2017

PexRAP inhibits PRDM16-mediated thermogenic gene expression

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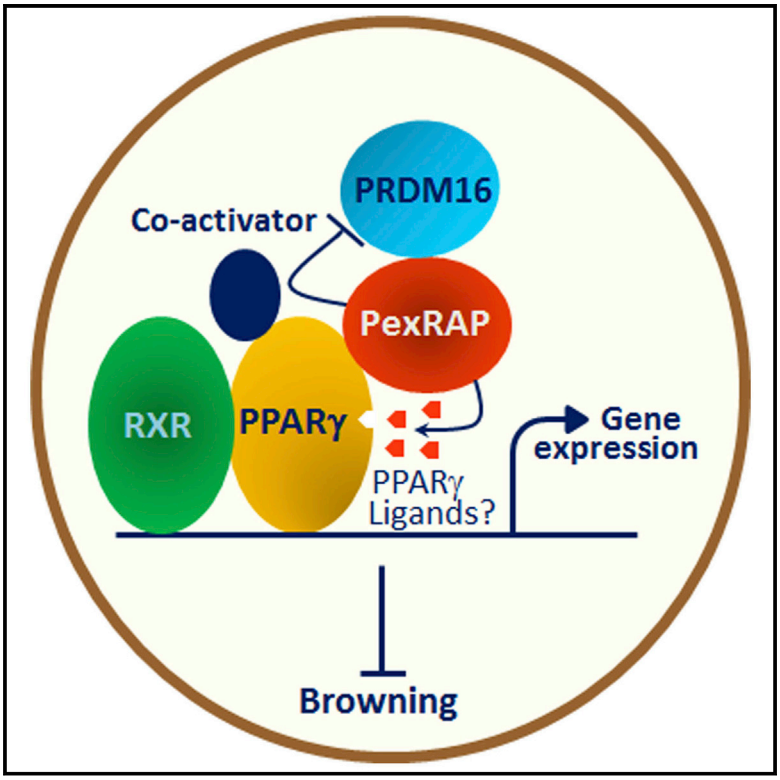
Graphical Abstract



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In Brief

Lodhi et al. find that the peroxisomal lipid synthetic enzyme PexRAP is also a nuclear protein that suppresses adipose tissue browning. PexRAP interacts with PPARγ and PRDM16 and inhibits PRDM16-mediated thermogenic gene expression.

Highlights

- PexRAP, a peroxisomal lipid synthetic enzyme, is also localized in the nucleus
- PexRAP protein levels are high in WAT but low in BAT
- Knockout of PexRAP in mice promotes adipose tissue browning
- PexRAP interacts with PPARγ and PRDM16 and disrupts PRDM16-mediated gene expression

Lodhi et al., 2017, Cell Reports *20*, 2766–2774
September 19, 2017
http://dx.doi.org/10.1016/j.celrep.2017.08.077

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Report

PexRAP Inhibits PRDM16-Mediated Thermogenic Gene Expression

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http://dx.doi.org/10.1016/j.celrep.2017.08.077

SUMMARY

How the nuclear receptor PPARγ regulates the development of two functionally distinct types of adipose tissue, brown and white fat, as well as the browning of white fat, remains unclear. Our previous studies suggest that PexRAP, a peroxisomal lipid synthetic enzyme, regulates PPARγ signaling and white adipogenesis. Here, we show that PexRAP is an inhibitor of brown adipocyte gene expression. PexRAP inactivation promoted adipocyte browning, increased energy expenditure, and decreased adiposity. Identification of PexRAP-interacting proteins suggests that PexRAP function extends beyond its role as a lipid synthetic enzyme. Notably, PexRAP interacts with importin-β1, a nuclear import factor, and knockdown of PexRAP in adipocytes reduced the levels of nuclear phospholipids. PexRAP also interacts with PPARγ, as well as PRDM16, a critical transcriptional regulator of thermogenesis, and disrupts the PRDM16-PPARγ complex, providing a potential mechanism for PexRAP-mediated inhibition of adipocyte browning. These results identify PexRAP as an important regulator of adipose tissue remodeling.

INTRODUCTION

The continued global rise in obesity and its associated insulin resistance and diabetes calls for alternative treatment approaches. Targeting adipose tissue function to decrease adiposity and improve insulin sensitivity represents such an approach. Adipose tissue is a complex organ that regulates whole-body energy balance (Rosen and Spiegelman, 2014). Two major types of adipose tissue are found in mammals: white fat and brown fat. White adipose tissue (WAT) primarily stores fat, which can be mobilized in times of need, whereas brown adipose tissue (BAT) transforms the chemical energy in food into heat through uncoupled respiration. In addition to classical brown adipocytes, clusters of brown adipocyte-like beige cells appear in subcutaneous WAT in response to prolonged cold exposure or beta adrenergic signaling (Wang and Seale, 2016). Brown and beige adipocytes are characterized by their expression of uncoupling protein-1 (UCP1), a long-chain fatty acid/H^+ symporter (Fedorenko et al., 2012) that mediates thermogenesis by allowing protons to leak across the inner mitochondrial membrane, bypassing ATP synthase. By promoting thermogenesis, this uncoupling of oxidation from ATP production increases energy expenditure. Thus, exploiting the thermogenic capacity of brown and beige adipocytes represents a potential strategy for treating obesity and diabetes.

Thermogenic gene expression in both types of UCP1-positive adipocytes is regulated by PRDM16, a large zinc finger-containing transcription factor (Cohen et al., 2014; Harms et al., 2014; Seale et al., 2007). Overexpression of PRDM16 in adipocyte precursors or in mice promotes brown gene expression (Seale et al., 2008). Conversely, adipose-specific deletion of PRDM16 inhibits browning of subcutaneous WAT and promotes obesity and insulin resistance (Cohen et al., 2014). PRDM16 does not appear to be required for embryonic development of the classical BAT but is necessary to repress white fat-selective genes within BAT and to maintain brown adipocyte function in adult mice (Harms et al., 2014; Ohno et al., 2013). PRDM16 regulates thermogenic gene expression by interacting with and modulating the activity of other transcription factors, including C/EBPβ, PGC1α, PPARα, and PPARγ (Kajimura et al., 2009; Seale et al., 2008).

The ligand-activated nuclear receptor PPARγ is a critical transcriptional regulator of both white and BAT development (Ahmadian et al., 2013). Ligand binding induces a conformational change in PPARγ, promoting dissociation of transcriptional repressors and recruitment of co-activators, resulting in activation of target gene expression. Because PPARγ can regulate development of the two functionally distinct types of adipose tissue, as well as browning of WAT, it is likely that PPARγ is activated by multiple endogenous partial agonists that in turn recruit different transcriptional co-activators controlling unique subsets of genes.

Our previous efforts to understand the role of adipose tissue lipogenesis in PPARγ signaling led to the identification of PexRAP (peroxisomal reductase activating PPARγ), a peroxisomal membrane protein that synthesizes ether-linked phospholipids, potential partial agonists for PPARγ (Lodhi et al., 2012). Here, we demonstrate that PexRAP is a multifunctional

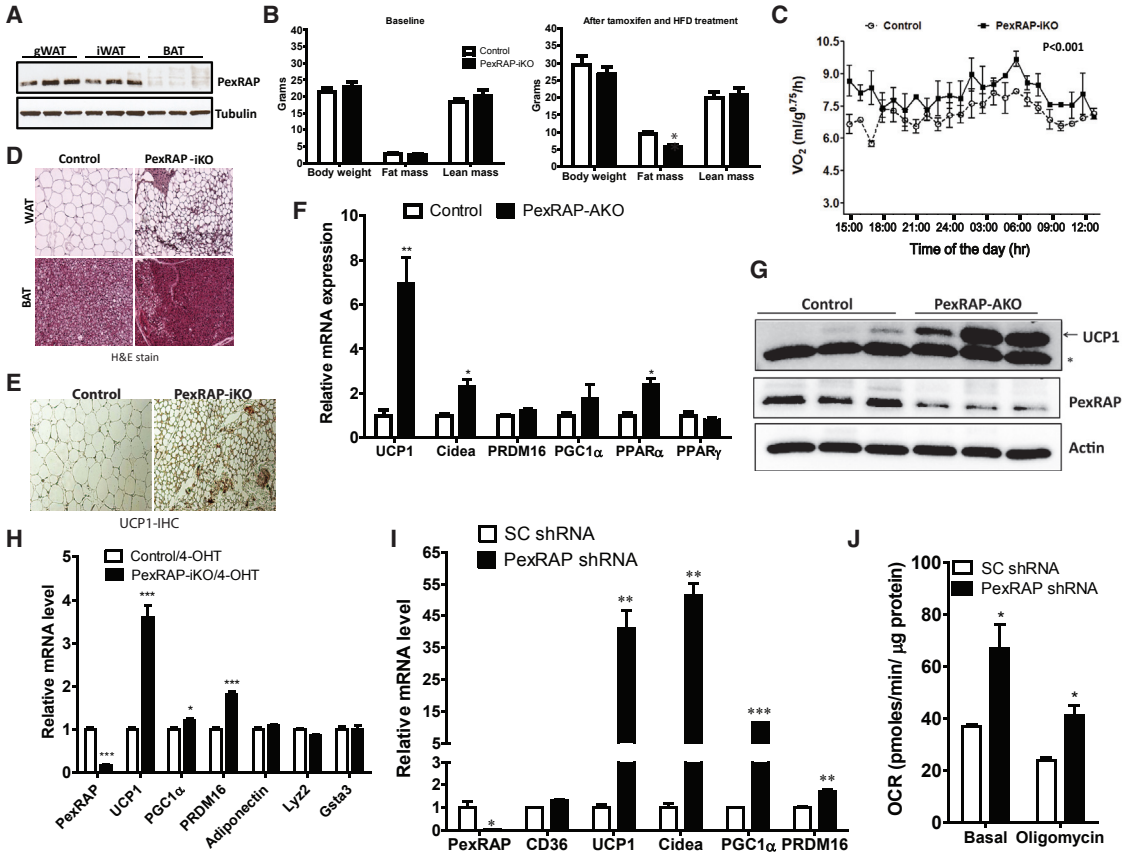

Figure 1. PexRAP Conditional Knockout Mice Have Increased Metabolism and Browning of Subcutaneous Adipose Tissue

(A) Western blot analysis of PexRAP expression in different adipose depots of wild-type (WT) C57 mice.

(B–E) PexRAP-iKO mice. (B) Body weight and body composition of control and PexRAP-iKO male mice at baseline and after tamoxifen treatment and 6 weeks of high-fat diet (HFD) (n = 4). (C) Oxygen consumption (V_{O_2}) by indirect calorimetry in HFD-fed mice (n = 4). (D) H&E staining of gWAT and BAT. (E) Immunohistochemical analysis of UCP1 expression in iWAT.

(F and G) PexRAP-AKO mice. (F) Gene expression in iWAT of control and PexRAP-AKO female mice following 3 day cold exposure (n = 3). (G) Western blot analysis of iWAT from cold-treated mice. *Non-specific band.

(H) Primary iWAT stromal vascular cells from control and PexRAP-iKO mice were treated with 3 μM 4-hydroxytamoxifen upon reaching 90% confluence. After 4 days, the cells were subjected to brown adipogenesis and analyzed for gene expression (n = 4).

(I and J) Gene expression analysis (I) and oxygen consumption rate (J) of PPARγ-MEFs subjected to brown adipogenesis after treatment with lentiviral SC or PexRAP shRNA (n = 5 or 6).

*p < 0.05, **p < 0.01, and ***p < 0.001. The results are presented as mean ± SEM. See also Figures S1 and S2.

protein that appears to act as a molecular switch to repress adipocyte browning.

RESULTS

PexRAP Expression Profile

We previously identified PexRAP as a protein involved in adipogenesis that is enriched in cellular fractions containing peroxisomal markers such as PMP70 and catalase (Lodhi et al., 2012). To determine the relative distribution of PexRAP in different adipose tissue depots, we isolated inguinal and gonadal WAT (iWAT and gWAT, respectively) as well as BAT from wild-type C57 mice fed chow diet (Figure 1). Western blot analysis indicated that the PexRAP protein was expressed in WAT depots, but its levels in BAT were low (Figure 1A). PexRAP protein levels were also decreased in iWAT of mice subjected to cold exposure for 1 week (Figure S1A). These data suggest that PexRAP might be a negative regulator of thermogenic gene expression and is maintained at low levels during BAT development or cold-induced browning of WAT.

Generation of PexRAP Knockout Mice

To determine its role in adiposity and metabolism, PexRAP (encoded by *Dhrs7b*) was knocked out in mice (Figure S1) using a targeting vector obtained from the EUCOMM repository (Figures S1B and S1C). Mice homozygous for the "knockout-first" allele manifested embryonic lethality, with only a small number of animals surviving into adulthood. Heterozygotes were viable and fertile and had no overt phenotype. Mass spectrometric analysis of lipid extracted from the postnuclear fraction of WAT

homogenate indicated a preferential decrease in phosphatidylcholine species with arachidonic acid at the *sn-2* position of the glycerol backbone in the knockout mice (Figure S1D). Ether-linked phospholipids, including plasmalogens, were present in low abundance in this fraction of the adipose tissue. The single plasmalogen species (p16:0/20:4-GPC) detected with an *m/z* ratio of 766.6 was decreased in the knockout (Figure S1D).

PexRAP Knockout Mice Have Increased Browning of Subcutaneous WAT

The surviving homozygous knockout mice were visibly smaller in size. Body weight measured in 1-month-old male knockout mice was approximately one-third less than in WT mice (Figure S1E). Gene expression analysis of gWAT and iWAT showed depot-specific effects of the knockout (Figure S1F). In gWAT, PexRAP knockout resulted in decreased expression of PPARγ and its target genes. In iWAT, PexRAP inactivation resulted in a striking increase in UCP1. PGC1α, Cidea, and PPARα messages were also increased, but PPARγ expression was minimally affected in iWAT (Figure S1F). Histologic analysis confirmed increased UCP1 expression in iWAT (Figure S1G). Together, these results suggest that PexRAP inactivation results in browning of subcutaneous WAT.

To study the effect on adipose tissue remodeling in detail, we converted the knockout-first allele into a conditional allele and crossed the floxed animals with Rosa-CreER mice to generate tamoxifen-inducible global PexRAP knockout (PexRAP-iKO) animals. We recently reported that PexRAP expression was decreased in multiple tissues of tamoxifen-treated PexRAP-iKO mice, including liver, bone marrow, and WAT, but not in the brain. Adult PexRAP-iKO mice treated with tamoxifen survive but have leukopenia because of the nearly complete loss of neutrophils (Lodhi et al., 2015).

Body composition analysis by EchoMRI indicated that PexRAP-iKO had significantly reduced adiposity when subjected to high fat feeding (Figure 1B). The decreased adiposity was observed despite hyperphagia (Figure S2A). Indirect calorimetry indicated that the knockout mice have elevated energy expenditure (Figure 1C). Histologic analysis showed that white adipocytes were smaller in PexRAP-iKO mice, and brown adipocytes had marked depletion of lipid droplets (Figure 1D). Morphometric analysis of the gonadal adipose depot confirmed that adipocyte size and number were significantly decreased in PexRAP-iKO mice compared with control animals (Figures S2B and S2C). Immunohistologic analysis using an anti-UCP1 antibody indicated robust browning of iWAT following tamoxifen-inducible knockout of PexRAP (Figure 1E). Together, these data suggest that knockout of PexRAP in precursor cells reprograms adipose tissue development and function.

To determine if the increased browning observed with PexRAP deficiency was due to effects intrinsic to adipose tissue, we generated adipose-specific PexRAP knockout (PexRAP-AKO) mice (Figures 1F and 1G) by crossing the floxed mice with adiponectin-Cre transgenic mice (Eguchi et al., 2011). PexRAP-AKO inactivation had no effect on body weight or body composition in mice fed chow diet or a high fat diet (not shown), perhaps suggesting that the protein is more important during an earlier stage of fat development and less so in mature adipose tissue. Adipose PexRAP deficiency also did not alter thermogenic gene expression at room temperature (not shown). However, when subjected to cold exposure for 3 days, PexRAP-AKO mice manifested a 7-fold increase in UCP1 expression in iWAT. Other markers of brown adipocytes, including Cidea and PPARα, were also significantly increased (Figure 1F). Western blot analysis confirmed the increased UCP1 and decreased PexRAP expression in knockout mice (Figure 1G).

Cell-Autonomous Effect of PexRAP Inactivation on Thermogenic Gene Expression

To determine if the effect of PexRAP inactivation on thermogenic gene expression was cell autonomous, we collected the stromal vascular fraction from iWAT of PexRAP-iKO and control mice and treated the cells with 4-hydroxytamoxifen (4-OHT) to knock out PexRAP. Following differentiation of these cells using a pro-brown adipogenesis cocktail (Fisher et al., 2012), thermogenic genes, such as UCP1, PGC1α, and PRDM16, significantly increased, whereas other adipocyte genes, such as adiponectin and white adipocyte-enriched genes, such as Lyz2 and Gsta3 (Siersbæk et al., 2012), were unchanged (Figure 1H). Knockdown of PexRAP in HIB1b brown preadipocytes (Figure S2D) or in PPARγ-overexpressing immortalized mouse embryonic fibroblasts (PPARγ-MEFs) (Figure 1I) using lentiviral short hairpin RNA (shRNA), a separate approach, also strikingly increased thermogenic gene expression. Basal and uncoupled rates of respiration were significantly increased with PexRAP knockdown in PPARγ-MEFs (Figure 1J), suggesting that the increase in thermogenic gene expression is physiologically relevant. Together, these data suggest that PexRAP is a cell autonomous regulator of UCP1 gene expression.

Identification of PexRAP-Interacting Proteins in Adipocytes Using Stable Isotope Labeling with Amino Acids of Cells in Culture

To understand the molecular mechanism of PexRAP function in adipocytes, we used SILAC (stable isotope labeling with amino acids of cells in culture), a mass spectrometry-based proteomics approach (Trinkle-Mulcahy et al., 2008), to identify PexRAP-interacting proteins in 3T3-L1 adipocytes, using the strategy depicted in Figure 2A. A complete list of interacting proteins identified by this approach is presented in Table S1. Analysis of protein-protein interactions using the STRING database (version 10.0) revealed that many of the potential PexRAP-interacting proteins interact with one another (Figure 2B), suggesting that some of these were probably pulled down as components of multi-protein complexes and are not necessarily direct binding partners of PexRAP. The interacting proteins were broadly categorized into several functional groups, including lipid and glucose metabolism, protein translation and targeting, and protein degradation (Figure 2C). The identification of proteins such as PMP70 (Abcd3), HSD17b4 (peroxisomal D-bifunctional protein), and AGPAT9 as PexRAP-binding proteins reinforced our notion that PexRAP is a peroxisomal protein involved in lipid metabolism. The pathway of PexRAP-mediated phospholipid synthesis starts in peroxisomes and is completed in the ER.

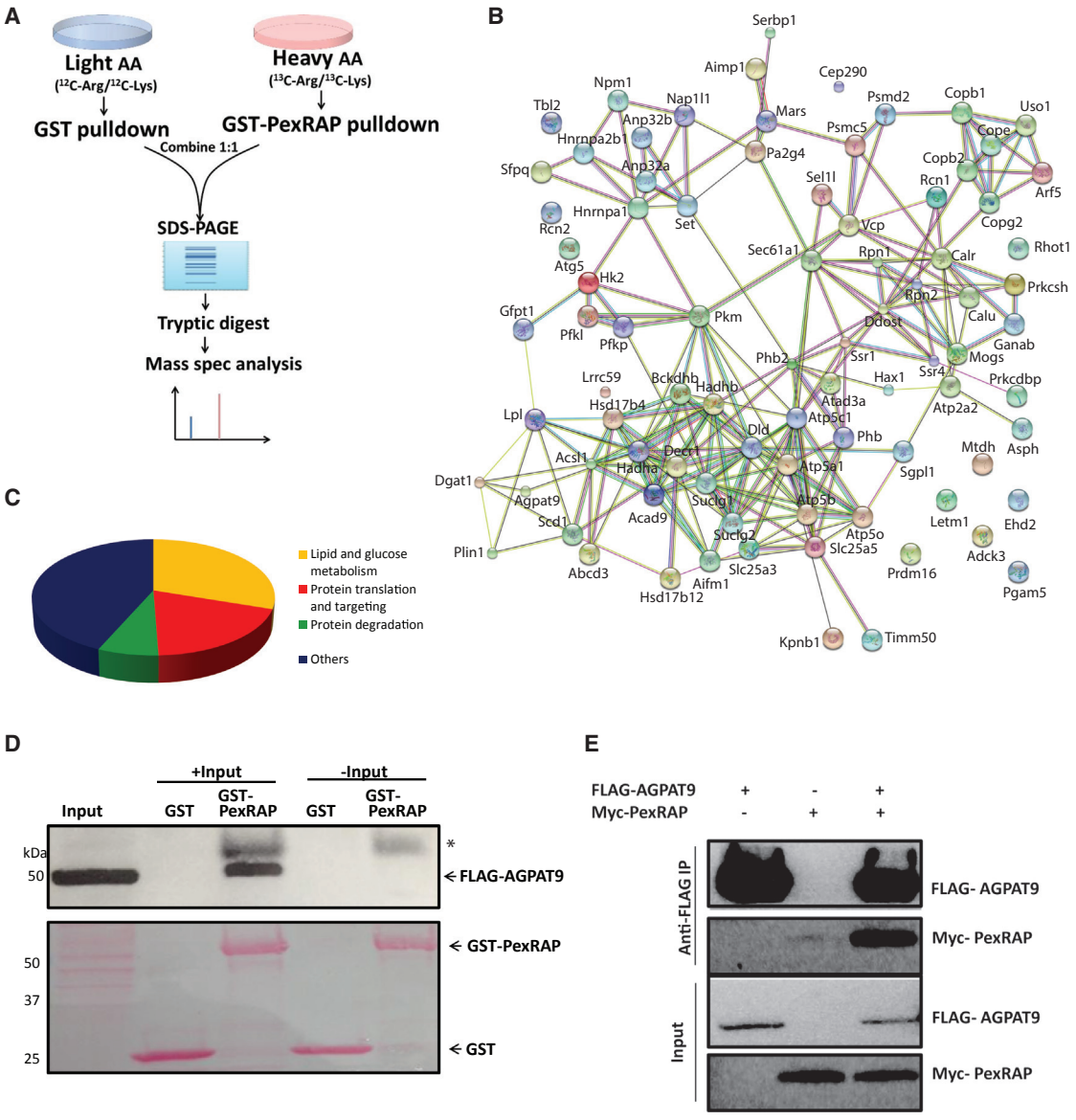

Figure 2. Identification of PexRAP-Interacting Proteins by SILAC in Adipocytes

(A) Strategy to identify PexRAP-interacting proteins using SILAC.

(B) STRING analysis of the proteins identified by SILAC.

(C) Classification of PexRAP-binding proteins in adipocytes.

(D) Pull-down assay of FLAG-AGPAT9 using GST-PexRAP. *GST-PexRAP band non-specifically detected by the FLAG antibody.

(E) Co-immunoprecipitation of myc-PexRAP with FLAG-AGPAT9.

See also Table S1.

PexRAP catalyzes the final peroxisomal step, generating lysophosphatidic acid (LPA) or its ether lipid equivalent, 1-*O*-alkyl glycerol 3-phosphate (AGP) (Dean and Lodhi, 2017). The identity of proteins that catalyze the subsequent steps is unclear. AGPAT9 is an ER-localized acylglycerolphosphate acyltransferase that might catalyze the next step in the PexRAP pathway. We confirmed the interaction of PexRAP with AGPAT9 using GST pull-down and co-immunoprecipitation assays (Figures 2D and 2E).

Nuclear Localization of PexRAP

Although these interactions might be predicted, we also found unexpected potential binding proteins. For example, the interaction with importin-β1 (Kpnb1), a karyopherin that binds to a non-classical nuclear localization sequence (NLS), was surprising and suggested that PexRAP may also be localized in the nucleus. Sequence analysis revealed a non-classical NLS in PexRAP similar to the importinβ-interacting NLS found in parathyroid hormone-related protein (PTHrP) (Cingolani et al., 2002)

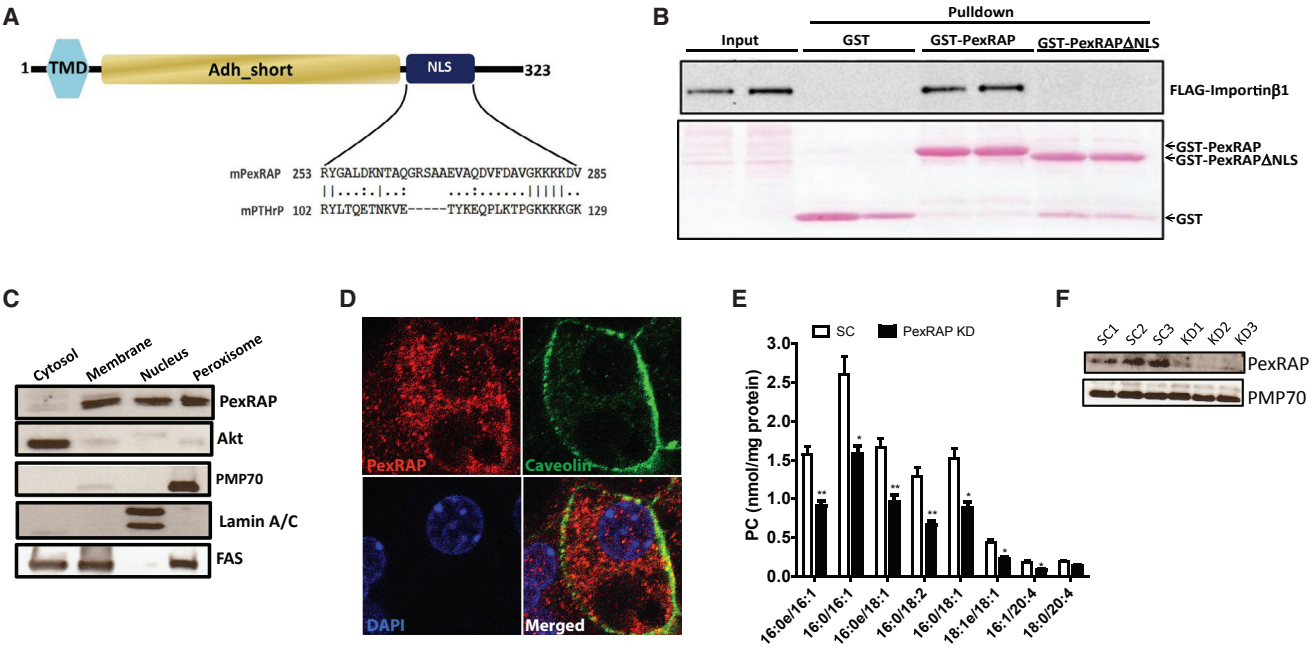

Figure 3. Nuclear Localization of PexRAP

(A) Identification of a non-classical NLS in PexRAP sharing homology with a known importin-β1-interacting NLS identified in PTHrP.

(B) GST-pull-down of FLAG-tagged importin-β1 using WT and ΔNLS-PexRAP.

(C) Subcellular fractionation of 3T3-L1 adipocytes.

(D) Immunofluorescence analysis using anti-PexRAP and anti-caveolin antibodies in PPARγ-MEFs differentiated into adipocytes.

(E) Mass spectrometric analysis of nuclear PC species in differentiated PPARγ-MEFs treated with scrambled or PexRAP shRNA. The "e" denotes ether-linked lipid species. *$p < 0.05$ and **$p < 0.01$; $n = 3$. The results are presented as mean ± SEM.

(F) Western blot analysis of PexRAP knockdown in the cells used in E.

(Figure 3). To determine if this sequence is required for interaction with importin-β, we generated a ΔNLS mutant of PexRAP. Although GST-tagged wild-type PexRAP interacted with importin-β, internal deletion of the NLS completely abolished the interaction (Figures 3A and 3B). Subcellular fractionation of 3T3-L1 adipocytes indicated that PexRAP is present in the nucleus in addition to its expected enrichment in peroxisome and membrane fractions (Figure 3C). Immunofluorescence analysis using an anti-PexRAP/DHRS7b antibody in differentiated PPARγ-MEFs revealed punctate cytosolic staining typically observed for peroxisomal proteins as well as nuclear staining (Figure 3D). To determine the role of PexRAP in nuclear phospholipid synthesis, we knocked down its expression in fully differentiated PPARγ-MEFs. Mass spectrometric analysis of lipids extracted from the nuclear fraction indicated that phosphatidylcholine (PC) levels were significantly reduced with PexRAP knockdown (Figures 3E and 3F). Interestingly, alkyl ether-linked PCs were abundantly present in the nucleus and reduced following PexRAP knockdown. Together, these data suggest that in addition to its localization in peroxisomes, PexRAP is present in the nucleus and may be involved in nuclear phospholipid synthesis.

PexRAP Interacts with PRDM16 and PPARγ and Inhibits Their Interaction with Each Other

Another surprise was the identification of PRDM16 as a potential PexRAP-binding protein (Figure 4). Because PRDM16 is expressed at very low levels in 3T3-L1 adipocytes (Seale et al., 2007), this is likely a specific interaction. We confirmed the interaction by western blot analysis of tagged PRDM16 pulled down with GST-PexRAP (Figure 4A). Using various GST-tagged fragments of PRDM16, we mapped a region encompassing residues 680–880 as the site of interaction with PexRAP (Figure 4B). Interestingly, this is also the region of PRDM16 through which it interacts with CtBP1 to repress white adipocyte genes (Kajimura et al., 2008).

PRDM16 regulates thermogenesis by interacting with and co-activating PPARγ and PPARα (Kajimura et al., 2009). Because PexRAP deficiency increases thermogenic gene expression, we hypothesized that PexRAP sequesters PRDM16 away from these nuclear receptors. While testing this possibility, we discovered that PexRAP itself directly interacts with the ligand-binding domains of PPARγ and PPARα, the interaction being stronger with PPARγ than PPARα (Figure 4C). Given these interactions, it is possible that PexRAP may be a transcriptional cofactor of PPARs.

Transcriptional cofactors frequently contain a leucine-rich stretch of amino acids called the LxxLL motif (Bulynko and O'Malley, 2011; Plevin et al., 2005). This motif is involved in interaction with the activation function-2 (AF-2) region of ligand-binding domain in nuclear receptors (Plevin et al., 2005). Sequence analysis of PexRAP revealed an LxxLL motif (^{38}LFRLL^{42} in the mouse sequence) near the N terminus. Internal deletion of these

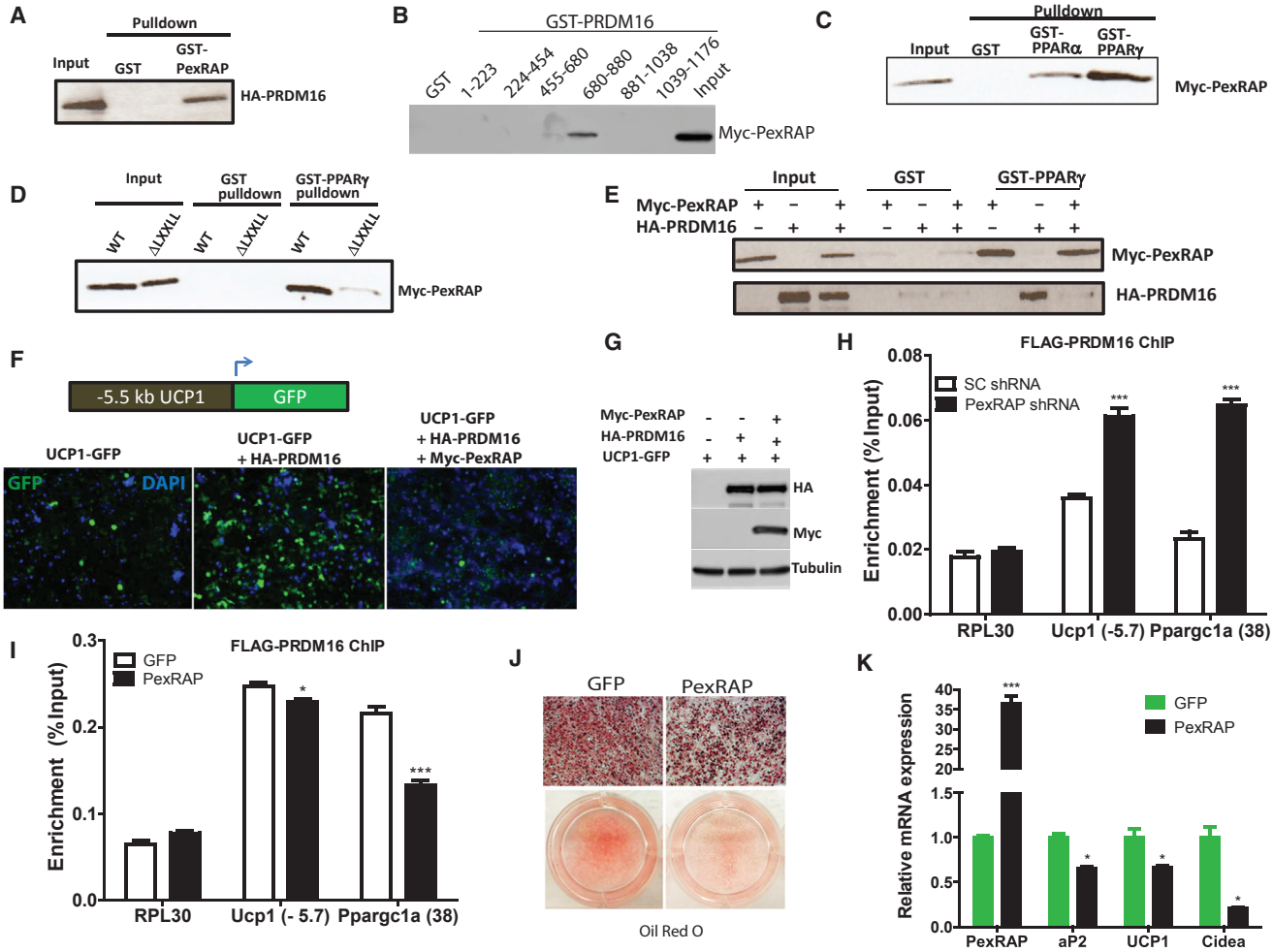

Figure 4. PexRAP Interacts with PRDM16 and PPARγ and Disrupts PRDM16-Mediated Gene Expression

(A) HA-PRDM16 pull-down using GST-PexRAP.

(B) Myc-PexRAP pull-down using various GST-tagged fragments of PRDM16.

(C) Myc-PexRAP pull-down using GST-LBD of PPARα and PPARγ.

(D) Deletion of an LxxLL motif in PexRAP abolishes the interaction with PPARγ.

(E) PexRAP competes with PRDM16 for interaction with PPARγ.

(F) Fluorescence microscopy analysis of 293T cells transfected with a GFP under the control of a −5.5 kb UCP1 promoter alone or together with HA-PRDM16 and Myc-PexRAP. A schematic of the UCP1-GFP construct is shown.

(G) Western blot analysis of protein expression of 293T cells in (F).

(H and I) Immortalized BAT SV cells expressing retrovirally encoded FLAG-PRDM16 were treated with scrambled or PexRAP shRNA (H) or with lentivirus expressing GFP or PexRAP (I). Following treatment with a brown adipogenesis cocktail for 2 days, chromatin immunoprecipitation was performed using an anti-FLAG antibody, followed by qPCR using primers for the indicated genes. N = 6.

(J) Oil red O staining in BAT SV cells differentiated into brown adipocytes following overexpression of lentiviral-encoded GFP or PexRAP.

(K) Gene expression analysis in the cells in (J) (n = 3).

*p < 0.05 and ***p < 0.001. The results are presented as mean ± SEM. See also Figure S3.

five residues in PexRAP abolished the interaction with PPARγ (Figure 4D), strongly suggesting a physiologically relevant PexRAP/PPARγ interaction.

To determine if PexRAP affects the interaction of PRDM16 with PPARγ, we ectopically expressed tagged PexRAP or PRDM16 alone or together and conducted pull-down assays using GST or GST-PPARγ. When either protein was expressed alone, it interacted with PPARγ. However, when PexRAP and PRDM16 were co-expressed, PexRAP inhibited the ability of PRDM16 to interact with PPARγ (Figure 4E).

PexRAP Regulates PRDM16-Mediated Transcriptional Activity

To determine whether PexRAP affects PRDM16-mediated UCP1 gene expression, we generated a GFP reporter driven by a −5.5 kb mouse UCP1 promoter (Figure 4F). Recently

ZFP516, a transcriptional activator of UCP1, was shown to drive the expression of a similar UCP1-GFP reporter in 293FT cells (Dempersmier et al., 2015). As shown in Figure 4F, overexpression of PRDM16 also strikingly promoted the expression of the UCP1-GFP in 293T cells. However, co-expression of PexRAP with PRDM16 markedly decreased the expression of the reporter, suggesting that PexRAP inhibits PRDM16-mediated thermogenic gene expression (Figure 4F). Western blot analysis of the overexpressed proteins is shown in Figure 4G.

To determine if PexRAP directly regulates recruitment of PRDM16 to brown adipocyte genes, we generated BAT SV cells that stably express FLAG-PRDM16 (Figure S3A) and performed chromatin immunoprecipitation (ChIP) assays using an anti-FLAG antibody followed by qPCR. Whereas knockdown of PexRAP in these cells significantly increased recruitment of PRDM16 to *Ppargc1a* and *Ucp1* (Figure 4H), lentivirus-mediated overexpression of PexRAP markedly decreased binding of PRDM16 to *Ppargc1a* and modestly but significantly decreased binding to *Ucp1* (Figure 4I). Western blot analysis of PexRAP knockdown or overexpression in these cells is shown in Figures S3B and S3C.

Next, we determined whether PexRAP overexpression affects thermogenic gene expression in BAT SV cells isolated from wild-type mice. Although overexpression of PexRAP in these cells following adipogenesis did not affect gene expression (Figure S3D), its overexpression prior to differentiation impaired adipogenesis and reduced thermogenic gene expression (Figures 4J and 4K). This is consistent with the possibility that PexRAP is likely involved in an earlier step of BAT development. The inhibition of brown adipogenesis with PexRAP overexpression suggests that the protein might have other functions independent of directly inhibiting PRDM16 function, perhaps modulating PPARγ activation.

DISCUSSION

Because beige fat might counteract obesity, adipose tissue remodeling has drawn considerable interest. Several factors have been identified that promote brown adipogenic programs, including PRDM16 and Ebf2 (Cohen et al., 2014; Harms et al., 2014; Rajakumari et al., 2013). Similarly, other factors have also been identified that inhibit brown and beige adipogenesis, including Rb/p107, MRTFA, TLE3, and Zfp423 (De Sousa et al., 2014; Hansen et al., 2004; McDonald et al., 2015; Shao et al., 2016; Villanueva et al., 2013). Previously, we identified PexRAP as a peroxisomal lipid synthetic enzyme that regulates adipogenesis (Lodhi et al., 2012). Here, we present evidence suggesting that PexRAP is a dual-function regulator of adipose tissue remodeling that inhibits thermogenic gene expression. Although PexRAP is abundantly present in WAT, PexRAP protein levels are very low in mature BAT. Inactivation of PexRAP in mice results in robust browning of subcutaneous white fat. Identification of PexRAP interacting proteins and characterization of its subcellular localization suggest that in addition to its role as a peroxisomal lipid synthetic enzyme, PexRAP is found in the nucleus, where it affects transcriptional regulation of adipose tissue browning. Together, these studies suggest that PexRAP might be part of a previously unrecognized peroxisome-to-nucleus regulatory circuit that controls adipose tissue remodeling.

PexRAP possesses a cryptic non-classical NLS through which it interacts with importin-β1. It is unclear whether PexRAP translocates from peroxisomes to the nucleus in response to a physiological stimulus or if there is a constitutively localized pool of PexRAP in the nucleus. There is precedent for transmembrane protein translocation to the nucleus in response to stimuli. For example, AEG-1 (astrocyte elevated gene-1), an endoplasmic reticulum (ER)-localized transmembrane protein, translocates to the nucleus in response to TNF-α treatment and functions as a transcriptional co-activator of nuclear factor κB (NF-κB) (Sarkar et al., 2008).

Our results suggest that nuclear localized PexRAP interacts with PRDM16 as well as PPARγ and prevents their interaction with each other, which could be the mechanism underlying the inhibitory role of PexRAP in adipocyte browning. However, it is possible that PexRAP interferes with PRDM16 function through various mechanisms. Although knockdown of PexRAP strikingly affects thermogenic gene expression, its overexpression in adipocytes has a relatively mild effect on brown fat genes. This presumably suggests that a cofactor might be required or other aspects of PexRAP function, including its lipid synthetic activity, might be involved in regulating gene expression.

TLE3, a white-selective transcriptional regulator, has been shown to inhibit brown adipocyte-selective gene expression by disrupting the interaction between PRDM16 and PPARγ (Villanueva et al., 2013). Moreover, the transcriptional regulator ZFP423 was recently shown to be important for maintaining white adipocyte identity by inhibiting thermogenic gene expression mediated by the brown-selective transcription factor Ebf2 (Shao et al., 2016). The need for seemingly redundant mechanisms for regulating adipose tissue remodeling remains unexplained. Perhaps these transcriptional regulators assume stage-specific roles during discrete steps in adipose tissue development. For example, Zfp423 is thought to function primarily at the level of the mature white adipocyte to inhibit thermogenic gene expression (Shao et al., 2016). Our studies suggest that PexRAP is likely more important during an earlier step of fat development. Notably, although ZFP423 can directly bind to DNA and regulate transcription, TLE3 and PexRAP lack DNA-binding domains and regulate transcription through their ability to interact with other transcription factors. TLE3 and PexRAP both interact with PPARγ, but PexRAP is unique in also possessing lipid synthetic activity that generates ether-linked phospholipids, potential partial agonists of PPARγ (Lodhi et al., 2012).

Although PPARγ could be activated by several different lipid ligands with variable effects on transcriptional activity, it is likely that the endogenous agonists of PPARγ in general are partial agonists that promote recruitment of distinct transcriptional cofactors controlling unique subsets of genes. This could be the mechanism allowing PPARγ to regulate the development of two functionally distinct types of adipose tissue, as well as the browning of WAT. In addition to the peroxisome-derived phospholipids that we identified, LPA and AGP, both products of PexRAP (Lodhi et al., 2012), have been reported to activate PPARγ (Stapleton et al., 2011; Tsukahara et al., 2006). The nuclear localization of PexRAP and its association with PPARγ

suggest that PexRAP might generate these lipid agonists locally, thus increasing efficacy and availability to PPARγ. Our results presented here and those of others (Albi and Viola Magni, 2004) suggest that ether lipids, including plasmalogens, are abundantly present in the nucleus.

PexRAP possesses transcriptional regulatory activity that extends beyond its established role as a peroxisomal lipid synthetic enzyme, suggesting a multifaceted role for the protein in adipose tissue remodeling. Pharmacological inhibition of PexRAP function in adipose tissue may be an appropriate strategy for treating obesity.

EXPERIMENTAL PROCEDURES

Animals

PexRAP (*DHRS7B*) knockout-first mice (C57BL/6J genetic background) were generated using a targeting vector obtained from the EUCOMM repository. Generation of PexRAP-iKO mice has been previously described (Lodhi et al., 2015). To induce knockout, 10- to 12-week-old control ($PexRAP^{lox/lox}$ without Cre) and PexRAP-iKO ($PexRAP^{lox/lox}$/Rosa-CreER) mice were treated daily with tamoxifen (50 μg/g body weight) for 5 consecutive days. To generate PexRAP-AKO animals, mice with a floxed PexRAP locus ($PexRAP^{lox/lox}$) were crossed with transgenic mice expressing Cre under the control of the adiponectin promoter (Eguchi et al., 2011). $PexRAP^{lox/lox}$ mice without Cre were used as a control for PexRAP-AKO mice. Genotyping was performed using previously described primer sets (Lodhi et al., 2015), and diets included PicoLab Rodent Diet 20 control chow and Harlan Teklad TD 88137 high-fat diet. Both male and female mice were used for experiments, as indicated in the figure legends. All protocols were approved by the Washington University Animal Studies Committee.

RT-PCR

Total RNA was extracted using PureLink RNA kit (Invitrogen) and reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). qPCR was performed using an Applied Biosystems StepOne Plus instrument using PowerUP SYBR Green reagent (Applied Biosystems). Prevalidated primers spanning exon-exon boundaries were used for amplifications. Assays were performed in duplicate and results were normalized to ribosomal protein L32 mRNA levels.

Pull-Down Assays

GST-pull-down assays were performed as previously described (Lodhi et al., 2012). Briefly, cells were lysed using a buffer containing 50 mM HEPES (pH 7.4), 4 mM EDTA, 2 mM EGTA, 2 mM sodium pyrophosphate, 1% Triton X-100, 10 mM NaF, and protease inhibitors (P8340; Sigma-Aldrich). Cell lysates were mixed with an equal volume of the same buffer containing no Triton X-100, then 5 μg of GST alone beads or GST fusion protein beads and rocked at 4C for 2 hr. Samples were spun down at 2,500 × *g* for 1 min and washed five times with lysis buffer containing 0.5% Triton X-100 before being resuspended in SDS sample buffer and subjected to SDS-PAGE.

Measurement of Oxygen Consumption Rate in Adipocytes

Oxygen consumption rate (OCR) in PPARγ-MEFs was measured using XF24 Extracellular Flux Analyzer with a FluxPak provided by Seahorse Bioscience. The cells were seeded and grown to confluence on XF24 cell culture microplates, followed by infection with lentiviral-encoded scrambled or PexRAP shRNA, as previously described (Lodhi et al., 2012). Media containing lentiviral shRNA were diluted 1:2 with fresh media prior to infection of cells. Eight days after the infection, the cells were exposed to browning cocktail to induce brown adipocyte differentiation for 6 days, as previously described (Fisher et al., 2012). OCR was measured at 3 min intervals at baseline and after the addition of oligomycin (3 μM final concentration).

UCP1-GFP Reporter Assay

To construct the UCP1-GFP reporter plasmid, a −5.5 kb UCP1 promoter was amplified by PCR using liver genomic DNA from a wild-type C57 mouse and cloned into the pCMV6-AC-GFP plasmid in place of the CMV promoter. HEK293T cells were grown on poly-l-lysine coated coverslips and transfected with either UCP1-GFP alone, UCP1-GFP and HA-PRDM16, or UCP1-GFP, HA-PRDM16, and Myc-PexRAP. Three days after transfection, coverslips were mounted and imaged with a Leica DMI4000B fluorescence microscope.

Statistics

Results are expressed as mean ± SEM. Comparisons between two groups were performed using two-tailed Student's t test. One-way ANOVA with Tukey's posttest was used for comparison of more than two groups. P values < 0.05 were considered to indicate statistically significant differences.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.08.077.

AUTHOR CONTRIBUTIONS

I.J.L. conceived the hypothesis, designed and conducted experiments, and wrote the manuscript. J.M.D. conducted experiments, interpreted data, and wrote the manuscript. A.H., H.P., M.T., and C.F. conducted experiments and interpreted data. H.S. performed SILAC analyses. F.-F.H. performed mass spectrometry analyses, assigned structures of lipid molecules, and interpreted data. C.F.S. designed experiments and wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by NIH grant R00 DK094874, startup funds from the Washington University Department of Medicine, and a Pilot & Feasibility Grant from the Washington University Diabetes Research Center (P30-DK020579) to I.J.L. and by NIH grants DK101392 and DK020579 to C.F.S. A.H. was supported by funds from the China Scholarship Council (201506140012).

Received: January 13, 2017
Revised: May 14, 2017
Accepted: August 23, 2017
Published: September 19, 2017

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