Hepatocyte DACH1 Is Increased in Obesity via Nuclear Exclusion of HDAC4 and Promotes Hepatic Insulin Resistance

Graphical Abstract

Highlights

- In obesity, CaMKII phosphorylates and blocks HDAC4 nuclear translocation in hepatocytes
- Lower nuclear HDAC4 decreases the SUMOylation and degradation of DACH1
- Increased nuclear DACH1 represses Atf6, which causes defective insulin signaling
- Silencing of hepatocyte DACH1 in obesity improves insulin sensitivity

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In Brief
Ozcan et al. find that CaMKII phosphorylates and blocks nuclear translocation of hepatocyte HDAC4 in obesity. Lower nuclear HDAC4 decreases the SUMOylation and degradation of the corepressor DACH1. Elevated nuclear DACH1 activates an ER stress pathway that causes defective insulin signaling, while silencing of hepatocyte DACH1 in obesity improves insulin sensitivity.
Hepatocyte DACH1 Is Increased in Obesity via Nuclear Exclusion of HDAC4 and Promotes Hepatic Insulin Resistance

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SUMMARY
Defective insulin signaling in hepatocytes is a key factor in type 2 diabetes. In obesity, activation of calcium/calmodulin-dependent protein kinase II (CaMKII) in hepatocytes suppresses ATF6, which triggers a PERK-ATF4-TRB3 pathway that disrupts insulin signaling. Elucidating how CaMKII suppresses ATF6 is therefore essential to understanding this insulin resistance pathway. We show that CaMKII phosphorylates and blocks nuclear translocation of histone deacetylase 4 (HDAC4). As a result, HDAC4-mediated SUMOylation of the corepressor DACH1 is decreased, which protects DACH1 from proteasomal degradation. DACH1, together with nuclear receptor corepressor (NCOR), represses Atf6 transcription, leading to activation of the PERK-TRB3 pathway and defective insulin signaling. DACH1 is increased in the livers of obese mice and humans, and treatment of obese mice with liver-targeted constitutively nuclear HDAC4 or DACH1 small hairpin RNA (shRNA) increases ATF6, improves hepatocyte insulin signaling, and protects against hyperglycemia and hyperinsulinemia. Thus, DACH1-mediated corepression in hepatocytes emerges as an important link between obesity and insulin resistance.

RESULTS
ATF6 Overexpression in Obese Mice Improves Insulin Sensitivity

While our previous work implicated suppression of ATF6 as a key mechanism of how CaMKII activation in HCs promotes insulin resistance, a key question relates to the molecular mechanism through which CaMKII suppresses ATF6. We show here that the corepressor DACH1 is responsible for silencing ATF6. DACH1 levels are increased in obesity by CaMKII-induced nuclear exclusion of HDAC4, which decreases HDAC4-mediated SUMOylation and degradation of DACH1. Accordingly, insulin sensitivity can be improved in obese mice by silencing either CaMKII or DACH1 or enforcing nuclear HDAC4 in HCs, without any change in food intake or body weight. These results identify DACH1 as a critical component of defective insulin action seen in obesity.
resistance in obesity (Ozcan et al., 2013), we asked whether genetically boosting hepatic ATF6 in obesity could improve metabolism. To this end, we treated diet-induced obese (DIO) mice with adeno-viral vectors encoding cleaved, nuclear ATF6 (ATF6N) or LacZ control. Adeno-ATF6N-treated mice had lower fasting blood glucose and lower plasma insulin levels and enhanced reduction of blood glucose in response to insulin stimulation (Figures S1A and S1B) in the absence of any change in body weight. Adeno-ATF6N treatment also improved acute insulin-induced p-Akt (Figure S1C), which is a dynamic measure of improved insulin signaling.

**CaMKII Suppresses HC Atf6 mRNA in Obesity**

Given the critical role of ATF6 in CaMKII-mediated improvement in insulin signaling, we next investigated whether CaMKII regulates ATF6 at the transcriptional level. For this purpose, we first analyzed the livers of Camk2gΔ/Δ obese mice treated with control AAV-TBG-LacZ versus AAV-TBG-Cre, which specifically deletes CaMKII in HCs and thereby increases insulin sensitivity (Ozcan et al., 2013). The livers of obese mice with HC-deleted CaMKII had higher ATF6 mRNA levels than the livers of the control obese mice (Figure S1D). We then isolated primary HCs from Camk2gΔ/Δ mice and treated them ex vivo with adeno-Cre to delete CaMKII or with adeno-LacZ control, followed by incubation with BSA (control) or palmitate. Palmitate mimics features of HC phosphorylation/nuclear exclusion of HDAC4 phosphorylation/nuclear exclusion of HDAC4 in obesity via nuclear exclusion of HDAC4 and promotes hepatic insulin resistance (Ozcan et al., 2013). Thus, we hypothesized that nuclear exclusion of HDAC4 repressed an Atf6 target, Dnajc3 (P58IPK), and abrogated the improvement in insulin-Akt signaling in CaMKII-deficient HCs upon palmitate treatment (Figures 1F and 1G). We then tested causation in vivo by silencing HDAC4 in DIO mice and found increased fasting blood glucose and plasma insulin levels and reduced glucose-disposal curves post-insulin (Figure S2) without a change in body weight. These data provide initial support for the role of an obesity-driven CaMKII-HDAC4 nuclear exclusion pathway in ATF6 suppression and defective insulin signaling in HCs.

To further establish causation, we took advantage of a phosphorylation-defective, constitutively nuclear HDAC4 mutant, HDAC4S3A (S246A/S467A/S632A) (Backs et al., 2006). Transfection of primary HCs with a relatively low titer of this mutant increased Atf6 and Dnaj3 mRNA levels and improved insulin-induced p-Akt after palmitate treatment (Figures 2A and 2B; compare white and black bars). To test the functional role of ATF6 in this improvement, we silenced Atf6 in palmitate-treated HDAC4S3A-overexpressing HCs using siRNA and found that this treatment lowered Dnaj3 and reduced insulin-induced p-Akt to the level of palmitate-treated control HCs (Figures 2A and 2B; compare black and gray bars). We then treated DIO mice with adenoviral vectors encoding the mutant HDAC4 versus LacZ control. Adeno-HDAC4S3A-treated mice had lower fasting blood glucose and lower plasma insulin levels in the absence of any change in body weight (Figures 2C and 2D). To link these findings to hepatic insulin signaling, we assayed p-Akt in the livers of mice injected with insulin through the portal vein. Similar to the case with palmitate-treated HCs, there was enhanced insulin-stimulated Akt phosphorylation in the livers of adenovirus-infected DIO mice (Figure 2E). Finally, acetylation of the key gluconeogenic transcription factor FoxO1, which suppresses its binding to DNA target sites, was shown to be increased in HCs and in the liver of lean mice with combined silencing of HDAC4, HDAC5, and HDAC7 (Mihaylova et al., 2011). However, we found that when hepatic HDAC4 alone was either silenced or rendered constitutively nuclear (HDAC4S3A) in obese mice, the level of acetylated FoxO1 in the liver was not altered (Figure S2F). These collective data indicate the presence of a CaMKII → HDAC4 phosphorylation/nuclear exclusion → ATF6 suppression pathway in obese liver, resulting in perturbed hepatic insulin signaling and disturbed glucose homeostasis.

**Obesity Induces HDAC4 Phosphorylation and Regulates Its Nuclear Localization**

The data above raise the key issue of how hepatic CaMKII lowers Atf6 transcription in obesity. Previous work has implicated CaMKII in transcriptional regulation through its ability to phosphorylate and promote the nuclear export of the class IIa histone deacetylase HDAC4 (Backs et al., 2006; Zhang et al., 2007). The possible relevance of CaMKII-induced phosphorylation and nuclear export of HDAC4 in obese mouse liver was suggested by the results of several experiments. First, phosphorylation of HDAC4 on Ser465 and Ser629 (corresponding to Ser467 and Ser632 in human HDAC4), two CaMKII phosphorylation sites (Backs et al., 2006), was significantly increased in the livers of obese mice compared with lean mice (Figure 1A). Second, in the livers of obese mice, nuclear HDAC4 levels were lower and cytoplasmic levels were higher (Figure 1B). Third and most importantly, liver-CaMKII1 deficiency or CaMKII inhibition markedly decreased p-Ser465 and p-Ser629-HDAC4 and promoted HDAC4 nuclear localization in the livers of obese mice (Figures 1C and 1D). Palmitate-treated CaMKII-deficient HCs also showed increased nuclear HDAC4 compared to HCs from control mice (Figure 1E). To determine causation, we silenced HDAC4 in CaMKII-deficient HCs using small interfering RNA (siRNA) and found that this lowered Atf6 and its downstream target, Dnaj3 (P58IPK), and abrogated the improvement in insulin-Akt signaling in CaMKII-deficient HCs upon palmitate treatment (Figures 1F and 1G). We then tested causation in vivo by silencing HDAC4 in DIO mice and found increased fasting blood glucose and plasma insulin levels and reduced glucose-disposal curves post-insulin (Figure S2) without a change in body weight. These data provide initial support for the role of an obesity-driven CaMKII-HDAC4 nuclear exclusion pathway in ATF6 suppression and defective insulin signaling in HCs.

**Nuclear Exclusion of HC HDAC4 in Obesity Elevates Nuclear DACH1, a Corepressor that Lowers Insulin Resistance**

We next turned our attention to the mechanism of how HDAC4 could induce Atf6 transcription. Because HDAC4 usually represses gene transcription (Parra, 2015), we considered the possibility that HDAC4 repressed an Atf6 gene repressor. Indeed, previous studies in skeletal muscle identified a transcriptional repressor called Dachshund homolog (DACH) as a target of HDAC4-mediated repression (Tang and Goldman, 2006; Tang et al., 2009). Thus, we hypothesized that nuclear exclusion of HDAC4 in HCs in obesity would result in the de-repression of DACH1, which would then repress Atf6. We first asked whether hepatic DACH1 is increased in obesity. DACH1 levels were increased in obese mice compared with lean mice (Figure 1A).

1. **CaMKII Suppresses HC Atf6 mRNA in Obesity**
2. **Obesity Induces HDAC4 Phosphorylation and Regulates Its Nuclear Localization**
3. **Nuclear Exclusion of HC HDAC4 in Obesity Elevates Nuclear DACH1, a Corepressor that Lowers Atf6 and Promotes Insulin Resistance**
indeed increased in the livers of both DIO and ob/ob mice compared with lean controls (Figure 3A, bottom). Moreover, this increase in hepatic DACH1 was significantly suppressed in obese mice in which liver CaMKII was deleted or inhibited (Figures 3B and S3B) or in obese mice subjected to treatment with adeno-HDAC4S3A to enforce nuclear HDAC4 (Figure 3C, top). Conversely, silencing hepatic DACH1 in obese HCs decreased hepatic nuclear HDAC4 and HDAC4-dependent gene expression (Figure 3E).}

See also Figure S1.
HDAC4 in obese mice further increased hepatic DACH1, suggesting that DACH1 lies downstream of HDAC4 (Figure 3C, bottom). We also tested the effect of CaMKII inhibition in metabolism-qualified primary human HCs and found that DACH1 was increased by palmitate treatment and that this increase was prevented by inhibition of CaMKII using adenovirus-43A-CaMKII (Figure S3C). Furthermore, analysis of 14 human liver biopsy specimens spanning body-mass indexes (BMIs) from 19 to 62 showed an overall trend for higher levels of DACH1 and p-Ser632-HDAC4 in the livers of obese versus leaner subjects (Figure 3D).

We next tested the importance of DACH1 in the regulation of the ATF6–TRB3–insulin signaling pathway using wild-type (WT) versus DACH1-deficient HCs treated with or without palmitate. For this experiment, we used primary HCs isolated from Dach1fl/fl mice and then treated the cells ex vivo with adenovirus-Cre or control adenovirus-LacZ. The data show that Atf6 mRNA was significantly higher and Trb3 mRNA was significantly lower in DACH1-deficient HCs (Figure 3E). Most importantly, DACH1 deficiency resulted in prevention of palmitate-induced suppression of insulin-induced p-Akt (Figure 3F).

To test the functional importance of DACH1 in glucose metabolism in obesity, we silenced hepatic DACH1 in DIO mice using adeno-associated virus-8 (AAV8)-mediated small hairpin RNA (shRNA) (Lisowski et al., 2014). This treatment, which lowered hepatic DACH1 levels by ~70% (Figure 4D) without a change in body weight, significantly lowered fasting blood glucose and plasma insulin (Figure 4A). DACH1 silencing also lowered plasma glucose in response to pyruvate challenge, which is a measure of hepatic glucose production (HGP); improved the blood glucose response to glucose stimulation; and enhanced reduction of blood glucose in response to insulin stimulation (Figures 4B and 4C). Consistent with our in vitro data, we observed increases in both Atf6 mRNA and insulin-stimulated Akt phosphorylation and a significant decrease in Trb3 mRNA levels in the DACH1-silenced DIO cohort (Figures 4D and 4E). Similar results were found in DIO Dach1fl/fl mice.
injected with AAV-8 encoding Cre recombinase driven by the HC-specific thyroxin binding globulin promoter (TBG-Cre) (Figure S4). These data are consistent with the hypothesis that hepatic DACH1 depletion improves glucose metabolism in obese mice by increasing hepatic insulin sensitivity.

DACH1 has a highly conserved N-terminal DACH Box domain, which shares ~35% amino acid identity to the Ski/Sno proteins and is therefore known as the DACH Ski/Sno (DS) domain (Li et al., 2002; Wu et al., 2003). The DS domain mediates the interaction of DACH1 with both DNA and other corepressors and is required for the ability of DACH1 to repress a number of genes (Sundaram et al., 2008; Wu et al., 2003, 2008). Accordingly, an engineered mutant of DACH1 in which the DS domain is deleted (ΔDS-DACH1) has been shown to act in a dominant-negative manner in blocking DACH1 gene repression (Sunde et al., 2006). As a further test of the importance of DACH1 in liver metabolism, we treated obese mice with adeno-ΔDS-DACH1 versus adeno-LacZ control. The ΔDS-DACH1 cohort had lower fasting blood glucose, lower plasma insulin levels, improved blood glucose response to glucose challenge, and enhanced reduction of blood glucose upon insulin stimulation, all without a change in body weight (Figures S5A–S5D). Adeno-ΔDS-DACH1 treatment also improved insulin-induced p-Akt and raised the level of P58IPK (Figure S5E). Similar results were obtained using ob/ob mice (Figure S6). These results further support the role of DACH1 in glucose homeostasis in obesity and indicate that the DS domain of DACH1 is required for this effect.

DACH1 Silencing Improves Glucose Metabolism in Obese Mice by Increasing ATF6

To test whether the metabolic improvement seen with hepatic DACH1 deficiency was dependent on the increase in ATF6, we

Figure 3. Hepatic DACH1 Is Increased in Obesity via CaMKII Activation and Nuclear Exclusion of HDAC4, Leading to Defective Insulin Signaling in HCs

(A) Extracts of total liver (top blot) and liver nuclei (bottom blot) from WT mice fed a chow or DIO diet for 12 weeks were assayed for DACH1, β-actin, and nucleophosmin (Np) by immunoblot.

(B) DACH1 and β-actin were assayed in liver extracts from ob/ob mice treated with adeno-LacZ or adeno-K43A-CaMKII.

(C) DACH1 and β-actin were assayed in livers from DIO mice treated with adeno-LacZ or adeno-HDAC4S3A (top blot) or DIO mice treated with adeno-LacZ or adeno-sh-HDAC4 (bottom blot).

(D) DACH1, β-actin, p-Ser632-HDAC4 and HDAC4 were assayed in liver extracts from human subjects with the indicated BMIs.

(E) HCs from Dach1fl/fl mice were transduced with adeno-LacZ or adeno-Cre. After 36 hr, cells were incubated with 0.3 mM palmitate for 11 hr, with the last 5 hr in serum-free media. Cells were then assayed for Dach1, Atf6, and Trb3 mRNA (n = 3; mean ± SEM, *p < 0.05).

(F) As in (E), except that some of the cells received BSA control instead of palmitate and were then treated with PBS control or 100 nM insulin for 5 min, as indicated by the minus and plus symbols. Cell lysates were then assayed for p-Akt, total Akt, and β-actin.

See also Figure S3.
silenced hepatic ATF6 in liver-DACH1-deficient obese mice using adeno-sh-ATF6 (Wang et al., 2009). The results show that all of the beneficial effects of DACH1 silencing—decreased blood glucose and plasma insulin, improved blood glucose response to glucose challenge, enhanced reduction of blood glucose in response to insulin, and increased insulin-induced p-Akt in liver—were abrogated by also silencing hepatic ATF6 (Figures 5A–5D). These data provide further evidence that hepatic DACH1 depletion improves insulin sensitivity and glucose metabolism in obese mice by de-repressing ATF6.

Recent work has identified a consensus DACH1 DNA-binding sequence using genome-wide in silico promoter analysis together with cyclic amplification and selection of targets (Zhou et al., 2010). Using this information, we identified an intronic region (intron 14) and an exon (exon 16) in the Atf6 gene that contain DACH1 consensus sequences. ChIP analysis in GFP-DACH1-transfected primary HCs showed significantly increased recruitment of DACH1 to both of these regions (Figures 5A, left graph, and 6B), whereas a control segment at the 3' end of the Atf6 gene gave no signal (not shown). Moreover, in view of the fact that corepressors bind to enhancer sites to suppress gene transcription, we found through additional ChIP experiments that the intronic site was enriched for two enhancer marks, histone H3 lysine 4 monomethylation (H3K4me1) and H3K27 acetylation (H3K27Ac) (Heintzman et al., 2007) (Figure 6A, middle and right graphs). We also observed significantly increased recruitment of DACH1 to the identified intronic and exonic regions in obese liver compared with lean liver, whereas a control segment (Rplp0) gave no signal (Figure 6C).

In view of previous work showing that DACH1 can interact with nuclear receptor corepressor (NCOR) to repress genes (Wu et al., 2003), we tested the interaction between DACH1
and NCOR. We observed that NCOR could be coimmunoprecipitated with DACH1 in liver from obese, but not lean, mice, suggesting that DACH1 and NCOR physically interact in the setting of obesity (Figure 6D). Consistent with the data in Figure 3A, very little DACH1 could be immunoprecipitated from lean liver. Moreover, ChIP analysis revealed the presence of NCOR on the intronic region where DACH1 binds Atf6, and this ChIP signal was markedly diminished in DACH1-deficient cells (Figure 6E). As further evidence, we showed that AAV8-sh-NCOR-treated obese mice had higher hepatic Atf6 mRNA levels compared with control mice (Figure 6F). These combined data are consistent with a model in which DACH1-NCOR complex binds to DACH1 consensus sites in the Atf6 gene in hepatocytes in the setting of obesity and represses Atf6.

**HDAC4-Mediated DACH1 SUMOylation Leads to Its Degradation**

To elucidate the molecular mechanism of DACH1 suppression by nuclear HDAC4, we first analyzed the livers of obese mice treated with adeno-LacZ versus adeno-HDAC4S3A and found no decrease in Dach1 mRNA in the HDAC4S3A cohort (Figure S7A). Similar results were obtained using palmitate-treated control versus HDAC4S3A-transfected primary HCs (Figure S7B). Using the HC model, we then explored the possibility that nuclear HDAC4 increases the proteasomal degradation of DACH1 protein. Consistent with this idea, the proteasome inhibitor MG132 partially prevented the decrease in DACH1 protein conferred by HDAC4S3A transfection (Figure 7A).

HDAC4 can act as an E3 ligase that affects protein SUMOylation (Grégoire and Yang, 2005; Zhao et al., 2005), which in turn can promote ubiquitination and proteasomal degradation of SUMOylated proteins (Miteva et al., 2010). To address whether a SUMOylation-dependent pathway was involved in the HDAC4-mediated suppression of DACH1, we first asked whether endogenous DACH1 gets SUMOylated. Consistent with this idea, the proteasome inhibitor MG132 partially prevented the decrease in DACH1 protein conferred by HDAC4S3A transfection (Figure 7A).

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Ubc9, which transfers the activated SUMO to protein substrates (Ghisletti et al., 2007; Hay, 2005), HDAC4-mediated DACH1 degradation was abrogated (Figure 7D). As direct evidence that HDAC4 promotes DACH1 SUMOylation, we found that silencing HDAC4 abrogated DACH1 SUMOylation in primary HCs (Figure 7E).

Based on the consensus SUMO-acceptor site sequence (Rodriguez et al., 2001) (http://www.abgent.com/sumoplot), we identified three high-probability sites for SUMOylation in murine DACH1: K341, K644, and K676. In order to determine whether these lysine residues are targets for SUMOylation and degradation of DACH1, we created a mutant of DACH1 with lysine to alanine substitutions at these sites (Figure 7F).

Figure 6. DACH1-NCOR Complex Represses Atf6
(A) Primary HCs from WT mice were transfected with an expression plasmid encoding GFP-DACH1. After 48 hr, the cells were incubated with 0.3 mM palmitate for 3 hr, and ChIP was performed using anti-GFP, anti-H3K4me1, anti-H3K27Ac, or immunoglobulin G (IgG) control antibodies. The region spanning a specific intron site containing a predicted DACH1-binding sequence (intron 14) was amplified by qRT-PCR and normalized to the values obtained from the input DNA (n = 3; mean ± SEM, *p < 0.05).

(B) Same as (A), except that region spanning a specific exon site containing a predicted DACH1-binding sequence (exon 16) was amplified by qRT-PCR and normalized to the values obtained from the input DNA (n = 3; mean ± SEM, *p < 0.05).

(C) ChIP was performed from liver extracts of lean and DIO mice with anti-DACH1 or IgG control antibodies. The region spanning specific intron and exon sites containing predicted DACH1-binding sequences (intron 14 and exon 16, respectively) and a non-specific region (Rplp0) were amplified by qRT-PCR and normalized to the values obtained from the input DNA (n = 3; mean ± SEM, *p < 0.05).

(D) DACH1 was immunoprecipitated (IP) from liver extracts and then probed for NCOR or DACH1 by immunoblot (B). Lanes 1–3 are lean mice, and lanes 4–8 are DIO mice. All DIO mice were treated with AAV8-con, except for lane 7, which was treated with AAV8-sh-DACH1. Lane 8 used control IgG instead of anti-DACH1 for the immunoprecipitation step.

(E) HCs from Dach1fl/fl mice were transduced with adeno-LacZ or adeno-Cre. After 48 hr, the cells were incubated with 0.3 mM palmitate for 3 hr, and ChIP was performed using an antibody against NCOR. The region spanning the aforementioned Atf6 intronic site was amplified by qRT-PCR and normalized to the values obtained from the input DNA (n = 3; mean ± SEM, *p < 0.05). Inset, ChIP from a parallel set of cells that was treated the same as above, up until the final post-immunoprecipitation wash. The beads were then boiled in loading buffer and assayed by immunoblot for NCOR and DACH1.

(F) Liver extracts from DIO mice treated with AAV8-con or AAV8-sh-NCOR were assayed for Atf6 mRNA levels by qRT-PCR (n = 6, mean ± SEM, *p < 0.05). Inset: protein extracts were probed for NCOR and β-actin by immunoblot to document NCOR silencing.
arginine substitutions at these three sites (DACH1-3KR mutant) and transfected DACH1-deficient HCs with this construct. In non-palmitate-treated HCs, WT DACH1 was SUMOylated as before, but this was not seen with DACH1-3KR (Figure S7D). Furthermore, HDAC4S3A was unable to decrease DACH1 levels in the DACH1-3KR-transfected cells, in contrast to the situation

Figure 7. HDAC4-Mediated SUMOylation of DACH1 Leads to Proteasomal Degradation of DACH1 and Defective Insulin Signaling in HCs

(A) Primary HCs from WT mice were transduced with adeno-LacZ or adeno-HDAC4S3A. After 24 hr, the cells were pretreated with either vehicle or MG132 for 1 hr followed by incubation with either BSA or 0.3 mM palmitate (palm) for 6 hr. Lysates were probed for DACH1 and β-actin by immunoblot. Densitometric quantification of the immunoblot data is shown in the bar graph (n = 3; mean ± SEM; *p < 0.05 versus all other groups except LacZ-con).

(B) Lysates from WT or DACH1 KO HCs transfected with poly-His-tagged SUMO1 and treated with BSA (Con) or palmate (palm) were immunoprecipitated using anti-poly-His and blotted for DACH1 or SUMO1. Arrows indicate SUMOylated proteins. The numbers below the SUMO1 immunoblot are the densitometric ratios of SUMO-DACH1:total SUMO. The lower blot shows DACH1 and β-actin in whole cell lysates.

(C) Primary HCs from WT mice treated with AAV8-TBG-DACH1 were transfected with HA-tagged SUMO1 and treated with BSA (Con) or palmate (palm) as indicated. Lysates were immunoprecipitated using anti-HA and blotted for DACH1 or SUMO1. Arrows indicate SUMOylated proteins.

(D) HCs were pretreated with either scrambled RNA (scr) or siRNA targeting Ubc9 (si-Ubc9). After 12 hr, the cells were transduced with adeno-LacZ or adeno-HDAC4S3A. After an additional 24 hr, the cells were incubated with BSA control or 0.3 mM palmitate (palm) for 5 hr as indicated by the minus and plus symbols. Lysates were then probed for DACH1 and β-actin by immunoblot. Densitometric quantification of the immunoblot data is shown in the bar graph (n = 3; mean ± SEM; *p < 0.05; bars 4 and 5 are not significantly different).

(E) Similar to (B), except that the cells were transduced with adeno-LacZ or adeno-sh-HDAC4.

(F) HCs from Dach1fl/fl mice treated with AAV8-TBG-Cre were transduced with plasmids encoding WT DACH1 or 3KR mutant DACH1. After 12 hr, the cells were transduced with adeno-LacZ or adeno-HDAC4S3A. After an additional 24 hr, the cells were incubated with 0.3 mM palmitate for 11 hr, followed by insulin stimulation for 5 min. Lysates were probed for DACH1, β-actin, p-Akt, and Akt.

(G) Summary scheme of a CaMKII-HDAC4-DACH1 pathway linking obesity to defective insulin signaling in HCs. Based on the data in this report and our previous publications (Ozcan et al., 2012, 2013), obesity-induced activation of CaMKII in HCs phosphorylates HDAC4, which promotes its exit from the nucleus. As a result, nuclear SUMOylation of the corepressor DACH1 is decreased, which prolongs its half-life and increases its level in the nucleus. DACH1, together with NCoR, decreases the transcription of Atf6, leading to activation of the PERK-TRB3 pathway and defective insulin signaling, as described previously (Ozcan et al., 2013). See also Figure S7.
with HCs expressing WT DACH1 (Figure 7F, top two blots). Most importantly, the improvement in insulin signaling conferred by HDAC4S3A in palmitate-treated HCs was abrogated when the cells expressed 3KR-DACH1 instead of WT DACH1 (Figure 7F, bottom two blots). These combined results show that nuclear HDAC4 decreases DACH1 through SUMOylation and subsequent proteasome-mediated degradation. With this finding, we present a summary of the pathway in which CaMKII activation in HCs in obesity leads to defective insulin signaling (Figure 7G).

**DISCUSSION**

Understanding how ATF6 is suppressed in HCs in obesity is a critical issue in view of its importance in both insulin signaling, supported by our previous study (Ozcan et al., 2013) and bolstered here, and HGP (Wang et al., 2009). Additionally, two independent studies reported associations between variants of the ATF6 gene in humans and disturbed glucose homeostasis and T2D (Meex et al., 2007; Thameem et al., 2006). The mechanism of ATF6 suppression revealed here involves a very interesting pathway in which the half-life of the corepressor DACH1 is prolonged, leading to decreased transcription of the Atf6 gene. Even though the key step in ATF6 activation is its post-translational proteolytic cleavage and nuclear translocation (Ye et al., 2000), the data here imply that the level of Atf6 transcription becomes rate limiting in HCs in the setting of obesity.

Our work shows that decreased levels of nuclear HDAC4 in HCs in obesity indirectly lowers Atf6 transcription by increasing the level of DACH1. In this context, a recent paper using an unbiased proteomics approach found that obesity in humans is associated with a decrease in HDAC4, which improves with physical exercise, suggesting the possibility that nuclear HDAC4 is protective in obesity (Abu-Farha et al., 2013). In contrast, Mihaylova et al. reported that combined silencing of hepatic HDAC4, 5, and 7 increases FoxO1 acetylation in fasted lean mice and lowers HGP in lean and obese mice (Mihaylova et al., 2011). In this regard, we found no difference in the level of acetylated FoxO1 in the livers of obese mice treated with HDAC3 represses genes involved in hepatic lipogenesis, and thus one of the effects of HC-targeted NCOR deletion is steatosis (Sun et al., 2013). In contrast, hepatic steatosis is improved by liver-targeted silencing of DACH1 in obesity (Sun et al., 2012).

Most studies on DACH1 have focused on its roles in development and tumorigenesis (Popov et al., 2010). Ironically, one of the developmental roles is related to perinatal pancreatic β cell proliferation, which is associated with DACH1-mediated repression of p27Kip1 (Kaloussova et al., 2010). However, this finding does not imply that hepatic NCOR silencing in obese mice would mimic the metabolic improvement seen with hepatic DACH1 silencing, because NCOR has functions in liver that are independent of DACH1. For example, NCOR in complex with HDAC3 represses genes involved in hepatic lipogenesis, and thus one of the effects of HC-targeted NCOR deletion is steatosis (Sun et al., 2013). In contrast, hepatic steatosis is improved by liver-targeted silencing of DACH1 (not shown) or its upstream effector, CaMKII (Ozcan et al., 2013). We provide evidence that suppression of Atf6 in HCs in obesity involves a DACH1-NCOR complex. However, this finding does not imply that hepatic NCOR silencing in obese mice would mimic the metabolic improvement seen with hepatic DACH1 silencing, because NCOR has functions in liver that are independent of DACH1. For example, NCOR in complex with HDAC3 represses genes involved in hepatic lipogenesis, and thus one of the effects of HC-targeted NCOR deletion is steatosis (Sun et al., 2013). In contrast, hepatic steatosis is improved by liver-targeted silencing of DACH1 (not shown) or its upstream effector, CaMKII (Ozcan et al., 2013).

In summary, our study reveals a role for DACH1 in obesity-induced glucose intolerance and insulin resistance in mice, with a striking correlation between liver DACH1 level and obesity in humans. While DACH1 upregulation in obesity may affect a number of different genes and pathways, its ability to suppress insulin signaling by repressing Atf6 transcription appears to be dominant. Nonetheless, future studies will likely reveal additional DACH1 targets in HCs that may shed additional light on gene expression changes in obesity. Finally, we recently published that drug-mediated inhibition of MK2, a kinase downstream of CaMKII in HCs in obesity, improves metabolism in obese mice (Ozcan et al., 2015), which is consistent with genetic studies conducted by both our group (Ozcan et al., 2013) and an independent laboratory (Ruiz et al., 2016). Given that the upstream kinase...
pathway promotes diabetes by suppressing ATF6, the findings in this report provide important mechanistic underpinnings for future therapeutic strategies that attempt to improve metabolism in obese T2D subjects by targeting this pathway.

**EXPERIMENTAL PROCEDURES**

**Mouse Experiments**

ob/ob and DIO mice were obtained from The Jackson Laboratory. DIO mice were fed a high-fat diet with 60% kcal from fat (Research Diet) and maintained on a 12-hr light-dark cycle. Dachfl/fl mice were generated as described previously (Chen et al., 2015). The detailed procedures are shown in Supplemental Experimental Procedures.

**Primary HCs**

Primary mouse HCs were isolated from 8- to 12-week-old mice as described previously (Ozcan et al., 2012). Unless indicated otherwise, the cells were cultured in DMEM containing 10% fetal bovine serum, treated as described in the figure legends, and then incubated for 5 hr in serum-free DMEM before harvesting. The detailed procedures are shown in Supplemental Experimental Procedures.

**Statistical Analysis**

All results are presented as mean ± SEM, and p values were calculated using the Student’s t test for normally distributed data and the Mann-Whitney rank sum test for non-normally distributed data. One-way ANOVA with post hoc Tukey test was used to evaluate differences among groups when three or more groups were analyzed.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.006.

**AUTHOR CONTRIBUTIONS**


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**REFERENCES**


