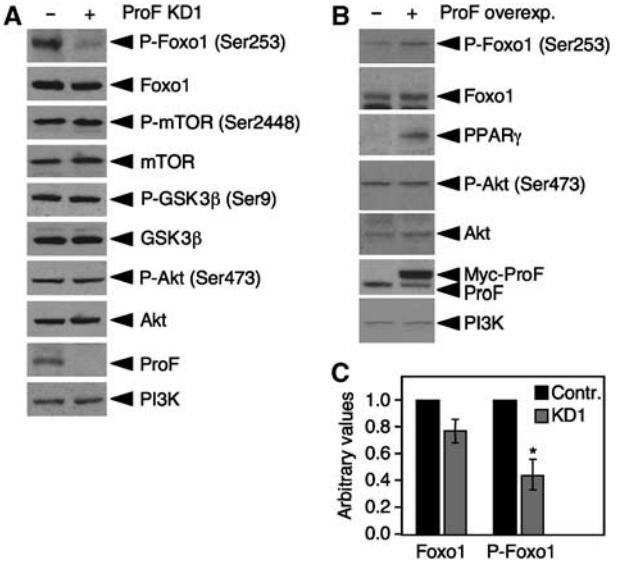


Figure 6 ProF specifically regulates Foxo1 phosphorylation. (**A**) Control cells (−) or ProF knockdown cells (+) at day 3 of differentiation. (**B**) Control cells (−) or cells expressing Myc-tagged ProF (+) at day 2 of differentiation. Phosphorylation of Akt and the Akt substrates Foxo1, mTOR, and GSK-3β, as well as expression of Foxo1, mTOR, GSK-3β, Akt, and ProF was detected by immunoblotting with antibodies for the indicated proteins. Expression of PPARγ shows the effect of ProF on adipogenesis. (**C**) Expression and phosphorylation of Foxo1 in control cells (Contr., black bars) and ProF knockdown cells (KD1, grey bars) were measured by densitometric scanning of Foxo1 and P-Foxo1 (Ser253) bands from immunoblots at day 3 of adipocyte differentiation. For analysis, control cells were normalized as 1. Data are mean values ± s.d. of four independent differentiation experiments ($*P<0.05$ of Foxo1 phosphorylation, relative to Foxo1 expression).

tion and differentiation in muscle cells, we performed ProF knockdown by stable transduction of C_2C_{12} myoblast cells with a lentiviral vector expressing either ProF KD1 or ProF KD2. shRNA against firefly luciferase (shGL2) was used as control. The cells were lysed at different stages of myogenesis, followed by SDS–PAGE for immunoblot analysis (Supplementary Figure 1A). Knockdown of ProF did not impair the expression of the differentiation marker proteins myogenin and myosin heavy chain, indicating that ProF is dispensable for this process. Furthermore, ProF did not affect Foxo1 expression and phosphorylation during myogenesis (Supplementary Figure 1A). These data correlate well with the low expression levels of ProF during myotube differentiation compared with ProF expression levels during adipogenesis (Supplementary Figure 1B). In summary, these data show that ProF is specifically affecting the differentiation of adipocytes.

Interaction of ProF with Foxo1

Next, we determined expression and phosphorylation of Foxo1 during adipocyte differentiation (Figure 7A). Foxo1 expression levels increased strongly during early differentiation (days 1–3 after induction) and declined during terminal differentiation (day 7). Foxo1 phosphorylation was found to slightly precede Foxo1 expression, whereas ProF protein levels peaked approximately 1 day before highest Foxo1 expression. These findings are consistent with earlier studies performed on Foxo1 expression and phosphorylation during adipogenesis (Nakae *et al*, 2003; Peng *et al*, 2003; Jing *et al*, 2007).

Next, we investigated the subcellular localization of both proteins in differentiating 3T3-L1 cells, because Foxo1 is known to shuttle between cytoplasm and nucleus dependent on its phosphorylation status (Kops and Burgering, 1999; Rena *et al*, 2001). Subcellular fractionation revealed that Foxo1 phosphorylation was detected exclusively in the cytoplasm and was strongly increased upon insulin stimulation (Figure 7B). The level of cytoplasmic Foxo1 increased after stimulation, whereas the level of nuclear Foxo1 was slightly decreased. ProF was mostly found in the cytoplasm. This is consistent with earlier studies (Fritzius *et al*, 2006; Hayakawa *et al*, 2006) and suggests that interaction of Foxo1 and ProF can take place in the cytoplasm.

To find out if ProF affects the localization of Foxo1, we performed subcellular fractionation in control cells (Contr.) and cells with ProF knockdown (KD1) (Figure 7C). ProF did not affect the activation or subcellular localization of Akt, whereas Foxo1 phosphorylation was decreased in KD1 cells compared to controls. Additionally, less Foxo1 was found in the cytoplasm of KD1 cells than in the cytoplasm of control cells, indicating that knockdown of ProF leads to an altered

subcellular localization of Foxo1 in 3T3-L1 cells during adipogenesis.

To test if Foxo1 and ProF can interact, we precipitated Myc-tagged ProF (Figure 7D, left) and Flag-tagged Foxo1 (Figure 7D, right) from lysates of HEK 293T cells and analysed co-precipitation of coexpressed proteins by immunoblotting. Myc-tagged ProF co-precipitated Flag-tagged Foxo1 (left) and Flag-tagged Foxo1 co-precipitated Myc-tagged ProF (right), indicating that ProF and Foxo1 can indeed physically interact.

Next, we analysed whether ProF, Foxo1, and Akt can form a trimeric complex by performing a sequential precipitation procedure. Immunoprecipitated Myc–ProF co-precipitated Flag–Foxo1 and HA–Akt (Figure 7E, IP1). The precipitated complex was thereafter eluted with a Myc peptide. Half of the eluate was used for immunoprecipitation of Flag–Foxo1, showing co-precipitation of HA–Akt and Myc–ProF (Figure 7E, IP2), whereas the other half was used for immunoprecipitation of HA–Akt, showing a weak co-precipitation of Flag–Foxo1 and co-precipitation of Myc–ProF (Figure 7E, IP3). Densitometric scanning of protein bands from direct lysates and immunoprecipitates revealed that ~1.5% of total cellular ProF was immunoprecipitated, co-precipitating ~0.2% of total cellular Foxo1 and ~0.15% of total cellular Akt (data not shown).

To test if hormonal stimulation of the cells regulates the formation of the trimeric Foxo1–Akt1–ProF complex, as has been shown before for the dimeric Akt1–ProF complex (Fritzius *et al*, 2006), HEK 293T cells were stimulated by insulin or left unstimulated. Immunoprecipitates of Myc–ProF from stimulated cells contained more Foxo1 and more Akt than immunoprecipitates from unstimulated cells (Figure 7F), suggesting a stronger interaction of ProF with the proteins after hormonal stimulation.

Ectopic expression of dominant-negative Foxo1 restores adipocyte differentiation of 3T3-L1 cells with ProF knockdown

To substantiate the role of ProF as a regulator of Foxo1 during adipocyte differentiation, we constructed a truncated Foxo1 mutant, lacking the C-terminal transactivation domain, but maintaining its DNA binding domain. This mutant, FoxoDN, has been shown to function as a dominant-negative regulator of Foxo1 and as an inducer of adipogenesis and PPARγ expression in mouse adipocytes (Nakae *et al*, 2003). Furthermore, FoxoDN restored adipocyte differentiation in fibroblasts from insulin receptor-deficient mice (Nakae *et al*, 2003), providing a link between insulin signalling, active Akt, and adipogenesis by Foxo1 phosphorylation.

Introduction of FoxoDN into ProF KD1 cells restored lipid accumulation in differentiated ProF KD1 cells (Figure 8A). Quantification of lipid accumulation showed that FoxoDN expression led to a complete recovery of lipid accumulation in differentiating ProF KD1 cells (Figure 8B). Furthermore, introduction of FoxoDN fully restored the expression of PPARγ in ProF knockdown cells (Figure 8C). These data show that a dominant-negative mutant of Foxo1 can compensate the effect of ProF knockdown on adipogenesis.

Next, we wanted to find out if introduction of active Akt could also restore lipid accumulation and PPARγ expression in ProF KD1 cells. Constitutively active myristoylated/palmitoylated Akt1 (m/p-Akt1) has been shown to cause spontaneous differentiation in 3T3-L1 cells in the absence of adipogenic treatment (Baudry *et al*, 2006). Interestingly, transduction of ProF KD1 cells with m/p-Akt1 caused only a limited recovery of lipid accumulation (Figure 8D) and an insignificant restoration of PPARγ expression (Figure 8E) in differentiated ProF KD1 cells. This might be due to the fact that we could only achieve a very low level of overexpression of m/p-Akt1 in our experiments, compared to FoxoDN overexpression (data not shown). Further experiments, performed with ProF KD1 cells with higher overexpression levels of m/p-Akt1, will reveal if constitutively active Akt is able to fully restore adipogenesis in cells with ProF knockdown.

Discussion

We have previously identified a WD-repeat propeller-FYVE protein, ProF, as an interaction partner for the kinases PKCζ and Akt in 3T3-L1 cells (Fritzius *et al*, 2006). Furthermore, we have shown that ProF forms a trimeric complex with PKCζ and its substrate VAMP2. In this complex, ProF was found to act as an adaptor-like protein for facilitated substrate phosphorylation of VAMP2 by the active kinase PKCζ (Fritzius *et al*, 2007).

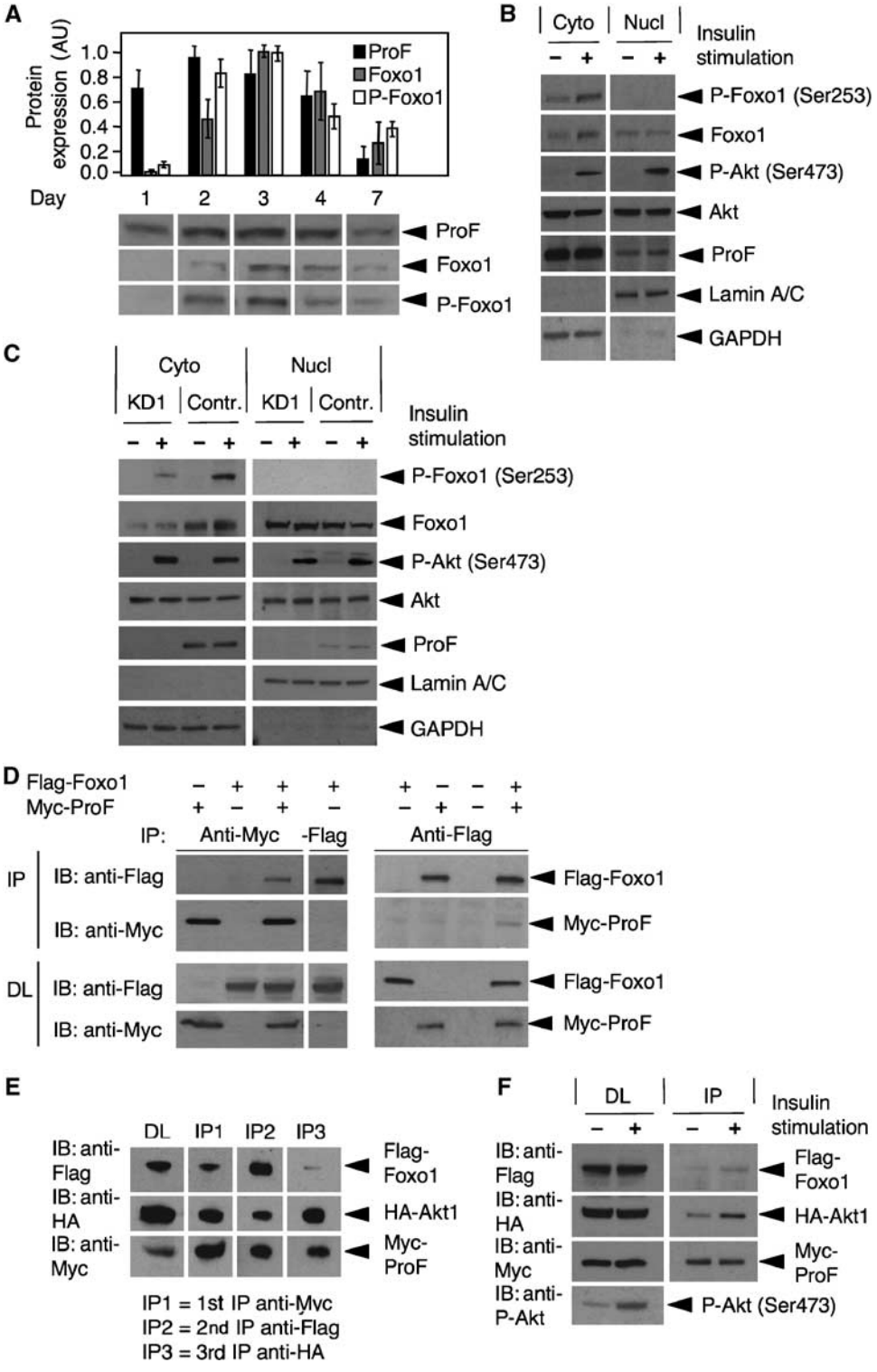
In this study, we identified a role for ProF during adipogenesis and showed complex formation of ProF with the kinase Akt and the kinase substrate Foxo1. The protein kinase Akt was found to affect adipogenesis after the expression of C/EBPβ and C/EBPδ, but before the expression of PPARγ and C/EBPα (Peng *et al*, 2003; Baudry *et al*, 2006), which resembled the effects of ProF on adipogenesis. Akt phosphorylation of Foxo1 at Ser253, in the Foxo1 DNA binding domain, has been demonstrated to decrease its transcriptional activity (Zhang *et al*, 2002). We have shown here that ProF increased the Akt-mediated Ser253 phosphorylation of Foxo1 during adipogenesis, whereas it did not affect the phosphorylation status and expression of Akt or the Akt substrates mTOR and GSK-3β.

We have found that the cytoplasmic ProF can interact with the transcription factor Foxo1, which is known to shuttle between the cytoplasm and the nucleus, dependent on its phosphorylation status (Van Der Heide *et al*, 2004). Furthermore, we have demonstrated in agreement with others (Nakae *et al*, 2003; Jing *et al*, 2007) that Foxo1 expression is transiently upregulated during adipogenesis. The highest ProF expression precedes Foxo1 expression by about 1 day. It is possible that ProF is required for the regulation of newly synthesized, cytoplasmic Foxo1 by Akt phosphorylation. When ProF expression decreases, Foxo1 may no longer be retained in the cytoplasm, but can translocate to the nucleus. There, it can downregulate PPARγ expression (Armoni *et al*, 2006). This multi-step process, involving ProF, Foxo1, and PPARγ, may also be responsible for the fact that the highest effect of ProF knockdown on PPARγ expression appears several days after maximal expression of ProF (Figure 2C). A recent publication supports the concept of Foxo1 regulation in the cytoplasm by showing that the deacetylase SIRT2, which is also a cytoplasmic protein with its highest expression ahead of Foxo1 expression, is a negative regulator of Foxo1 Ser253 phosphorylation during adipogenesis by regulating the acetylation/deacetylation status of Foxo1 (Jing *et al*, 2007). Thus, regulation of Foxo1

phosphorylation by Akt can also take place outside of the nucleus.

Taken together, we have found that ProF binds Akt and Foxo1 and facilitates Foxo1 Ser253 phosphorylation by insulin-stimulated Akt. Additionally, dominant-negative Foxo1, which is known to restore adipocyte differentiation in cells with disturbed insulin signalling (Nakae *et al*, 2003), also restored adipogenesis in ProF knockdown cells, showing that ProF is a positive regulator of insulin-induced adipogenesis. The proposed roles of ProF, Akt, and Foxo1 during adipocyte differentiation are summarized schematically in a model (Figure 8F).

Furthermore, ProF could have additional effects on fat cell differentiation. Although it did not affect the phosphorylation of the Akt substrates mTOR and GSK-3β (Figure 6A), it may modulate other Akt-regulated adipogenetic pathways, such as



Akt phosphorylation of the transcription factor GATA2, a negative regulator of PPARγ expression and adipogenesis (Menghini *et al*, 2005). ProF might also have a role outside of the Akt signalling pathway. For example, ProF could modulate phosphorylation and stabilization of β-catenin, a negative regulator of PPARγ/C/EBPα expression and adipocyte differentiation (Ross *et al*, 2000), similarly to the proposed role of ProF in Foxo1 phosphorylation. Further investigation of the function of ProF in signal transduction during adipogenesis may lead to a better understanding of the molecular mechanism of differentiation.

Moreover, we found that ProF knockdown decreased glucose uptake in adipocytes as a result of impaired adipogenesis and reduced GLUT4 expression (Figure 3D). Decreased glucose uptake into adipose tissue contributes to type II diabetes, as it results in elevated blood glucose levels, which causes many of the complications of type II diabetes (summarized in Bryant *et al*, 2002). ProF may function as an anti-diabetic protein by promoting the generation of new adipocytes by adipogenesis. This increases glucose uptake and thus regulates blood glucose levels.

Therefore, an in-depth study of the role of ProF in adipocyte differentiation could also lead to a better comprehension of metabolic diseases such as type II diabetes.

Materials and methods

Materials

Cell culture media, supplements, Tris-glycine gradient gels, and Lipofectamin 2000 were purchased from Invitrogen. Insulin was from Novo Nordisk, 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone (DMX) were from Sigma, and troglitazone was from Calbiochem. Mouse monoclonal antibody (B-7) against tubulin, rabbit polyclonal (A-14) against Myc, goat polyclonal (C-20) against GLUT4, rabbit polyclonal (H-100) against PPARγ, mouse monoclonal (E-8) against PPARγ, rabbit polyclonal (14AA) against C/EBPα, mouse monoclonal (H-7) against C/EBPβ, rabbit polyclonal (C-22) against C/EBPδ, goat polyclonal (N-18) against lamin A/C, rabbit polyclonal (FL-335) against GAPDH, mouse monoclonal (C4) against β-actin, and mouse monoclonal (M-225) against myogenin were obtained from Santa Cruz Biotechnology. Mouse monoclonal (M2) against Flag and mouse monoclonal against HA (12CA5) were from Roche, and p85 subunit PI3K antibody was from Upstate. Antibodies against P-Foxo1 (Ser253), Foxo1, P-Akt (Ser473), Akt, P-mTOR (Ser2448), mTOR, P-GSK3β (Ser9), and GSK3β were from Cell Signaling Technology. Anti-MF20 mouse monoclonal antibody against myosin heavy chain was from the Developmental Studies Hybridoma Bank (University of Iowa).

Mice treatment

Four-week-old male C57BL/6J mice were housed in a temperature-, humidity-, and light-controlled room (12-h light and 12-h dark cycle) and allowed free access to water and chow. After 1 week, mice were fed either the standard chow (D12450B, 10% energy as fat; Research Diets) or HDF (D12492, 60% energy as fat; Research Diets) for 12 weeks. All animal studies were carried out according to Swiss Animal Rights in the facility of the Institute of Medical Virology with the permission of the Zurich Veterinary office.

Cell culture

The murine fibroblastic cell line 3T3-L1 (ATCC number CL-173) and the human embryonic kidney cell line HEK 293T (ATCC number CRL-11268) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS; Seratec). Mouse C_2C_{12} myoblast cells (ATCC number CRL-1772) were grown in DMEM under high-serum conditions with 20% FCS and were passaged every second day at a 1:10 dilution to maintain $<50\%$ confluency. Primary cultures of HWP-c, derived from the abdomen of female patients, were purchased from PromoCell and grown in preadipocyte growth medium (PromoCell) following the manufacturer's instructions. Trypsinized cells in single-cell suspension were mixed with Trypan blue (0.8 mM in PBS), incubated at room temperature for 5 min, loaded onto a haemocytometer, and counted under a light microscope.

Generation of stably transduced 3T3-L1 cells

Retroviruses containing the construct pRTP-Myc-ProF (Myc–ProF) or the empty pRTP vector (Control) were produced as described earlier (Fritzius *et al*, 2007). For shRNA downregulation, the sequences used were as follows: for ProF KD1: nt 1154–1172 of ProF open reading frame (5′-GAACTGACAAGGTAATTAA-3′) and for ProF KD2: nt 131–149 of ProF 3′ untranslated region (5′-CCACTGTTACCGCAATCTA-3′). For shRNA downregulation in HWP-c (ProF KDh), the sequence used was as follows: nt 1154–1172 of ProF open reading frame (5′-GAACTGACAAGGTTATTAA-3′). The control shRNA shGL2 targets the sequence 5′-AACGTACGCG GAATACTTCGA-3′ in the mRNA of the American firefly (*Photinus pyralis*) luciferase. Dominant-negative FoxO1 (FoxoDN) was generated by introducing a stop codon (tag) and an *Asc*I restriction site 3′ of Asp258 and by introducing an *Xba*I restriction site 5′ of the Flag tag using pcDNA3-Flag-FoxO1, kindly provided by Dr Kun-Liang Guan, as template and the following primers: forward primer: 5′-GCTCTAGAGCCACCATGGACTACAAGGACGACGATGAC-3′; reverse primer: 5′-TTGGCGCGCCCTACTAGTCCATGGATGCAGCTCTTCTCC-3′. Similarly, m/p-Akt1 was generated by introducing an *Asc*I restriction site after bp 48 of 3′-untranslated region and by introducing an *Xba*I restriction site 5′ of the HA tag using m/p-Akt1, a highly active form of Akt1 described earlier (Andjelkovic *et al*, 1997), as template and the following primers: forward primer:

Figure 7 Interaction of ProF with Foxo1. (**A**) ProF expression (black bars), Foxo1 expression (grey bars), and Foxo1 phosphorylation at Ser253 (white bars) at the indicated days of adipocyte differentiation were measured by densitometric scanning of protein expression and phosphorylation bands. Highest levels were normalized as 1. One typical blot of three experiments shows ProF expression as well as Foxo1 expression and Ser253 phosphorylation during adipocyte differentiation. (**B, C**) Subcellular localization of ProF and Foxo1 in control cells (B) and in control (Contr.) and ProF knockdown (KD1) cells (C). At day 3 of differentiation, 3T3-L1 cells were serum-starved for 4 h and stimulated with 100 nM insulin for 15 min (+) or left unstimulated (−) before subcellular differentiation. Equal amounts of protein from the cytoplasmic (Cyto) and nuclear (Nucl) fraction were analysed by SDS–PAGE and immunoblotting with antibodies for the indicated proteins. The purity of the cytosolic and the nuclear fraction was determined with antibodies against lamin A/C and GAPDH. (**D**) Interaction of ProF with Foxo1. HEK 293T cells were transiently transfected with Flag–Foxo1 in the presence or absence of Myc–ProF. Interaction of ProF with Foxo1 was analysed by immunoprecipitation (IP) with an antibody to Myc (left) or Flag (right) tag, followed by immunoblotting (IB) with antibodies against Flag and Myc epitopes. Direct lysates (DL) are shown as expression controls. (**E**) Trimeric complex formation of Flag–Foxo1, HA–Akt1, and Myc–ProF was shown by transient coexpression of the three proteins in HEK 293T cells (DL). The complex comprising the overexpressed proteins was subjected to immunoprecipitation using anti-Myc (IP1). The complex was eluted by addition of excess of competing Myc peptide followed by immunoprecipitation using an antibody directed against the Flag epitope (IP2) or the HA epitope (IP3). Immunoprecipitates of the different steps were analysed by immunoblot against the indicated proteins. Samples were loaded onto one gel and separating lanes were induced later for clarity. (**F**) Stimulation-dependent complex formation of Flag–Foxo1, HA–Akt1, and Myc–ProF was shown by transient coexpression of the three proteins in HEK 293T cells. Cells were stimulated with 200 nM insulin for 15 min (+) or left unstimulated (−) before lysis. Stimulation-dependent interaction of ProF with Foxo1 and Akt1 was analysed by immunoprecipitation (IP) with an antibody to Myc followed by immunoblotting (IB) with antibodies against Flag, HA, and Myc epitopes. Direct lysates (DL) are shown as expression controls, and P-Akt1 (Ser473) demonstrates insulin stimulation of the cells.

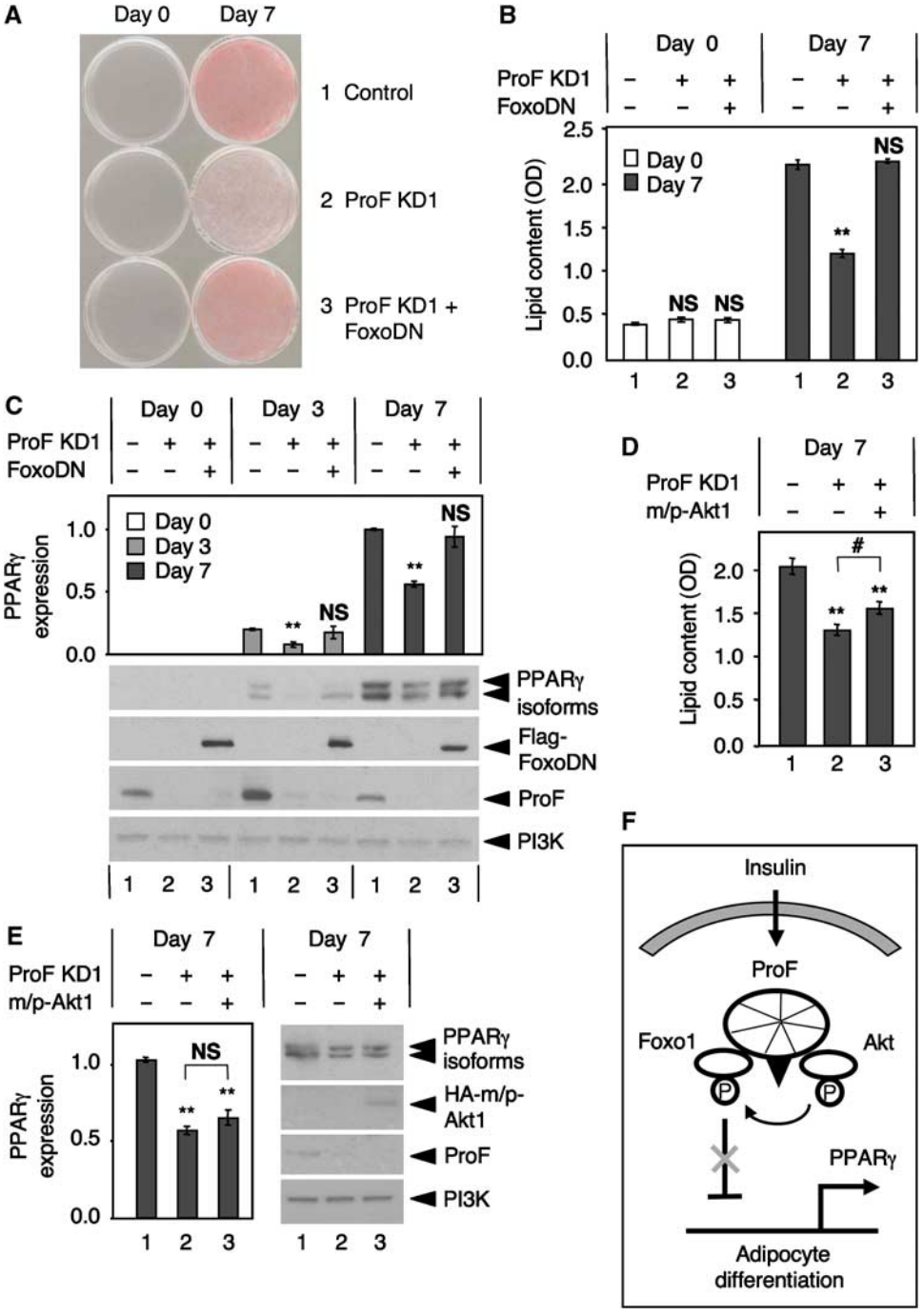


Figure 8 Ectopic expression of dominant-negative Foxo1 restores adipocyte differentiation of 3T3-L1 cells with ProF knockdown. (**A**) Oil Red O staining of control-vector-transduced cells (1), cells with knockdown of ProF by ProF KD1 (2), and cells cotransduced with ProF KD1 and dominant-negative Foxo1, FoxoDN, at days 0 and 7 of differentiation (3). (**B**) Triglyceride content of control cells (1), ProF KD1 cells (2), and cells cotransduced with ProF KD1 and FoxoDN (3) was measured spectrophotometrically at the indicated days of differentiation. Data are mean values ± s.d. of three individual points (NS = nonsignificant, $^{**}P<0.01$). (**C**) Control cells (1), ProF KD1 (2), and cells cotransduced with ProF KD1 and FoxoDN (3) were lysed at the indicated days. The expression of PPARγ was measured by densitometric scanning of PPARγ protein bands from immunoblots shown below (top). Data are mean values ± s.d. of three individual points (NS = nonsignificant, $^{**}P<0.01$). One representative blot shows protein expression, performed by immunoblotting with the indicated antibodies. (**D**) Triglyceride content of control cells (1), ProF KD1 cells (2), and cells cotransduced with ProF KD1 and m/p-Akt1 (3) was measured spectrophotometrically at day 7 of differentiation. Data are mean values ± s.d. of three individual points (NS = nonsignificant, $^{**}P<0.01$, relative to controls, $^{\#}P<0.05$, relative to ProF KD1). (**E**) Control cells (1), ProF KD1 cells (2), and cells cotransduced with ProF KD1 and m/p-Akt1 (3) were lysed at day 7 of differentiation. The expression of PPARγ was measured by densitometric scanning of PPARγ protein bands from immunoblots (left). Data are mean values ± s.d. of three individual points (NS = nonsignificant, $^{**}P<0.01$, relative to control cells, NS = nonsignificant, relative to ProF KD1). One representative blot shows the protein expression (right). (**F**) Model for the role of ProF in adipogenesis. Stimulation of cells by insulin triggers a signal transduction cascade, which leads to phosphorylation (P) and activation of Akt. ProF interacts with (P)-Akt (Fritzius *et al*, 2006) and Foxo1 (Figure 7) in a trimeric complex and facilitates the phosphorylation of the anti-adipogenetic transcription factor Foxo1 (Figure 6). Phosphorylated Foxo1 loses its DNA binding ability and becomes inactive as a transcription factor (grey cross), which allows expression of PPARγ (Figure 3) and thus adipocyte differentiation (Figure 2).

5′-GCTCTAGAGCCACCATGGGCTGCGTTTGCTCGTCGAATCC-3′; reverse primer: 5′-TTGGCGCGCCTCTCCATCCCTCCAAGCTATC-3′. Subsequently, the amplicon was digested with *Asc*I and *Xba*I restriction enzymes to excise Flag–FoxoΔ259 or HA–m/p-Akt1. FUW–Flag–FoxoDN or FUW–HA–m/p-Akt1 was cloned using cFUW linearized with *Asc*I and *Xba*I and the *Asc*I–*Xba*I fragment of Flag–FoxoΔ259 or HA–m/p-Akt1. Essentially, all viral transductions were performed as described earlier (Fritzius *et al*, 2006), yielding a pool of shRNA- or construct-expressing cells.

Adipocyte differentiation

For adipocyte differentiation, early-passage 3T3-L1 fibroblasts were grown in growth medium (DMEM supplemented with 10% FCS) to confluency followed by medium change. After 72 h, at day 0, differentiation was induced by growth medium supplemented with 166 nM insulin, 0.1 μg/ml DMX, and 112 μg/ml IBMX. Daily replacement of the hormonal induction medium was performed for 3 days. Then, at day 3, the medium was replaced by growth medium containing 166 nM insulin. For all subsequent experiments, days 1–3 after induction were considered as early differentiation, days 4–6 as late differentiation, and day 7 and later as terminal differentiation. To obtain differentiated HWP-c, HWP-c were first grown in growth medium (PromoCell) until confluence, followed by preadipocyte differentiation medium (PromoCell) for 72 h and adipocyte nutrition medium (PromoCell) for 2 weeks, following the supplier's instructions. After 17 days, cells were considered to be terminally differentiated.

C_2C_{12} myotube differentiation

For differentiation, early-passage C_2C_{12} myoblast cells were grown under high-serum conditions (DMEM supplemented with 20% FCS) to confluency. At day 0, differentiation was induced by low-serum-containing growth medium (DMEM supplemented with 2% horse serum (Invitrogen)) and incubated for 5 days without further medium change.

Oil Red O staining and triglyceride content determination

For Oil Red O staining, cells were washed two times with PBS for 3 min. After fixation with 5% paraformaldehyde in PBS for 15 min at room temperature, cells were washed three times with PBS for 3 min each. Then, a 0.08% (w/v) dilution of Oil Red O (Sigma) in 60% isopropanol was applied for 1 h at room temperature with continuous shaking. Subsequently, the cells were washed three times with PBS and analysed by light microscopy.

For quantification of lipid content, triglycerides were extracted with 20% isopropanol containing 5% SDS. The amount of triglyceride was measured spectrophotometrically at 518 nm.

RT–PCR

RNA was extracted using TRI Reagent and RT–PCR was performed with 1 μg of total RNA. Primers for murine ProF were as follows: forward (nt 658–678): GATCACTCTGTCATCATGTGG; reverse (nt 894–915): CTTACTGTCCCACATTTGCTTG. Primers for the late differentiation markers C/EBPα and PPARγ were designed as described (Jensen *et al*, 2004): for C/EBPα: forward (nt) AGGTGCTGGAGTTGACCAGT, reverse (nt) CAGCCTA GAGATCCAGCG; for PPARγ: forward (nt) CCAGAGCATGG TGCCTTCGCTG, reverse (nt) CAGCAACCATTGGGTCAGCTC; for GLUT4: forward (nt) CTTCTTTGAGATTGGCCCTGG, reverse (nt) AGGTGAAGATGAAGAAGCCAAGC. Primers for murine β-actin as an internal control were described earlier (Fritzius *et al*, 2006). Primers for first-strand cDNA were synthesized at 48°C for 45 min and amplified by 2 min at 94°C and 22 cycles (for β-actin) or 30 cycles (for ProF) for 30 s at 94°C, 1 min at 68°C, and 2 min at 68°C.

Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblot analysis were performed as described earlier (Fritzius *et al*, 2006). Briefly, cells were lysed in NETN buffer containing 100 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.5% Nonidet P-40 (NP-40), and 1 mM EDTA. Lysates were solubilized by shaking for 15 min at 4°C. The protein complexes were studied as described earlier (Fritzius *et al*, 2007). Murine tissues were extracted and homogenized as described previously (Fritzius *et al*, 2006).

Deoxyglucose uptake measurements

Differentiated adipocytes (day 7) were starved for 20 h in DMEM containing 0.5% FCS, incubated for 30 min at 37°C with 1 ml of DMEM containing 100 nM insulin, washed three times with 2 ml of warm HEPES-buffered Krebs-Ringer phosphate buffer (120 mM NaCl, 5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 30 mM HEPES, pH 7.2), and incubated for 10 min in the same buffer in the presence of 1 ml of 1 μCi/ml 2-DG (Amersham) with a specific activity of 0.8 Ci/mmol. Cells were lysed in radioimmunoprecipitation assay buffer as described (Fritzius *et al*, 2006) and lysates were subjected to scintillation counting analysis for measurement of 2-DG uptake.

Subcellular fractionation

Cells were lysed in ice-cold cytoplasmic extraction buffer (0.5% NP-40, 10 mM Tris–HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA, 1 mM DTT, and 3 mM $MgCl_2$) and then incubated for 6 min on ice with vortexing every 2 min, followed by low-speed centrifugation (1100 *g*) for 5 min. The pellet was washed with cytoplasmic extraction buffer without NP-40, centrifuged (1100 *g*) for 5 min, resuspended in nuclear extraction buffer (20 mM HEPES–KOH (pH 7.9), 350 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 1.5 mM $MgCl_2$, 10 mM NaF, 25% glycerol), incubated on ice for 15 min with occasional vortexing every 5 min, and thereafter centrifuged at 16 000 *g* for 15 min at 4°C.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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