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SETDB2 links glucocorticoid to lipid metabolism through *Insig2a* regulation

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SUMMARY

Transcriptional and chromatin regulations mediate the liver response to nutrient availability. The role of chromatin factors involved in hormonal regulation in response to fasting is not fully understood. We have identified SETDB2, a glucocorticoid-induced putative epigenetic modifier, as a positive regulator of GR-mediated gene activation in liver. *Insig2a* increases during fasting to limit lipid synthesis, but the mechanism of induction is unknown. We show *Insig2a* induction is GR-SETDB2-dependent. SETDB2 facilitates GR chromatin enrichment and is key to glucocorticoid dependent enhancer-promoter interactions. INSIG2 is a negative regulator of SREBP and acute glucocorticoid treatment decreased active SREBP during refeeding or in livers of *Ob/Ob* mice; both systems of elevated SREBP-1c driven lipogenesis. Knockdown of SETDB2 or INSIG2 reversed the inhibition on active SREBPs. Overall, these studies identify a GR-SETDB2 regulatory axis of hepatic transcriptional reprogramming and identify SETDB2 as a potential target for metabolic disorders with aberrant glucocorticoid actions.

Graphical Abstract

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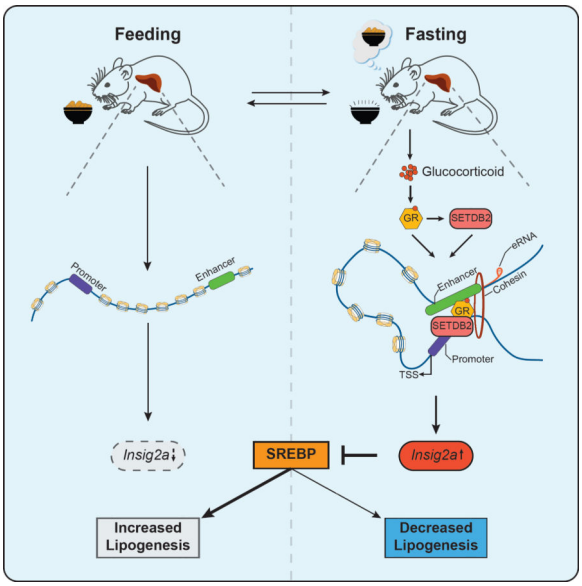
AUTHOR CONTRIBUTIONS

M.R.R., S.K., and T.F.O. developed the study concept and experimental design. M.R.R., R.M.E., P.E.P., and K.S., performed experiments, B.D. helped design and interpret the 3C study, F.F. provided reagents, J.D. and X.L. performed bioinformatics analyses, and M.R.R., S.K., and T.F.O. interpreted data and wrote the manuscript.



Keywords

SETDB2; glucocorticoid receptor; insig2; SREBP; fasting liver

INTRODUCTION

There are fifty SET-domain containing proteins in the human genome and studies over the last decade have shown they function as histone lysine methyltransferases and are key players in epigenetic regulatory processes (Volkel and Angrand, 2007). Methyltransferases also have non-histone substrates that impact many cellular and metabolic processes (Biggar and Li, 2015; Teperino et al., 2010). Some of these methyltransferases regulate hepatic metabolic homeostasis. For example, a deficiency in the H3K4 methyltransferase MLL3, results in resistance to high fat diet-induced hepatic steatosis (Lee et al., 2008a). The MLL3 methyltransferase is part of a larger complex that is a selective coactivator for the lipogenic transcription factor LXR (Lee et al., 2008b). Depletion of a histone arginine methyltransferase, PRMT5, was also shown to decrease hepatic glucose production. Mechanistically, PRMT5 interacts with the co-regulatory factor CRTC2 and the two are recruited to regulatory sites for gluconeogenic genes in response to glucagon signaling in the liver (Tsai, 2013). These studies confirm the relevance of protein methyltransferases in maintaining liver metabolic homeostasis. However, outside of these few examples, whether any other lysine or arginine methyltransferases contribute directly to liver physiology is unknown.

SETDB2 is classified in the KMT1 sub-family of SET domain-containing lysine methyltransferases that includes H3K9 methyltransferases such as SUV39H1, G9a and SETDB1. H3K9 methylation is a common histone mark associated with gene silencing and recent work in macrophages has linked SETDB2 to an antiviral and anti-inflammatory response through negative regulation of LPS and IFNβ-induced genes (Kroetz et al., 2015;

Schliehe et al., 2015). Consistent with it participating in gene silencing, a loss of SETDB2 resulted in a decrease in H3K9 trimethylation levels at promoters of several proinflammatory genes.

In contrast, we have identified SETDB2 can also perform as a positive regulator of gene expression, and reveal a novel underlying regulatory mechanism for glucocorticoid receptor (GR)-mediated gene activation during fasting in the liver. Our mouse liver microarray and RNA-seq analyses comparing genes differentially expressed by fasting versus feeding revealed *Setdb2* was expressed significantly higher in the fasted state among all transcripts encoding SET-domain containing proteins. We show SETDB2 gene expression is directly regulated by glucocorticoids and the protein interacts with nuclear GR to facilitate long-range GR-dependent chromatin interactions to induce a subset of GR target genes during fasting and in response to acute dexamethasone (Dex) challenge. Interestingly, SETDB2 recruitment is accompanied by a decrease in H3K9 methylation suggesting this scaffolding role is independent of SETDB2 H3K9 methyltransferase activity.

One of the GR-SETDB2 target genes is *Insig2a*. The *Insig2* gene encodes for two transcripts, *Insig2a* and *Insig2b*, which differ only in their non-coding first exon. *Insig2a*, but not *Insig2b*, was found to increase significantly in the liver with fasting and decrease with refeeding (Yabe et al., 2003). The mechanism for the induction of *Insig2a* by fasting however has not been characterized. INSIG2 resides at the endoplasmic reticulum trapping the SREBP-SCAP complex to prevent SREBP translocation to Golgi (the site of SREBP proteolytic activation). This mechanism prohibits SREBP nuclear translocation and activation of lipogenic genes. Thus, *Insig2a* induction by GR-SETDB2 contributes to the negative regulation of lipogenesis during fasting. We also show that SETDB2 induces *Insig2a* in response to an acute glucocorticoid challenge both during the refeeding cycle and in the livers of *Ob/Ob* mice. In both of these situations, SREBP levels are significantly elevated to increase lipogenesis and the glucocorticoid dependent increase in *Insig2a* results in a decrease in the nuclear accumulation of SREBPs. Thus, our studies link glucocorticoids and GR directly to lipid metabolism through SETDB2 and INSIG2.

RESULTS

The hepatic transition between the fed and fasted states represents a robust nutritional regulatory switch and is dependent on changes in gene expression required to adapt to the two different extreme metabolic conditions. We performed a microarray comparison of genes differentially expressed under fasting versus feeding in mouse liver and plotted the data to emphasize genes that were expressed at higher levels during fasting (Fig. S1A, Table S1). As expected, genes encoding enzymes involved in fatty acid oxidation and glucose production were high on the list. In searching for understudied genes that might play key roles in regulating the differential gene patterns we noted that *Setdb2*, a putative histone H3 lysine 9 methlytransferase, was expressed at significantly higher levels during fasting. This was unusual because it was the only putative epigenetic modifying enzyme that exhibited a similar pattern of expression. In support of our finding, *Setdb2* is indeed significantly induced by fasting but downregulated by refeeding in a liver RNA-seq analysis by Zhang et al (Zhang et al., 2011), while transcripts corresponding to other SET-domain lysine

methyltranferases were only moderately altered between fasted and refed states (Table S2). SETDB2 is a member of the SET-domain family of lysine methyltransferases that have been linked to both positive and negative regulation of gene expression (Glaser et al., 2006; Mozzetta et al., 2015). There is abundant literature on many of the SET domain enzymes but comparatively little was known about SETDB2 when we started our work.

Liver SETDB2 levels increase in response to glucocorticoids

We first confirmed that *Setdb2* mRNA was elevated by gene-specific qPCR and also found nuclear SETDB2 protein was elevated in livers of fasted mice compared to a refed state (Fig. 1A). The robust induction of *Setdb2* in liver suggested it performs an important regulatory role during fasting. In order to evaluate the role of SETDB2 in liver, we sought to first identify the mechanism for elevated *Setdb2* expression during fasting by treating mouse primary hepatocytes (MPH) with various compounds that mimic stress-related signals that regulate genes relevant to fasting. These include the fasting hormone glucagon, a catecholamine (isoproterenol), a PKA activator (forskolin), a PPAR-α agonist (WY14653), and the synthetic glucocorticoid dexamethasone (Dex) (Fig. 1B, S1B–C). Interestingly, Dex was the only compound that induced *Setdb2* mRNA. When Dex-treated MPH were exposed to RU486, a GR antagonist, the glucocorticoid-mediated increase of *Setdb2* was blunted (Fig. S1D). Furthermore, *Setdb2* RNA and nuclear protein were also robustly increased when *ad lib* fed mice were injected with Dex. In support of these observations, GR knockdown prevented the *Setdb2* induction by Dex in mice that were pre-infected with an adenovirus expressing a sh-GR construct (Fig.1C). We also identified a glucocorticoid responsive DNase sensitive region with a GR binding site at −5kb relative to the *Setdb2* transcriptional start site (TSS) in a previously reported genome-wide analysis of GR binding in the liver (Grontved et al., 2013) (Fig. S1E). Next, we showed that GR was robustly enriched at the predicted site within the *Setdb2* 5' flanking region in response to either Dex treatment (Fig. S1F) or fasting for 24 hour (Fig. S1G). These results are consistent with GR activating *Setdb2* expression in liver during fasting or following Dex treatment. This SETDB2 increase by Dex was also observed in human Hepatocytes (Corning) and the HepG2 cell line (Fig. S1H). Nuclear accumulation of the SETDB2 protein in response to elevated glucocorticoids parallels the translocation of GR from the cytoplasm to the nucleus (Fig. 1E). Therefore, we assessed whether SETDB2 associates with nuclear GR by co-immunoprecipitation (Fig. 1D), and by confocal immunofluorescence co-localization for nuclear SETDB2 and GR (Fig. 1E), and found SETDB2 and GR interact in nucleus in response to glucocorticoid. Thus, we hypothesized that SETDB2 may work together with GR to activate glucocorticoid-regulated genes during fasting in the liver, which would represent a previously unrecognized GR-SETDB2 stress-responsive axis.

SETDB2 knockdown blunts glucocorticoid-mediated induction of a subset of GR targets

To evaluate the relevance of the SETDB2-GR interaction, we assessed GR activation of gene expression by Dex in MPH where *Setdb2* was knocked down using an sh-adenovirus approach (sh-DB2). The sh-DB2 treatment resulted in an 80% knockdown of *Setdb2* mRNA. An RNA-seq analysis identified 2678 differentially expressed genes in the Dex-treated cells infected with the sh-DB2 adenovirus relative to the Dex-treated control cells (Table S3). The most down-regulated genes in the sh-DB2 cells corresponded to known

Dex-responsive GR targets, such as *Tat* and *Cyp2b10*. When we compared the SETDB2-regulated genes to GR target genes identified by comparison of wild type and a liver knockout of GR (Wong et al., 2010) there were 34 Dex-induced genes that overlapped (36%; 34 of the 95) (Fig. 2A). Therefore, a significant set of glucocorticoid-responsive genes is under the control of the GR-SETDB2 regulatory axis.

A list of these putative GR-SETDB2 targets is presented in Table S4 and includes *Tat, Cyp2b10*, *Insig2*, *Igfbp1*, and *Gdf15*, while Dex induction of other known GR targets including *Fkbp5, Mt2*, and *Sult1e1* were not affected by SETDB2 knockdown. To test if this subset of GR targets is also regulated by SETDB2 *in vivo*, we studied the impact of SETDB2 deficiency in mice infected with a sh-control or sh-DB2 adenovirus followed by Dex treatment. SETDB2 and GR accumulated in the nucleus in response to Dex in the control (Fig. 2B, lanes 1–2), while only GR was detected in the nucleus of sh-DB2 infected mice (Fig. 2b, lane3). Therefore, SETDB2 knockdown did not impair nuclear accumulation of GR, but did compromise Dex induction of *Tat* and *Cyp2b10* (Fig. 2C) as we observed in MPH. Accordingly, GR binding to *Tat* and *Cyp2b10* promoters analyzed by ChIP-qPCR was also decreased by SETDB2 knockdown (Fig. 2D). In contrast, Dex induction of GR targets *Mt2* and *Sult1e1*, which were refractory to SETDB2 knockdown in MPH, was not affected by SETDB2 knockdown in the mouse liver (Fig. S2A), and GR binding to the promoters of *Mt2* and *Sult1e1* was not affected (Fig. S2B). Thus, SETDB2 is required for glucocorticoid-induced GR binding and activation of a subset of GR target genes in the liver.

GR and SETDB2 mediate glucocorticoid-induced transcription of *Insig2a* via GR binding at the *Insig2* locus

Along with *Tat* and *Cyp2b10*, *Insig2* is also on the list of putative GR-SETDB2 co-regulated genes. This is notable because the INSIG2 protein, which localizes to the endoplasmic reticulum, is a negative regulator of SREBP proteolytic activation required for nuclear translocation (Radhakrishnan et al., 2007). SREBPs activate genes of lipid synthesis and they are efficiently shut off during fasting in the liver (Horton, 1998). This is particularly relevant for SREBP-1c, which is a major regulator of insulin-dependent lipogenic gene expression, thus, SREBP-1c mRNA and protein levels decline rapidly upon fasting (Horton, 1998). Expression of the *Insig2a* isoform is specifically induced by fasting (Yabe et al., 2003) to limit SREBP maturation as reported over a decade ago, but the mechanism involved has not been determined. Based on our results, we hypothesized that GR and SETDB2 co-regulate *Insig2a* during fasting. Similar to the results for *Setdb2*, *Insig2a* expression in MPH was induced by Dex but not by the other compounds that mimic stress pathways that are activated by fasting (Fig. S3A). Additionally, RU486 prevented the Dex-mediated induction of *Insig2a* (Fig. S3B). *Insig2a* expression was also induced by Dex treatment in liver and this was blunted by infection with sh-GR or sh-DB2 (Fig. 3A). As predicted, sh-DB2 treatment blunted the fasting dependent induction of *Insig2a* in the liver (Fig. S3C) which was accompanied by a two-fold increase in hepatic triglyceride levels in *ad lib* chow fed mice (Fig. S3D) suggesting this regulatory mechanism plays a key physiologic role *in vivo*.

When we evaluated the *Insig2* gene locus within a genome wide data set of Dex-regulated DNase 1 hypersensitive regions and GR binding in the liver (Grontved et al., 2013), we

found both Dex-responsive DNase 1 hypersensitive sites and GR binding sites near the TSS for *Insig2a* (Fig. 3B). Thus, we performed GR-ChIP using primers to interrogate these sites and showed that GR binding was both Dex-responsive and dependent on SETDB2 at 3 sites near the *Insig2a* TSS (Figs. 3C and S3E–F). GR enrichment was most robust at the −1.6kb TSS site (Fig. 3C). Interestingly, Dex-responsive SETDB2 enrichment was also observed at the −1.6kb site (Fig. 3D), further supporting SETDB2-GR co-regulation of *Insig2a* by GR. Additionally, GR and SETDB2 binding were also increased at the *Insig2a* promoter by fasting (Fig. S3G), suggesting that endogenous glucocorticoid action is sufficient to drive GR and SETDB2 binding and activation of hepatic *Insig2a* expression during fasting.

GR-SETDB2 co-regulation in liver is associated with a decrease in H3K9 methylation

Our data so far suggest SETDB2 cooperates with GR to activate a specific set of GR target genes in response to stress (Figs. 4A–B). SETDB2 is predicted to be a H3K9 methyltransferase, and its knockdown during interferon stimulation in macrophages results in reduced H3K9me3 at promoters for cytokine genes (Kroetz et al., 2015; Schliehe et al., 2015). Thus, we analyzed H3K9me3 levels at GR-SETDB2 target promoters in livers of Dex-treated mice. Surprisingly, H3K9me3 was significantly reduced by Dex treatment at promoters of the GR-SETDB2 targets *Insig2a, Tat*, and *Cyp2b10* in wild type mice (Fig. 4C). H3K9me1 (Fig. S4A) and H3K9me2 (Fig. S4B) levels were also significantly reduced, without any significant changes in total H3 (Fig. S4C). However, Dex treatment did result in a significant increase in H3K4me3 (Fig. 4D). The increase in H3K4me3 along with the decrease in H3K9 methylation are consistent with gene activation, but they are inconsistent with SETDB2 acting as a H3K9 methyltransferase in this context. In accordance with SETDB2 contributing to gene activation, treatment with sh-DB2 blunted the Dex dependent decrease in H3K9me3 at SETDB2-GR promoter targets (Fig. S4E–H).

Interestingly, G9a, another member of the KMT1 subfamily functions as a molecular scaffold in activation of a subset of GR target genes and the scaffold role was not affected by an inhibitor of G9a methlytransferase activity (Bittencourt, 2012). Furthermore, the H3K9 demethylase JMJD1a participates as a cAMP-induced scaffold protein to stimulate enhancer-promoter looping of the *Adrb1* gene in brown adipose tissue in response to catecholamines (Abe et al., 2015) and this bridging function is also independent of the demethylase activity of JMJD1a. Based on these two examples, we hypothesized that SETDB2 might regulate GR-dependent enhancer-promoter interactions playing a scaffolding role to activate SETDB2-GR target genes independent of its putative H3K9 methylation activity.

Long-range chromatin interaction of enhancer-promoter at the *Lcn2* locus requires SETDB2

To test the hypothesis that SETDB2 activates gene expression with GR through long-range chromatin looping, we used the 3C (chromosome conformation capture) method (Dekker, 2002) focusing first on *Lcn2* because previous studies described long-range chromatin interactions at the *Lcn2* gene locus that contributed to *Lcn2* activation by glucocorticoids in a cell culture model (Hakim et al., 2009). In liver, Dex robustly increased *Lcn2* expression and this induction was significantly blunted when SETDB2 was knocked down (Fig. 5A).

GR and SETDB2 both associated with the *Lcn2* promoter, and GR enrichment was dependent on SETDB2 (Fig. 5B). Similar to the promoters of other SETDB2-GR gene targets, H3K9me3 at the *Lcn2* promoter was not decreased in Dex-treated liver infected with sh-DB2 (Fig. S4H), suggesting a Dex-dependent gene activation role for SETDB2 independent of its putative H3K9me3 activity. Therefore, we used *Lcn2* to test our hypothesis that SETDB2 facilitates GR-dependent chromatin looping to activate target gene expression. The proposed mechanism for the strong activation of *Lcn2* expression by glucocorticoids involves long-range chromatin interactions between an enhancer site near the neighboring *Ciz1* gene and a GRE site within the *Lcn2* proximal promoter (Hakim et al., 2009). We noted there is a Dex responsive DNase 1 site at +26kb relative to the *Lcn2* TSS, which is close to the previously reported enhancer region for *Lcn2* in cell lines (Hakim et al., 2009). We measured GR binding by ChIP-qPCR at this enhancer site and found GR enrichment was SETDB2 dependent (Fig. 5C). RAD21 is a component of the Cohesin complex and is known to participate in chromatin looping (Mishiro et al., 2009). We found RAD21 was significantly enriched at the Lcn2 promoter in response to Dex and this enrichment was also significantly reduced by SETDB2 knockdown (Fig. 5D).

Next, we evaluated interactions between the *Lcn2* promoter/enhancer using chromatin from livers of mice treated with Dex. This revealed a Dex-dependent increase in association of chromatin from the +26kb region with the TSS site. Furthermore, this putative looping decreased significantly in mice containing a hypomorphic gene trap construct (DB2GT, Fig. S5A) inserted at position 59423974 of Chromosome 14 upstream of exon 6 in the SETDB2 gene (Fig. 5E). SETDB2 gene expression was significantly reduced in livers of the SETDB2 gene trap mice (DB2GT) (Fig. S5B) and *Lcn2* induction in response to Dex was also blunted (Fig. S5B). Taken together, these results suggest SETDB2 acts as a scaffold protein to facilitate long-range enhancer-promoter interactions required for activation of a subset of GR target genes.

To further evaluate the role of SETDB2 in the Dex-dependent long range chromatin looping, we crossed the DB2GT mouse with a mouse expressing the FLP recombinase which results in excision of the gene trapped construct leaving a floxed allele at the otherwise wild type gene locus (Fig. S5A). Endogenous expression of *Setdb2* and *Lcn2* was rescued by this approach (Fig. S5C), in addition to the enhancer-promoter interaction observed at the *Lcn2* locus by 3C-qPCR (Fig. 5F). Binding of GR, SETDB2, and RAD21 to the *Lcn2* promoter and GR binding at the *Lcn2* enhancer were also restored (Fig. S5D).

Long-range enhancer-promoter at the *Insig2* locus requires SETDB2

In order to determine whether the SETDB2-dependent looping is a more general mechanism, we also evaluated long-range interactions within the *Insig2a* locus. A Dex-responsive DNase hypersensitive site was identified in the Insig2a locus at +41kb from the TSS in a previous data set (Grontved et al., 2013). Similar to *Lcn2*, GR binding to this site was SETDB2-dependent (Fig. 6A). We also detected a Dex-dependent enhancer RNA (eRNA) transcribed from the +41kb site which was dependent on both SETDB2 and GR (Fig. 6B). Interestingly, the +41kb eRNA also increased robustly in the fasted state (Fig. S6A), which was accompanied by GR and SETDB2 enrichment as well (Figs. S6B–C). 3C-

qPCR analyses of livers revealed a Dex-responsive long-range interaction between the +38/+48kb region and the *Insig2a* TSS that was SETDB2 dependent (Fig. 6C).

Similar to what was observed for *Lcn2*, mating of the DB2GT with the FLP expresser rescued the blunted induction of *Insig2a* expression (Fig. S6D), as well as restoring GR and SETDB2 binding (Fig. S6E). Dex-mediated induction of SETDB2-GR gene targets was also rescued in MPH from DB2GT mice when SETDB2 expression was restored by adenovirus transduction (Fig. S6F). Interestingly, induction was also restored by an adenovirus construct that expresses a mutant form of SETDB2 with point mutations at critical regions within the SET domain that are required for S-adenosylmethionine binding and catalytic activity (N639A and H640A) (Fig. S6F). This suggests that similar to G9a and JMJD1a, the scaffold role for SETDB2 does not require its enzymatic activity.

SETDB2 mediates Dex dependent SREBP regulation through *Insig2a*

INSIG2 is a negative regulator of SREBP maturation and conditions that result in an increase in INSIG levels are predicted to decrease nuclear accumulation of SREBPs (Jeon and Osborne, 2012). This occurs in liver during fasting through the increased expression of *Insig2a*; however, SREBP-1c gene expression is also suppressed rapidly by fasting because of the acute decrease in insulin. So whether the glucocorticoid dependent induction of *Insig2* expression would be sufficient to decrease nuclear SREBP levels was unclear because of the rapid loss in SREBP-1c gene expression upon fasting. Thus, we evaluated SREBP expression in mice that were refed following a fast where SREBP levels are robustly induced. Two hours prior to refeeding, we treated one group of mice with a vehicle control and three groups with increasing concentrations of Dex, and food was then added back and all mice were sacrificed after 8 hr of refeeding. The nuclear levels of both SREBP-1 and SREBP-2 were very low in mice harvested immediately following 24 hr of fasting and, as expected, both were significantly increased by refeeding (Fig. 7A, lanes 5–9). Interestingly, Dex treatment significantly reduced the induction of both SREBPs by refeeding (Fig. 7A–B, lanes 10–14). The acute Dex effect on SREBP processing is also concentration-dependent with SREBP-1 appearing to be more sensitive (Fig. S7). Parallel to the inhibition of SREBP processing, the Dex treatment resulted in accumulation of membrane bound INSIG2 protein, which is consistent with our model and would result in the retention of the SCAP-SREBP complex in the endoplasmic reticulum to prevent SREBP nuclear accumulation. To further evaluate the mechanism, we repeated this experiment but we also pre-infected cohorts of mice with sh-adenoviruses for SETDB2 or INSIG2 (Fig. 7B) to probe their roles in blunting SREBP regulation. Knockdown of either INSIG2 or SETDB2 resulted in decreased INSIG2 protein at the membrane and both blunted the Dex inhibition of SREBP accumulation. These results demonstrate that SETDB2 is required to mediate the acute Dex effect on nuclear SREBP. They also demonstrate that SETDB2 is upstream of INSIG2 because whereas knockdown of SETDB2 reduced both SETDB2 and INSIG2, knockdown of INSIG2 restored SREBP but SETDB2 expression was not affected.

Dex-dependent activation of *Insig2* a reduces SREBP-1 levels in livers of *Ob/Ob* mice

In addition to their obesity, *Ob/Ob* mice accumulate excess hepatic triacylglycerol (TAG) which is mediated through an elevated level of SREBP-1 and an increase in *de novo*

lipogenesis (Shimomura, 1999). When *Ob/Ob* mice were crossed with SREBP-1c knockout mice, the accumulation of liver TAG was significantly reduced without an effect on body weight (Yahagi et al., 2002). Thus, increased hepatic SREBP-1c is a key driver of fatty liver in the *Ob/Ob* mouse. *Ob/Ob* mice also have elevated circulating glucocorticoids but reduced levels of hepatic 11-β hydroxysteroid dehydrogenase (11β-HSD), an enzyme responsible for the local production of active corticosterone from inactive 11-keto forms (Liu, 2003). Additionally, expression of TAT, a sensitive GR target gene is also significantly reduced in *Ob/Ob* (Blake, 1970a; Blake, 1970b). Thus, *Ob/Ob* mice exhibit partial hepatic "glucocorticoid resistance" and this might contribute to elevated levels of SREBP-1c and excess TAG accumulation. Indeed, upon treating *Ob/Ob* mice with Dex, there was a robust increase in INSIG2 along with a decrease in the very high levels of nuclear SREBP-1c (Fig. 7C). Importantly, this was accompanied by a significant decrease in liver TAG as well (Fig. 7D). The decrease in TAG occurred without a change in total body weight (data not shown) and highlights the importance of the GR-SETDB2 axis for hepatic glucocorticoid regulation in a common pathophysiologic model.

DISCUSSION

The mammalian liver has evolved and integrated metabolic network to help maintain whole body energy homeostasis. It is a dynamic and robust process and incudes mechanisms to adapt rapidly to regulatory signals that change in response to fluctuations in nutrient demand and availability. A failure of the system leads to metabolic diseases ranging from simple NAFLD to steatohepatitis or NASH. Glucocorticoids play an important role in liver metabolism in part through local GR signaling. In the current study, we uncovered a new GR-associated protein, called SETDB2, that modulates glucocorticoid-responsiveness of a subset of GR target genes, which includes *Insig2*, an important negative regulator of SREBP and lipid metabolism (Radhakrishnan et al., 2007). Yabe et al. originally reported that INSIG2 was encoded by two mRNAs with distinct 5' terminal non-coding exons expressed from two different promoters (Yabe et al., 2003). The upstream promoter drives expression of the *Insig2a* isoform, which Yabe et al. showed was inducible by fasting in the liver whereas *Insig2b* is constitutively expressed from the second promoter. The mechanism for fasting-induced expression from the *Insig2a* promoter and whether it plays a key role in regulating SREBP levels has not been established. We show that *Insig2a* induction during fasting is strongly dependent on the combined actions of GR and SETDB2.

There is abundant literature on a number of SET domain lysine methyltransferases contributing to cellular signaling pathways, but comparatively little is known about the physiological and biochemical functions of SETDB2 in liver. SETDB2 is a member of the KMT1 sub-family of SET domain containing lysine methyltransferases that includes SUV39H1, G9a, and SETDB1 (Volkel and Angrand, 2007). The similarity in primary structure of SETDB2 and SETDB1 has led to the assumption that SETDB2 is a H3K9 methyltransferase, but evidence is lacking. A study by the Gozani lab showed SETDB1, but not SETDB2, is capable of transferring a chemical label to an H3 tail peptide consistent with H3K9 methyltransferase activity for SETDB1 but not SETDB2 (Binda et al., 2011). Another study found SETDB2 displayed no methylation activity with the unmodified or the H3K9 monomethylated peptide substrates (Falandry et al., 2010), but the authors noted a weak

SETDB2 methylation activity when the H3K9 dimethylated peptide was used as a substrate. SETDB2 and SETDB1 share only 36% sequence identity in their active site SET domains which are bifurcated and have distinct inserted sequences; this and other sequence differences between these proteins is consistent with their distinct epigenetic roles.

Two recent studies have shown that SETDB2 is induced by interferon signaling in macrophages in the context of acute respiratory viral infection (Kroetz et al., 2015; Schliehe et al., 2015). In this setting, SETDB2 loss of function experiments suggest it is induced to turn off a select set of pro-inflammatory and anti-viral genes during the resolution phase of the acute proinflammatory response. Cytokine gene expression remained high and H3K9me3 at the corresponding gene promoters was lower during the resolution phase in SETDB2-deficient macrophages. Thus, it is interesting that our liver studies show glucocorticoids induce SETDB2 recruitment to hepatic chromatin along with GR, and this is associated with gene activation. Additionally, the co-enrichment of GR and SETDB2 to target gene loci in liver was accompanied by a decrease in H3K9me3, which was the predicted epigenetic mark for SETDB2. Levels of H3K9me1 and H3K9me2 were also reduced. This is a consistent pattern for H3K9me changes that accompany gene activation and suggests the putative H3K9me3 activity of SETDB2 is not involved in co-enrichment and activation with GR. Recent studies have found epigenetic modifiers can positively and negatively contribute to gene expression programming in a context-dependent manner. For example, G9a, known for its H3K9me2 activity in gene silencing, also acts as a positive transcriptional coregulator for GR and estrogen receptor (ERα) in cancer cells (Bittencourt, 2012; Zhang et al., 2016). G9a can dimethylate a non-histone substrate, the estrogen receptor (ERα), creating a docking site for an epigenetic reader (PHF20 tudor domain) for the recruitment of the MOF acetyltransferase complex to cancer-associated ER-responsive promoters (Zhang et al., 2016).

Overall, our studies suggest SETDB2 drives the transcriptional activation of genes relevant to the metabolic stress of fasting in liver, a condition when circulating glucocorticoids are elevated. Therefore, we predicted that altered SETDB2 levels in the liver could affect metabolic homeostasis through deregulated expression of GR-SETDB2 targets, such as *Insig2a*. This is notable because INSIG2 can inhibit lipogenesis via retention of the inactive SCAP-SREBP complex within the ER membrane. We therefore evaluated the impact of glucocorticoid-induced hepatic *Insig2a* expression on SREBP processing during the fasting to refeeding transition. During fasting, SREBPs are very low in part due to INSIG2, while refeeding inhibits *Insig2a* expression and drives SREBP nuclear accumulation to activate the lipogenic and cholesterogenic gene programs. When we injected Dex into mice just before the refeeding phase was initiated, the levels of INSIG2 protein were maintained at a high level and SREBP nuclear accumulation was dramatically reduced. This suggests that acute Dex treatment *in vivo* leads to a GR-SETDB2-mediated induction of hepatic *Insig2a* and an increase in membrane INSIG2 protein that effectively inhibits SREBP nuclear translocation. When this was repeated under conditions where either INSIG2 or SETDB2 protein levels were knocked down, there was a blunting of the Dex inhibition of SREBP accumulation. However, the sh-DB2 and sh-Insig2 blunting of the Dex-mediated suppression of SREBP-1 does not fully restore SREBP-1 processing to control refed levels suggesting additional Dex-dependent target genes also influence SREBP-1 protein levels.. Interestingly, the knockdown

experiments also demonstrate that SETDB2 is upstream of INSIG2 because while knockdown of either one resulted in partial restoration of SREBP, the knockdown of INSIG2 restored SREBP but SETDB2 levels were unaffected.

Insufficient activation of *Insig2a* through GR signaling may contribute to NAFLD in *Ob/Ob* mice. SREBP-1c levels are elevated in livers of *Ob/Ob* mice (Shimomura, 1999) and likely contribute to hepatic steatosis in this model because when the SREBP-1c knockout mouse was crossed with *Ob/Ob*, body weight remained high but hepatic triglyceride levels were significantly reduced (Yahagi et al., 2002). This apparent hepatic glucocorticoid resistance is also supported by the significant reduction in expression and activity of 11β-HSD1 (Liu, 2003) and TAT in *Ob/Ob* liver (Blake, 1970b). 11β-HSD1 produces locally active corticosterone from the inactive 11-dehydrocorticosterone in order to amplify local glucocorticoid action. Mice deficient in 11β-HSD1 have a blunted induction of glucocorticoid-induced genes in liver during fasting, while SREBP targets are increased in the refed state (Kotelevtsev, 1997; Morton et al., 2001). Thus, we posited that liver SREBP-1c levels in *Ob/Ob* mice might be elevated in part because of ineffective regulation of *Insig2a* by GR. Consistent with this hypothesis, when we injected Dex into *Ob/Ob* mice, there was an increase in membrane localized INSIG2 in the liver that was matched by a reciprocal decline in the excessively high SREBP-1c nuclear protein levels. Importantly, this was also accompanied by a significant reduction in liver TAG that was independent of changes in total body weight.

Mechanistically, we showed that SETDB2 influences chromatin looping at the *Lcn2* and *Insig2* loci. This occurs through dynamic enhancer-promoter interactions that increase in response to glucocorticoids, and decrease with deficiencies in either SETDB2 or GR. We found a previously undocumented enhancer-promoter interaction between an enhancer at +41kb and the proximal TSS of *Insig2*. The +41kb enhancer site coincides with a Dex-responsive DNase hypersensitive region in liver (Grontved et al., 2013) that we show expresses a Dex-responsive and SETDB2 dependent eRNA that is also induced by fasting. DNase hypersensitive sites and eRNA are both markers for active enhancers and they are associated with chromatin looping as reported previously in an estrogen responsive system (Hah et al., 2013). Epigenetic modifiers, like SETDB2, have previously been reported to contribute to chromatin looping-mediated gene regulation independent of their enzymatic activity, as is the case for G9a and JMJD1a (Abe et al., 2015; Bittencourt, 2012). G9a was described as a molecular scaffold for transcriptional coactivators in an alveolar epithelial carcinoma cell line, a function which was maintained in the presence of an inhibitor of G9a methylation activity (Bittencourt, 2012). JMJD1a is an H3K9 demethylase that regulates expression of a subset of gene targets within the beta-adrenergic system of brown adipose. JMJD1a facilitates chromatin looping that is dependent on cAMP phosphorylation of JMJD1a. However, a JMJD1a mutation that eliminates its demethylase activity did not affect its role in looping (Abe et al., 2015). Like G9a and JMJD1a, we demonstrate SETDB2 works as a putative molecular scaffold, independent of its putative methyltransferase activity, to dynamically regulate signal dependent long-range enhancer-promoter interactions. The scaffold function for SETDB2 in chromatin looping is relevant to activation of stress-responsive GR targets within the liver. The fasting-related GR-cistrome in liver shows binding sites are clustered with other transcription factor motifs within open chromatin

domains and this arrangement is predicted to confer a rapid induction of stress-responsive genes (Goldstein and Hager, 2015), like *Insig2a*. Thus, future work will evaluate if the synergy between GR and other transcription factors relies on SETDB2 to facilitate looping and regulate expression of fasting-induced genes in liver.

In summary, we have uncovered a novel function for SETDB2 as a GR-interacting protein that is both glucocorticoid-responsive at the transcriptional level and is co-enriched at the protein level along with GR at select glucocorticoid regulated target sites. This provides mechanistic insight into GR's mode of action for transcriptional regulation in liver and makes SETDB2 a potential therapeutic target to modulate glucocorticoid action in metabolic diseases associated with altered glucocorticoid sensitivity such as obesity and diabetes, or in patients undergoing chronic glucocorticoid treatment. These conditions are associated with liver dysfunction and can lead to NAFLD, NASH, fibrosis or liver cancer (Ahmed et al., 2012; Kadmiel and Cidlowski, 2013; Quax et al., 2013; Rose et al., 2010). Furthermore, there are single nucleotide polymorphisms (SNPs) within the human SETDB2 locus that are associated with metabolic disease traits including glycosylated hemoglobin (HGVPM569), systolic blood pressure (HGVPM563), fasting plasma insulin (HGVPM822), and serum cholesterol (HGVPM568). Additionally, SNPs near the human INSIG2 locus have been associated with obesity and lipid metabolism (Do et al., 2010; Herbert, 2006; Hotta et al., 2008; Kaulfers et al., 2015). Also, SETDB2 is induced by Dex in other metabolic tissues such as white adipose and skeletal muscle (data not shown). Thus, SETDB2 may contribute to a wide array of GR-associated diseases, which increases the translational relevance and therapeutic potential for understanding the mechanism of SETDB2 action more thoroughly.

EXPERIMENTAL PROCEDURES

Animals and treatments

All animal experiments were performed in accordance with accepted standards of animal welfare and with permission of the Sanford Burnham Prebys Medical Discovery Institute, Lake Nona's International Animal Care and Use Committee (protocol 2012-0088). We used 8 to 12-week-old male C57BL/6J mice from the Jackson Laboratory and maintained them on a chow diet (Teklad Diets, #2016) with a 12-hour light (7AM-7PM), 12-hour dark cycle (7PM-7AM). For fasting and refeeding studies, mice were sacrificed after a 24-hour fast (7AM-7AM), or after a 24-hour fast followed by either 2 hour refeeding (ChIP and 3C analysis) or 8-hour refeeding (protein analysis). Equal amounts of total liver RNA pooled from 6 mice per group (24 hour fasted versus fed) were used for triplicate microarray analysis as previously described (Shin et al., 2012). Dexamethasone (Dex) (Steraloids Inc., P0519-000) was delivered intraperitoneally at a dose of 10mg/kg, food was removed at the time of injection, and tissues were harvested 4 hours after treatment for RNA analysis or 2 hours for ChIP and 3C analysis. In refeeding studies, Dex was delivered 1 hour prior to refeeding. For adenoviral-mediated gene knockdown studies, adenovirus overexpressing short-hairpin (sh) Control, shSETDB2, shGR (Lemke et al., 2008), or shINSIG2 constructs (Haas et al., 2012) were delivered by intravenous retro-orbital injections at a dose of 2×10^9 plaque forming units (PFU) per mouse seven days prior to harvest.

Setdb2^{tm1a} mice containing a hypomorphic gene trap construct inserted at nucleotide position 59423974 of Chromosome 14, upstream of exon6 of SETDB2, were created from an embryonic stem (ES) cell clone EPD0164_4_E09 (JM8.N4 C57BL/6N) obtained from the Knockout Mouse Project (KOMP) repository. Germ line transmission of the Setdb2^{tm1a} allele was achieved by breeding male chimeric mice with C57BL/6J females and confirmed by genotyping along with the presence of black-coat pups. Setdb2^{tm1a} mice were bred with FLPeR mice (Jackson Laboratory, stock no. 009086) to excise the gene trap construct and result in the Setdb2^{flox} conditional allele with *loxP* sites flanking exon 6 of the *Setdb2* locus.

B6.Cg-Lep $^{ob/ob}$ mice (Jackson Laboratory, No. 000632) were treated with PBS or DEX (10mg/kg body weight) via intraperitoneal injection at ZT=10.

Chromosome Conformation Capture (3C) analysis

3C-qPCR analysis of mouse liver was performed as described previously (Hagege et al., 2007) with minor modifications. Liver (1.0 gram) was minced and fixed with 1% formaldehyde for 10min, treated with 125 mM glycine/PBS for 5 min, and washed with PBS. Liver pellets were processed as described above in the ChIP assay section in order to obtain intact nuclei. Nuclei were lysed in CutSmart Digestion buffer (NEB) containing 0.3% SDS for 1 hour at 37°C while shaking at 900rpm followed by addition of 2% Triton X-100 (final concentration) and continued incubation at 37°C for 1 hour. The chromatin DNA was then subject to restriction endonuclease digestion by addition of 400U of restriction enzyme (Hind III for *Lcn2* gene locus analysis or EcoR1 for Insig2 analysis). After overnight enzymatic digestion, chromatin samples were adjusted to 1.6% SDS and incubated at 65°C for 20 min. Sample buffers were adjusted to a ligation buffer composition as described in Hagehe et al., 2007 in a total volume of 6.125 mL containing 1% Triton-X100 (final concentration) followed by 1-hour incubation at 37°C. Chromatin fragments were ligated for 5 hours at 16°C and maintained at room temperature for 30 min prior to addition of proteinase K and overnight incubation at 65°C. Samples were treated with Riboshredder RNAse (Epicentre) for 45 minutes followed by phenol-chloroform DNA purification. Re-ligated fragments were further purified with the Roche High Pure PCR production purification kit and the resulting 3C template DNA was adjusted to 100 ng/uL. Standard curves were prepared using GAPDH for reference and promoter-enhancer interactions were assessed by TaqMan real-time quantitative PCR. Bacterial artificial chromosome (BAC) clone RP23-61N22 (CHORI BacPac Resource Center) spanning the *Ciz1-Lcn2* locus, and a BAC clone RP23-260H4 (CHORI BacPac Resource Center) spanning the Insig2 locus were used to prepare the control template for normalization of relative PCR amplification efficiencies. Primers and TaqMan probe sequences are provided in supplemental material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- SETDB2 is required for GR-mediated activation of select GR targets in liver.
- *Insig2a* induction in liver is SETBD2-GR dependent.
- SETDB2 is key to GR-mediated enhancer-promoter interaction at *Insig2* and *Lcn2* loci.
- SETDB2 knockdown blunts glucocorticoid-mediated inhibition of SREBP processing.

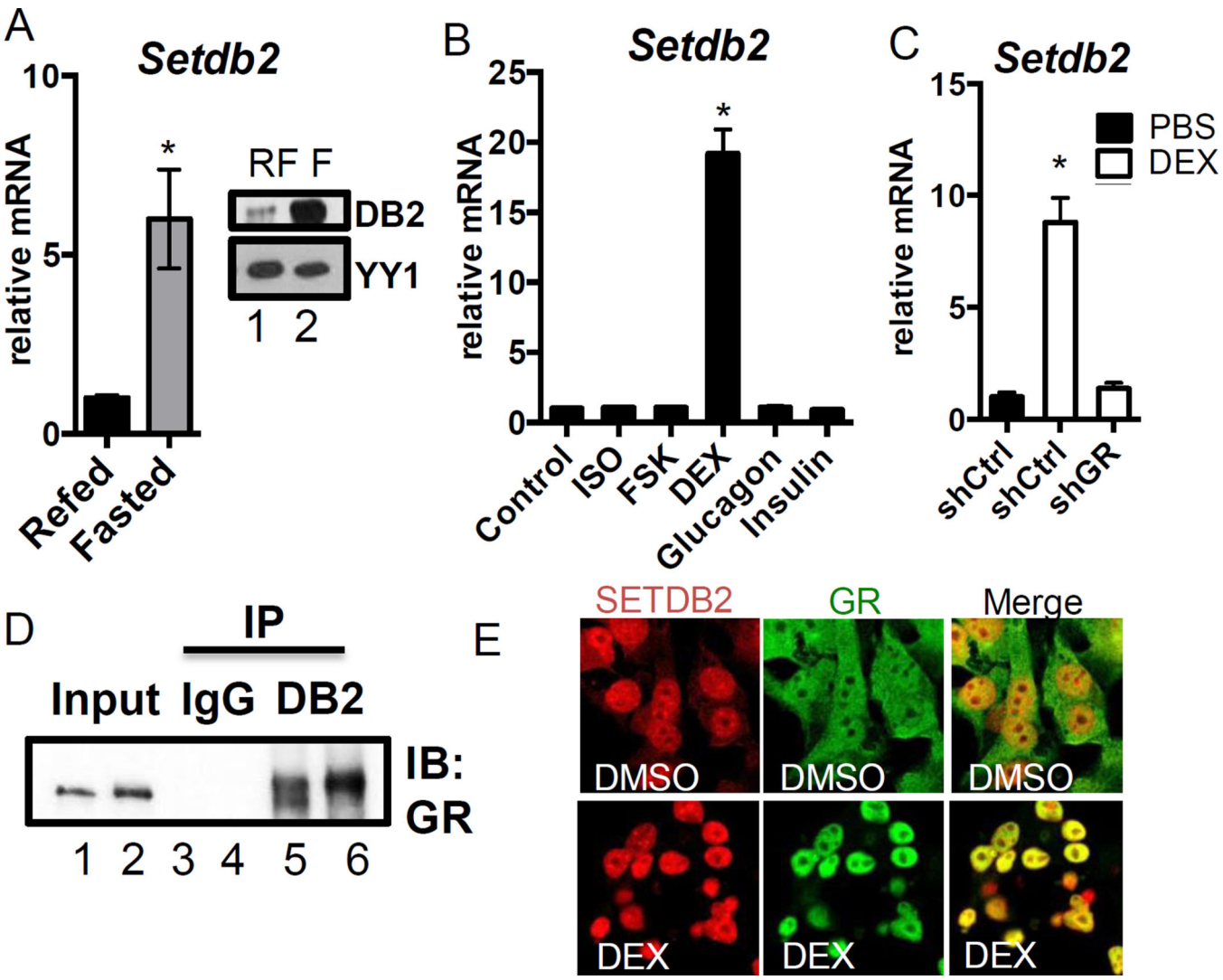

Figure 1. SETDB2 is a glucocorticoid responsive gene

A) Liver *Setdb2* mRNA and SETDB2 nuclear immunoblot in refed versus fasted liver. B) *Setdb2* mRNA in mouse primary hepatocytes treated with various stress/fasting-related compounds. C) Liver *Setdb2* induction by DEX is GR dependent. D) GR and SETDB2 interact in MPH. E) SETDB2 and GR immunofluorescence in Hepa1-6. *$p<0.05$ relative to control, (n=4–6).; RF=Refed, F=Fast, DB2=SETDB2. Data are represented as mean ± SEM.

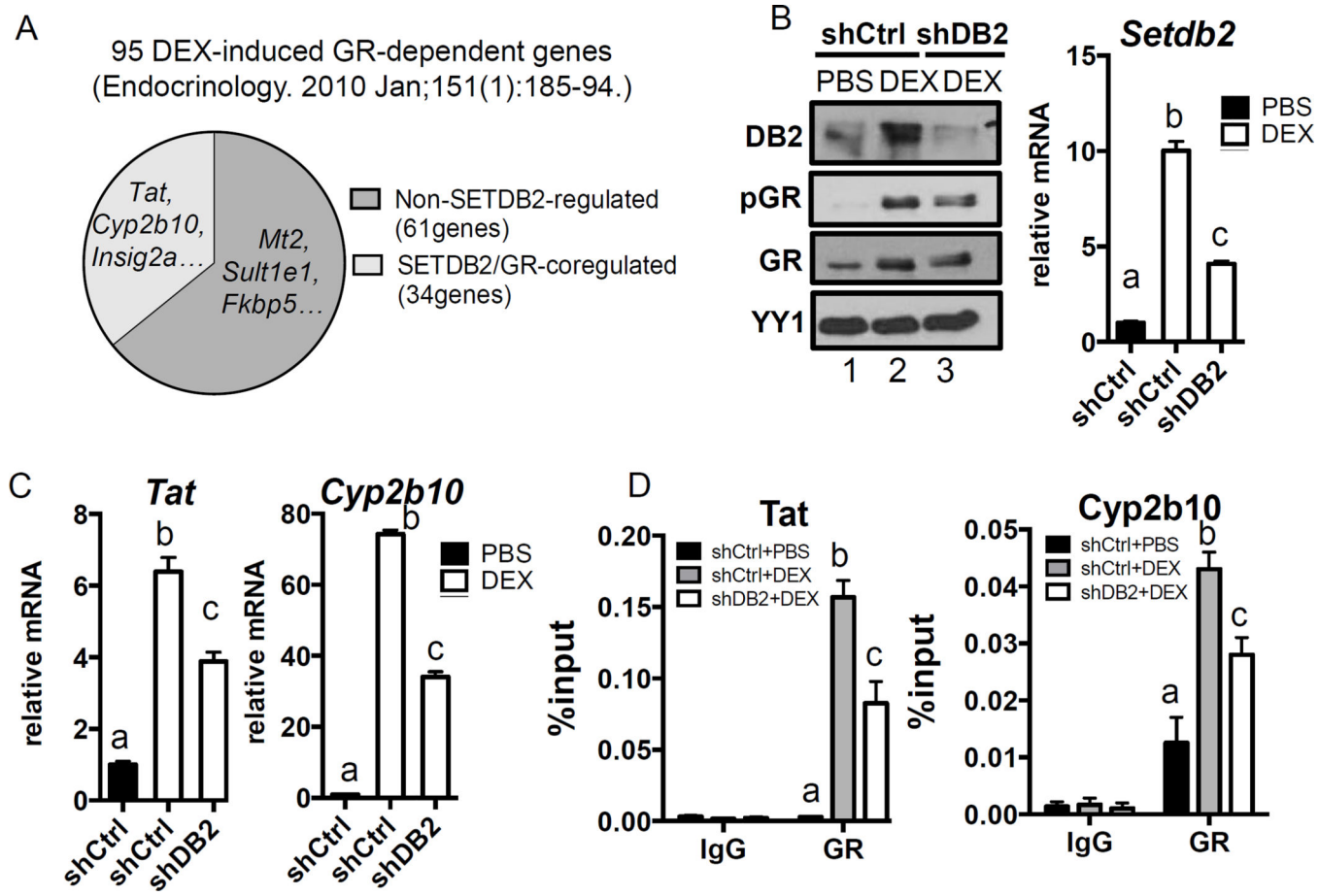

Figure 2. SETDB2 is required for Dex-mediated activation of select GR targets

A) Pie chart of SETDB2-GR co-regulated genes. B) SETDB2 immunoblot of liver nuclear protein and mRNA in mice infected with shControl (shCtrl) or shSETDB2 (shDB2) adenovirus. Liver mRNA and GR-ChIP qPCR at promoters of C) *Tat* and D) *Cyp2b10*. n=3. Groups with different letters are statistically different, $p<0.05$. Data are represented as mean ± SEM.

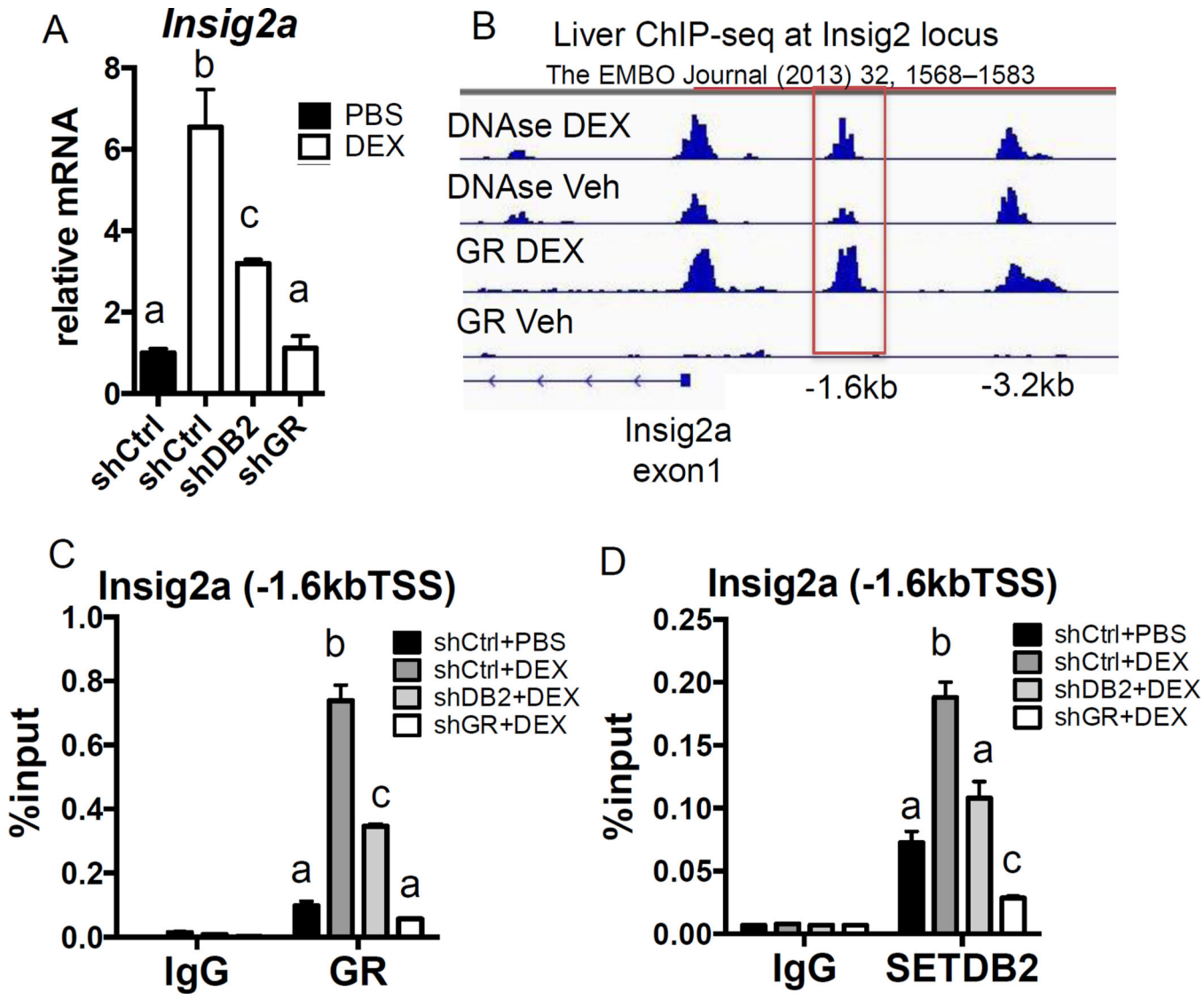

Figure 3. SETDB2 and GR are required for Dex-mediated activation of *Insig2a*

A) Liver *Insig2a* mRNA from mice infected with Ad-shControl (shCtrl), shSETDB2 (shDB2), shGR in presence or absence of Dex. B) *Insig2* gene track showing Dex-responsive DNase and GR binding site at −1.6kbTSS. C) GR ChIP-qPCR and D) SETDB2 ChIP-qPCR at *Insig2a* promoter. Groups with different letter are statistically different, $p<0.05$. Data are represented as mean ± SEM.

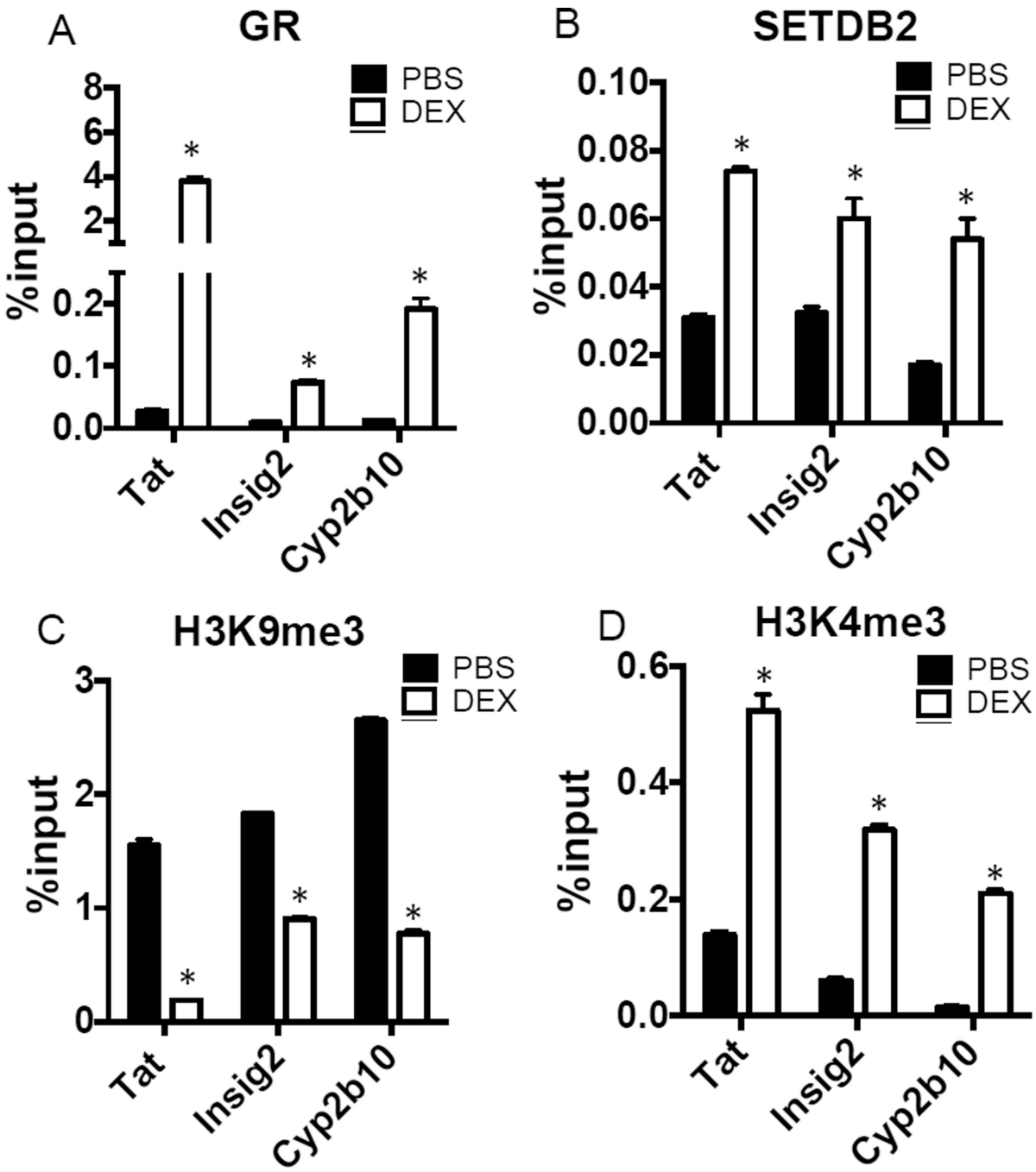

Figure 4. SETDB2 does not correlate with H3K9methylation at promoters of Dex-induced GR targets

ChIP-qPCR at promoters of SETDB2-GR regulated genes. A) GR. B) SETDB2. C) H3K9me3. D) H3K4me3. *p<0.05 relative to vehicle treatment. Data are represented as mean ± SEM.

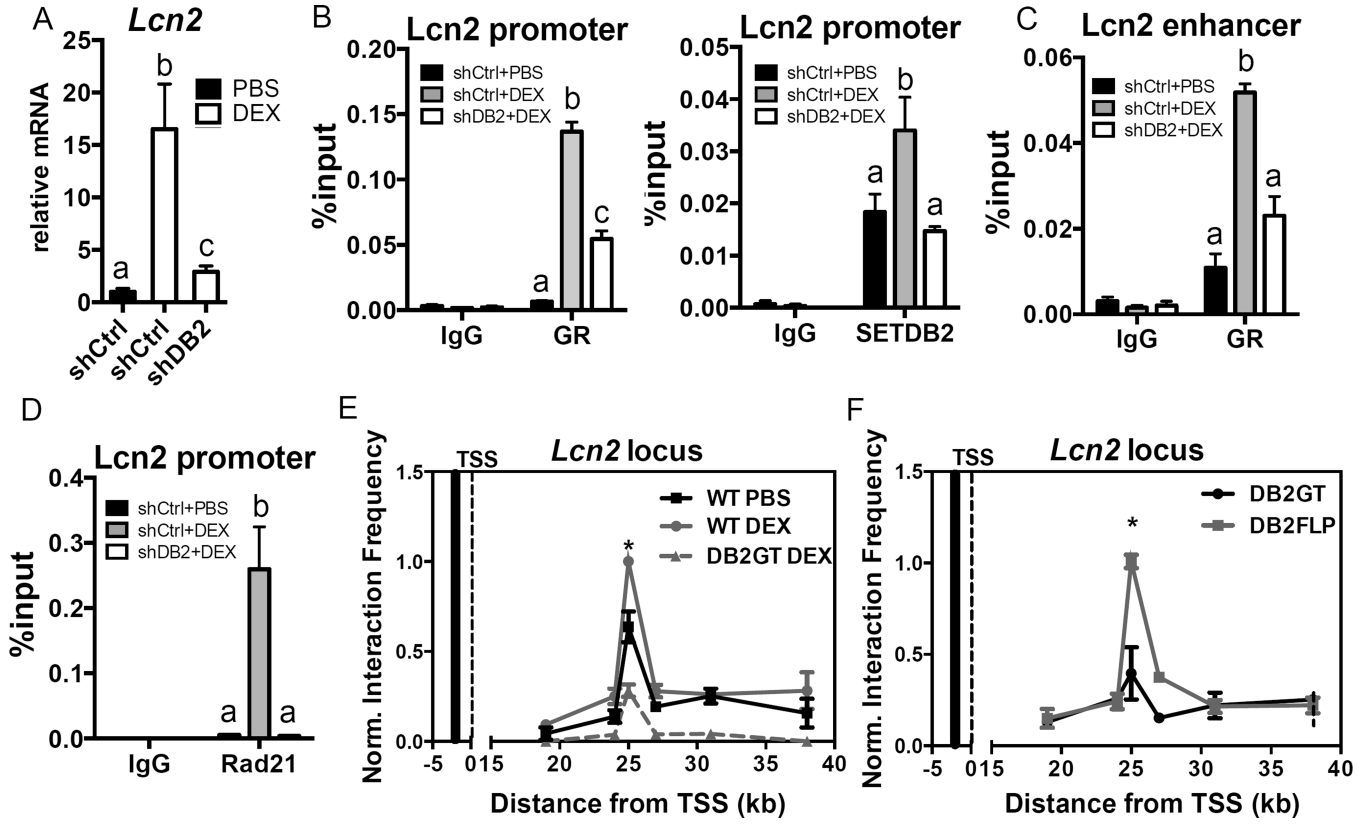

Figure 5. Long range chromatin interaction of enhancer-promoter at *Lcn2*

A) Liver *Lcn2* mRNA. B) GR and SETDB2 ChIP-qPCR at *Lcn2* promoter. C) GR-ChIP qPCR at *Lcn2* enhancer. D) RAD21-ChIP qPCR at *Lcn2* promoter. E) 3C-qPCR at *Lcn2* locus from wild type (WT) and SETDB2-deficient mouse livers (DB2GT) +/− DEX. F) 3C-qPCR at *Lcn2* locus from mouse liver with restored SETDB2 (DB2FLP) in presence of Dex. Groups with different letter are statistically different, $p<0.05$. *$p<0.05$ versus control. Bait for 3C-qPCR spans *Lcn2* promoter from TSS to −1.5kbTSS. Data are represented as mean ± SEM.

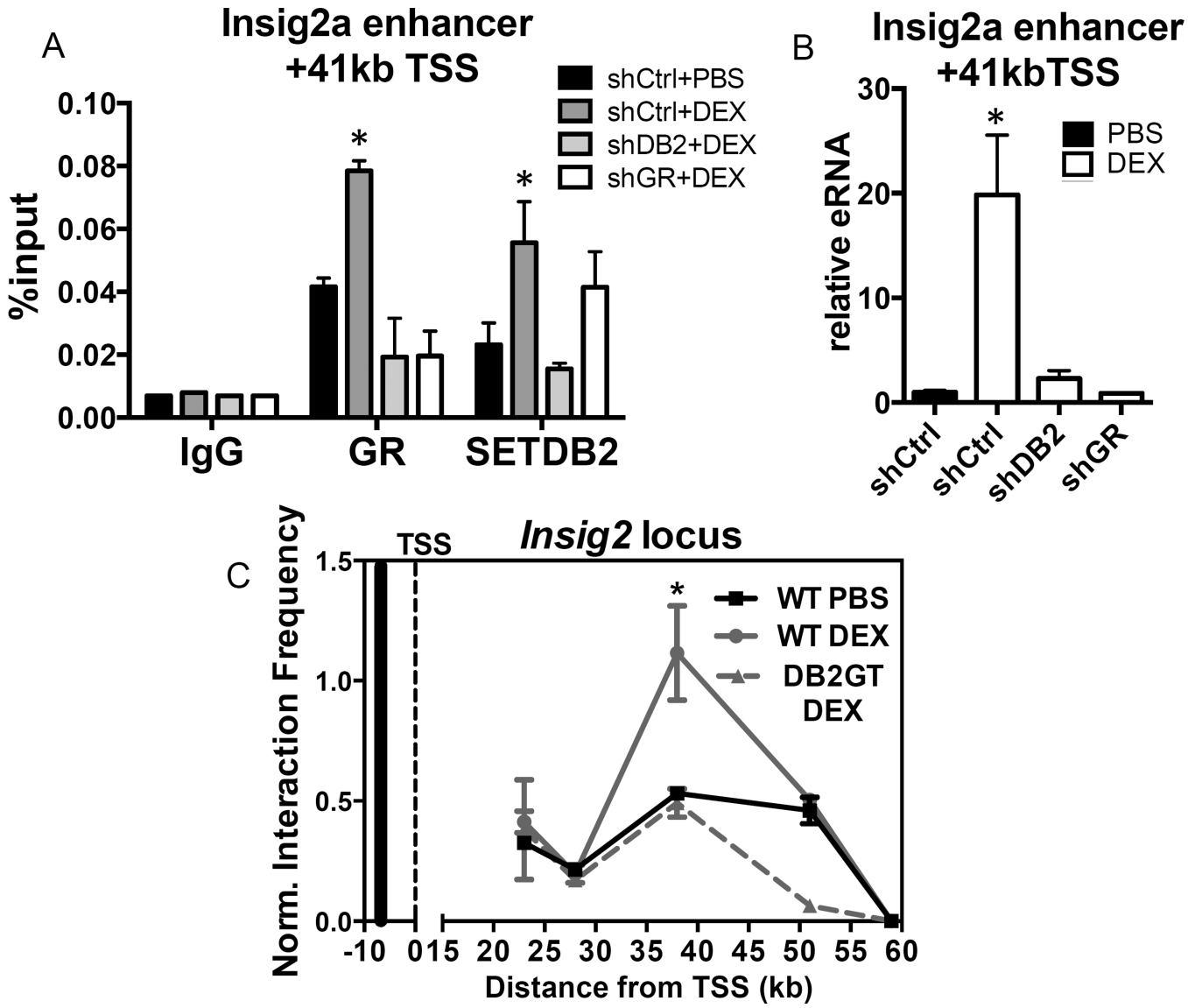

Figure 6. Long range chromatin interaction of enhancer-promoter at *Insig2*

A) GR and SETDB2 ChIP-qPCR at *Insig2* enhancer in livers lacking SETDB2 or GR. B) *Insig2* enhancer RNA of mouse liver in response to Dex. C) 3C qPCR at *Insig2* locus from wild type and SETDB2 gene trap mouse livers +/−DEX. *p<0.05 relative to sh-Control +PBS or WT PBS. Bait for 3C-qPCR spans *Insig2a* promoter from TSS to −8kbTSS. Data are represented as mean ± SEM.

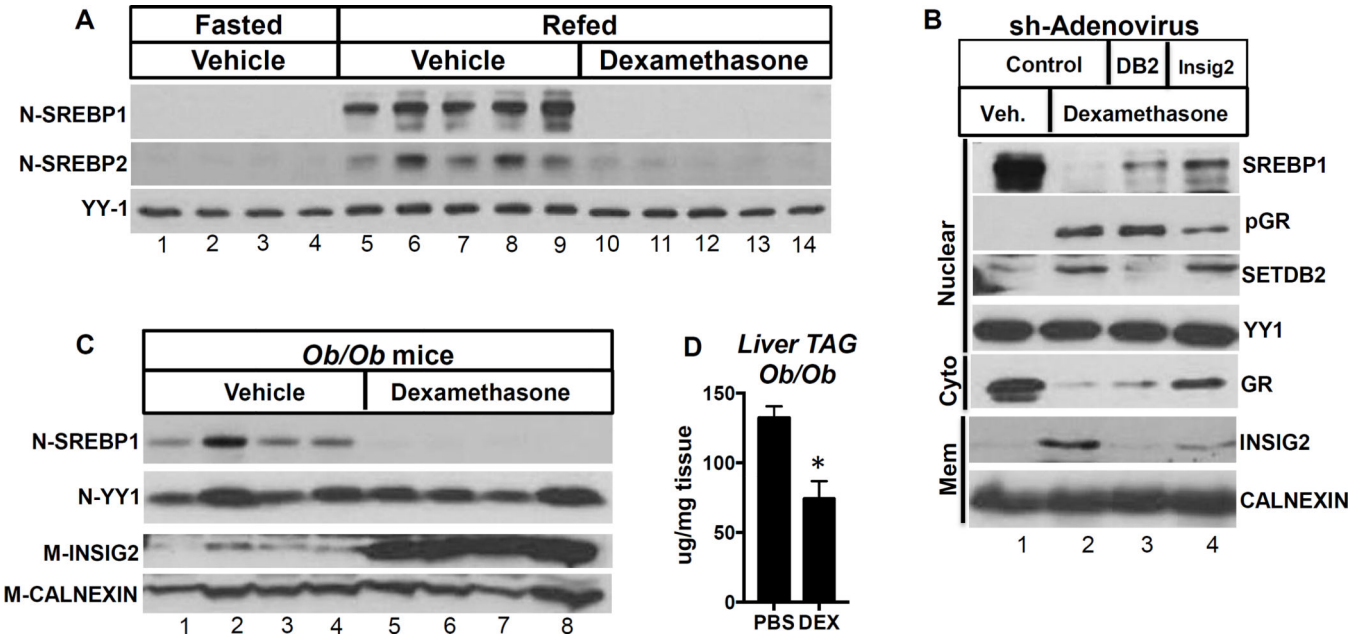

Figure 7. Dex inhibition of SREBP processing during refeeding

A) Liver immunoblot of nuclear SREBP1/2, during a fasted or refed state +/− Dex. B) Liver immunoblot during refed state +/−DEX in mice infected with shControl (shCtrl), shSETDB2 (shDB2), shINSIG2. n= pool 3 mice. C) Dex inhibition of SREBP processing in *Ob/Ob* liver. N-Nuclear, M-Membrane. Each lane in Figs. 7A and 7C represents protein from individual mice. D) Liver TAG (n=4) from 10-week old *Ob/Ob* mice treated with PBS or Dex (10mg/kg body weight) for 3 days via intraperitoneal injection at ZT=10; *p<0.05. Data are represented as mean ± SEM.