Additional Sex Comb-like (ASXL) Proteins 1 and 2 Play Opposite Roles in Adipogenesis via Reciprocal Regulation of Peroxisome Proliferator-activated Receptor γ*[S]

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Our previous studies have suggested that the mammalian additional sex comb-like 1 protein functions as a coactivator or repressor of retinoic acid receptors in a cell-specific manner. Here, we investigated the roles of additional sex comb-like 1 proteins in regulating peroxisome proliferator-activated receptors (PPARs). In pulldown assays *in vitro* and in immunoprecipitation assays *in vivo*, ASXL1 and its paralog, ASXL2, interacted with PPARα and PPARγ. In 3T3-L1 preadipocyte cells, overexpression of ASXL1 inhibited the induction of PPARγ activity by rosiglitazone, as shown by transcription assays, and completely suppressed adipogenesis, as shown by Oil Red O staining. In contrast, overexpression of ASXL2 greatly enhanced rosiglitazone-induced PPARγ activity and enhanced adipogenesis. Deletion of the heterochromatin protein 1 (HP1)-binding domain from ASXL1 caused the mutant protein to enhance adipogenesis similarly to ASXL2, indicating that HP1 binding is required for the adipogenesis-suppressing activity of ASXL1. Adipocyte differentiation was associated with a gradual decrease in ASXL1 expression but did not affect ASXL2 expression. Knockdown of ASXL1 and ASXL2 had reciprocal effects on adipogenesis. In chromatin immunoprecipitation assays in 3T3-L1 cells, ASXL1 occupied the promoter of the PPARγ target gene *aP2* together with HP1α and Lys-9-methylated histone H3, whereas ASXL2 occupied the *aP2* promoter together with histone-lysine *N*-methyltransferase MLL1 and Lys-9-acetylated and Lys-4-methylated H3 histones. Finally, microarray analysis demonstrated that ASXL1 represses, whereas ASXL2 increases, the expression of adipogenic genes, most of which are PPARγ targets. These results suggest that members of the additional sex comb-like family provide complex regulation of adipogenesis via differential modulation of PPARγ activity.

Peroxisome proliferator-activated receptors (PPARs)[3] are ligand-dependent transcription factors that belong to the nuclear receptor superfamily. Extensive studies have suggested that PPARs play major roles in various physiological processes, including lipid metabolism, inflammation, and angiogenesis (reviewed in Refs. 1–3). One member of the PPAR family, PPARγ, functions primarily in adipocyte differentiation; it is induced during adipogenesis and is both necessary and sufficient for adipocyte differentiation to occur (4). When bound to ligands with agonist activity, such as rosiglitazone, a synthetic thiazolidinedione, PPARγ, forms a heterodimer with a retinoid X receptor; this dimer regulates the expression of specific subsets of genes containing a PPARγ-response element in their promoters (4). These genes include those encoding adipocyte lipid-binding protein 2 (aP2), fatty acid-binding protein 4, and lipoprotein lipase. Recent genome-wide microarray and chromatin immunoprecipitation (ChIP) analyses have identified several other PPARγ target genes (5–9).

Like that of other nuclear receptors, the activity of PPARγ is differentially regulated by its association with various coregulators (10–12). In the absence of ligand, PPARγ associates with corepressors, such as nuclear receptor corepressor or silencing mediator for retinoid and thyroid hormone receptors, to repress PPARγ-mediated transcription, leading to adipogenic blockage in 3T3-L1 cells, a mouse embryonic fibroblast-like preadipose cell line (13). Binding of the NAD-dependent deacetylase Sirt1 to PPARγ represses PPARγ transcriptional activity by recruiting nuclear receptor corepressor and silencing mediator for retinoid and thyroid hormone receptors to its target genes, thereby repressing fat accumulation in adipocytes (14). Ligand binding induces a conformational change in the PPARγ ligand-binding domain, leading to the release of corepressors; as a result, a variety of coactivators with histone lysine acetyltransferase activity are recruited to PPARγ-responsive promoters, thereby activating transcription (12, 15). One PPARγ coactivator, coactivator-1α (also known as PGC-1α), modulates energy expenditure by regulating mitochondrial biogenesis and adaptive thermogenesis in brown adipocytes (16, 17).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[3] The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; ASXL, additional sex comb-like; IP, immunoprecipitation; qPCR, quantitative PCR.

PPARγ participates in regulation at the epigenetic level through its direct association with histone-modifying enzymes or by its indirect association with these enzymes via coregulators (18, 19). Cyclin D1 recruits histone deacetylases and histone methyltransferase Suv39H1 to PPARγ-responsive gene promoters through protein-protein interactions, leading to decreased acetylation and increased methylation of Lys-9 of histone H3 (H3K9), thereby inhibiting adipogenic gene expression and adipogenesis (20). Histone deacetylases are down-regulated during adipocyte differentiation (21). Recently, activation of the histone methyltransferase SETDB1 via Wnt-NLK signaling was reported to induce H3K9 methylation at PPARγ target genes; activated SETDB1 forms a complex with PPARγ, inactivating PPARγ and inducing osteoblastogenesis (22). By binding to methylated H3K9, heterochromatin protein 1 (HP1)-α appears to participate in gene silencing by inducing the formation of higher order chromatin structure (23, 24). Other studies have suggested that methylation of Lys-4 of histone H3 (H3K4), probably by histone-lysine *N*-methyltransferase MLL3, at adipogenic genes is critical for gene activation and adipogenesis in 3T3-L1 cells (25, 26). These histone modifications may provide for diverse regulation of PPARγ at the chromatin level during adipocyte differentiation.

We previously showed that additional sex comb-like 1 (ASXL1), a mammalian homolog of the *Drosophila* protein Asx, both enhances the transcriptional activity of retinoic acid receptors by associating with steroid receptor coactivator 1 (SRC-1) and represses the activity of these receptors by recruiting lysine-specific demethylase 1 (LSD1) and HP1 to the target gene in a cell type-specific manner (27, 28).

Here, we investigated the different roles of ASXL1 and its paralog ASXL2 in the regulation of other PPARs and in adipocyte differentiation. We found that ASXL1 suppresses the transactivation activity of ligand-bound PPARγ, as well as its adipocyte differentiation-inducing activity, whereas ASXL2 promotes these activities. Consistent with these observations, ASXL1 occupies the *aP2* promoter together with HP1α and methylated H3K9, whereas ASXL2 occupies the *aP2* promoter together with acetylated H3K9, methylated H3K4, and the histone-lysine H3K4 methyltransferase MLL1. We also examined the genome-wide response of ASXLs to PPARγ regulation using microarray analysis. Our results establish that ASXL1 and ASXL2 play different roles in PPARγ regulation, providing additional insight pertinent to the treatment of fat-related disorders.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—3T3-L1 and HEK293 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and antibiotic/antimycotic agents (all from Invitrogen) in a 5% CO_2 atmosphere at 37 °C. For use with cells to be treated with PPAR ligands, the FBS was pretreated with charcoal.

Plasmids and Cloning—All cDNAs were constructed according to standard methods and verified by sequencing. Deletion or point mutants of the desired genes were created by PCR amplification. DNA sequences encoding murine ASXL1,

a murine ASXL1 mutant defective for HP1 binding (ASXL1ΔHP1), and human ASXL2 were inserted into the G418-resistant pcDNA3 vector (Invitrogen), which inserted a 2× FLAG epitope tag in front of the encoded proteins. The resulting vectors expressed 2× FLAG-tagged proteins, designated FLAG-ASXL1, FLAG-ASXL1ΔHP1, and FLAG-ASXL2, respectively. DNA sequences encoding amino acids 961–1514 of murine ASXL1 or amino acids 916–1435 of human ASXL2 were inserted into the pGEX4T-1 vector (Amersham Biosciences), which fused the glutathione *S*-transferase (GST) coding sequence to those of ASXL1 and ASXL2, creating the GST-ASXL1 and GST-ASXL2 fusion proteins.

GST Pulldown Assays—GST-ASXL1 and GST-ASXL1 were expressed in *Escherichia coli* and purified on glutathione-Sepharose beads (Amersham Biosciences) by standard methods. FLAG-tagged PPARα and FLAG-tagged PPARγ were translated *in vitro* in rabbit reticulocyte lysate (Promega, Madison, WI). Then approximately equal amounts of GST or GST fusion protein and FLAG-tagged PPARα or FLAG-tagged PPARγ were mixed, and bound proteins were detected by Western blotting using anti-FLAG antibody (Sigma).

Western Blotting and Immunoprecipitation (IP)—Primary antibodies against HP1α, trimethylated H3K4 (H3K4me3), trimethylated H3K9 (H3K9me3), and acetylated H3K9 (H3K9Ac) were from Upstate (Chicago, IL; catalog numbers 05-689, 07-473, 07-442, and 07-352, respectively); anti-PPARγ antibody was from Santa Cruz Biotechnology (Santa Cruz, CA; catalog number sc-7273); anti-MLL1 antibody was from Bethyl Laboratories (Montgomery, TX; catalog number A300-374A); and anti-FLAG M2 and anti-β-actin antibodies were from Sigma (catalog numbers F3165 and A1978, respectively). Anti-ASXL1 and -ASXL2 antibodies were affinity-purified from rabbit polyclonal serum raised against amino acids 233–247 of mouse ASXL1 and mouse monoclonal ascites fluid raised against amino acids 1213–1396 of human ASXL2, respectively. Both antibodies were able to recognize human and mouse ASXLs.

For Western blotting, cells were lysed in a lysis buffer (27) supplemented with protease inhibitor mixture (Roche Applied Science), and proteins in the lysates were subjected to SDS-PAGE on 8–12% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies as indicated and then with peroxidase-conjugated mouse or rabbit IgG secondary antibodies (Amersham Biosciences). The protein bands were detected with an ECL system (Amersham Biosciences).

For IP, HEK293 cells were treated with dimethyl sulfoxide (DMSO) with or without 1 μM rosiglitazone (a PPARγ-specific agonist; BioVision, Mountain View, CA) overnight and lysed in RIPA buffer supplemented with a protease inhibitor mixture (Roche Applied Science). The lysates were incubated overnight at 4 °C with a 1:200 dilution of anti-PPARγ antibody and then with A/G-agarose beads (Santa Cruz Biotechnology) for 2 h at 4 °C. The beads were washed three times with radioimmunoprecipitation assay buffer, and the bound immune complexes were released from the beads by boiling and analyzed by Western blotting. For study of the ASXL2-PPARγ interaction, the HEK293 cells were transfected with

FLAG-ASXL2 cDNA prior to DMSO or rosiglitazone treatment.

Transient Transfection and Luciferase Assay—HEK293 cells were seeded in 12-well culture plates (1.5×10^5 cells/well). Lipofectamine Plus reagent (Invitrogen) was used to cotransfect the cells with an *aP2* promoter-luciferase reporter vector and various amounts of ASXL1 or ASXL2 expression vector. After a 6-h incubation, the cells were washed, fed with medium containing 5% charcoal-stripped FBS, and incubated for an additional 20 h in the presence of 1 μM rosiglitazone. The cells were then washed with ice-cold PBS, collected, resuspended in 100 μl of luciferase lysis buffer (Promega), and subjected to three freeze-thaw cycles. The luciferase activity was measured and normalized to the β-galactosidase activity as described previously (28).

Knockdown of ASXL1 and ASXL2 Expression by RNAi—For RNAi experiments, ASXL1- and ASXL2-specific siRNA duplexes were synthesized by Invitrogen. The ASXL1 duplex had the sense and antisense sequences 5′-CCUCCAUCCAG-UGACAAAUCCCAUU-3′ and 5′-AAUGGGAUUUGUCAC-UGGAUGGAGG-3′, respectively, and the ASXL2 duplex specific had the sense and antisense sequences 5′-GAGCUUUA-GGAGGACCCAUUCUGUA-3′ and 5′-UACAGAAUGGGU-CCUCCUAAAGCUC-3′, respectively. The control siRNA was a duplex of the sense sequence 5′-CCUACGCCACCAA-UUUCGU-3′ and the antisense sequence 5′-ACGAAAUUG-GUGGCGUAGG-3′. HEK293 or 3T3-L1 cells were transfected with siRNA using Lipofectamine 2000 in Opti-MEM I reduced serum medium (Invitrogen) according to the manufacturer's instructions. Knockdown of gene expression was verified by Western blotting using anti-ASXL antibodies.

Stably Transfected Cell Lines—3T3-L1 preadipocytes were transfected with pcDNA3 derivatives encoding FLAG-ASXL1, FLAG-ASXL1ΔHP1, or FLAG-ASXL2 or with empty pcDNA3 encoding the FLAG tag only using Lipofectamine Plus reagent (Invitrogen). After 48 h, the cells were treated with 0.6 mg/ml G418 (Invitrogen). After 5–7 days, the culture medium was replaced with fresh culture medium containing G418, and G418-resistant colonies were selected for 2 weeks. Stably transfected cells were verified by Western blotting using anti-FLAG antibody.

Real Time RT-PCR—For studies of the effects of overexpression of ASXL1 and ASXL2, HEK293 cells were transfected with pcDNA3 derivatives encoding FLAG-ASXL1 or FLAG-ASXL2, or with empty pcDNA3 encoding the FLAG tag only. For studies of the effect of knockdown of ASXL1 and ASXL2 expression, HEK293 cells were transfected with the corresponding ASXL1- or ASXL2-specific siRNA duplexes. Transfected cells were treated with 1 μM rosiglitazone, and the total RNA was extracted using Easy-Blue Reagent (Intron Biotechnology, Kyunggi, Korea). Then 2 μg of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and random primers (Invitrogen). Quantitative PCRs (qPCR) were performed using the iQTM SYBR Green Supermix and Icycler CFX96 real time PCR detection system (Bio-Rad) with the primers as shown in Table 1. The mRNA expression data were normalized to the level of

TABLE 1
Primer sequences used for real time PCR

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
aP2γ	AAAGACAGCTCCTCCTCGAAGGTT	TGACCAAATCCCCATTTACGC
LPL	ATCCATGGATGGACGGTAACG	CTGGATCCCAATACTTCGACCA
GAPDH	CGGCTACCACATCCAAGGAA	AGCCACATCGCTCAGACACC

TABLE 2
Primer sequences used for normal PCR

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
ASXL1γ	CAAATGAAGCGCAACAGAGG	TGATTCTTCTTTGGTCAAACC
ASXL2	CAGCACCAGCAGCCATTTCAG	GGAAAACGAGCCCTGGGAGAG
adipsin	CCTGAACCCTACAAGCGATG	GGTTCCACTTCTTTGTCCTCG
GAPDH	GTGGATATTGTTGCCATCA	GACTCCACGACGTACTCA

GAPDH mRNA expression and expressed as fold-increases relative to the controls.

For normal RT-PCR, stably transfected 3T3-L1 cells were grown in DMEM containing 10% charcoal-stripped FBS and treated with or without 1 μM rosiglitazone. Total RNA was extracted and reverse-transcribed as described above. The cDNA for *aP2* and *LPL* was amplified by PCR using the primer pairs shown above for qPCR. The cDNAs for *ASXL1*, *ASXL2*, *adipsin*, and *GAPDH* were amplified by PCR using the primers as shown in Table 2. The PCR products were visualized by 1.5% agarose gel electrophoresis.

Differentiation of 3T3-L1 Cells—Stably transfected 3T3-L1 cell lines were cultured until confluent. Two days later (on day 0), the cells were treated with a differentiation-inducing mixture containing 1 μM rosiglitazone, 0.5 μM dexamethasone, 100 μM 3-isobutyl-1-methylxanthine, and 1 μg/ml insulin (day 0). The medium was removed every 2 days and replaced with fresh medium containing DMEM plus 10% FBS, 1 μg/ml insulin, and 1 μM rosiglitazone. Cells were harvested for Western blotting, RT-PCR, or ChIP analysis every 2 days.

Oil Red O Staining—Stably transfected 3T3-L1 cells induced to differentiate as described above under "Differentiation of 3T3-L1 Cells" for a total of 8 days were washed twice with PBS (pH 7.4) and fixed with 2 ml of 10% formalin in PBS for 30 min at room temperature. The cells were stained with 0.5% Oil Red O (Sigma) for 30 min with gentle agitation, and the excess stain was removed using 60% isopropyl alcohol. The cells were washed with water and photographed under a light microscope.

To assess the effect of ASXL knockdown on adipogenesis, the 3T3-L1 cells were transfected with *ASXL* siRNA before differentiation was induced, as described above. The siRNA was also added to the fresh medium replaced every 2 days during differentiation.

ChIP Analysis—ChIP analysis was performed as described previously (28). Mock-transfected 3T3-L1 cells or 3T3-L1 cells stably expressing FLAG-ASXL1 or FLAG-ASXL2 were induced to differentiate, as described above. Cross-linked, immunoprecipitated chromatin complexes were recovered by IP with anti-FLAG and other indicated antibodies. Cross-linking was then reversed according to the protocol provided by Upstate. The DNA pellets were recovered and analyzed by PCR or real time qPCR using a primer pair encompassing the *aP2* promoter region, 5′-AAATTCAGAAGAAAGTAA-ACACATTATT-3′ (forward) and 5′-ATGCCCTGACCAT-
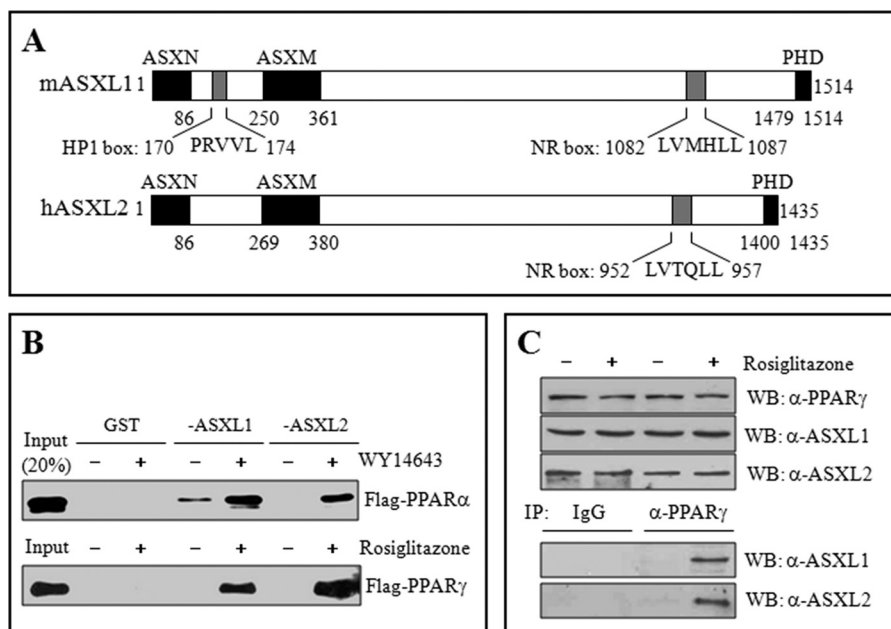
FIGURE 1. **ASXL1 and ASXL2 interact with ligand-bound PPARγ.** *A*, schematic representation of mouse ASXL1 (*mASXL1*) and human ASXL2 (*hASXL2*), which were used in this study. The ASXN, ASXM, and PHD domains, which are conserved in the ASXL family, are indicated by *black boxes*. The previously identified nuclear receptor binding motif (nuclear receptor (*NR*) box) (27) and HP1 binding region (*HP1 box*) (28) are indicated by *gray boxes*. *B*, ligand-dependent, direct interactions between ASXLs and PPARs. FLAG-PPARα and FLAG-PPARγ were translated *in vitro* and incubated with 2 μg of GST, GST-ASXL1 (−*ASXL1*), or GST-ASXL2 (−*ASXL2*) in the presence of 1 μM WY-14643, a specific activator of PPARα, or rosiglitazone, a specific activator of PPARγ. Bound proteins were visualized by SDS-PAGE and Western blotting using anti-FLAG antibody. *C*, endogenous interactions between ASXLs and PPARγ. HEK293 cells were transfected with a FLAG-PPARγ expression vector in the presence or absence of 1 μM rosiglitazone, and cell lysates were prepared. *Upper panel*, the expression of PPARγ, ASXL1, and ASXL2 was confirmed by Western blotting (*WB*). *Lower panel*, the cell lysates were immunoprecipitated (*IP*) with preimmune serum (*IgG*) or anti (*α*)-PPARγ antibody, and the precipitated proteins were analyzed by Western blotting with anti-ASXL1 and anti-ASXL2 antibodies.

GTGA-3′ (reverse). Fold enrichment ratios were calculated from *Ct* values normalized against *Ct* of IgG control. Percentage of input was calculated compared with input sample used in qPCR. Relative occupancy is the fold-increase in the percentage of input over that of day 0 (set to 1).

Microarray and Data Analysis—TRIzol reagent (Invitrogen) was used to extract RNA from 3T3-L1 cells stably expressing FLAG, FLAG-ASXL1, or FLAG-ASXL2 after differentiation for 8 days as described above. The 3T3-L1/FLAG and 3T3-L1/FLAG-ASXL1 or -ASXL2 RNA samples were labeled with Cy3 (FLAG and FLAG-ASXL1) or Cy5 (FLAG-ASXL2). Labeled samples were hybridized to an Agilent Mouse 44k 4plex microarray (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. The Lowess (locally weighted linear regression curve fit) and dye-swap normalization methods were applied to the Cy5/Cy3 ratio of the microarray signal intensities, and the results were filtered with a cutoff at $p < 0.05$. Genes exhibiting significant differences in expression level were classified by referring to the Gene Ontology, KEGG, and DAVID Bioinformatics Resources databases.

Statistical Analysis—Results were evaluated with Student's *t* test for the two groups and are presented as means ± S.D.

RESULTS

ASXL1 and ASXL2 Interact with PPARs—Our previous findings concerning the roles of ASXL1 in retinoic acid receptor regulation (27, 28) prompted us to investigate whether ASXL1 and its paralog ASXL2 also regulate another family of nuclear receptors, the PPARs. The functional domains of

ASXL1 and ASXL2 are shown in Fig. 1*A*. Whereas both proteins contain nuclear receptor-binding motifs, only ASXL1 harbors an HP1-binding motif. This motif is critical for retinoic acid receptor repression (28), and the ASXL1ΔHP1 mutant, which lacks this motif, is defective in HP1 binding (28).

To examine the physical interactions between ASXL and PPAR proteins *in vitro* and *in vivo*, we performed GST pulldown and co-IP assays, respectively. For GST pulldown assays, GST-ASXL1 and GST-ASXL1 fusion proteins were expressed in *E. coli*, purified, and mixed with *in vitro* translated FLAG-PPARα or FLAG-PPARγ in the absence and presence of ligands. Subsequent Western blotting with anti-FLAG antibody indicated that ASXL1 and ASXL2 both interact with PPARα and PPARγ in a ligand-dependent manner (Fig. 1*B*).

For co-IP assays, HEK293 cells, which express very low levels of PPARγ, were transfected with a vector expressing FLAG-PPARγ. After the expression of PPARγ, ASXL1, and ASXL2 was confirmed by Western blotting (Fig. 1*C*, *upper panel*), the PPARγ-linked complexes were immunoprecipitated with anti-PPARγ antibody and analyzed by Western blotting using anti-ASXL1 and -ASXL2 antibodies. As shown in the *lower panel* of Fig. 1*C*, this analysis showed that PPARγ interacts with ASXL1 and ASXL2 in a rosiglitazone-dependent manner. No precipitation of ASXL1 or ASXL2 was detectable when an IgG control antibody was used for the immunoprecipitation.

PPARγ Activity Is Negatively Regulated by ASXL1 but Positively Regulated by ASXL2—To determine the significance of the physical interactions between ASXL proteins and PPARγ,
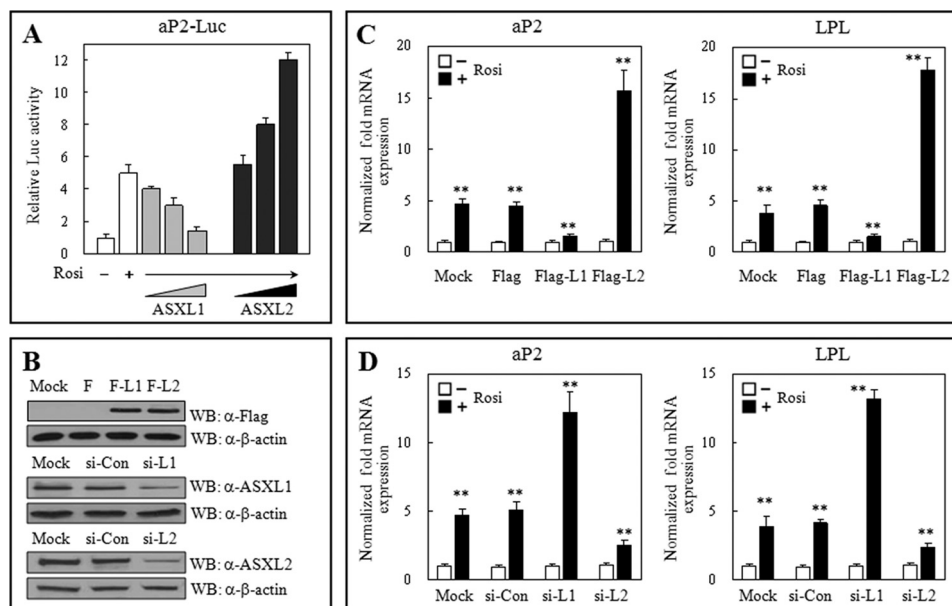
FIGURE 2. **PPARγ transcriptional activity is repressed by ASXL1 but enhanced by ASXL2.** *A*, effect of ASXLs on PPARγ-driven luciferase reporter gene activity. HEK293 cells were transfected with 0.1 μg of a luciferase reporter gene under control of the PPAR-responsive *aP2* promoter (*aP2-Luc*) and increasing amounts (0, 0.1, 0.2, or 0.5 μg) of ASXL1 or ASXL2 expression vector in the presence of 1 μM rosiglitazone (*Rosi*). Extracts of transfected cells were subjected to luciferase activity assays. Relative luciferase activity is shown as means ± S.D. of three independent experiments. *B*, overexpression and knockdown of ASXL1 and ASXL2 in HEK293 cells. *Upper panel*, for overexpression, cells were transfected with 1 μg of FLAG-ASXL1 (*F-L1*) or FLAG-ASXL2 (*F-L2*) expression vector or empty FLAG vector (*F*; control), and expression of the FLAG-tagged proteins was confirmed by Western blotting (*WB*) with anti (α)-FLAG antibody. *Lower two panels*, for knockdown, HEK293 cells were transfected with 200 pmol of ASXL1-specific (*si-L1*) or ASXL2-specific (*si-L2*) siRNA or control (*si-Con*) siRNA, and levels of ASXL1 and ASXL2 expression were analyzed by Western blotting (*WB*) with anti-ASXL1 and -ASXL2 antibodies. *C* and *D*, effects of ASXL overexpression (*C*) and knockdown (*D*) on the expression of the endogenous PPARγ target genes *aP2* and *LPL*. Total RNA was extracted from transfected HEK293 cells and subjected to real time qPCR. Fold-increases in mRNA expression were normalized to the level of *GAPDH* RNA. Data shown represent means ± S.D. of three independent experiments. **, $p < 0.01$.

we examined the roles of ASXL1 and ASXL2 in PPARγ-mediated transcription. In luciferase reporter assays carried out in transfected HEK293 cells, ASXL1 strongly inhibited the rosiglitazone induction of PPARγ transcriptional activity, whereas ASXL2 significantly increased it, and both effects were dose-dependent (Fig. 2*A*).

To confirm the reciprocal effects of ASXL1 and ASXL2 on PPARγ-mediated luciferase reporter activity, we used real time RT-qPCR to monitor the expression of two endogenous PPARγ-responsive genes, *aP2* and *LPL*, in HEK293 cells under ASXL overexpression or knockdown conditions. ASXL1 and ASXL2 expressions were monitored by Western blotting using anti-FLAG, anti-ASXL1, and anti-ASXL2 antibodies (Fig. 2*B*). As shown in Fig. 2*C*, the rosiglitazone-induced expression of *aP2* and *LPL* mRNA was almost completely abolished in ASXL1-overexpressing cells but was greatly increased in ASXL2-overexpressing cells. Conversely, siRNA-mediated knockdown of ASXL1 expression significantly increased PPARγ-mediated gene expression, whereas knockdown of ASXL2 expression significantly decreased it (Fig. 2*D*). Overall, these results demonstrate that the rosiglitazone-induced transcriptional activity of PPARγ is negatively regulated by ASXL1 but positively regulated by ASXL2.

ASXL1 and ASXL2 Oppositely Regulate Adipogenesis—To assess whether the differential regulation of PPARγ by ASXLs is functionally linked to their roles in adipocyte differentiation, 3T3-L1 cell lines constitutively expressing FLAG-ASXL1, FLAG-ASXL1ΔHP1, or FLAG-ASXL2 were generated by stable transfection. Expression of these constructs was

confirmed by Western blotting with anti-FLAG antibody (Fig. 3*A*). Then the effect of ASXL expression on PPARγ activity was monitored by RT-PCR 5 days after adipogenesis was induced using a differentiation-inducing mixture containing rosiglitazone (Fig. 3*B*). As expected from the results shown in Fig. 2, ASXL1 overexpression repressed rosiglitazone induction of expression of the PPARγ target genes *aP2* and *LPL*, whereas ASXL2 overexpression greatly enhanced this induction (Fig. 3*B*). Interestingly, deletion of the HP1 box from ASXL1 greatly enhanced PPARγ transcriptional activity, suggesting that the HP1 box is required for the repression activity of ASXL1 and that the ASXL1ΔHP1 mutant functions as a dominant-negative regulator or as a coactivator like ASXL2.

Consistent with the RT-PCR analyses, Oil Red O staining under conditions identical to those described above indicated that the stable expression of FLAG-ASXL1, but not FLAG alone, blocked 3T3-L1 cell differentiation into adipocytes (Fig. 3*C*). However, the stable expression of FLAG-ASXL2 or FLAG-ASXL1ΔHP1 resulted in significantly enhanced differentiation.

To verify the physiological roles of ASXL1 and ASXL2 in adipogenesis, we examined ASXL levels every other day during 3T3-L1 cell differentiation by Western blotting. Whereas the ASXL1 level decreased gradually during adipocyte differentiation, the ASXL2 level remained relatively constant, and the PPARγ protein level increased (Fig. 4*A*). The amount of *aP2* mRNA also increased during differentiation (Fig. 4*A*).

These results prompted us to test the effect of RNAi-mediated ASXL knockdown on adipogenesis of 3T3-L1 preadipo-
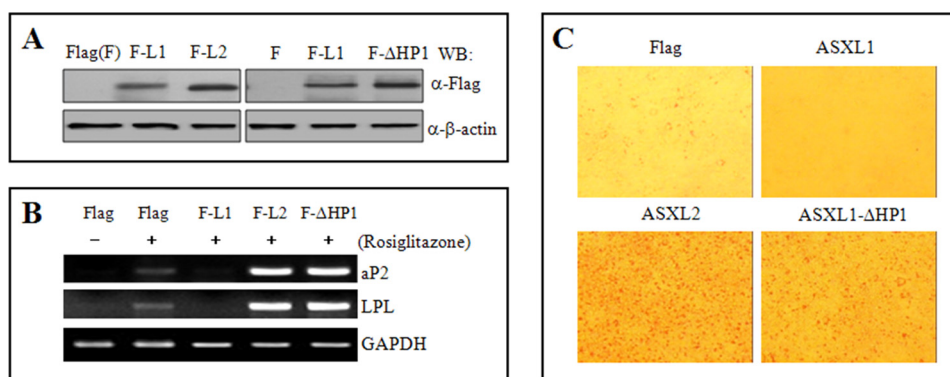
FIGURE 3. **ASXL1 and ASXL2 reciprocally regulate adipogenesis.** *A*, generation of cell lines stably expressing mouse ASXL1 and human ASXL2. 3T3-L1 cells were transfected with FLAG-ASXL1 (*F-L1*), FLAG-ASXL2 (*F-L2*), or FLAG-ASXL1ΔHP1 (*F-ΔHP1*). Stable transfectants were selected using G418, and expression of the FLAG-tagged proteins was confirmed by Western blotting (*WB*) using anti-FLAG antibody. β-Actin was used as an internal control. *B*, effects of stable overexpression of ASXL1 (*F-L1*), FLAG-ASXL2 (*F-L2*), and FLAG-ASXL1ΔHP1 (*F-ΔHP1*) on the expression of *aP2* and *LPL*. Total RNA was extracted from stably transfected 3T3-L1 cells cultured in the presence of 1 μM rosiglitazone and subjected to RT-PCR using primer pairs specific for the *aP2* and *LPL* coding sequences. *GAPDH* mRNA was used as an internal control. *C*, effect of stable overexpression on the adipogenic potential of 3T3-L1 cells. The stable cell lines from *B* were cultured in differentiation-inducing medium, including rosiglitazone for 8 days and subjected to Oil Red O staining to visualize lipid droplets.
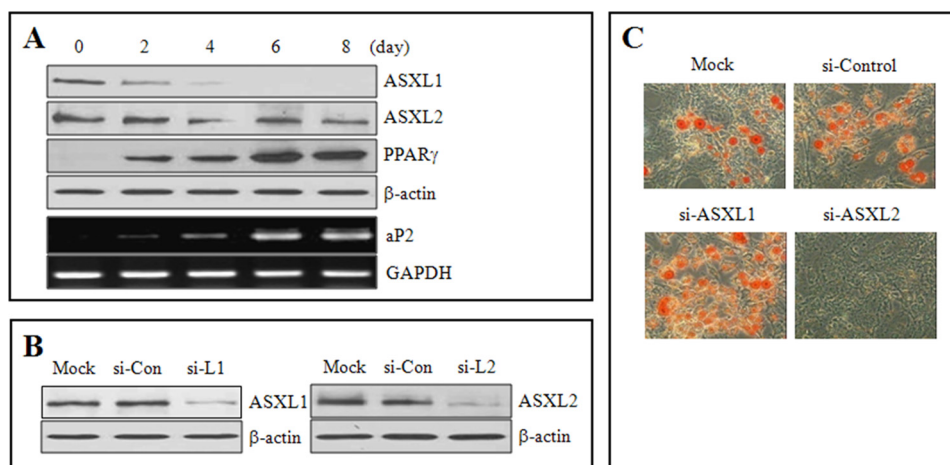

FIGURE 4. **Down-regulation of ASXL2 abolishes adipogenesis.** *A*, expression of ASXL1 and ASXL2 during adipogenesis. 3T3-L1 cells were cultured in differentiation-inducing medium, including rosiglitazone for 8 days. Cells were harvested every 2 days and subjected to Western blotting to detect ASXL1, ASXL2, and PPARγ, with β-actin as a control (*four upper panels*) and RT-PCR to detect *aP2* mRNA, with *GAPDH* mRNA as an internal control (*two lower panels*). *B*, knockdown of ASXL1 and ASXL2. 3T3-L1 cells were transfected with ASXL1- or ASXL2-specific siRNA (*si-L1* and *si-L2*, respectively) or control siRNA (*si-Con*), and knockdown of ASXL1 and ASXL2 expression was confirmed by Western blotting. *C*, effect of ASXL knockdown on lipid accumulation. During growth of 3T3-L1 cells in differentiation-inducing medium, including rosiglitazone, they were treated with ASXL1- or ASXL2-specific siRNA (*si-ASXL1* and *si-ASXL2*, respectively) every 2 days. The cells were subjected to Oil Red O staining at day 8.

cytes. The depletion of ASXL1 and ASXL2 by their respective siRNAs was confirmed by Western blotting (Fig. 4*B*). Subsequent Oil Red O staining showed that ASXL1 knockdown increased intracellular lipid accumulation, whereas ASXL2 knockdown completely blocked it (Fig. 4*C*). Collectively, these data suggest that adipocyte differentiation of 3T3-L1 preadipocytes is differentially regulated by ASXL1 and ASXL2 *in vivo*, presumably because the two proteins oppositely modulate PPARγ target gene expression.

ASXL1 and ASXL2 Recruit Histones with Repressing (ASXL1) or Activating (ASXL2) Modifications to the aP2 Gene Promoter—To determine the mechanism underlying differential regulation of PPARγ activity by ASXL1 and ASXL2 at the chromatin level, we performed ChIP assays and examined the recruitment of chromatin proteins (PPARγ, HP1α, and MLL1) and modified histone proteins to the endogenous PPARγ-responsive *aP2* gene promoter (Fig. 5). When 3T3-L1 cells stably expressing ASXL1 and ASXL2

were examined 5 days after the induction of adipogenesis by treatment with a rosiglitazone-containing differentiation-inducing mixture, ASXL1 and ASXL2 were recruited to the promoter together with PPARγ. HP1α binding to the promoter was dependent on ASXL1 but not on ASXL2. Consistent with this recruitment of HP1α, H3K9me3 modification at the promoter was increased. In contrast, when ASXL2 was overexpressed, histones with activating modifications, such as H3K9ac and H3K4me3, were enhanced to the promoter, along with the histone H3K4 methyltransferase MLL1. These ChIP data show that ASXL1 overexpression leads to the recruitment of more HP1α and increased H3K9me3 modification, but less H3K9ac, H3K4me3, and MLL1, to the *aP2* promoter, whereas ASXL2 overexpression has the opposite effect.

Next, to assess how ASXL1 and ASXL2 are associated with histone modifications at the chromatin level *in vivo* during adipocyte differentiation, real time qPCR was used to quantify

the precipitated DNA from ChIP assays (Fig. 6). As in our earlier IP analysis (Fig. 4*A*), PPARγ binding to the *aP2* promoter gradually increased during adipogenesis of 3T3-L1 cells. In contrast, ASXL1 binding to the promoter decreased during adipogenesis, and ASXL2 binding to the promoter remained constant. Consistent with the decrease in ASXL1 binding, HP1α binding and H3K9me3 modification at the promoter progressively decreased. This reduction in ASXL1 and H3K9me3 at the promoter occurred concomitantly with

elevation in the activating histone modifications H3K9ac and H3K4me3 and MLL1. Binding of the histone demethylase LSD1 to the promoter was unchanged during adipogenesis. Together, these data provide clues to the mechanisms underlying the opposing regulatory effects of ASXL1 and ASXL2 on rosiglitazone-induced PPARγ activity and adipocyte differentiation.

ASXL1 and ASXL2 Oppositely Regulate Adipogenic Genes— To identify and compare the genes targeted for PPARγ regulation by ASXL1 and ASXL2 in response to rosiglitazone in 3T3-L1 cells, we performed expression profiling using microarrays. To this end, stably transfected 3T3-L1 cells overexpressing FLAG, FLAG-ASXL1, or FLAG-ASXL2 were generated. Adipogenesis was induced by treatment with rosiglitazone-containing differentiation-inducing mixture for 8 days, and the gene expression profiles were compared. Compared with untreated cells, a total of 1760 genes were changed >2-fold by rosiglitazone, 998 genes in ASXL1 cells and 762 genes in ASXL2 cells (Fig. 7*A*). Among these genes, 312 genes were regulated by both ASXL1 and ASXL2. Cluster analysis of these common genes showed surprising results; most genes are similarly regulated (Fig. 7*B*). Of note, only a subset of genes involved in adipogenesis, lipid metabolism, and insulin sensitivity was suppressed by ASXL1 and induced by ASXL2, including Kruppel-like factor (*KLF*) 15, GPR43, G_0/G_1 switch gene (*G0S*) 2, *adipsin*, hexokinase-2, glucose transporter type 4 (*GLUT4*), plasminogen activator inhibitor (*PAI*)-1, glycerophosphate dehydrogenase (*GPDH*), and stearoyl-CoA desaturase (*SCD1*) 1. Further data analysis identified a number of
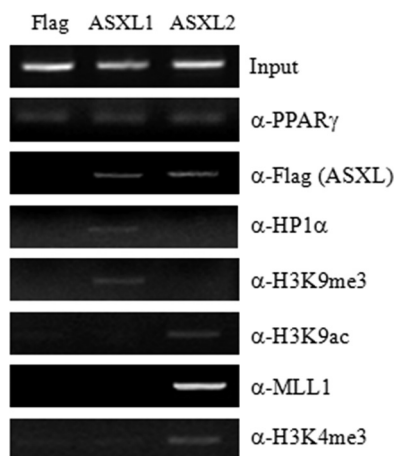

FIGURE 5. **Effect of ASXL1 and ASXL2 on chromatin occupancy by modified histones.** After 3T3-L1 cells stably expressing FLAG-ASXL1 or FLAG-ASXL2 were induced to differentiate as described in Fig. 3*B*, cross-linked, sheared chromatin was prepared from the cells. The chromatin was subjected to IP using the antibodies indicated at *right*, and the precipitates were subjected to PCR analysis using primer pairs spanning the *aP2* gene promoter. *Input*, PCR products obtained from the chromatin samples before IP.
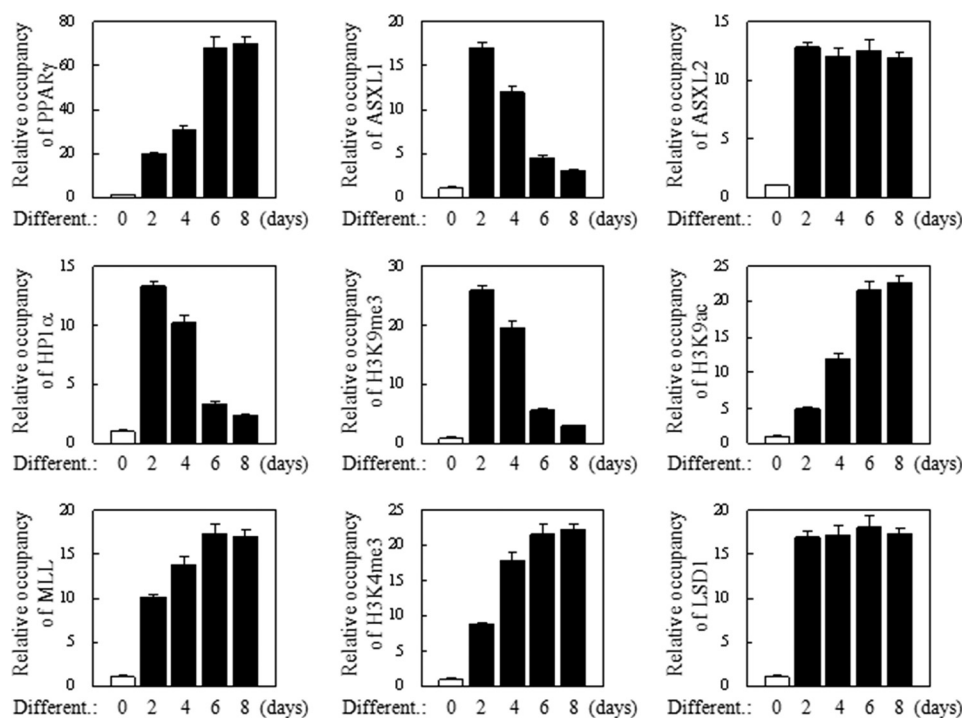

FIGURE 6. **Chromatin occupancy by modified histones during adipogenesis.** 3T3-L1 cells were cultured in a differentiation-inducing medium. Cell samples were removed every 2 days, and the DNA was cross-linked and sheared. The resulting chromatin samples were then immunoprecipitated with antibodies specific for PPARγ, ASXL1, ASXL2, HP1α, H3K9me3, H3K9ac, MLL1, H3K4me3, or LSD1 as indicated. The precipitates were subjected to PCR analysis using primer pairs spanning the *aP2* gene promoter. Relative occupancy is the fold-increase in the percentage of input over that of day 0 (set to 1). *Error bars* indicate S.D. ($n = 3$).
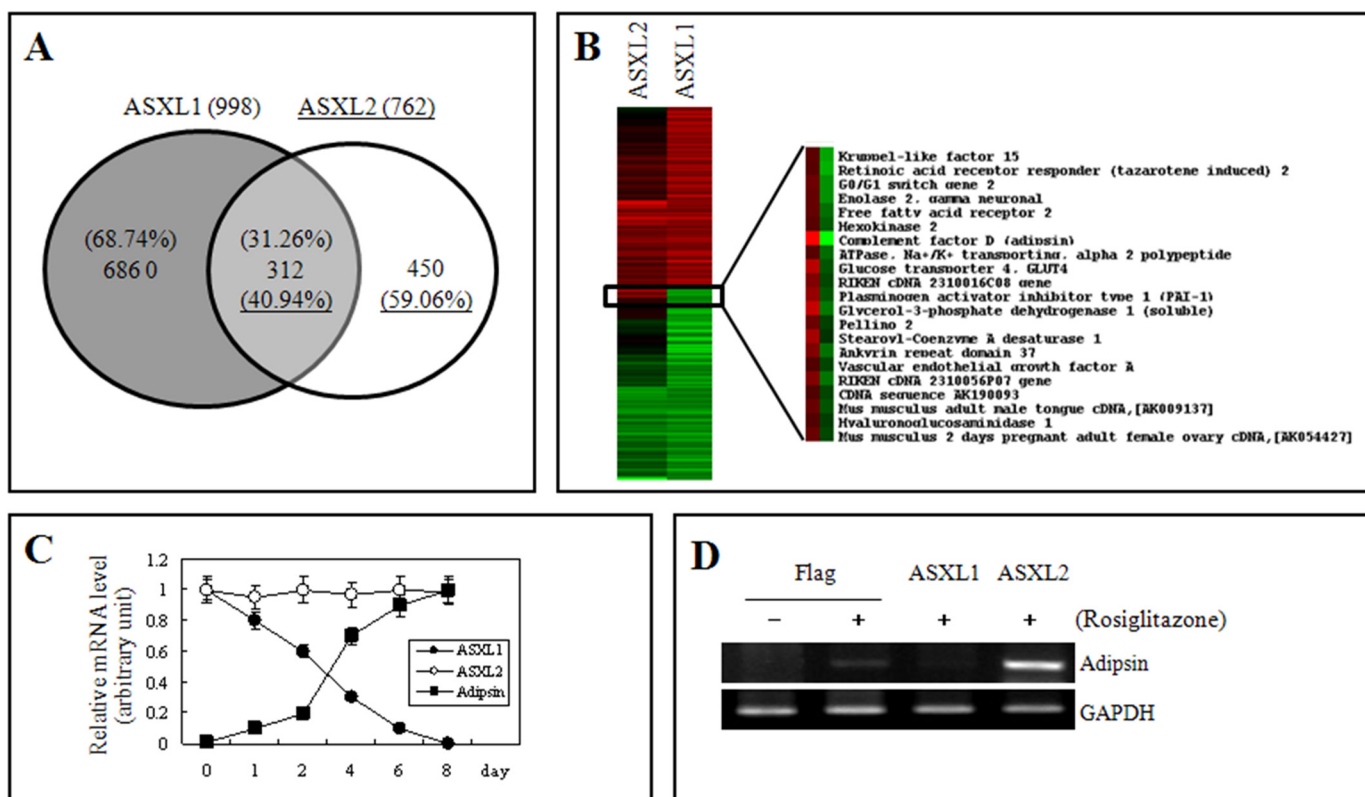
FIGURE 7. **Microarray analysis of genes regulated by ASXL1 and ASXL2.** 3T3-L1 cells stably expressing FLAG, FLAG-ASXL1, or FLAG-ASXL2 were induced to differentiate as described in Fig. 3*B*. Total RNA was extracted, labeled with Cy3 (FLAG and 3T3-L1/FLAG-ASXL1) or Cy5 (FLAG-ASXL2), and hybridized to an Agilent Mouse 44k 4plex microarray. *A,* number of genes regulated by ASXL1 and ASXL2. Genes changed >2-fold in expression are shown by numbers and percentages. *B,* cluster analysis of adipogenesis-related gene expression in differentiated 3T3-L1 cells stably expressing FLAG-ASXL1 (*green*, down-regulated) and FLAG-ASXL2 (*red;*, up-regulated). *C,* expression of *ASXL1*, *ASXL2*, and *adipsin* mRNA during adipogenesis was quantified by real time qPCR. Results shown are normalized to *GAPDH* mRNA levels. *D,* validation of *adipsin* mRNA expression. 3T3-L1 cells stably expressing FLAG, FLAG-ASXL1, or FLAG-ASXL2 were induced to differentiate in the presence of 1 μM rosiglitazone. Total RNA was extracted and subjected to RT-PCR using primer pairs spanning the *adipsin* coding sequence. *GAPDH* was used as an internal control.

other genes that exhibited at least 2-fold changes in expression levels by two ASXLs, including genes regulated by PPARγ, namely *adipsin* (29), *LPL* (30), hormone-sensitive lipase (31, 32), *GPDH* (33), *SCD1* (34), *perilipin* (35, 36), *GLUT4* (37, 38), *PAI-1* (39), and *G0S2* (Table 3) (40).

The *adipsin* gene was selected for further analysis by RT-PCR and densitometry. As shown in Fig. 7*C*, *ASXL1* and *adipsin* mRNA levels varied inversely during adipocyte differentiation. As expected, no change in *ASXL2* mRNA expression was observed. Moreover, after differentiation induction in the presence of rosiglitazone, *adipsin* expression was highly elevated in FLAG-ASXL2-overexpressing 3T3-L1 cells, whereas it was decreased in FLAG-ASXL1-overexpressing 3T3-L1 cells (Fig. 7*D*). These microarray data suggest that ASXL1 is an authentic PPARγ corepressor, whereas ASXL2 is a PPARγ coactivator, and that both fine-tune adipogenesis via differential regulation of PPARγ *in vivo*.

DISCUSSION

Although a variety of PPAR coregulators have been analyzed for their roles in PPAR signaling, the mechanistic underpinnings of the complexities of PPAR function at the chromatin and physiological levels are not yet fully understood. In this study, we characterized two mammalian homologs of the *Drosophila* Asx protein, ASXL1 and ASXL2, as novel PPARγ coregulators.

TABLE 3

List of genes oppositely regulated by ASXL1 and ASXL2

Genes	ASXL1	ASXL2	PPARγ target
Adipokine			
adipsin	0.083	10.69	O
Transcription factors			
PPARγ	0.285	0.866	
PGC1β	0.434	1.321	
Kruppel-like factor 15 (*KLF15*)	0.241	2.213	O
Lipid metabolic enzymes			
Hydoxysteroid 11-β dehydrogenase 1	0.275	1.727	
Phosphoglycerate kinase 1 (*PGK1*)	0.453	1.287	
Lipoprotein lipase (*LPL*)	0.455	1.853	O
Hormone-sensitive lipase (*HSL*)	0.287	1.916	O
Glycerol-3-phosphate dehydrogenase 1 (*GPDH*)	0.352	5.454	O
Stearoyl-coenzyme A desaturase 1 (*SCD1*)	0.644	4.155	O
Cytochrome *c* oxidase, subunit VIIIb	0.522	5.562	
Fatty acid-binding proteins			
Free fatty acid receptor 2 (*GPR43*)	0.384	2.38	
Perilipin (lipid droplet-associated protein)	0.156	1.764	O
Glucose homeostasis			
Glucose transporter 4 (*GLUT4*)	0.480	4.491	O
Hexokinase 2 (*HK2*)	0.441	2.163	
Others			
Plasminogen activator inhibitor 1 (*PAI-1*)	0.472	3.649	O
G0/G1 switch gene 2 (*G0S2*)	0.287	2.558	O
Apolipoprotein C-I	0.713	2.405	
Ankyrin repeat domain 37 (*LRP2BP*)	0.376	3.033	
Retinoic acid receptor responder 2	0.218	2.140	

Previously, we showed that ASXL1 can enhance the transcriptional activity of retinoic acid receptors by associating with SRC-1 and can repress their activity by recruiting LSD1

and HP1 to target genes in a cell type-specific manner (27, 28). Genetic studies in *Drosophila* have indicated that Asx is a member of the "enhancer of the trithorax and polycomb" gene family, sometimes up-regulating and sometimes down-regulating transcription (41). In contrast to *Drosophila*, in which a single Asx isoform plays a dual function in transcription, mammals have three ASXL isoforms (ASXL1, ASXL2, and ASXL3) (42–44).

Our present data show that ASXL1 acts as a corepressor in PPAR-mediated transcription, whereas ASXL2 acts as a coactivator. Furthermore, ASXL2 is a coactivator of retinoic acid and estrogen receptors (data not shown), suggesting that it is a general coactivator of nuclear hormone receptors. However, mammalian ASXL1 appears to have multiple regulatory functions that are specific to the cell context and the type of transcription factor; thus, it functions as an enhancer of the trithorax and polycomb, like Asx. Overall, our observations suggest a complex transcriptional regulatory scheme carried out by various mammalian ASXL homologs, in contrast to the simple and efficient regulation carried out by the single *Drosophila* Asx protein. The evolution of a mechanism for transcriptional regulation in which two isoforms of the same family have reciprocal functions might be a rare event.

Based on their roles in position-effect variegation and the regulation of retinoic acid receptors through interactions with histone-modifying enzymes, *Drosophila* Asx and mammalian ASXLs appear to be chromatin-associated proteins (27, 28, 45). In this study, we found that the overexpression of ASXL1 in 3T3-L1 preadipocytes induced to differentiate in the presence of rosiglitazone led to the increased HP1α recruitment and H3K9me3 modification and the decreased MLL1 recruitment, H3K9ac and H3K4me3 modifications to the PPARγ-responsive *aP2* gene promoter, whereas the overexpression of ASXL2 had the opposite effect. These findings were confirmed by quantitative ChIP analysis of 3T3-L1 cells during adipogenesis.

Our previous studies indicated that HP1 binding to ASXL1 is critical for retinoic acid receptor repression (28), suggesting that binding of ASXL1 to HP1 interferes with PPARγ activity by increasing the level of methylated H3K9, which acts as an HP1-binding site on PPARγ target promoters, resulting in decreased expression of adipogenic genes. On the other hand, ASXL2, which does not bind HP1, promotes differentiation by binding to PPARγ and increasing the level of methylated H3K4, leading to the elevation of PPARγ activity. Various histone modifications on PPARγ-targeted adipogenic genes have been reported in both preadipocytes and adipocytes (recently reviewed in Ref. 46). These modifications occur either through the direct association of PPARγ with histone-modifying enzymes or through the indirect association of PPARγ with these enzymes via coregulators, resulting in differential PPARγ regulation and adipogenesis. Positive PPARγ cofactors include the histone acetyltransferases SRC-1 and CBP/p300, coactivator-associated arginine methyltransferase 1 (CARM1), Pax transactivation domain-interacting protein (PTIP), the histone H3K4 methyltransferases MLL3 and MLL4, and the H3K9 demethylase JMJD1A (47–51), whereas the negative cofactors include nuclear receptor corepressor/

silencing mediator for retinoid and thyroid hormone receptors, cyclin D1, histone deacetylases, the histone H3K9 methyltransferases Suv39H1 and SETDB1, and retinoblastoma protein (13, 20–22, 52). The histone modifications mediated by various ASXL-associated cofactors appear to provide diversity in the regulation of PPARγ at the chromatin level during adipocyte differentiation.

How is differential PPARγ regulation by ASXL1 and ASXL2 physiologically relevant in the regulation of adipogenesis? Our Western blot analysis showed that ASXL1 expression decreases gradually, whereas ASXL2 expression remains constant, during adipogenesis of 3T3-L1 cells. Knockdown of ASXL1 increased the adipogenic potential of the cells, whereas ASXL2 knockdown abolished it. When overexpressed, ASXL1 and ASXL2 had opposite effects, consistent with the knockdown findings. Therefore, we speculate that, at the early differentiation stage, ASXL1 binds to PPARγ and blocks its transcriptional activity; then, after adipogenesis has progressed, ASXL1 levels diminish, leaving PPARγ free to interact with ASXL2 and activate transcription. These reciprocal roles of ASXL1 and ASXL2 in PPARγ regulation are reminiscent of the reciprocal roles of cyclin D1 *versus* cyclin D3 and of retinoblastoma protein-associated HDAC3 *versus* CBP/p300 (20, 52, 53).

Our genome-wide analysis confirmed the physiological roles of ASXL1 and ASXL2 in adipogenesis at the molecular level, supporting the hypothesis that ASXL1 is an authentic corepressor of PPARγ, whereas ASXL2 is a PPARγ coactivator, and that together ASXL1 and ASXL2 fine-tune adipogenesis via differential regulation of PPARγ. Interestingly, as exhibited by cluster analysis of microarray data, most genes except adipogenesis-associated genes were similarly regulated by ASXL1 and ASXL2. Although the roles of these genes remained to be investigated, the specific regulation of a subset of adipogenic genes may guarantee the physiological roles of ASXLs in adipogenesis.

To investigate the role of ASXL1 and ASXL2 in adipogenesis *in vivo*, we examined the expression of ASXLs in the subcutaneous adipose tissue of mice fed normal or high fat diets. The high fat diet was associated with down-regulation of ASXL1 without affecting ASXL2 expression (supplemental Fig. 1). Consistent with our other findings shown in 3T3-L1 cells, these results suggest that ASXL1 suppresses adipogenesis in the mouse adipose tissue and that ASXL2 enhances it. To further examine the differential roles of ASXL1 and ASXL2 in adipogenesis, we are currently generating transgenic mice expressing exogenous ASXL1 or ASXL2 in the adipose tissue. Together with our current findings, our future studies using these mice will provide data to ensure the discovery of new targets for the treatment of obesity and associated metabolic diseases.

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