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UBE3A suppresses overnutrition-induced expression of the steatosis target genes of MLL4 by degrading MLL4

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Abstract

Regulation of the protein stability of epigenetic regulators remains ill-defined despite its potential applicability in epigenetic therapies. The histone H3-lysine 4-methyltransferase MLL4 is an epigenetic transcriptional coactivator that directs overnutrition-induced obesity and fatty liver formation, and *MLL4*^{+/-} mice are resistant to both. Here we show that the E3 ubiquitin ligase UBE3A targets MLL4 for degradation thereby suppressing high fat diet (HFD)-induced expression of the hepatic steatosis target genes of MLL4. In contrast to *MLL4*^{+/-} mice, *Ube3a*^{+/-} mice are hypersensitive to HFD-induced obesity and fatty liver development. *Ube3a*^{+/-};*MLL4*^{+/-} mice lose this hypersensitivity, supporting roles of increased MLL4 levels in both phenotypes of *Ube3a*^{+/-} mice. Correspondingly, our comparative studies with wild-type, *Ube3a*^{+/-} and *Ube3a*^{-/-} and UBE3A-overexpressing transgenic mouse livers demonstrate inverse-correlation of UBE3A protein levels with MLL4 protein levels, expression of the steatosis target genes of MLL4, and their decoration by H3-lysine 4-monomethylation, a surrogate marker for the epigenetic action of MLL4. Therefore, UBE3A indirectly exerts an epigenetic regulation of obesity and steatosis by degrading MLL4. This UBE3A-MLL4 regulatory axis provides a potential therapeutic venue for treating various MLL4-directed pathogenesises, including obesity and hepatic steatosis.

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Author contributions

J.K., B.L., D.H.K., Y.P., Y.L., J.G.Y., and J.K.L. conducted the experiments, S.L. and J.W.L. designed the experiments, and S.L., S.K.L. and J.W.L. wrote the paper.

Supplemental information

Supplemental Information includes three supplemental figures, two Excel files listing significantly upregulated and downregulated genes in *Ube3a*^{+/-} and *Ube3a*-tg livers relative to their respective littermate WT livers.

Introduction

Epigenetic features, heritable molecular determinants of phenotype independent of DNA sequences, involve different mechanisms, including DNA methylation and histone modifications (1). Disruption of these processes is a major contributor to many disorders, and the enzymatic activities of epigenetic regulators have been targeted for development of small molecular inhibitors for epigenetic therapies (1). However, alternative pathways to regulate their activities, such as modulation of the protein stability of epigenetic regulators, remain poorly understood.

Methylation of histone H3 lysine 4 (H3K4), H3K36 and H3K79 marks gene activation, while methylation of H3K9, H3K27 and H4K20 marks gene repression (2). Tri-methylation of H3K4 (H3K4me3) marks promoters, while mono- and di-methylations of H3K4 (H3K4me1 and H3K4me2) mark enhancers (2). In lower eukaryotes, all methylations of H3K4 are performed by the sole H3K4-methyltransferase (H3K4MT) Set1, which forms a large steady-state Set1-complex (2). In higher eukaryotes, H3K4-methylations are performed by multiple H3K4MTs, including the six Set1-like complexes containing the H3K4MTs Set1 α , Set1 β , MLL1, MLL2 (aka WBP7), MLL3 (aka KMT2C) or MLL4 (aka KMT2D) (3). Each Set1-like complex contains a unique H3K4MT enzyme, complex-specific subunits, and a common subcomplex, consisting of RBBP5, ASH2L, WDR5, and DPY-30, that facilitates the H3K4MT activity of Set1-like complexes (3). We identified the first two mammalian Set1-like complexes containing MLL3 or MLL4 (4, 5). The MLL3/4-complexes also contain the H3K27-demethylase UTX (6). Specific subunits in these two complexes also include PA1, PTIP, and ASC-2 (4, 5). ASC-2 and PTIP serve as adaptors that recruit the epigenetic coactivators MLL3/4 and UTX to enhancers of their target genes by interacting with specific transcription factors bound to those enhancers (2, 3, 6–9). ASC-2, using its two LXXLL motifs that recognize the activated conformation of nuclear receptors, tethers MLL3/4-complexes to their target enhancers occupied by several nuclear receptors (5, 10–17), triggering their target genes to form open chromatin for transactivation. Interestingly, epigenetic regulation of diverse metabolic processes has been identified as one of the key physiological functions of MLL3/4-complexes, including our recent report for their essential roles in overnutrition-directed obesity and fatty liver formation (11, 13–16, 18, 19). The liver steatogenic function of MLL3/4-complexes involves their coactivator action for the lipogenic peroxisome proliferator-activated receptor- γ 2 (PPAR γ 2) (19). Importantly, MLL3/4 decorate the enhancers with H3K4me1 and H3K4me2 (20–24), and we have confirmed that MLL3/4-complexes direct H3K4me1 modification of the enhancers associated with the lipogenic target genes of PPAR γ 2 and MLL3/4 (19).

UBE3A (aka E6AP) is an E3 ubiquitin ligase that conjugates a small marker protein ubiquitin to its specific substrate proteins for degradation by the ubiquitin-proteasome system (25). *UBE3A/Ube3a* are paternally inactivated through genomic imprinting in the brain but not in the peripheral tissues, and deletion or inactivation of a maternal copy of *UBE3A* results in the absence of functional UBE3A protein in the brain, causing Angelman syndrome (AS), which is a neurodevelopmental disorder characterized by lack of speech, cognitive impairments, happy demeanor, motor deficits and seizures, and other symptoms (26). Duplication of the chromosomal region containing the *UBE3A* gene results in Dup15q

syndrome, which is associated with autism spectrum disorders (ASDs) (27). Most Dup15q syndrome cases results from a maternally inherited duplication and therefore overexpression of UBE3A also occurs in the brain (27). However, the impact of altered expression of UBE3A in patients of Angelman and Dup15q syndromes as well as their animal models on the function of peripheral organs remains ill-defined.

Obesity is common with Angelman syndrome (28–31), and *Ube3a*^{+/-} mice, developed as an animal model for Angelman syndrome (32), also show obesity due to enhanced adiposity (33). Obesity is a major risk factor for non-alcoholic fatty liver disease (34), and indeed *MLL4*^{+/-} mice are resistant to development of both obesity and hepatic steatosis (14, 19). Here we report MLL4 as a new substrate of UBE3A. Consistently, *Ube3a*^{+/-} mice are hypersensitive to HFD-induced obesity and fatty liver development, and these hypersensitivities result from increased MLL4 levels and upregulation of the steatosis target genes of MLL4 (19). This UBE3A-MLL4 regulatory axis may provide a unique therapeutic venue for treating various MLL4-directed pathogenesises, including obesity and hepatic steatosis.

Materials and Methods

Ubiquitylation assay

To detect ubiquitylated MLL4 proteins, cells were transfected with Flag-MLL4^C, Myc-Ube3a WT and HA-ubiquitin (HA-Ub), and lysed by boiling in a buffer containing 2% SDS, 150 mM NaCl, and 10 mM Tris-HCl [pH 8.0]. For immunoprecipitation, these lysates were diluted with 4 volumes of a buffer containing 150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM DTT, and 1% Triton X-100, followed by preclearing with protein G-agarose beads and incubation with anti-Flag antibody. HA-Ub conjugates in immunoprecipitated MLL4 proteins were analyzed by immunoblot analysis with antibodies against the HA epitope.

Animals

All mouse works were performed under approved protocols by the Institutional Animal Care and Use Committee of the Oregon Health & Science University and Seoul National University. *Ube3a*^{+/-} and *Ube3a*-tg mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed in a pathogen-free animal facility under standard 12 hr light/12 hr dark cycle, and allowed to ad libitum access to water and fed normal chow diet or HFD (42% fat, 0.2% cholesterol, TD.88137, Harlan Teklad, North America) for up to 9 weeks or intraperitoneally (IP)-injected daily with either vehicle (5% DMSO and 95% corn oil) or 30 mg/kg of rosiglitazone (R2408, Sigma Chemicals) for 7 days.

Statistical analyses

Statistical differences were determined by Student's t-test and one-way ANOVA followed by Tukey's test using Prism 6 (GraphPad) software. Statistical significance is displayed as *P* < 0.05 (one asterisk), *P* < 0.01 (two asterisks), *P* < 0.001 (three asterisks) or *P* < 0.0001 (four asterisks). 'ns' indicates not-significant (*P* > 0.05).

Accession Number

GSE103553 contains all our RNAseq results.

Results

UBE3A regulates the protein stability of MLL4

To discover proteins that modulate the H3K4MT activity of MLL4, we performed a yeast two hybrid screening with a cDNA library from embryonic day 13.5 (E13.5) mouse brains using Gal4 fusion to the C-terminal region of MLL4 (aa 4774–5262, herein referred to as MLL4^C), which contains the catalytic SET domain of MLL4, as bait (S-Fig. 1A). We used the interaction between p53 and SV40 large T (35) as a positive control and another SET domain-containing protein SET7 (36) as a negative control to discriminate against proteins that recognize all SET domain-containing proteins. Proteins identified to bind MLL4 but not SET7 include the C-terminal region of UBE3A (aa 617–849) (S-Fig. 1A). In HEK293 cells, both WT UBE3A and the catalytically inactive C843A point-mutant version of UBE3A (37) (UBE3A-mt) interacted with MLL4^C (S-Fig. 1B), and UBE3A-mt interacted with MLL4^C but not SET7 (S-Fig. 1C). We further mapped the interaction interfaces to the SET domain of MLL4 (aa 5113–5262) and a subregion within the catalytic HECT domain of UBE3A (aa 704–790) (S-Fig. 1, D and E).

To test interactions between endogenous full-length MLL4 and UBE3A, we immunoprecipitated MLL4 in several mouse cell types and tissues, including mouse embryonic stem cells (ESCs), murine Hepa1c1c7 cultured liver cells, and postnatal day 5 (P5) mouse whole brains, with anti-MLL4 antibody, followed by immunoblotting with anti-UBE3A antibody. We found association of endogenous MLL4 and UBE3A in all three cases (S-Fig. 1F, Fig. 1A), suggesting that these interactions occur in a variety of different cell types.

To test if MLL4 is a substrate of UBE3A in vitro, we employed the UBE3A-interacting MLL4^C fragment, because the full-length MLL4 is difficult to visualize for ubiquitylation due to its gigantic size (e.g., 5262 aa for hMLL4). In HEK293 cells, MLL4^C was ubiquitylated and this ubiquitylation was enhanced by overexpressed UBE3A (Fig. 1B). Using the powerful yeast genetic system that rapidly isolates interaction-null missense mutants (38), we identified several UBE3A point-mutants, which interact with p53 but not MLL4^C, including UBE3A-Q784H. In HEK293 cells, we confirmed that WT UBE3A bound both MLL4^C and p53, while UBE3A-Q784H bound only p53 (Fig. 1C). Consistently, WT UBE3A reduced levels of both MLL4^C and p53 in a dose-dependent manner, while UBE3A-Q784H affected only p53 levels (Fig. 1D). The half-life of MLL4^C was clearly reduced by WT UBE3A but not UBE3A-mt (S-Fig. 2A). Moreover, transient transfection of shRNA against Ube3a (sh-Ube3a) reduced UBE3A levels while increasing endogenous MLL4 levels in HEK293 cells (S-Fig. 2B). These results identify MLL4 as a substrate of UBE3A for degradation.

MLL3 and MLL4 often play redundant roles and share their target genes, while MLL4 is often a dominant player (6). To determine if MLL3 is targeted by UBE3A, we examined if MLL3 interacts with UBE3A. In HEK293 cells expressing Myc-UBE3A-mt and Flag-

MLL3^C (similarly constructed as MLL4^C), UBE3A-mt was co-immunopurified with MLL3^C (S-Fig. 2C). In HEK293 cells transiently transfected with Flag-MLL3^C-expressing vector and either expression vector alone or vector for HA-UBE3A, HA-UBE3A reduced the protein levels of Flag-MLL3^C (S-Fig. 2D). These results identify MLL3 as a potential substrate of UBE3A.

Control of MLL4 stability by UBE3A in the liver

MLL4 is critical for lipid and bile acid homeostasis in the liver (14, 19). Therefore, to investigate if MLL4 is a substrate of UBE3A in vivo, we prepared the livers of *Ube3a*^{+/-}, *Ube3a*^{-/-} and UBE3A-overexpressing *Ube3a*-tg mice (32, 39) as well as their respective littermate wild-type (WT) controls. We confirmed the down- and up-regulation of UBE3A proteins in these mice (S-Fig. 2E). We measured MLL4 protein levels and the global levels of H3K4me1, a surrogate marker for the H3K4MT activity of MLL4 (20–24). MLL4 levels were significantly higher in *Ube3a*^{-/-} and *Ube3a*^{+/-} livers and lower in *Mll4*^{+/-} and *Ube3a*-tg livers relative to their WT controls, as determined by immunohistochemistry with anti-MLL4 antibody (S-Fig. 3A-D). Similar results were found for MLL3 (S-Fig. 3B,D). In direct confirmation of our results for MLL3/4, our immunoblotting analysis with anti-MLL3/4 antibody revealed that MLL4 levels in *Ube3a*-tg livers are lower than those in WT livers while no differences were observed with the control proteins RBBP5 and actins, and that MLL3 levels in *Ube3a*^{+/-}/*Ube3a*^{-/-} livers are higher than those in WT livers while no differences were observed with the control protein RBBP5 (Fig. 2A). Also our immunohistochemistry results with anti-H3K4me1 antibody revealed that H3K4me1 levels are significantly reduced in both *Mll4*^{+/-} and *Ube3a*-tg livers in comparison to their WT controls (S-Fig. 4A,B). In contrast, H3K4me1 levels were enhanced in *Ube3a*^{-/-} and *Ube3a*^{+/-} livers relative to their WT controls (S-Fig. 4C). Levels of H3 were comparable between WT and either *Mll4*^{+/-}, *Ube3a*-tg, *Ube3a*^{-/-} or *Ube3a*^{+/-} livers (S-Fig. 4A-C). Consistent with the findings that MLL4 mainly produces H3K4me1/2 (20–24), we found that H3K4me2 levels, but not H3K4me3 levels, were reduced in *Ube3a*-tg livers (S-Fig. 4D). These results suggest that UBE3A alters the chromatin landscape and gene expression by degrading MLL4 in the liver.

Notably, immunoblotting to measure endogenous MLL4 levels in the liver has been challenging, which was the main reason why we employed immunohistochemical approaches. This problem likely involves its huge size as well as other liver-specific complications, given our immunoblotting efforts for H3K4me1/2/3 have also been difficult. However, we noted that our immunohistochemical results are semi-quantitative and should be interpreted with caution. For instance, for H3K4me1 levels in *Ube3a*-tg livers relative to controls, our immunoblotting results showed ~20% reduction (Fig. 2B), while our immunohistochemical approach revealed ~46% reduction (S-Fig. 4B). To further investigate the inverse correlation between MLL3/4 and UBE3A in the livers, we performed two additional experiments. First, using anti-MLL4 antibody, we immunoprecipitated MLL4 from liver lysates of WT, *Ube3a*^{+/-} or *Ube3a*^{-/-} mice, followed by immunoblotting for co-immunopurified RBBP5. These experiments showed that more RBBP5 is coimmunopurified with MLL4 in *Ube3a*^{-/-} livers relative to WT control livers while RBBP5 levels do not change in input lanes (S-Fig. 3E), suggesting that *Ube3a*^{-/-} livers likely have more MLL4-

complex than WT controls. Second, we performed dot blot analysis for MLL3 and MLL4 for liver lysates of WT, *Ube3a*^{+/-} or *Ube3a*^{-/-} mice. While UBE3A levels in *Ube3a*^{-/-} livers were lower than those in WT controls and RBBP5 levels were comparable between WT and *Ube3a*^{-/-} livers, levels of both MLL3 and MLL4 were significantly increased in *Ube3a*^{-/-} livers relative to WT controls (S-Fig. 3F).

Taken together, we concluded that MLL3 and MLL4 are targeted by UBE3A for degradation in the liver.

UBE3A antagonizes obesity and hepatic steatosis by targeting MLL4 for degradation

To assess the physiological consequences of MLL4 degradation by UBE3A, we focused on the well-defined liver function of MLL4 (14, 19). Because obesity is a major risk factor for non-alcoholic fatty liver disease (34) and given the higher levels of the steatogenic factor MLL4 (19) in *Ube3a*^{+/-} livers (S-Fig. 3C), we reasoned that *Ube3a*^{+/-} mice would be more susceptible to HFD-induced fatty liver development relative to WT controls. Indeed, male *Ube3a*^{+/-} mice developed HFD-induced obesity significantly more efficiently than their WT controls (Fig. 3A). *Ube3a*^{+/-} mice were much more prone to develop HFD-induced hepatic steatosis than their WT controls (Fig. 3B). In MRI analyses, *Ube3a*^{+/-} mice were ~132% heavier than their WT controls after 8 weeks of a HFD (~42 g for *Ube3a*^{+/-} vs. ~32 g for WT), and ~70% of this gain came from an increase in whole body fat mass (~16.5 g for *Ube3a*^{+/-} vs. ~9.5 g for WT) (Fig. 3C). The livers of *Ube3a*^{+/-} mice were ~170% heavier than those of their WT control mice after 8 weeks of a HFD (~2.5 g for *Ube3a*^{+/-} vs. ~1.5 g for WT), and ~60% of this gain came from an increase in liver fat mass (~0.76 g for *Ube3a*^{+/-} vs. ~0.14 g for WT) (Fig. 3C). We obtained similar results with female *Ube3a*^{+/-} mice (S-Fig. 5A-C). These results link UBE3A to fat mass and suggest crucial roles of UBE3A in both fat tissues (i.e., adipocytes) and hepatocytes in antagonizing obesity and hepatic steatosis, respectively.

To test if the enhanced susceptibility of *Ube3a*^{+/-} mice to obesity and fatty liver development (Fig. 3A-C, S-Fig. 5A-C) is due to the increased level of MLL4 (S-Fig. 3C), we established *Ube3a*^{+/-};*Mll4*^{+/-} double mutants. Both phenotypes of *Ube3a*^{+/-} mice were normalized in *Ube3a*^{+/-};*Mll4*^{+/-} mice (Fig. 3D,E), suggesting that the increased level of MLL4 in *Ube3a*^{+/-} mice (S-Fig. 3C) is responsible for their sensitivity to HFD-induced obesity and hepatic steatosis. These results provide in vivo evidence for critical roles of MLL4 in mediating the ability of UBE3A to antagonize obesity and hepatic steatosis.

Ube3a-tg mice (39), developed as an animal model for UBE3A-overexpressing Dup15q syndrome (27), express lower levels of MLL4 (Fig. 2A, S-Fig. 3D). Therefore, we expected them to be resistant to HFD-induced development of obesity and hepatic steatosis, like *Mll4*^{+/-} mice (14, 19). However, *Ube3a*-tg mice (both males and females) were similar to their WT controls in both phenotypes (S-Fig. 5D,E). Notably, both Dup15q syndrome patients and *Ube3a*-tg mice do not develop obesity (27, 39).

UBE3A inhibits expression of the steatosis target genes of MLL4

To understand the enhanced susceptibility of *Ube3a*^{+/-} mice as well as the lack of resistance of *Ube3a*-tg mice to HFD-induced hepatic steatosis, we performed RNAseq experiments

with the livers of WT vs. *Ube3a*^{+/-} female mice as well as WT vs. *Ube3a*-tg female mice, all fed a HFD for 8 weeks. Our bioinformatics analyses identified 89 downregulated and 188 upregulated genes in *Ube3a*^{+/-} livers relative to their WT controls as well as 214 downregulated and 268 upregulated genes in *Ube3a*-tg livers relative to their WT controls (upregulated by more than 1.5 fold or downregulated by less than 0.66 with *P* values less than 0.05) (Fig. 4A). In our gene ontology (GO) analyses, lipid and phospholipid metabolic processes emerged as GO terms with the two highest *P* values for 188 upregulated genes in *Ube3a*^{+/-} livers, while lipid metabolism was the only significant GO term for 214 downregulated genes in *Ube3a*-tg livers (Fig. 4B). We previously identified 16 steatotic genes as the most strongly downregulated genes in *Mll4*^{+/-} livers, which belong to these GO terms, consistent with the resistance of *Mll4*^{+/-} mice to HFD-induced fatty liver development (19). Remarkably, 13 of these genes were identified to be ‘most strongly’ upregulated in *Ube3a*^{+/-} livers and 6 of these genes were found to be ‘most strongly’ downregulated in *Ube3a*-tg livers (Fig. 4A). These results were independently validated (Fig. 5A,B). Unlike *Ube3a*^{+/-} livers, the steatogenic target genes of MLL4 (19) were no longer upregulated in *Ube3a*^{+/-};*Mll4*^{+/-} livers relative to WT controls (Fig. 5C), demonstrating that the increased level of MLL4 in *Ube3a*^{+/-} livers (S-Fig. 3C) directs their enhanced levels of expression of the steatogenic target genes of MLL4 (Fig. 5A). These results suggest that the steatogenic target genes of MLL4 (19) are the primary targets of UBE3A in the liver, and their upregulation underlies the enhanced susceptibility of *Ube3a*^{+/-} mice to fatty liver development (Fig. 3).

Elovl2- and *Socs2*-deficient mice are resistant to hepatic steatosis (40, 41), and *Elovl2*- and *Socs2* are upregulated in *Ube3a*-tg livers (* in Fig. 4A). Therefore, upregulation of at least two pro-steatotic genes in *Ube3a*-tg mice may have contributed at least in part to their lack of resistance to HFD-induced hepatic steatosis despite downregulation of the six steatogenic target genes of MLL4 (Fig. 4A), although other possibilities also exist. It is unclear why *Elovl2* and *Socs2* are upregulated in *Ube3a*-tg livers. Both genes are not regulated by MLL4 (14, 19), and therefore this upregulation may involve reduced levels of other substrates of UBE3A.

Because *Ube3a*^{+/-} mice showed an enhancement in both adipose tissue development and fatty liver formation (Fig. 3) via enhanced MLL4 levels (S-Fig. 3C) and MLL4 is a critical effector of both hepatic steatosis and adipogenesis via regulating a similar set of genes (15, 16, 19, 23, 42), we examined whether expression of the steatosis target genes of MLL4 is altered in the white adipose tissue (WAT) of *Ube3a*^{+/-} mice. Expression of *Scd1* was significantly upregulated in *Ube3a*^{+/-} WAT relative to their WT controls, and a similar trend (*P*=0.06) was observed with *Cidec* (Fig. 5D). These results suggest, coupled with the fact that MLL4 is also a critical effector of adipogenesis (16, 23, 42), that the UBE3A-MLL4 axis also operates in the WAT to impinge on adipogenesis.

Overall, our results demonstrate that UBE3A indirectly suppresses expression of the steatosis target genes of MLL4 in the liver by degrading MLL4 protein.

UBE3A blocks MLL4-recruitment to the steatosis target genes of MLL4 and their decoration by H3K4me1 in the liver

PPAR γ 2 recruits MLL4-complex to the steatogenic target genes of MLL4 via their PPAR-response elements (PPREs), and rosiglitazone, a ligand for PPAR γ , induces their expression by facilitating MLL4's interaction with PPAR γ 2 (19). Therefore, the susceptibility of *Ube3a*^{+/-} mice to HFD-induced obesity and fatty liver development (Fig. 3A-C, S-Fig. 5A-C) may at least in part involve HFD-induced upregulation of PPAR γ 2 and its recruitment of MLL4-complex to the steatogenic target genes of MLL4. In HEK293T cells expressing Flag-tagged PPAR γ 2 and vector alone or an increased dose of UBE3A-expression vector, PPAR γ 2 levels were not reduced by UBE3A (S-Fig. 6A), suggesting that PPAR γ 2 is not a substrate of UBE3A. To test if UBE3A epigenetically modulates expression of the steatogenic target genes of MLL4 in the liver, we compared expression of several representative steatosis target genes of MLL4 (19) as well as their recruitment of MLL4 and H3K4me1 decoration in *Ube3a*-tg livers relative to their WT controls, including *Pparg2*, *Scd1*, *Cd36* and *Cidec*. Expression of *Pparg2*, *Cd36*, *Scd1* and *Cidec* was induced upon daily IP injection of 30 mg/kg of rosiglitazone for 7 days in WT livers (Fig. 6A). This induction was significantly blunted in *Ube3a*-tg livers while the expression of a negative control (PPAR γ 1) was neither induced by rosiglitazone nor different between WT and *Ube3a*-tg livers (Fig. 6A). Consistently, rosiglitazone triggered recruitment of MLL4 to the PPREs of *Pparg2*, *Cd36*, *Scd1* and *Cidec* in WT livers but significantly less in *Ube3a*-tg livers (Fig. 6B). Rosiglitazone also increased levels of H3K4me1 in the PPREs of *Pparg2*, *Cd36*, *Scd1* and *Cidec* in WT livers but significantly less in *Ube3a*-tg livers (Fig. 6C). For both experiments, we used *Untr6* as a negative control locus, which does not recruit PPAR γ 2 (14). These results suggest that UBE3A, via degradation of MLL4, decreases the recruitment of MLL4 to its target genes thereby antagonizing their open chromatin formation marked by H3K4me1 and expression.

Discussion

While modulation of the protein stability of epigenetic regulators has been relatively less extensively explored in comparison to inhibitors of their catalytic activities, it provides a unique and an additional opportunity to regulate their epigenetic gene regulatory activities. In this report, we identified the H3K4MT MLL4 as a new substrate of the E3 ubiquitin ligase UBE3A (Fig. 7). This UBE3A-MLL4 axis suggests a previously unsuspected association among Angelman, Dup15q, and Kabuki syndromes, as they result from reduced expression of UBE3A (and likely enhanced MLL4 levels) due to inactivating mutations in the maternal copy of *UBE3A* (26), overexpression of UBE3A (and likely reduced MLL4 levels) with duplication of the chromosome 15q11.2–13.1 region containing the *UBE3A* gene (27), and reduced expression of MLL4 from inactivating mutations in a single copy of *MLL4* (43), respectively.

Kabuki and Dup15q syndromes show a unique facial feature and dwarfism (26, 43). Given our finding of the UBE3A-MLL4 axis, reduced MLL4 levels may be responsible for both features in these disorders. We observed deficits in apical dendrite outgrowth and dendritic branching in pyramidal neurons in our *Mll4* conditional knockout mouse brains (our

unpublished results). Selective inhibition of apical dendrite outgrowth in pyramidal neurons is reported in UBE3A-deficient mouse brains (44, 45), and both decreased and increased expression of UBE3A reduces dendritic branching in *Drosophila* (46), indicating that the proper level of UBE3A is important for the normal dendritic patterning. These results suggest that dendrite growth deficits in UBE3A-deficient mouse brains (and possibly *Ube3a*-tg brains as well as Angelman and Dup15q human brains) involve dysregulated levels of MLL4. Overall, altered MLL4 levels are expected to trigger at least some clinical features of Angelman and Dup15q syndromes. Therefore, inhibitors of the catalytic activities of UBE3A may remedy some of the clinical features of Kabuki syndrome via normalizing MLL4 levels. Similarly, inhibitors of the catalytic activities of MLL4 may help improve some of the clinical features of Angelman syndrome.

Most studies on Angelman and Dup15q syndromes have been focused on neurodevelopmental deficits and roles of UBE3A in the brain, except the findings that obesity is common with Angelman syndrome (28–31). Consistent with the well-defined adipogenic (15, 16, 23, 42) and steatogenic (19) actions of MLL4, *Ube3a*^{+/-} mice showed enhanced susceptibility to HFD-induced obesity and fatty liver development via increased adiposity and hepatic steatosis relative to their controls (Fig. 3A-C; S-Fig. 5A-C). These phenotypes were rescued by removal of a single copy of *MLL4* (Fig. 3D,E). These results, together with the inverse correlation between UBE3A and MLL4 levels in the liver (Fig. 2A, S-Fig. 3), suggest that UBE3A in the liver and fat tissues likely antagonizes the known function of MLL4 in steatogenesis (19) and adipogenesis (15, 16, 23, 42), respectively, by degrading MLL4 and enhanced MLL4 levels direct the obesity phenotype of Angelman syndrome patients (28–31). *Ube3a*^{+/-} mice show a lower motor activity due to altered circadian rhythm but no differences in food intake (33). The former may have also contributed to the obese phenotype of *Ube3a*^{+/-} mice by limiting energy consumption. Overall, future investigation is warranted to examine if Angelman syndrome patients are prone to overnutrition-induced steatosis development, like *Ube3a*^{+/-} mice (Fig. 3B,C; S-Fig. 5B,C).

By comparatively analyzing *Ube3a*^{+/-}, *Ube3a*^{-/-}, and UBE3A-overexpressing *Ube3a*-tg livers (32, 39), we found that UBE3A levels are inversely correlated with MLL4 levels (Fig. 2A; S-Fig. 3; Fig. 7) and levels of H3K4me1, a surrogate marker for the epigenetic activity of MLL4 (Fig. 2B; S-Fig. 4), as well as specific expression of the steatosis target genes of MLL4 (19) and their recruitment of MLL4 and H3K4me1 decoration (Fig. 4–6). While our ChIP results confirmed reduced MLL4-recruitment to the steatosis target genes of MLL4 (19) and their H3K4me1 decoration (Fig. 6B,C) in *Ube3a*-tg livers, ChIP experiments with *Ube3a*^{+/-} livers were challenging, likely due to their unusually fatty nature. We were able to demonstrate, under chow diet condition, increased recruitment of MLL4 to *Nr1d1*, one of the strongest target genes of MLL4 that we use as a positive control for MLL4-ChIP experiments (14), in *Ube3a*^{+/-}/*Ube3a*^{-/-} livers relative to WT controls (S-Fig. 6B).

Although we mostly analyzed the hepatic roles of UBE3A in regulating expression of the steatosis target genes of MLL4 (19) in this report, UBE3A likely antagonizes adipogenesis by suppressing the expression of an identical set of genes via degrading MLL4. This prediction is based on the findings that MLL4 serves as an essential coactivator of PPAR γ 2

in both adipogenesis (16, 23, 42) and hepatic steatosis (19). Indeed, our findings for increased adiposity in *Ube3a*^{+/-} mice (Fig. 3; S-Fig. 5B,C) as well as their enhanced expression of Scd1 and Cidec in the WAT (Fig. 5D) lend strong support for this notion.

While our results for upregulation of two potentially pro-steatotic genes *Elovl2* and *Socs2* (40, 41) may contribute to the absence of resistance of *Ube3a*-tg mice to HFD-induced fatty liver formation, other explanations also exist. For instance, this may involve downregulation of a substrate or substrates other than MLL4 in the liver and/or other tissues. In this regard, it is interesting to note that, while *Ube3a*^{+/-} mice show no gross sexual differences, *Ube3a*-tg mice show sexual dimorphism (Fig. 5B). Downregulation of an unknown substrate of Ube3a may interfere with the downregulation of the lipogenic target genes of MLL4 (due to reduced MLL4 levels) only in *Ube3a*-tg males but not in females. In contrast, this substrate may have no such interference when it is overexpressed in *Ube3a*^{+/-} mice. Identification of additional substrates of UBE3A that modulate the steatotic/adipogenic action of MLL4 either directly or indirectly will be an interesting future investigation.

In summary, we conclude that UBE3A indirectly exerts an epigenetic regulation of hepatic steatosis through targeting MLL4 for degradation. This UBE3A-MLL4 regulatory axis may provide a unique therapeutic venue for treating various MLL4-directed pathogenesises, including obesity and hepatic steatosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. UBE3A binds and ubiquitylates MLL4.

(A) Immunoprecipitation of Hepa1c1c7 liver cell lysates by anti-MLL4 antibody, but not by IgG, co-immunopurifies UTX and RBBP5, two subunits of MLL4-complex, as well as UBE3A, revealing that endogenous UBE3A associates with endogenous MLL4-complex in Hepa1c1c7 cells. (B) HEK293 cells expressing HA-Ub, Myc-UBE3A and Flag-MLL4^C were subject to immunoprecipitation by anti-Flag antibody followed by immunoblotting with anti-HA antibody, revealing that MLL4^C is ubiquitylated and this ubiquitylation is enhanced by overexpressed UBE3A. (C) HEK293 cells expressing HA-UBE3A WT or HA-UBE3A-Q784H as well as Flag-MLL4^C or Flag-p53 were subject to immunoprecipitation by IgG (control) or anti-Flag antibody followed by immunoblotting with anti-HA antibody, revealing that UBE3A WT interacts with both MLL4^C and p53, while UBE3A-Q784H binds only p53. (D) HEK293 cells expressing HA-UBE3A WT or HA-UBE3A-Q784H as well as Flag-MLL4^C or Flag-p53 were subject to immunoblotting with anti-Flag antibody or anti-β-tubulin (for loading controls), revealing that UBE3A WT degrades both MLL4^C and p53 in a dose-dependent manner, while UBE3A-Q784H degrades only p53.

Figure 2. UBE3A levels inversely correlate with levels of MLL4 and H3K4me1 in the liver.

(A) Liver lysates of WT, *Ube3a*-tg, *Ube3a*^{+/-} and *Ube3a*^{-/-} male mice were subject to immunoblotting with antibodies against MLL3/4, RBBP5, UBE3A and actins.

Quantification of each signal measured by ImageJ is shown below each band, showing that MLL3/4 levels, but not those of RBBP5 and actins, are altered in *Ube3a*-tg/*Ube3a*^{+/-}/*Ube3a*^{-/-} livers relative to WT control livers. A decrease in UBE3A in *Ube3a* mutants were also shown as control. (B) Liver lysates of WT and *Ube3a*-tg male mice were subject to immunoblotting with antibodies against actins, H3, H2B, and H3K4me1. Quantification of each signal measured by ImageJ is shown below each band, showing that H3K4me1 levels, but not those of actins, H3, and H2B are lower in *Ube3a*-tg livers relative to WT control livers.

Figure 3. *Ube3a*^{+/-} mice are more susceptible to fatty liver development than WT control mice due to enhanced MLL4 levels.

(A) ~2-months-old WT and *Ube3a*^{+/-} male mice were subject to a HFD for 8 weeks with their body weights measured each week. *Ube3a*^{+/-} mice showed an enhanced susceptibility to HFD-induced obesity. (B,C) Representative images of these mice sacrificed after 8-weeks of a HFD (B) and MRI measurements of fat and lean mass (C) revealed that *Ube3a*^{+/-} mice show enhanced fat storage in both the liver and fat depots relative to their WT control mice. (D, E) Deletion of a copy of *Mll4* rescued the enhanced susceptibility of *Ube3a*^{+/-} mice to HFD-induced obesity and hepatic steatosis, as shown by our findings that the enhanced % increase in body weight (D) as well as the increase in both whole body fat mass and liver fat mass (E) of *Ube3a*^{+/-} mice in comparison to WT control mice upon a HFD for 8 weeks were restored in *Ube3a*^{+/-}; *Mll4*^{+/-} mice to similar levels observed with WT control mice. All mice were male and ~2-months-old. Statistical differences were determined by Student's t-test (A,C) and one-way ANOVA followed by Tukey's test (D,E) using Prism 6 (GraphPad) software; $P < 0.05$ (one asterisk), $P < 0.01$ (two asterisks), and $P < 0.001$ (three asterisks), and 'ns' indicates not-significant ($P > 0.05$).

Figure 4. UBE3A modulates the expression of the steatogenic target genes of MLL4. (A) Heat maps for 89 downregulated and 188 upregulated genes in *Ube3a*^{+/-} livers as well as 214 downregulated and 268 upregulated genes in *Ube3a*-tg livers relative to their respective WT control livers (all female mice only). (B) GO terms with significant *P* values for 89 upregulated genes in *Ube3a*^{+/-} livers as well as 214 downregulated genes in *Ube3a*-tg livers are as shown. The previously defined steatogenic target genes of MLL4 in the liver (19) are included in the most strongly upregulated genes in *Ube3a*^{+/-} livers and the most strongly downregulated genes in *Ube3a*-tg livers. Intriguingly, two additional steatogenic genes (*Elovl2*, *Socs2*) are also upregulated in *Ube3a*-tg livers.

Figure 5. UBE3A modulates expression of the steatogenic target genes of MLL4 via degrading MLL4.

(A-C) Modulation of expression of the steatosis target genes of MLL4 by UBE3A was examined in *Ube3a*^{+/-} (A), *Ube3a*-tg (B) and *Ube3a*^{+/-}; *MLL4*^{+/-} livers (C) relative to their respective control livers after 8 weeks of a HFD. (D) The expression of two steatosis target genes of MLL4 was also examined in the WAT of *Ube3a*^{+/-} mice relative to WT control mice. Statistical differences were determined by Student's t-test (A,B,D) and one-way ANOVA followed by Tukey's test (C) using Prism 6 (GraphPad) software; $P < 0.05$ (one asterisk), $P < 0.01$ (two asterisks), $P < 0.001$ (three asterisks), and $P < 0.0001$ (four asterisks), and 'ns' indicates not-significant ($P > 0.05$).

Figure 6. UBE3A epigenetically regulate expression of the steatogenic target genes of MLL4.

(A-C) Rosiglitazone-enhanced expression of *Pparg2*, *Cd36*, *Scd1* and *Cidec* as determined by qRT-PCR (A), as well as rosiglitazone-enhanced recruitment of MLL4 to the PPREs in these genes (B) and their decoration by H3K4me1 (C) as determined by ChIP, are significantly impaired in *Ube3a*-tg livers relative to their WT control livers. The experiments were done with male mice. n=3 for vehicle-treated WT mice; n=3 for rosiglitazone-treated WT mice; n=3 for vehicle-treated *Ube3a*-tg mice; n=4 for rosiglitazone-treated *Ube3a*-tg mice. Statistical differences were determined by Student's t-test; $P < 0.05$ (one asterisk), $P < 0.01$ (two asterisks), and $P < 0.001$ (three asterisks).

Figure 7. The working model.

Degradation of MLL4 by UBE3A results in an inverse correlation of UBE3A levels with MLL4 levels, MLL4 occupancy of its target genes, and open chromatin marked by H3K4me1/2 (indicated as green dots). As a consequence, relative to WT control livers, *Ube3a*-tg livers show suppressed expression of the steatosis target genes of MLL4, while *Ube3a*^{+/-} livers show the opposite.