GPS2/KDM4A Pioneering Activity Regulates Promoter-specific Recruitment of PPARγ

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Abstract

Timely and selective recruitment of transcription factors to their appropriate DNA-binding sites represents a critical step in regulating gene activation; however the regulatory strategies underlying each factor’s effective recruitment to specific promoter and/or enhancer regions are not fully understood. Here, we identify an unexpected regulatory mechanism by which promoter-specific binding, and therefore function, of PPARγ in adipocytes requires G protein Suppressor 2 (GPS2) to prime the local chromatin environment via inhibition of the ubiquitin ligase RNF8 and stabilization of the H3K9 histone demethylase KDM4A/JMJD2. Integration of genome-wide profiling data indicates that the pioneering activity of GPS2/KDM4A is required for PPARγ-mediated regulation of a specific transcriptional program, including the lipolytic enzymes ATGL and HSL. Hence, our findings reveal that GPS2 exerts a biologically important function in adipose tissue lipid mobilization by directly regulating ubiquitin signaling and indirectly modulating chromatin remodeling to prime selected genes for activation.

Introduction

Members of the nuclear receptor superfamily of transcription factors play critical roles in a variety of developmental processes and in maintaining homeostasis of different organs.
Nuclear receptors transcriptional activity is mediated by the recruitment of specific cofactors that are responsible for promoting chromatin remodeling events conducive to gene activation or repression, with the exchange between corepressors and coactivators being a highly regulated process often induced by ligand binding (Glass and Rosenfeld 2000; Perissi and Rosenfeld 2005; Hager et al. 2009). Among the nuclear receptors, the Peroxisome Proliferator-Activator Receptors (PPARs) and Liver X Receptors (LXRs) are critical for the development and the functional regulation of key metabolic organs, the adipose tissue and the liver respectively, with both receptors forming functional heterodimers with the Retinoid X Receptor (RXR). PPARγ, in particular, is known as the master regulator of adipocyte differentiation and a critical factor for the regulation of lipid metabolism, immunity and insulin sensitivity (Rosen and Spiegelman 2001; Hong and Tontonoz 2008; Sonoda et al. 2008).

Genome wide studies of transcription factors binding, histone modifications and chromatin remodeling events have revealed that an epigenomic transition state is initiated within hours of stimulating adipogenesis. This dramatic reorganization of the preadipocyte chromatin landscape includes early transcription factors (C/EBPβ, GR, RXR and STAT5) binding to DNA, transient chromatin opening and changes in histone marks (Lefterova et al. 2008; Nielsen et al. 2008; Mikkelsen et al. 2010; Steger et al. 2010; Siersbaek et al. 2011). Interestingly, the so-called “adipogenic hotspots” where these changes occur during the very first few hours of differentiation are often found occupied by PPARγ in mature adipocytes, suggesting that early remodeling events could affect PPARγ binding at later stages (Steger et al. 2010).

Among others, lysine methylation is a prominent post-translational modification of histones that regulates chromatin structure, with H3K9 and H3K27 tri-methyl marks being recognized as hallmarks of gene repression. H3K9me3 in particular correlates with constitutive heterochromatin, whereas demethylation of H3K9 is associated with gene activation (Hublitz et al. 2009). KDM4/JMJD2 is a family of Jmjc-domain containing demethylases responsible for H3K9 and H3K36 demethylation. Overexpression of the KDM4 proteins associates with changes in chromatin remodeling that modulate gene expression and promote cell proliferation, invasion and other oncogenic properties (Berry and Janknecht 2013; Black et al. 2013; Young and Hendzel 2013). Members of this family have been associated with transcriptional activation mediated by nuclear receptors, such as estrogen and androgen receptors, or by other transcription factors that play critical roles during adipocyte differentiation (Zhang et al. 2005; Guo et al. 2012; Berry and Janknecht 2013).

G-protein Suppressor 2 (GPS2) is a small protein, originally identified while screening for suppressors of Ras activation in the yeast pheromone response pathway, that exerts critical anti-inflammatory roles in adipocytes and macrophages and is significantly downregulated in human obesity (Spain et al. 1996; Zhang et al. 2002; Cardamone et al. 2012; Toubal et al. 2013). While GPS2 is known to interact with various transcriptional regulators, including histone acetyltransferases, DNA repair proteins and DNA-binding transcription factors (Peng et al. 2000; Peng et al. 2001; Lee et al. 2006; Sanyal et al. 2007; Zhang et al. 2008; Jakobsson et al. 2009), a clear understanding of the molecular mechanism of GPS2...
transcriptional function remains strikingly incomplete. Moreover, whereas GPS2 is thought to act as a coactivator for some transcription factors, its identification as an intrinsic component of the NCoR/SMRT nuclear receptor corepressor complex is suggestive of a repressive role (Zhang et al. 2002). Accordingly, recent studies indicate that GPS2, together with the associated corepressor SMRT, is downregulated in the adipose tissue of obese individuals, where it plays a critical role in the regulation of a pro-inflammatory gene program (Toubal et al. 2013).

In addition to its transcriptional activity, GPS2 plays an important role in maintaining basal regulation of JNK activity by inhibiting the enzymatic activity of the TRAF2/Ubc13 ubiquitin complex and preventing hyper-activation of the TNFα signaling pathway (Zhang et al. 2002; Cardamone et al. 2012). In vivo relevance of this anti-inflammatory role was confirmed in aP2-GPS2 transgenic mice by a protective effect against diet-induced insulin resistance in adipose tissue, and by inhibition of TNFα target genes activation in macrophages. However, aP2-driven overexpression of GPS2 is not sufficient to ameliorate systemic insulin resistance in obese mice and promotes hepatic steatosis (Cardamone et al. 2012). Accordingly, GPS2 transgenic mice present elevated levels of Resistin (RETN), an adipokine first identified as a mediator of insulin resistance in murine obesity model (Steppan et al. 2001; Cardamone et al. 2012). Because the regulation of RETN expression in the adipose tissue is driven by a combination of PPARγ and C/EBPα response elements (Tomaru et al. 2009), we proposed that GPS2 might be acting as a coactivator for PPARγ (Cardamone et al. 2012).

Here, we have taken advantage of the functional connection between GPS2 and PPARγ to gain new insights into strategies of selective transcription factor recruitment to regulatory elements in controlling gene transcription programs. Genome-wide localization of GPS2 binding to chromatin in differentiating adipocytes was instrumental to identify an unexpected regulatory strategy based on GPS2 acting as a priming factor for PPARγ recruitment to a selected cohort of target genes, including the lipolysis rate-limiting enzymes ATGL and HSL. Our findings indicate that regulation of PPARγ recruitment by GPS2 is specific to promoter regions and depends on the inhibition of RNF8/Ubc13 enzymatic activity and consequent stabilization of the histone demethylase KDM4A/JMJD2.

**Results**

**GPS2 transcriptional role in adipocytes as a coactivator for PPARγ**

Our previous work indicates that GPS2 regulates Resistin (RETN) gene expression by modulating PPARγ transcriptional activity (Cardamone et al. 2012). Based on these results, we hypothesized that GPS2 may play a widespread regulatory role in the adipose tissue as a PPARγ co-activator. To investigate the transcriptional role of GPS2 and determine its genome-wide localization during adipogenesis, we performed chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) in undifferentiated (d0) and differentiated (d6) 3T3-L1 cells. A dramatic increase in GPS2 binding to DNA upon differentiation was observed, with n=7652 GPS2 peaks detected in undifferentiated cells compared with n=19793 peaks in differentiated cells (Figure 1A and S1A). This 2–3 folds increase in GPS2 binding to chromatin is consistently observed in each class when peaks are...
divided among promoters, enhancers and other locations in the genome based on position in respect to RefSeq genes definition and published H3K4me1 epigenetic signature in the 3T3-L1 cellular model (Mikkelsen et al. 2010) (Figure 1A and S1A). However, GPS2 binding at promoter regions is found to be stronger than at enhancers or other genomic locations, as shown by increased median peak intensity (Figure 1B). Also, although we detected a significant number of binding events at gene promoters (33–43%) and enhancer regions (17–20%), most GPS2 peaks were detected at non-promoter/non-enhancer regions (40–47%), which leaves open the interesting question of the role of GPS2 in these regions of unknown functions that probably include other tissue-specific regulatory elements (Figure S1A). Importantly, genes located nearby GPS2 peaks were associated with a significant enrichment in adipose-specific functions as indicated by GREAT analysis (Figure S1B), thus further supporting a putative role for GPS2 in adipogenesis.

Because GPS2 had been previously identified both as a component of the NCoR/SMRT corepressor complex, and as a coactivator for a number of transcription factors, including the nuclear receptors PPARγ, LXR and FXR (Zhang et al. 2002; Zhang et al. 2008; Jakobsson et al. 2009; Cardamone et al. 2012), we reasoned that GPS2 could be responsible for both repressive and activating events during adipogenesis. Accordingly, GPS2 binding is significantly enriched in proximity of genes that are both up- and down-regulated during adipogenesis, as defined based on Pol2 occupancy or H3K36me3 pattern (Mikkelsen et al. 2010)(Figure S1C and S1D). To address whether this dual transcriptional role corresponds to GPS2 association with different binding partners, we overlapped the genome-wide localization profile of GPS2 with comparable ChIP-seq datasets for NCoR, SMRT, PPARγ and RXR as available in 3T3-L1 cells (Nielsen et al. 2008; Lefterova et al. 2010; Raghav et al. 2012). As predicted, a strong co-localization was observed with both corepressors and nuclear receptors, with GPS2 peaks overlapping with NCoR/SMRT localization predominantly in undifferentiated cells (GPS2-NCoR common peaks decrease from 1196 to 804 on promoters and 333 to 224 on enhancers, GPS2-SMRT common peaks decrease from 1125 to 531 on promoters and 366 to 115 on enhancers) (Figure 1C and S1E). The co-localization of GPS2 and PPARγ/RXR binding to regulatory regions instead greatly increases upon differentiation (GPS2-RXR common peaks increase from 612 to 3591 on promoters and from 253 to 1272 on enhancers, and GPS2-PPARγ common peaks increase from 73 to 1385 on promoters and 77 to 877 on enhancers) (Figure 1D and S1F). In both instances the overlapping is more significant on promoters than on enhancers and, as expected, is absent on other genomic locations. Thus, these results together confirm that GPS2 can be recruited to genes regulated by nuclear receptors both as part of the corepressors complex or independently of NCoR/SMRT.

While our initial hypothesis of GPS2 acting as a co-activator for PPARγ was supported by the increase in overlapping between GPS2 and RXR/PPARγ binding to promoter regions in differentiated adipocytes, the relatively low number of common peaks, as compared to the extensive gene program regulated by PPARγ, suggested a specificity for a dedicated subset of target genes. To investigate this hypothesis and uncover the molecular signature defining the common target genes, we re-analyzed PPARγ binding profile by distinguishing peaks located at promoters, enhancers or other locations based on the same definition of positioning and epigenetic marks used for the GPS2 ChIP-seq dataset. Remarkably, this
analysis revealed that co-localization of GPS2 and PPARγ binding is significantly concentrated on promoter regions, with almost 60% (1141 out of 2012) of PPARγ-bound promoters presenting overlapping GPS2 peaks, whereas no significant overlapping is observed over enhancers and other locations (Figure 1E and 1F).

Hence, integrative analyses of GPS2 genome wide localization confirmed a dual role for GPS2 in the regulation of nuclear receptor-mediated gene expression and indicated the existence of a specific transcriptional program co-regulated by GPS2 and PPARγ that is characterized by PPARγ binding to promoter regions, rather than to distal sites.

**GPS2 is required for the transcriptional regulation of lipolytic genes in adipocytes**

Intriguingly, gene ontology analysis of the identified transcriptional program indicated a significant enrichment for GPS2 and PPARγ co-localization to genes that are important in the regulation of lipolysis, hepatic steatosis, fatty acid oxidation and circulating free fatty acids level, whereas PPARγ-dependent regulation of genes involved in brown fat differentiation, regulation of glucose metabolism and response to insulin appear to be GPS2-independent (Supplemental Table 1).

Among the genes that are co-bound by GPS2 and PPARγ, we selected for further investigation two known targets of PPARγ-transcriptional regulation, namely the Hormone-Sensitive Lipase (HSL) and the Adipose Triglyceride Lipase (ATGL), due to their critical roles as master regulators of lipolysis (Deng et al. 2006; Kim et al. 2006; Kershaw et al. 2007; Lass et al. 2011). To confirm that GPS2 regulates the expression of these genes in vivo, we took advantage of the aP2-GPS2 transgenic mice (Cardamone et al. 2012). Both RNA and protein expression of HSL and ATGL were found upregulated in the adipose tissue of GPS2 transgenic mice when compared to wildtype littermates, similarly to the increase previously reported for RETN (Figure 2A–B). Conversely, downregulation of GPS2 by siRNA significantly impairs their expression in differentiated 3T3-L1 cells (Figure 2C). As expected, the expression of pro-inflammatory cytokines, such as IL6, is significantly upregulated, whereas the expression of other known targets of PPARγ regulation is not affected by changes in GPS2 expression, further suggesting the specificity of GPS2-PPARγ functional interaction on a selected subset of regulatory regions (Figure 2D).

Based on the observation that ectopic upregulation of ATGL in mice is sufficient to drive elevated basal lipolysis (Ahmadian et al. 2009), we reasoned that lipolysis in the adipose tissue of aP2-GPS2 transgenic mice might be increased due to GPS2-mediated upregulation of rate-limiting lipolytic enzymes. Indeed, basal activation of HSL by phosphorylation is observed in the adipose tissue of aP2-GPS2 mice (Figure 2E), confirming activation of the lipolytic pathway. Similarly, induced activation of the lipolytic pathway by beta-adrenergic stimulation is increased in primary adipocytes isolated from epididimal fat of aP2-GPS2 transgenic mice, as measured by phosphorylation of HSL (Figure 2F).

In addition to being induced during adipogenesis, HSL and ATGL gene expression is known to be upregulated upon lipolysis induction (Festuccia et al. 2006; Chakrabarti et al. 2011), thus we asked whether GPS2 participates in their transcriptional activation in response to the beta-adrenergic receptor agonist isoproterenol in 3T3-L1 cells. Also in this setting, GPS2
downregulation by siRNA significantly impaired the induced upregulation of both HSL and ATGL (Figure 2G). To our surprise, ChIP-qPCR experiments in isoproterenol-stimulated 3T3-L1 cells indicated that recruitment of PPARγ to the HSL and ATGL promoters is severely impaired upon siGPS2 transfection (Figure 2H). Thus, our results confirm that GPS2 is a critical component of the transcriptional machinery regulating basal and induced lipolysis in the adipose tissue via modulation of the expression of two rate-limiting enzymes, and suggest that loss of ATGL and HSL gene activation in absence of GPS2 is due to impaired recruitment of PPARγ.

**GPS2 is required for chromatin remodeling of PPARγ target genes**

To further dissect the molecular mechanism of GPS2 transcriptional actions on PPARγ-regulated promoters, we first confirmed that PPARγ occupancy on RETN, HSL and ATGL promoters was indeed greatly diminished upon GPS2 downregulation (Figure 3A). These results together suggest that GPS2 is not being recruited to DNA by PPARγ as a “classic” coactivator, but rather plays a function in priming regulatory regions for the nuclear receptor binding. Indeed comparison between GPS2 and PPARγ profiling indicated that GPS2 was already bound at d0 to most promoter regions that were later marked by PPARγ at d6 (Figure 1F). Thus, we asked whether GPS2 could be required for dictating local changes in histone modifications necessary to regulate the permissiveness of the promoter for transcription factor binding. Because demethylation of H3K9 is a critical step towards gene activation, we first tested whether the promoter methylation status was dependent on GPS2. In concert with our hypothesis, a significant increase in the repressive mark tri-methyl H3K9 (H3K9me3) was observed on target promoters upon GPS2 downregulation by siRNA (Figure 3B). We next investigated the recruitment of the machinery responsible for histone demethylation. Specific recruitment of KDM4A (JMJD2A/JHDM3A), an H3K9 demethylase previously shown to interact with NCoR/SMRT corepressorcomplex (Zhang et al. 2005), was observed on the RETN promoter and found to be dependent on GPS2 (Figure 3C). Furthermore, in differentiating 3T3-L1 cells, siRNA mediated knockdown of KDM4A significantly impaired the recruitment of PPARγ (Figure 3D) without affecting GPS2 (Figure S3A). As a result, expression of the ATGL, HSL, and RETN genes is significantly downregulated upon siKDM4A transfection (Figure 3E), thus confirming that KDM4A is required for PPARγ-dependent gene activation. Together these results support the hypothesis that GPS2 indirectly regulates PPARγ activity on three key metabolic genes by modulating its recruitment via the pioneering activity of KDM4A-dependent H3K9 demethylation. Because this strategy would imply that both GPS2 and KDM4A are already present on the ATGL, HSL, and RETN promoters prior to PPARγ recruitment, we performed a kinetic analysis of the occupancy of these target promoters during adipocyte differentiation. As predicted, GPS2 and KDM4A presence precedes PPARγ recruitment on each regulatory region (Figure 3F–G), thus confirming their role in priming a specific subset of promoters for subsequent gene activation.

**GPS2 regulate Genome-wide localization of KDM4A**

Based on the consistent results observed on the regulatory regions of HSL, ATGL, and RETN, we hypothesized that the same strategy could be employed to modulate the expression of a larger cohort of genes. Thus, we determined KDM4A genome wide...
localization in differentiated 3T3-L1, in the presence or absence of GPS2, and overlapped it with GPS2 binding profile. Despite the low number of binding sites detected (1657 in cells transfected with siCTL and 1349 in cells transfected with siGPS2) (Figure S3A), a significant interaction was observed between GPS2 and KDM4A, with almost 50% of KDM4A peaks overlapping with GPS2 binding (Figure 4A). KDM4A binding profile appeared to be equally divided among intergenic regions and promoters/intronic regions (Figure S3B). Notably, we found that KDM4A binding to GPS2-bound promoters was stronger than KDM4A binding to GPS2-bound enhancers or other locations, with a very large percentage of KDM4A-regulated promoters being enriched for GPS2 binding (163 out of 203 peaks) (Figure 4A). Most importantly, KDM4A binding to promoters, but not to other regions, was specifically reduced upon GPS2 downregulation by siRNA transfection, as indicated by a general reduction in the overall number of peaks and a significant reduction in tag density and read counts over each peak (Figure 4B, 4C and S3C). Thus, our findings indicate that GPS2 is required on a number of genes to promote KDM4A binding to regulated promoters. To further confirm that this strategy is employed for regulating PPAR\(\gamma\) transcriptional activity, we asked to which extent the KDM4A program overlaps with PPAR\(\gamma\) binding sites. Again whereas only 35% of overall KDM4A peaks were in common with PPAR\(\gamma\) peaks (568 out of 1657 peaks), almost 50% of KDM4A-bound promoters were in common with PPAR\(\gamma\) peaks (93 out of 203 peaks). On this subset, a striking overlap with GPS2 binding was observed, with more than 80% of the common KDM4A/PPAR\(\gamma\) promoter peaks being co-occupied by GPS2 (75 out of 93 peaks) (Figure 4E). Thus, our data together suggest the existence of a defined set of promoters that are co-regulated by PPAR\(\gamma\)/KDM4A/GPS2 based on a dedicated regulatory strategy, which depends on GPS2-mediated regulation of KDM4A occupancy to promote histone demethylation and allow PPAR\(\gamma\) recruitment. To validate this hypothesis on a representative subset of the 93 candidate target genes identified by genome wide analyses, we picked six random genes and measured the effect of downregulating either GPS2 or KDM4A on their expression. As shown in Figure 4E, in either conditions, the expression of all but one gene was severely downregulated, including syntaxin-16 (STX16), pantothenate kinase 1 (PANK1), LETM1 Domain Containing 1 protein (LETM1D1), insulin receptor 1 (INSR1), limb development membrane protein 1 (LMBRI), and retinoblastoma-like 2/p130 (RBL2). Also, downregulating GPS2 by siRNA is sufficient to loose PPAR\(\gamma\) recruitment to each of their promoters as measured by ChIP (Figure 4F), thus confirming that our genome wide approach has identified a specific transcriptional program regulated via the priming strategy we have dissected on the promoters of RETN, HSL and ATGL.

Finally, comparison between the promoter locations that are marked by GPS2, KDM4A and PPAR\(\gamma\) in differentiated adipocytes with locations bound by NCoR and/or SMRT in preadipocytes also revealed an extensive overlapping, with more than 70% of the peaks that are co-regulated by GPS2/KDM4A/PPAR\(\gamma\) being marked by corepressors prior to differentiation (Figure 4G). This suggests that recruitment of both KDM4A and GPS2 to the specific target promoters that need to be primed for PPAR\(\gamma\) binding may occur in the preadipocyte state via the NCoR/SMRT corepressor complex. Demethylation of these regulatory regions by KDM4A depends on the presence of GPS2 and is required for later recruitment of PPAR\(\gamma\).
GPS2 inhibition of RNF8 stabilizes the histone demethylase KDM4A

Next we addressed the molecular mechanism of GPS2-mediated regulation of KDM4A promoter occupancy and histone demethylation. Based on our recent findings that cytosolic GPS2 modulates inflammatory responses via inhibition of the ubiquitin machinery required for activation of the TNFα pathway (Cardamone et al. 2012), we asked whether GPS2 actions in the nucleus could reflect a similar mechanism. Intriguingly, the E2 ubiquitin-conjugating enzyme regulated by GPS2 in the cytosol, Ubc13, also functions as a chromatin-modifying factor for DNA damage-induced ubiquitination of histone H2A in the nucleus. The major difference being that in the cytoplasm Ubc13 preferentially associates with the TRAF family of E3 ligases to regulate inflammatory responses, whereas in the nucleus it partners with the E3 RING ligases RNF8 and RNF168 (Huen et al. 2007; Mailand et al. 2007; Stewart et al. 2009). Because RNF8 was previously reported to function as a putative co-activator for the PPARγ heterodimeric partner, RXR (Takano et al. 2004) and was shown to regulate the ubiquitin-dependent degradation of KDM4A at DNA damage sites (Bohgaki et al. 2011), we asked whether GPS2 could regulate chromatin remodeling and promoter accessibility via inhibition of Ubc13/RNF8-dependent ubiquitination and degradation of KDM4A.

First, we investigated whether RNF8 and Ubc13 were bound to DNA, as both enzymes would have to be recruited on the same genomic locations regulated by GPS2 and PPARγ for GPS2 to function as a local inhibitor of the RNF8/Ubc13 ubiquitin-conjugating machinery. In agreement with this hypothesis, found that both RNF8 and Ubc13 are recruited on the RETN, HSL and ATGL promoters in differentiated 3T3-L1 (Figure 5A). Moreover, their binding to chromatin is not affected by GPS2 downregulation by siRNA, as would be expected if their enzymatic activities, rather than their recruitment to chromatin, were regulated by GPS2. Importantly, RNF8 downregulation by itself does not affect HSL, ATGL and RETN gene expression, however it is sufficient to rescue the expression of the same genes when impaired by siGPS2 transfection (Figure 5B). These results confirm that GPS2 is required to prevent the expression of these genes to be inhibited by promoter-bound RNF8.

Next, we investigated by ChIP analysis whether the recruitment of KDM4A to the regulatory regions of the genes under examination was affected by downregulating RNF8 or by GPS2-mediated regulation of RNF8. These experiments confirmed: i) that KDM4A binding to the ATGL and RETN promoters significantly increased upon RNF8 downregulation (Figure 5C), ii) that KDM4A binding to these promoters was highly downregulated in the absence of GPS2 (Figure 5D), iii) that the loss of KDM4A occupancy in absence of GPS2 was dependent on RNF8, as indicated by the full rescue observed upon RNF8 downregulation (Figure 5D). As expected, PPARγ binding was similarly restored upon RNF8 downregulation as a consequence of KDM4A recovered recruitment to the selected promoters (Figure 5C).

Finally, we tested the interaction between GPS2 and RNF8 in vivo and in vitro by co-immunoprecipitation in 293T cells and by GST pull-down using purified proteins. A significant interaction was observed in both experimental settings (Figure S4A), with RNF8
binding specifically to the N terminus domain of GPS2 (aa 2–99) (Figure S4B), even in absence of Ubc13 (Figure S4C). To address the functionality of such interaction we performed in vitro ubiquitination in a fully reconstituted system, which indicated that RNF8/Ubc13-dependent ubiquitin chain synthesis was inhibited by GPS2 in a concentration dependent manner (Figure 5E). In addition, to directly address whether GPS2 presence is required for protecting KDM4A from Ubc13-dependent ubiquitination, we investigated KDM4A protein stability and its ubiquitination in vitro. Whereas KDM4A stability was only slightly decreased in nuclear extracts from 3T3-L1 cells transfected with siRNA against GPS2 (Figure S4D), KDM4A poly-ubiquitination by Ubc13/RNF8 in vitro was strongly inhibited by recombinant GPS2 (Figure 5F). Combined, these results show that GPS2 binds directly to RNF8, in addition to Ubc13, and by doing so inhibits their enzymatic activity. They also suggest that GPS2 is not responsible for regulating the global level of KDM4A in the cell, but is rather required at the chromatin level to regulate ubiquitin-dependent dismissal of KDM4A from specific regulatory regions.

Discussion

A critical step in the regulation of adipocyte differentiation is an extensive reprogramming of gene expression that includes the activation of a large cohort of adipogenic genes, which are kept under negative regulation in undifferentiated cells. A wealth of studies has contributed to the identification of the complex network of transcription factors and regulatory complexes that drive these changes (Farmer 2006; Rosen and MacDougald 2006). Among others, genome-wide analysis of corepressors binding to DNA during the progression of adipogenesis has revealed a model in which the corepressors SMRT, and NCoR to a lesser extent, function in concert with two DNA-binding partners, namely C/EBPβ on distal sites and Kaiso on proximal sites, to block adipogenic genes expression in undifferentiated cells (Raghav et al. 2012). Here, genome-wide localization analysis in murine adipocytes of GPS2, a component of the NCoR and SMRT corepressor complexes, reveals that GPS2 plays complementary roles in gene repression and gene activation during adipogenesis In particular, our findings define a specific transcriptional program co-regulated by GPS2 and PPARγ which is characterized by recruitment of PPARγ to promoter-specific binding sites rather than distal sites as observed for the large majority of PPARγ regulated genes (Everett and Lazar 2013). On these target promoters GPS2 acts as a pioneering factor for PPARγ recruitment based on its ability to inhibit the E3 ubiquitin ligase RNF8, protect KDM4A from degradation and therefore promote histone H3K9 demethylation.

Intriguingly, mammalian KDM4A has been linked to both transcriptional repression, in association with the NCoR complex, and activation functions, mediated by nuclear receptors, in a fashion very similar to GPS2 itself (Zhang et al. 2005; Shin and Janknecht 2007). As Drosophila KDM4A plays an essential function in mediating ecdysone hormone signaling during larva development (Tsurumi et al. 2013), epigenetic regulation by KDM4s demethylases may be a conserved strategy to modulate changes in chromatin structure at promoters regulated by nuclear receptors. Future studies will indicate whether the regulatory component mediated by GPS2 also represents a conserved mechanism contributing to the regulation of other nuclear receptors’ activity, including LXR, ER and...
AR. Importantly, regulatory mechanisms based on stabilization of histone demethylases may represent unexplored druggable targets in the treatment of human diseases as indicated by the recent finding of KDM4B stabilization by Hsp90 in tumors (Ipenberg et al. 2013).

One important difference compared to previously reported models of KDM-mediated regulation of gene expression is that the regulatory strategy that emerges from our results is based on H3K9 demethylase activity being required to prime chromatin for nuclear receptor binding rather than being brought in by the liganded nuclear receptor as a chromatin remodeling cofactor (Garcia-Bassets et al. 2007; Wang et al. 2007; Guo et al. 2012; Tsurumi et al. 2013). While together these results confirm the importance of H3K9me as a mechanism for defining groups of commonly coordinated genes, it is possible that the different nature of regulation achieved via demethylation reflects the fact that we have analyzed PPARγ-mediated events in a developmental program, rather than in response to hormonal signaling cues. Indeed, during the early phases of adipogenesis PPARγ is not expressed, and other transcription factors, such as C/EBPβ, RXR and GR, are required to promote gene expression changes that will determine cell fate determination. As a result, PPARγ recruitment to the regulatory regions governing adipogenic gene expression is likely influenced by changes in chromatin status determined prior to its expression (Steger et al. 2010; Siersbaek et al. 2012; Everett and Lazar 2013). Thus, in the context of adipogenesis, our results support the idea of an “assisted loading” model in which priming of a “de novo” regulatory unit is achieved by remodeling events that are required to promote a chromatin open state permissive for PPARγ binding (Voss et al. 2011; Madsen et al. 2014).

Intriguingly, a different member of the KDM/JMJD2 family, KDM4B, is recruited by C/EBPβ during the early mitotic clonal expansion phase of adipocyte differentiation to demethylate its own target genes (Guo et al. 2012). Because C/EBPβ and PPARγ often work in tandem in the regulation of pro-adipogenic gene expression, it is tempting to speculate that similar epigenetic strategies, with different demethylases might be employed to regulate priming of different subsets of PPARγ target genes.

Accordingly, our findings indicate that GPS2/KDM4A coregulatory activity is targeted towards a specific subset of PPARγ target genes, including the two master regulators of lipolysis, ATGL and HSL. Increased expression of these genes in adipocytes from aP2-GPS2 mice is associated with increased HSL phosphorylation, thus suggesting that both basal and induced lipolysis is hyper-activated in the adipose tissue of GPS2 transgenic mice. Additional studies will be required to confirm whether the increase in ATGL and HSL expression described here translates in to an effective increase in the rate of lipolysis in vivo, and to address how the increase in lipolysis contributes to the general metabolic profiling of aP2-GPS2 transgenic mice. Nonetheless, we speculate that increased mobilization of lipids from the adipose tissue due to upregulated lipolysis might be contributing to the observed excessive fat deposition in peripheral organs (Cardamone et al. 2012). Intriguingly, genetic inactivation of another component of the NCoR corepressor complex, TBLR1, blunts the lipolytic response of white adipocytes through the impairment of cAMP-dependent signal transduction (Rohm et al. 2013), thus suggesting that GPS2 and associated cofactors play a critical role in the regulation of lipid mobilization.
Finally, the identification of a regulatory strategy for PPARγ-mediated transcription based on the control of the local chromatin architecture via stabilization of a histone demethylase together with our recent report of GPS2-mediated inhibition of JNK activation in adipose tissue and macrophages, indicates that the ability of GPS2 to regulate Ubc13-dependent ubiquitin signaling, both inside and outside the nucleus, plays a central role in key metabolic organs. As a recent study in human adipose tissue indicates that GPS2-mediated transcriptional repression is also critical for the regulation of inflammatory genes (Toubal et al. 2013), together these observations suggest that GPS2 may become an interesting target for novel therapeutic approaches towards metabolic diseases.

Materials and Methods

Reagents and Antibodies

Anti-GPS2 rabbit antibody was generated against a C’terminal peptide (Cardamone et al., 2012). Other antibodies are purchased from Santa Cruz: anti-PPARγ (H-100), anti-Ubc13 (YD-16), anti-Ub (P4D1), anti-HDAC2 (H54); or from Sigma: anti-RNF8 (AV40071), anti-Ubc13 (AV43437). Also anti-Flag M2 and mouse anti-HA (Upstate Biotechnology), rat anti-HA (Roche) and anti-KMD4A (Abcam ab47984) are used. siRNAs specific for GPS2, RNF8 and KDM4A were purchased from Invitrogen (Silencer® Select siRNAs).

Lipolysis Assay

Lipolysis assays were performed in mice primary adipocytes, isolated as described (Carswell et al. 2012), or in mature 3T3-L1 cells after 6–8 day of induced differentiation. 3T3-L1 cells were grown, transfected and induced to differentiate as previously described (Cardamone et al. 2012). To measure induced lipolysis cells were pre-incubated in DMEM without phenol red and supplemented with 4% BSA fatty acid free for 5h prior to 1h stimulation with 10 μM of isoproterenol (Calbiochem, Merck).

Protein interaction studies and In vitro ubiquitination

Nuclear and whole cell protein extraction was performed as previously described (Cardamone et al., 2012). For co-IP experiments, extracts were incubated with the specific antibody overnight at 4°C and isolated on protein A/G agarose beads (Invitrogen). GST-fusion proteins containing different regions of GPS2 (GPS2 A: aa 2–99; GPS2 B: aa 2–137; GPS2-C: aa 2–155; GPS2-D: aa 2–187; GPS2-E: aa 155–327; GPS2-F: aa 212–327) or GPS2 full-length were expressed in BL21 bacteria and purified as described (Perissi et al. 2004). For GST pull down studies, the immobilized GST-fusion proteins were incubated with TnT RNF8 (Promega). Ubiquitination assays were carried out as previously described using 50nM E1, 5μg ubiquitin and 200nM Ubc13 Uev1a (Cardamone et al., 2012). RNF8 and GPS2 were produced as GST or His fusion proteins and purified from bacterial lysates. Full-length KDM4A was produced using the Promega TnT kit and purified by anti-Flag immunoprecipitation.

ChIP assay, RNA Isolation and RT-PCR Analysis

Chromatin immunoprecipitation (ChIP) was performed as described (Perissi et al. 2004). For ChIP-Seq sample preparation, 3T3-L1 cells were subjected to standard ChIP prior to library
preparation. For expression experiments, RNA was isolated using the RNeasy Kit (Qiagen). First strand cDNA synthesis from total RNA template was performed with the IScript cDNA Synthesis System (Biorad), followed by SYBR-green qPCR amplification. Normalization was performed using specific amplification of CyclophilinA and qPCRs were performed in triplicate for each biological duplicate experiment. All ChIP and qPCRs were repeated at least three times and representative results were shown. Primers used are specific for the regions indicated and their sequences are available on request. Data are shown as averages between the triplicates plus standard deviation. Significance is calculated by paired student’s T-test.

**ChIP-sequencing and Bioinformatic analysis of ChIP-seq datasets**

ChIP-seq samples were subjected to standard ChIP, libraries were prepared and sequenced on a GAII sequencing machine according to Illumina’s standard protocol. ChIP-seq datasets for GPS2 and KDM4A can be downloaded from the NCBI GEO repository (Series GSExxxx). PPARγ, RXR, and Pol2 ChIP-seq datasets were downloaded from NCBI GEO Series GSE13511 (Lim et al. 2013), NCoR and SMRT ChIP-seq data were downloaded from ArrayExpress E-MTAB-103 (Raghav et al. 2012). H3K36me3, H3K4me1, H3K4me2 and H3K27ac ChIP-seq datasets were downloaded from NCBI GEO Series GSE20752 (Mikkelsen et al. 2010). Sequence alignment of ChIP-seq samples was performed by using Bowtie or BFAST to mm8, or mm9 assembly of the mouse genome. The HOMER software suite was used to call the peaks for GPS2, NCoR, SMRT, RXR and PPARγ ChIP-seq datasets. GPS2 peaks are considered on promoters if the peak summit is within −1kb and +400bp around the TSS site. To define GPS2 peaks on enhancers, we extended the H3K4me1 and GPS2 peaks +/-1kb around the peak summit and the intersections were computed by using BedTools. The heatmaps of ChIP-seq datasets were displayed in TM4/MeV. GO/pathway analysis was computed by DAVID/EASE, ToppGENE, HOMER and GREAT tools. The enrichment analysis of GPS2 binding near adipogenesis-regulated genes was based on hypergeometric distribution and was computed in R. To compute the differentially expressed genes based on POL2 and H3K36me3 ChIP-seq datasets, we used mm9 RefSeq gene annotations and BedTools to count the sequencing reads on each gene. Statistically significant differentially expressed genes were defined using edgeR (FDR < 0.01) and a supplementary read density (RD) criteria of at least 2 read/kb gene length.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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| HIGHLIGHTS |
|----------------|
| • PPARγ binding to promoter-specific locations depends on H3K9 demethylase KDM4A |
| • GPS2 stabilizes KDM4A on target promoters by inhibiting Ubc13/RNF8 ubiquitination |
| • Genome wide studies reveal a specific program regulated via GPS2/KDM4A actions |
| • Rate-limiting lipolysis enzymes ATGL and HSL are regulated by GPS2/KDM4A/PPARγ |
Figure 1. GPS2 genome-wide localization in 3T3-L1 adipogenesis

(A) Venn diagram of the distribution of GPS2 ChIP-seq peaks before (d0) and after (d6) differentiation. Peaks are divided among promoters (−1kb/+400bp from RefSeq TSS), enhancers (distal sites positive for H3K4me1 mark) and other locations (distal sites negative for H3K4me1 mark) (Mikkelsen et al. 2010). (B) Boxplot showing GPS2 binding intensity on promoters, enhancers, other locations. (C–D) Unclustered heatmaps displaying the localization of NCoR and SMRT peaks (C) (Raghav et al. 2012), or PPARγ and RXR peaks (D) (Lefterova et al. 2008) around GPS2 peaks on GPS2-bound promoters and enhancers. (E) Boxplot of GPS2 binding intensity on PPARγ-bound promoters, enhancers, other locations at day 6 of differentiation. (F) Unclustered heatmaps showing the localization of GPS2 binding sites within +/- 1kb of PPARγ peaks on promoter and enhancer regions.
Figure 2. GPS2 is required for transcriptional regulation of lipolytic genes

(A) RT-qPCR analysis of ATGL, HSL and RETN gene expression in the WAT from wt and aP2-GPS2 transgenic mice. (B) WB showing increase of ATGL and HSL protein expression in the WAT of transgenic mice. (C–D) RT-qPCR analysis showing decreased expression of ATGL, HSL and RETN in 3T3-L1 cells transfected with siRNA against GPS2. No significant changes are observed for PPARγ, aP2 and AdPLA, while IL6 expression is upregulated in absence of GPS2. (E) Phosphorylation of Ser660 on HSL is increased in aP2-GPS2 mice. (F) Increased basal and induced phosphorylation of HSL in WAT of aP2-GPS2 mice. (G) Impaired transcriptional activation of ATGL and HSL upon isoproterenol (ISO) treatment in absence of GPS2 as shown by RT-qPCR analysis in 3T3-L1. (H) ChIP for PPARγ and GPS2 on the ATGL and HSL promoters. All graphic data is +/- SD, with corresponding P values indicated as follow: * if P<0.08, ** if P<0.05, *** if P<0.01.
Figure 3. GPS2 is required for PPARγ binding and chromatin remodeling of target genes
(A) ChIP for PPARγ on ATGL and HSL promoters and for GPS2 and PPARγ on RETN promoter. (B) Increased H3K9Me3 in absence of GPS2 as measured by ChIP on HSL, ATGL and RETN promoters. (C) ChIP assay analysis of KDM4A recruitment on RETN promoter. (D) ChIP assay analysis of PPARγ recruitment on RETN and ATGL promoter. (E) RT-qPCR analysis of HSL and ATGL expression in differentiated 3T3-L1 cells with or without siRNA against GPS2. (F–G) Time dependent recruitment of PPARγ, KDM4A and GPS2 to the RETN (F), ATGL and HSL (G) promoters at day 0 (d0), day 2 (d2), day 4 (d4), and day 6 (d6) of 3T3-L1 differentiation. All graphic data is +/− SD, with corresponding P values indicated as follow: * if P<0.08, ** if P<0.05, *** if P<0.01.
Figure 4. Genome-wide analysis of GPS2-mediated regulation of KDM4A binding to chromatin

(A) Overlapping between GPS2 and KDM4A ChIP-seq datasets in differentiated 3T3-L1 cells. (B) KDM4A binding is specifically reduced on promoters co-occupied by GPS2 and KDM4A as shown by box plot and tag density profile. The reduction is statistically significant (Welch two-sample t-test: p-value = 1.73e-05). (C) No statistically significant difference is observed on non-promoter regions. (D) Heatmaps showing the overlapping of KDM4A peaks with GPS2 and PPARγ peaks on promoters. (E) qPCR analysis showing decreased expression of six representative genes upon KDM4A or GPS2 transient downregulation in differentiated 3T3-L1 cells. (F) ChIP analysis showing dismissal of PPARγ from their promoters upon GPS2 downregulation. (G) Heatmaps showing overlapping of GPS2, N-CoR and SMRT peaks around the KDM4A peaks located on promoter regions.
Figure 5. Inhibition of RNF8 enzymatic activity is required for KDM4A stabilization and lipolytic genes expression

(A) ChIP for RNF8 and Ubc13 on the ATGL, HSL and RETN promoters. (B) Transient transfection of siRNA for RNF8 rescues siGPS2-dependent downregulation of ATGL, HSL and RETN gene expression (left panel). No significant change is observed upon siRNF8 transfection (right panel). (C) Increased recruitment of KDM4A on ATGL and RETN promoters is observed after RNF8 downregulation via specific siRNA. (D) ChIP analysis in differentiated 3T3-L1 showing that dismissal of PPARγ and KMD4A from ATGL, HSL and RETN regulatory units is rescued by ablation of RNF8 via specific siRNA. (E) In vitro ubiquitination assay with recombinant E1, E2 (Ubc13/Uev1a) and bacterially expressed and purified E3 ligase (RNF8) showing that GPS2 inhibits RNF8 enzymatic activity in a dose dependent manner. (F) In vitro ubiquitination assay showing that poly-ubiquitination of KDM4A by RNF8/Ubc13 is inhibited by GPS2. All graphic data is +/- SD, with corresponding P values indicated as follow: * if P<0.08, ** if P<0.05, *** if P<0.01.