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

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SUMMARY

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 peroxisome proliferator-activator receptor γ (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 adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (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 receptor 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 factor 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 (CCAAT-enhancer-binding protein β [C/EBPβ], glucocorticoid receptor [GR], RXR, and signal transducer and activator of transcription 5 [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; Siersbæk et al., 2011). Interestingly, the so-called "adipogenic hot spots" 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 posttranslational modification of histones that regulates chromatin structure, with Histone 3 Lysine 9 (H3K9) and Lysine 27 (H3K27) trimethyl 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

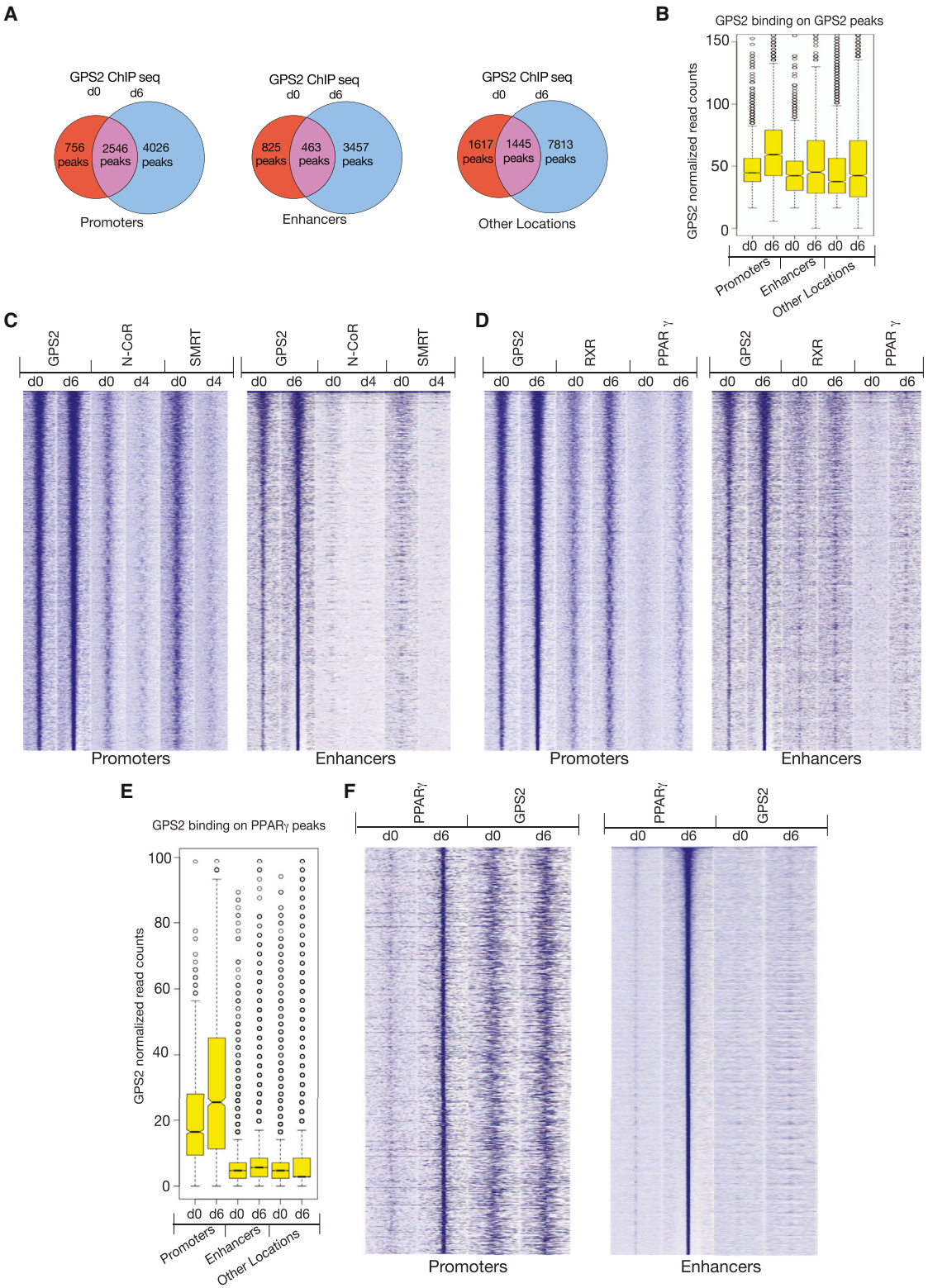

Figure 1. GPS2 Genome-wide Localization during 3T3-L1 Adipogenesis

(A) Venn diagram of the distribution of GPS2 ChIP-seq peaks before (day 0) and after (day 6) differentiation. Peaks are divided among promoters (−1 kb/+400 bp from RefSeq TSS), enhancers (distal sites positive for H3K4me1 mark), and other locations (distal sites negative for H3K4me1 mark) (Mikkelsen et al., 2010).
(B) Box plot showing GPS2-binding intensity on promoters, enhancers, other locations.

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androgen receptors (ERs and ARs, respectively), or by other transcription factors that play critical roles during adipocyte differentiation (Zhang et al., 2005; Guo et al., 2012; Berry and Jan-knecht, 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). Although GPS2 is known to interact with various transcriptional regulators, including histone acetyltransferases, DNA repair proteins, and DNA-binding transcription factors (Peng et al., 2000, 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 nuclear receptor corepressor (NCoR)/silencing mediator for retinoid and thyroid hormone receptors (SMRT) 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 proinflammatory gene program (Toubal et al., 2013).

In addition to its transcriptional activity, GPS2 plays an important role in maintaining basal regulation of c-Jun N-terminal kinase (JNK) activity by inhibiting the enzymatic activity of the tumor necrosis factor (TNF)-receptor associated factor 2 (TRAF2)/Ubc13 ubiquitin complex and preventing hyperactivation of the TNF-α 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 TNF-α target gene 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 a 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 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 *adipose triglyceride lipase* (*ATGL*) and *hormone-sensitive lipase* (*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 *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 coactivator. 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 (day 0) and differentiated (day 6) 3T3-L1 cells. A dramatic increase in GPS2 binding to DNA upon differentiation was observed, with 7,652 GPS2 peaks detected in undifferentiated cells compared with 19,793 peaks in differentiated cells (Figures 1A and S1A). This 2- to 3-fold 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 gene definition and published H3K4me1 epigenetic signature in the 3T3-L1 cellular model (Mikkelsen et al., 2010) (Figures 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 nonpromoter/nonenhancer 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 Genomic Regions Enrichment of Annotations Tool (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 complex and as a coactivator for a number of transcription factors, including the nuclear receptors PPARγ, LXR, and FXR (Zhang et al., 2002, 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

(C and 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) Box plot of GPS2-binding intensity on PPARγ-bound promoters, enhancers, other locations at day 0 and day 6 of differentiation.

(F) Unclustered heatmaps showing the localization of GPS2-binding sites within ±1 kb of PPARγ peaks on promoter and enhancer regions.

is significantly enriched in proximity of genes that are both up- and downregulated during adipogenesis, as defined based on Pol2 occupancy or H3K36me3 pattern (Mikkelsen et al., 2010) (Figures 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 data sets 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 colocalization 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 1,196 to 804 on promoters and 333 to 224 on enhancers; GPS2-SMRT common peaks decrease from 1,125 to 531 on promoters and 366 to 115 on enhancers) (Figures 1C and S1E). The colocalization of GPS2 and PPARγ/RXR binding to regulatory regions instead greatly increases upon differentiation (GPS2-RXR common peaks increase from 612 to 3,591 on promoters and from 253 to 1,272 on enhancers; GPS2-PPARγ common peaks increase from 73 to 1,385 on promoters and 77 to 877 on enhancers) (Figures 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 corepressor complex or independently of NCoR/SMRT.

Although our initial hypothesis of GPS2 acting as a coactivator 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 reanalyzed 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 data set. Remarkably, this analysis revealed that colocalization of GPS2 and PPARγ binding is significantly concentrated on promoter regions, with almost 60% (1,141 out of 2,012) of PPARγ-bound promoters presenting overlapping GPS2 peaks, whereas no significant overlapping is observed over enhancers and other locations (Figures 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 coregulated 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 (GO) analysis of the identified transcriptional program indicated a significant enrichment for GPS2 and PPARγ colocalization to genes that are important in the regulation of lipolysis, hepatic steatosis, fatty acid oxidation, and circulating levels of free fatty acids (FFA), whereas PPARγ-dependent regulation of genes involved in brown fat differentiation, regulation of glucose metabolism, and response to insulin appears to be GPS2 independent (Table S1).

Among the genes that are cobound by GPS2 and PPARγ, we selected for further investigation two known targets of PPARγ transcriptional regulation, namely *HSL* and *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 expression and protein expression of *HSL* and *ATGL* were found upregulated in the adipose tissue of GPS2 transgenic mice when compared to wild-type (WT) littermates, similarly to the increase previously reported for *RETN* (Figures 2A and 2B). Conversely, downregulation of GPS2 by small interfering RNA (siRNA) significantly impairs their expression in differentiated 3T3-L1 cells (Figure 2C). As expected, the expression of proinflammatory cytokines, such as interleukin-6 (IL-6), 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 β-adrenergic stimulation is increased in primary adipocytes isolated from epididymal 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 β-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 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

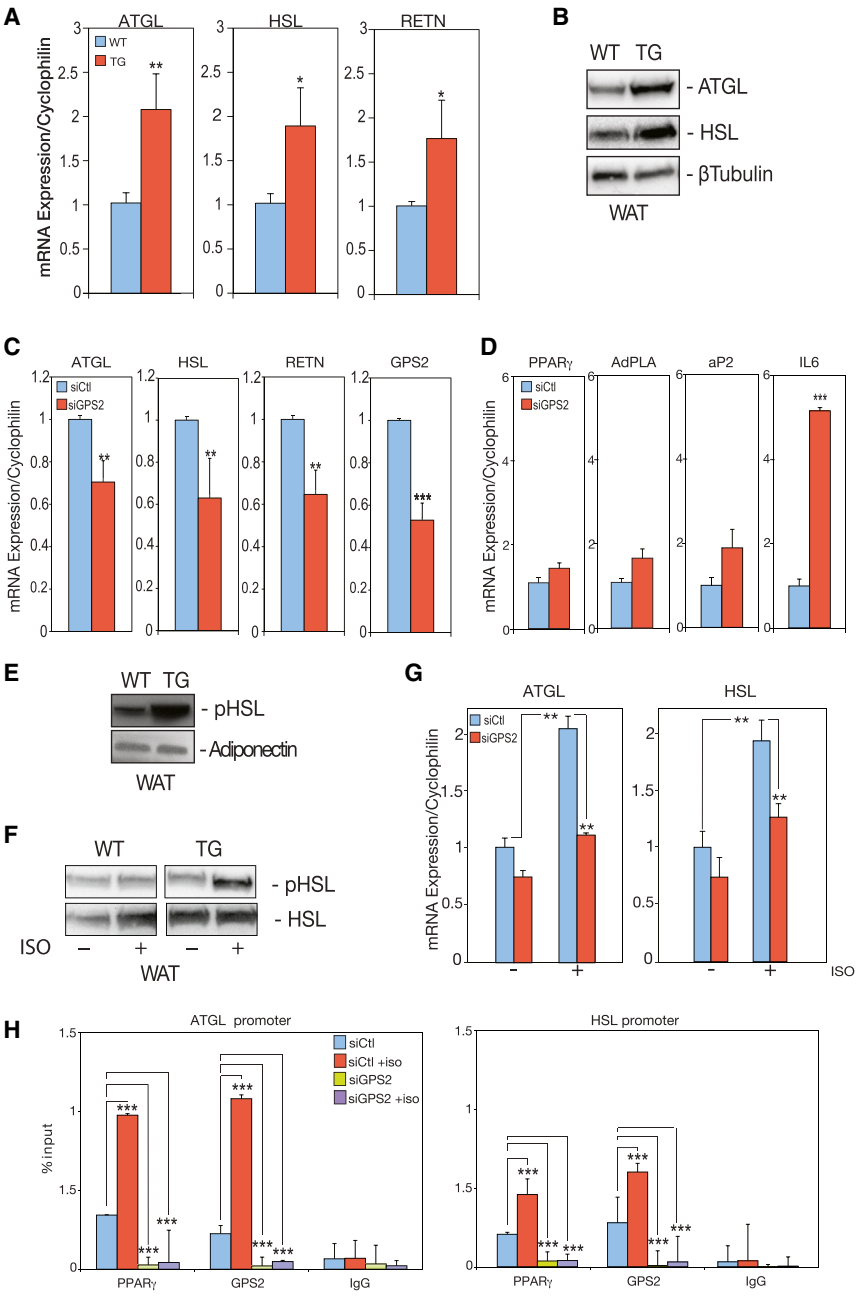


Figure 2. GPS2 Is Required for Transcriptional Regulation of Lipolytic Genes

(A) RT-qPCR analysis of *ATGL*, *HSL*, and *RETN* gene expression in the white adipose tissue (WAT) from WT and aP2-GPS2 transgenic mice.

(B) Western blot showing increase of ATGL and HSL protein expression in the WAT of transgenic mice.

(C and 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*, whereas *IL-6* 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. ISO, isoproterenol.

(G) Impaired transcriptional activation of *ATGL* and *HSL* upon 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. IgG, immunoglobulin G.

All graphic data are ±SD, with corresponding p values indicated as follow: *$p < 0.08$; **$p < 0.05$; ***$p < 0.01$.

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 day 0 to most promoter regions that were later marked by PPARγ at day 6 (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 toward 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 trimethyl 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 complex (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 S2A). 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

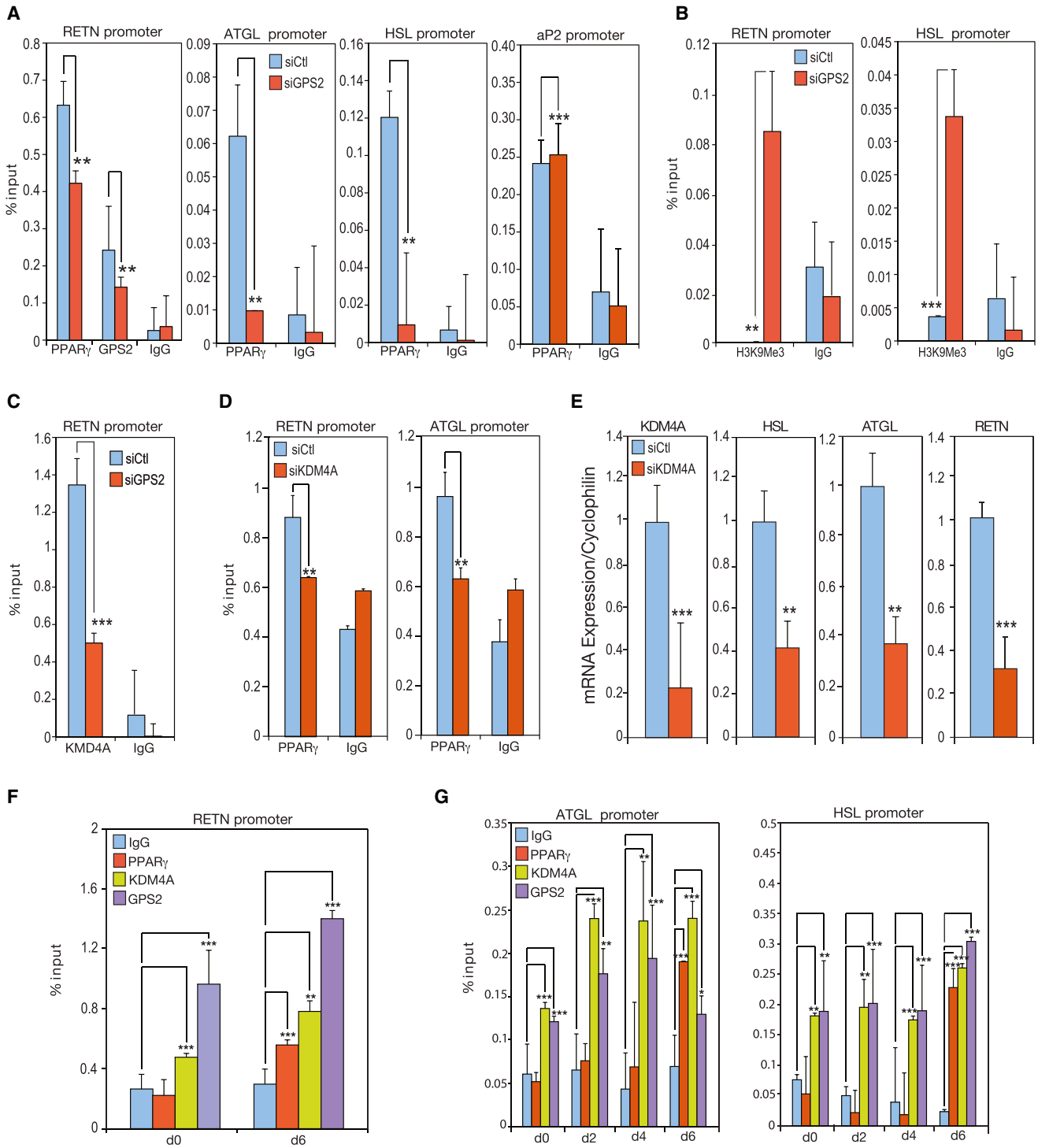


Figure 3. GPS2 Is Required for PPARγ Binding and Chromatin Remodeling of Target Genes

(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* 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 KDM4A, RETN, HSL, and ATGL expression in differentiated 3T3-L1 cells with or without siRNA against KDM4A.

(F and G) Time-dependent recruitment of PPARγ, KDM4A, and GPS2 to the *RETN* (F), *ATGL*, and *HSL* (G) promoters at days 0, 2, 4, and 6 of 3T3-L1 differentiation.

All graphic data are ±SD, with corresponding p values indicated as follow: *p < 0.08; **p < 0.05; ***p < 0.01.

during adipocyte differentiation. As predicted, GPS2 and KDM4A presence precedes PPARγ recruitment on each regulatory region (Figures 3F and 3G), thus confirming their role in priming a specific subset of promoters for subsequent gene activation.

GPS2 Regulates 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 (1,657 in cells transfected with siCTL and 1,349 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 (Figures 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 PPARγ transcriptional activity, we asked to which extent the KDM4A program overlaps with PPARγ-binding sites. Again, whereas only 35% of overall KDM4A peaks were in common with PPARγ peaks (568 out of 1,657 peaks), almost 50% of KDM4A-bound promoters were in common with PPARγ 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/PPARγ 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 coregulated by PPARγ/KDM4A/GPS2 based on a dedicated regulatory strategy, which depends on GPS2-mediated regulation of KDM4A occupancy to promote histone demethylation and allow PPARγ recruitment. To validate this hypothesis on a representative subset of the 93 candidate target genes identified by genome-wide analyses, we picked 6 random genes and measured the effect of downregulating either GPS2 or KDM4A on their expression. As shown in Figure 4E, in either condition, the expression of all but one gene was severely downregulated, including syntaxin-16 (*STX16*), pantothenate kinase 1 (*PANK1*), LETM1 Domain Containing 1 protein (LETMD1), insulin receptor 1 (*INSR1*), limb development membrane protein 1 (*LMBR1*), and retinoblastoma-like 2/p130 (*RBL2*). Also, downregulating GPS2 by siRNA was sufficient to loose PPARγ 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*.

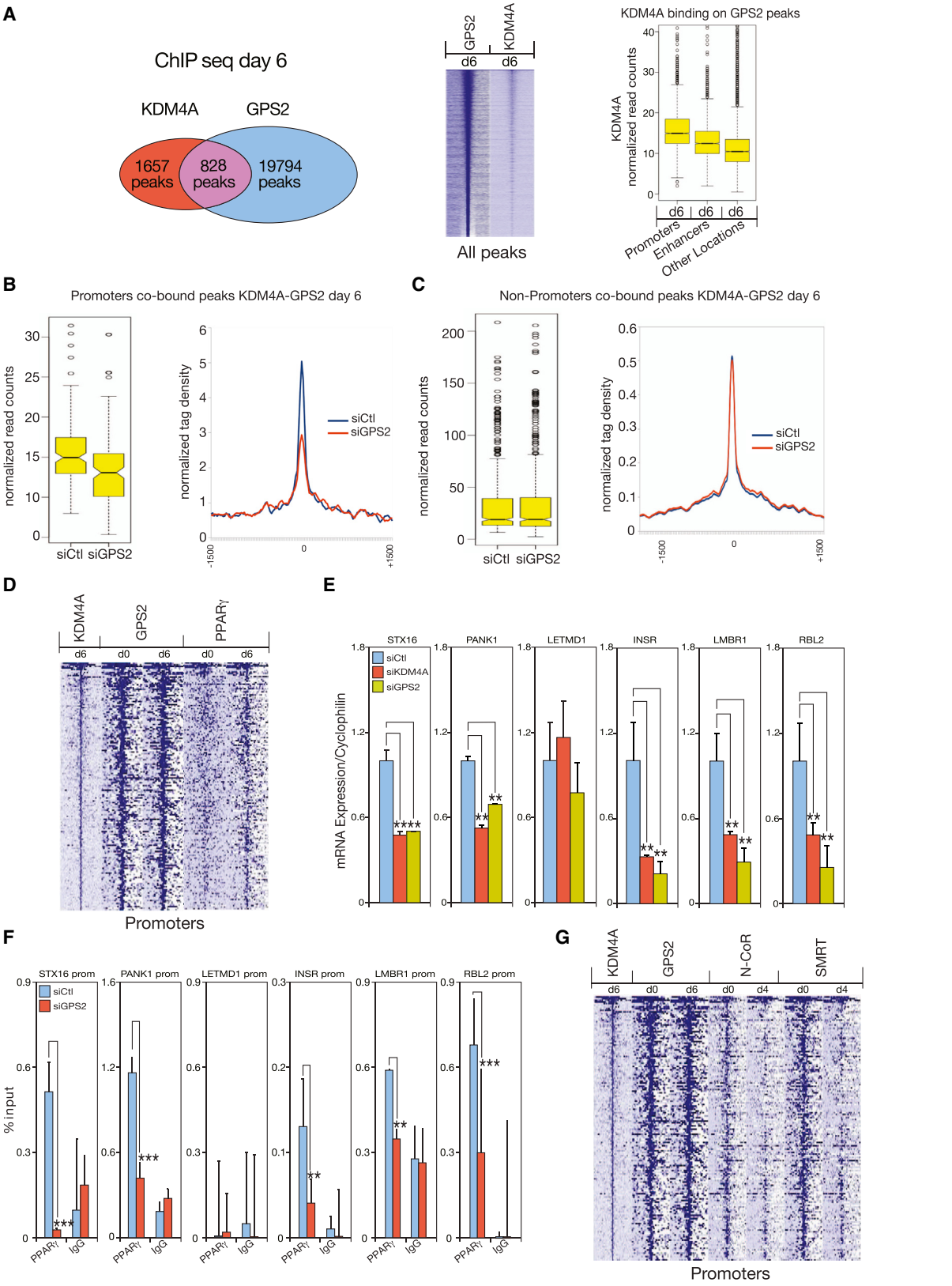
Furthermore, comparison between the promoter locations that are marked by GPS2, KDM4A, and PPARγ in differentiated adipocytes with locations bound by NCoR and/or SMRT in preadipocytes also revealed an extensive overlapping, with more than 70% of the peaks that are coregulated by GPS2/KDM4A/PPARγ being 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 PPARγ binding may occur in the preadipocyte state via the NCoR/SMRT complex. Demethylation of these regulatory regions by KDM4A depends on the presence of GPS2 and is required for later recruitment of PPARγ.

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 TNF-α 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 coactivator 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 because 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, we 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



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GPS2-mediated regulation of RNF8. These experiments confirmed that (1) KDM4A binding to the *ATGL* and *RETN* promoters significantly increased upon RNF8 downregulation (Figure 5C), (2) KDM4A binding to these promoters was highly downregulated in the absence of GPS2 (Figure 5D), and (3) 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 coimmunoprecipitation (coIP) in 293T cells and by glutathione S-transferase (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 protein stability was only slightly decreased in nuclear extracts from 3T3-L1 cells transfected with siRNA against GPS2 (Figure S4D), KDM4A polyubiquitination 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, to a lesser extent, NCoR function in concert with two DNA-binding partners, namely C/EBPβ on distal sites and Kaiso on proximal sites, to block adipogenic gene 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 coregulated by GPS2 and PPARγ that 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). Because *Drosophila* KDM4A plays an essential function in mediating ecdysteroid hormone signaling during larva development (Tsurumi et al., 2013), epigenetic regulation by KDM4 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). Although 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

Figure 4. Genome-wide Analysis of GPS2-Mediated Regulation of KDM4A Binding to Chromatin

(A) Overlapping between GPS2 and KDM4A ChIP-seq data sets 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: $p = 1.73 \times 10^{-5}$, Welch two-sample t test.

(C) No statistically significant difference is observed on nonpromoter 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, NCoR, and SMRT peaks around the KDM4A peaks located on promoter regions.

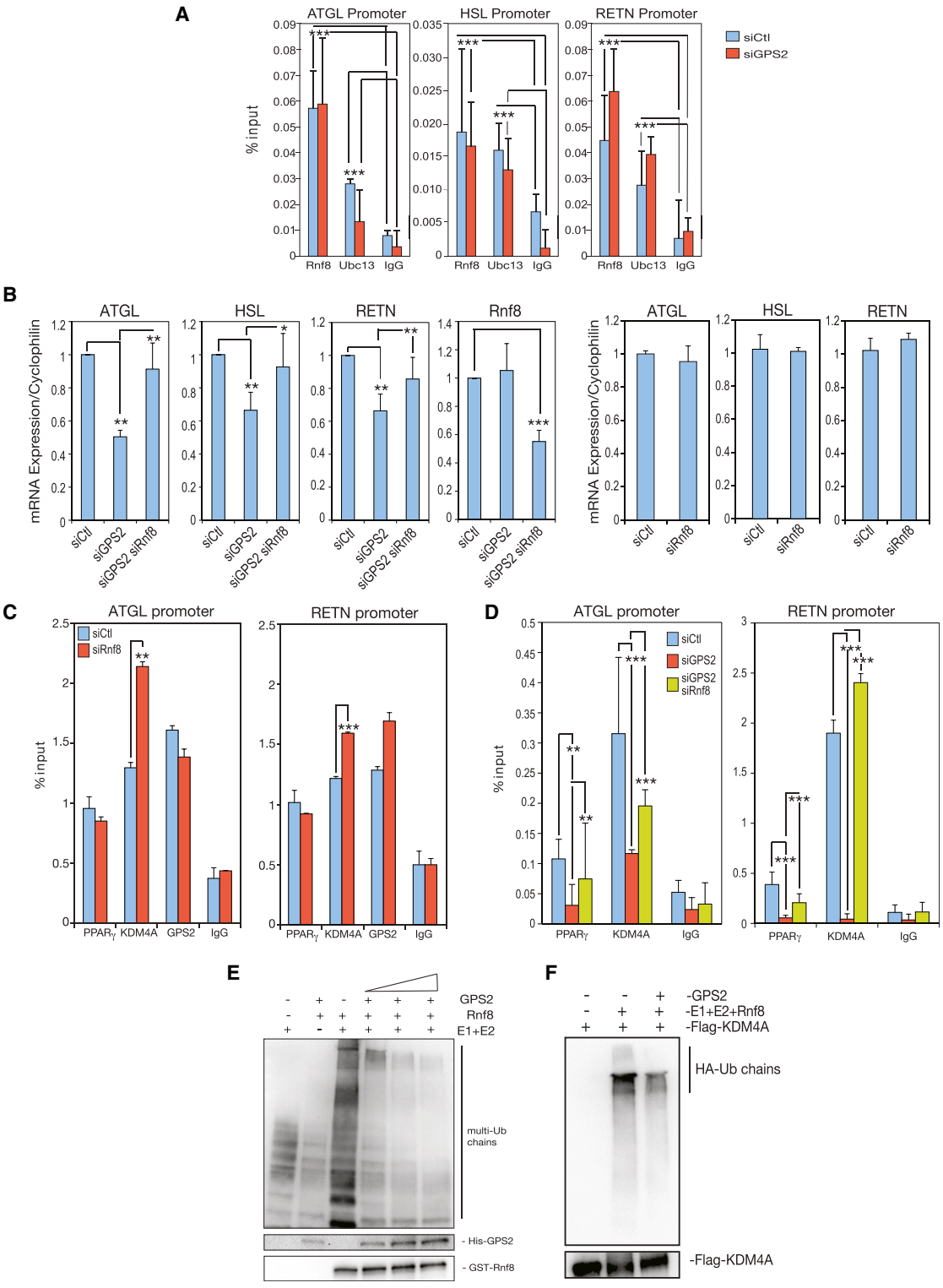

Figure 5. Inhibition of RNF8 Enzymatic Activity Is Required for KDM4A Stabilization and Lipolytic Gene Expression

(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.

(legend continued on next page)

signaling cues. Indeed, during the early phases of adipogenesis, when PPARγ is not yet expressed, other transcription factors, such as C/EBPβ, RXR, and GR, are required to promote gene expression changes that will determine cell fate. 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; Siersbæk 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 proadipogenic 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 toward 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 lipolysis and induced lipolysis are hyperactivated 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 into 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 complex, TBLR1, blunts the lipolytic response of white adipocytes through the impairment of cyclic AMP-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. Because 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 toward metabolic diseases.

EXPERIMENTAL PROCEDURES

Mice

The aP2-GPS2 transgenic mice were previously described (Cardamone et al., 2012). All animal experiments were performed in accordance with the NIH guide for the care and use of laboratory animals and with the approval of the Institutional Animal Care and Use Committee of Boston University.

Reagents and Antibodies

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

Lipolysis Assay

Lipolysis assays were performed in mouse primary adipocytes, isolated as described by Carswell et al. (2012), or in mature 3T3-L1 cells after 6–8 days 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 preincubated in Dulbecco's modified Eagle's medium without phenol red and supplemented with 4% BSA fatty acid-free for 5 hr prior to 1 hr 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 coIP 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; and 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 50 nM E1, 5 μg ubiquitin, and 200 nM 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

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 (Bio-Rad), followed by SYBR Green quantitative PCR (qPCR) amplification. Normalization was performed using specific amplification of Cyclophilin A, and qPCRs were performed in triplicate for each biological duplicate experiment. All ChIP and

(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 and 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 polyubiquitination of KDM4A by RNF8/Ubc13 is inhibited by GPS2.

All graphic data are ±SD, with corresponding p values indicated as follow: *p < 0.08; **p < 0.05; ***p < 0.01.

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 upon request. Data are shown as averages between the triplicates plus SD. Significance is calculated by paired Student's t test.

ChIP-Seq and Bioinformatic Analysis of ChIP-Seq Data Sets

ChIP-seq samples were subjected to standard ChIP; libraries were prepared and sequenced on a GAII sequencing machine according to Illumina's standard protocol. PPARγ, RXR, and Pol2 ChIP-seq data sets were downloaded from NCBI Gene Expression Omnibus (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 data sets 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 data sets. GPS2 peaks are considered on promoters if the peak summit is within −1 kb and +400 bp around the transcription start site (TSS). To define GPS2 peaks on enhancers, we extended the H3K4me1 and GPS2 peaks ±1 kb around the peak summit, and the intersections were computed by using BedTools. The heatmaps of ChIP-seq data sets were displayed in TM4/MeV. GO/pathway analysis was computed by DAVID/EASE, ToppGENE, HOMER, and GREATs. 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 data sets, 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 (false discovery rate <0.01) and a supplementary read density criterion of at least two reads per kilobase gene length.

ACCESSION NUMBERS

The NCBI GEO repository accession number for the GPS2 and KDM4A ChIP-seq data sets reported in this paper is GSE57779.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.05.041.

AUTHOR CONTRIBUTIONS

M.D.C. and V.P. conceived the project and designed and analyzed all the experiments. M.D.C. performed most of the experiments with the help of M.C., C.T.C., and J.A. Deep sequencing experiments were run in the laboratory of M.G.R., and B.T. performed all bioinformatics analyses. V.P. supervised the project and wrote the manuscript with critical input from M.D.C. and M.G.R.

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