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Reciprocal regulation of LXRα activity by ASXL1 and ASXL2 in lipogenesis

Ui-Hyun Park [a], Mi-ran Seong [a], Eun-Joo Kim [b], Wonhee Hur [b], Sung Woo Kim [b], Seung Kew Yoon [c], Soo-Jong Um [a,*]

[a] Department of Bioscience & Biotechnology, Institute of Bioscience, BK21 Graduate Program, Sejong University, Seoul 143-747, South Korea
[b] Department of Molecular Biology, BK21 Graduate Program, Dankook University, Gyeonggi-do 448-701, South Korea
[c] The Catholic University Liver Research Center & WHO Collaborating Center of Viral Hepatitis, The Catholic University, College of Medicine, Seoul 137-701, South Korea

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ABSTRACT

Liver X receptor alpha (LXRα), a member of the nuclear receptor superfamily, plays a pivotal role in hepatic cholesterol and lipid metabolism, regulating the expression of genes associated with hepatic lipogenesis. The additional sex comb-like (ASXL) family was postulated to regulate chromatin function. Here, we investigate the roles of ASXL1 and ASXL2 in regulating LXRα activity. We found that ASXL1 suppressed ligand-induced LXRα transcriptional activity, whereas ASXL2 increased LXRα activity through direct interaction in the presence of the ligand. Chromatin immunoprecipitation (ChIP) assays showed ligand-dependent recruitment of ASXLs to ABCA1 promoters, like LXRα. Knockdown studies indicated that ASXL1 inhibits, while ASXL2 increases, lipid accumulation in H4IIE cells, similar to their roles in transcriptional regulation. We also found that ASXL1 expression increases under fasting conditions, and decreases in insulin-treated H4IIE cells and the livers of high-fat diet-fed mice. Overall, these results support the reciprocal role of the ASXL family in lipid homeostasis through the opposite regulation of LXRα.

1. Introduction

Liver X receptors (LXRs) are ligand-dependent transcription factors that belong to the nuclear receptor (NR) superfamily [1]. LXRα (NR1H3) is expressed in tissues, such as the liver, spleen, kidney, intestine, and adipose tissue, while LXRβ (NR1H2) is ubiquitously expressed [2,3]. Extensive studies have established that LXRα is a major factor maintaining homeostasis of lipid and cholesterol metabolism by regulating target gene expression [3–5]. LXRα forms a heterodimer with retinoid X receptors (RXRs) and binds to LXR response elements (LXREs) in the promoter of target genes, including sterol regulatory element-binding protein-1c (SERBP-1c), fatty acid synthase (FAS), steroyl CoA desaturase 1 (SCD1), ATP-binding cassette trans porter A1 (ABCA1), and ABCG5 [6–9]. The transcriptional activity of LXRα can be stimulated by natural oxysterol and synthetic ligands, including T0901317 and GW3965 [10], through the concerted dissociation of corepressors (NCoRs) and association of coactivators such as SRC1, ASC2, and PGC-1 [3]. In addition to these classic coregulators, the presence of corepressors of agonist-bound nuclear receptors, including RIP140, LCoR, PRAME, REA, MTA1, NSD1, and COPR1, was recently reported [11]. However, the roles of these corepressors in LXR regulation largely remain unexplored.

Additional sex comb (Asx) was originally studied in *Drosophila* as Enhancer of Trithorax (TrxG) and Polycomb (PcG) group protein [12]. Three paralogs of Asx-like (ASXL) were found in mammals, encoding ASXL1, ASXL2, and ASXL3 [13–15]. Recently, we reported that ASXL1 acts as a dual-function regulator of the retinoic acid (RA) receptor, cooperating with SRC1 for activation or LSD1 and HP1 for repression in the presence of RA [16,17]. Mutations of ASXL1 are often found in humans, mostly linked to chronic myelomonocytic leukemia, myelodysplastic syndromes, and Bohring-Opitz syndrome [18–20]. A recent study using leukemia cells obtained from human patients with ASXL1 mutations showed that ASXL1 is associated with polycomb repressive complex 2, increasing histone H3K27 methylation [21]. Our recent study demonstrated that ASXL1 interacts with ligand-bound PPARγ and inhibits adipogenesis by suppressing PPARγ activity through the reduction of active histone and enrichment of repressive histone in the PPARγ target promoter. In contrast, ASXL2 plays an opposite role in PPARγ-associated adipogenesis [22].

In general, obesity is associated with high adipogenesis in adipose tissues and lipogenesis in the liver. In this regard, our previous findings [22] prompt us to investigate the roles of ASXL1 and

Abbreviations: ASXL, additional sex comb-like; LXRα, liver X receptor alpha; WB, Western blotting; Luc, luciferase; shRNA, small hairpin RNA; KD, knockdown; ChIP, chromatin immunoprecitation; HFD, high fat diet.

* Corresponding author. Fax: +822 3408 4334.
E-mail address: umsj@sejong.ac.kr (S.-J. Um).

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ASXL2 in the regulation of LXRα activity and lipogenesis in liver cells. Here, we found that both ASXLs interact with ligand-bound LXRα, but oppositely regulate the transcriptional activity of LXRα at the target promoter. Consistently, ASXL1 depletion in H4IIE liver cells promoted palmitate-induced accumulation of lipids, while lipid deposition was impaired in ASXL2- and LXRα-depleted cells. Together with low ASXL1 expression in the livers of high-fat diet (HFD)-fed mice, our data suggest that ASXL1 and ASXL2 oppositely regulate LXRα activity to maintain lipid homeostasis in the liver.

2. Materials and methods

2.1. Cell culture

Rat hepatoma H4IIE cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and an antibiotic–antimycotic (Gibco, Grand Island, NY) in a 5% CO_2 atmosphere at 37 °C. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and an antibiotic–antimycotic (Gibco) in a 5% CO_2 atmosphere at 37 °C. For treatment with the LXRα ligand, FBS was pretreated with charcoal.

2.2. Plasmid cloning

All cDNA was constructed according to standard methods and verified by sequencing. DNA sequences encoding murine ASXL1, LXRα, and human ASXL2 were inserted into the modified pcDNA3 vector (Invitrogen, Carlsbad, CA), which harbors the 2 × Flag epitope tag in front of the encoded genes. For the GST-fusion protein, DNA sequences encoding amino acids (aa) 961–1514 of murine ASXL1 or aa 916–1435 of human ASXL2 were inserted into the pGEX4T-1 vector (GE Healthcare, Piscataway, NJ). For His-fusion proteins, murine LXRα full-length cDNA was inserted into the pET15b vector (Novagen, Madison, WI).

2.3. GST pull-down assays

For the GST pull-down assay, GST-fused ASXL1 (aa 961–1514) and ASXL2 (aa 916–1435) were expressed in *Escherichia coli* and purified on glutathione-Sepharose beads (Novagen) by standard methods [23]. GST, GST-ASXL1, or GST-ASXL2 (each 0.2 μg) was mixed with 0.2 μg of His-LXRα and treated with 10 μM of synthetic LXRα ligand T0901317 for 30 min at 30 °C. Pre-equilibrated glutathione-Sepharose beads were added and further incubation was allowed for 1 h. Bound proteins were eluted with 40 μl of 2 × SDS loading buffer by boiling for 10 min and visualized by Western blotting (WB) using an anti-His antibody (Abcam, Cambridge, UK).

2.4. Western blotting (WB) and immunoprecipitation (IP) assays

WB and IP were performed as reported previously [23]. Briefly, H4IIE cells were transfected with the indicated plasmid DNA using the Lipofectamine plus reagent (Invitrogen) overnight and treated with 10 μM of T0901317. For WB, lysates were separated by SDS-PAGE on 6–8% gels, transferred to PVDFs membrane, and blotted with the indicated antibodies. For IP, lysates were pre-cleared by incubating with protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min at 4 °C. Pre-cleared lysates were then mixed with normal IgG, anti-ASXL1 [16], or ASXL2 (Bethyl Laboratories, Montgomery, TX) antibodies. After incubation with protein A/G beads for 4 h at 4 °C, the immune complexes were released from the beads by boiling in sample buffer for 5 min and analyzed by WB using anti-LXRα antibody (Abcam).

2.5. Luciferase reporter gene assays

HEK293 cells were seeded on 12-well plates and transfected with LXRα, LXRE-tk-luciferase reporter, increasing amounts of ASXL1 or ASXL2, and SV40-driven β-galactosidase expression vectors using Lipofectamine (Invitrogen). After transfection for 4 h, cells were fed with DMEM containing 5% charcoal-stripped FBS and incubated overnight in the presence of T0901317 (10 μM). Luciferase activity was measured as described previously [24].

2.6. RNA interference (RNAi)

For depletion of ASXL1, ASXL2, and LXRα using small hairpin RNA (shRNA), the synthetic oligonucleotides were annealed, digested with *Hin*dIII and *Bam*HI, and ligated into the digested pSilencer 2.1-U6 hygro (Ambion, Austin, TX). The H4IIE-derived knockdown stable cell lines were generated by transfecting shRNA expression vectors and selecting resistant colonies against hygromycin (AG Scientific, San Diego, CA) at 0.1 mg/ml. The knockdown efficiency was monitored by WB analysis using anti-ASXL1, anti-ASXL2, and anti-LXRα antibodies. pSilencer hygro luciferase was used as a control (shLuc).

2.7. RNA extraction and reverse-transcription (RT) quantitative-PCR (RT-qPCR)

Total RNA was extracted from H4IIE cells stably depleted with ASXL1, ASXL2, or LXRα using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized with 1 μg of total RNA using MMLV reverse transcriptase and random primers (Invitrogen). Quantitative real-time PCR (qPCR) reactions were performed using the iQ™ SYBR Green Supermix and Icycler CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). All expression levels were normalized using GAPDH as an internal standard in each well. Fold expression was defined as the fold increase relative to the control.

2.8. Chromatin immunoprecipitation (ChIP) assays

ChIP assay was performed as described previously [16]. H4IIE cells were adapted in 5% charcoal stripped-FBS plus MEM media for 24 h and treated with 10 μM of T0901317 for 30 min. Cross-linked, sheared chromatin complexes were recovered by IP with anti-LXRα, ASXL1, and ASXL2 antibodies. Cross-linking was then reversed according to the protocol from Millipore (Billerica, MA). The DNA pellets were recovered and analyzed by qPCR using a primer pair that encompasses the LXRE of the ABCA1 promoter region [5′-CCCAACTCCCTAGATGTGTC-3′ (forward) and 5′-CCACTCACTCTC GCTCGCA-3′ (reverse)]. Ratios of fold enrichment from each antibody were calculated from Ct values normalized against the Ct of IgG using qPCR. Percentages of input were calculated compared to the input sample used in the qPCR reaction.

2.9. Nile Red staining

H4IIE cells stably depleted with ASXL1, ASXL2, LXRα, or luciferase control (shLuc) were treated with 0.1 mM of sodium palmitate (Sigma–Aldrich, St. Louis, MO) for 24 h. Cells were washed in phosphate-buffered saline (PBS), fixed with 2 ml of 4% formaldehyde in PBS for 5 min, stained with 1 μg/ml Nile Red (Sigma–Aldrich) for 5 min with gentle agitation, and photographed under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) at a wavelength of 488 nm for excitation and greater than 550 nm for emission.

2.10. Immunohistochemical staining

Paraffin sections were used for immunohistochemistry of ASXL1 and ASXL2. The sections were performed using an EnVision Plus system according to the manufacturer's instructions (Dako, Carpinteria, CA). Samples with >5% positive staining in a given area for a particular antibody were considered to be positive. The intensity of staining was graded semiquantitatively as negative, weak, moderate, or strong positivity. Each slide was read independently by two pathologists.

3. Results and discussion

3.1. LXRα interacts with ASXL1 and ASXL2 in a ligand-dependent manner

To investigate the role of the ASXL family in lipogenesis, we first addressed whether ASXL1 or ASXL2 interacts with LXRα, a critical transcription factor involved in lipogenesis. As depicted in Fig. 1A, both ASXL1 and ASXL2 contain the conserved nuclear receptor box (NR box), which is responsible for the interaction with the ligand-binding domain of NRs in the presence of ligands [16,17,22]. The physical interaction between LXRα and ASXLs was demonstrated by GST pull-down assays *in vitro* and IP assays *in vivo*. For GST pull-down assays, purified His-tagged LXRα and GST, and GST-fused ASXL1 or ASXL2 were mixed in the absence and presence of the synthetic LXRα ligand, T0901317. Subsequent WB analysis using an anti-His antibody revealed that LXRα directly interacts with ASXL1 (Fig. 1B, top) and ASXL2 (Fig. 1B, bottom) in the presence of the ligand. For IP assays, rat hepatoma H4IIE cells were cultured in the absence and presence of T0901317. The endogenous expression levels of LXRα, ASXL1, and ASXL2 were monitored by WB using each antibody. Immunocomplexes were pulled down with anti-ASXL1 or ASXL2 antibody and analyzed by WB using anti-LXRα antibody. As shown in Fig. 1C, we observed the endogenous interaction of LXRα with ASXL1 and ASXL2 in a ligand-dependent manner, whereas LXRα was undetectable when an IgG control antibody was used for the IP. These data suggest that LXRα interacts with ASXL1 and ASXL2 *in vitro* and *in vivo* with ligand dependency.

3.2. Transcriptional activity of LXRα is oppositely regulated by ASXL1 and ASXL2

To determine the implication of the physical interactions between LXRα and ASXLs, we first performed luciferase reporter gene assays. HEK293 cells were transiently transfected with vectors expressing Flag-mLXRα, RXRα, and increasing amounts of Flag-mASXL1 or Flag-hASXL2 in the presence of the ligand. Upon overexpression, ASXL1 repressed the ligand-induced transcriptional activity of LXRα in a dose-dependent manner, whereas ASXL2 strongly increased LXRα (Fig. 2A). To confirm the opposite effects of ASXL1 and ASXL2 on LXRα activity, we examined the mRNA expression of the LXRα-responsive genes, SREBP-1c and FAS, upon ligand stimulation under stable knockdown conditions, shown by WB, of ASXL1 and ASXL2 in H4IIE cells (Fig. 2B). As determined by RT-qPCR (Fig. 2C), the expression of both LXRα target genes was enhanced upon depletion of ASXL1 and reduced upon depletion of ASXL2 and LXRα compared to the control (shLuc), supporting the reciprocal roles of ASXL1 and ASXL2 in LXRα activation.

3.3. ASXL1 and ASXL2 are enriched on the LXRα target promoter ligand-dependently

To substantiate the role of ASXL1 and ASXL2 in LXRα activation and to determine whether the ASXLs are recruited to the LXRα target gene promoter, ChIP assays were performed using the *ABCA1* gene in H4IIE cells treated with ligand T09012317 (Fig. 3A). As shown in Fig. 3B, ligand treatment increased the occupancies of ASXL1 and ASXL2. Further ChIP–qPCR data supported the

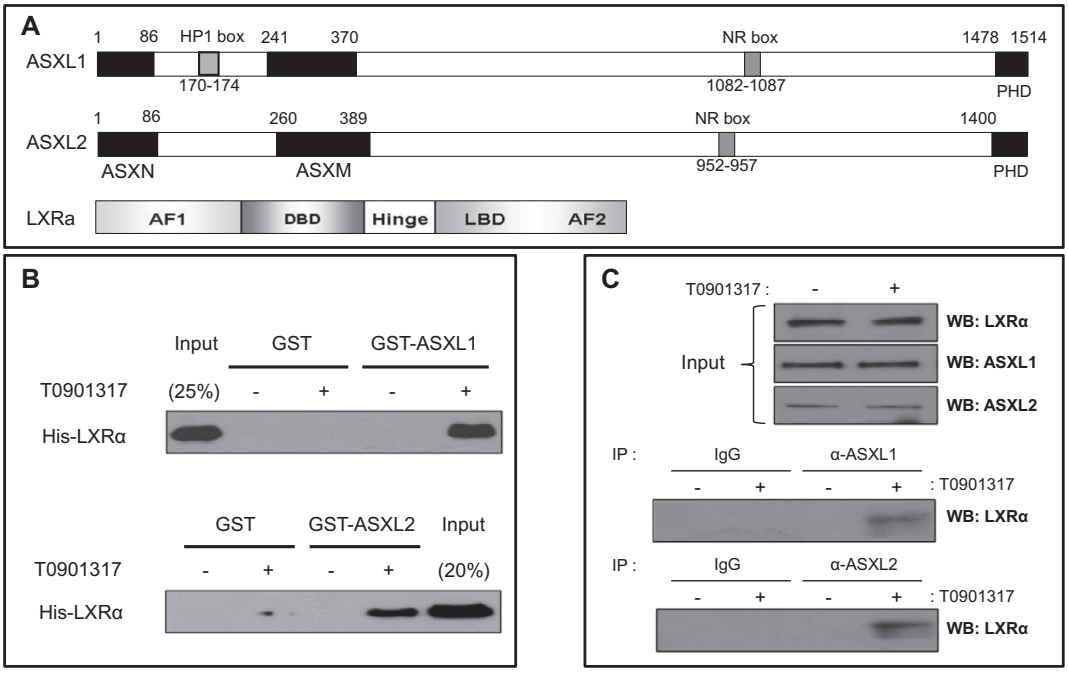

Fig. 1. Ligand-dependent interactions of ASXL1 and ASXL2 with LXRα. (A) Schematic representation of mouse ASXL1 (mASXL1), human ASXL2 (hASXL2), and mouse LXRα (mLXRα). Conserved ASXN, ASXM, and PHD domains are shown in black. The NR box and HP1 box are presented in gray boxes. (B) GST pull-down assays. Purified GST-fused ASXL1 and ASXL2 proteins were incubated with His-mLXRα proteins in the presence of 10 μM of T0901317, a synthetic ligand of LXRα. Bound proteins were visualized by an anti-His antibody. (C) Immunoprecipitation (IP) assays. H4IIE cells were treated with T0901317. Lysates were pulled down by the IgG control, anti-ASXL1 or anti-ASXL2 antibody. Immune complexes were eluted and visualized by Western blotting (WB) using an anti-LXRα antibody.

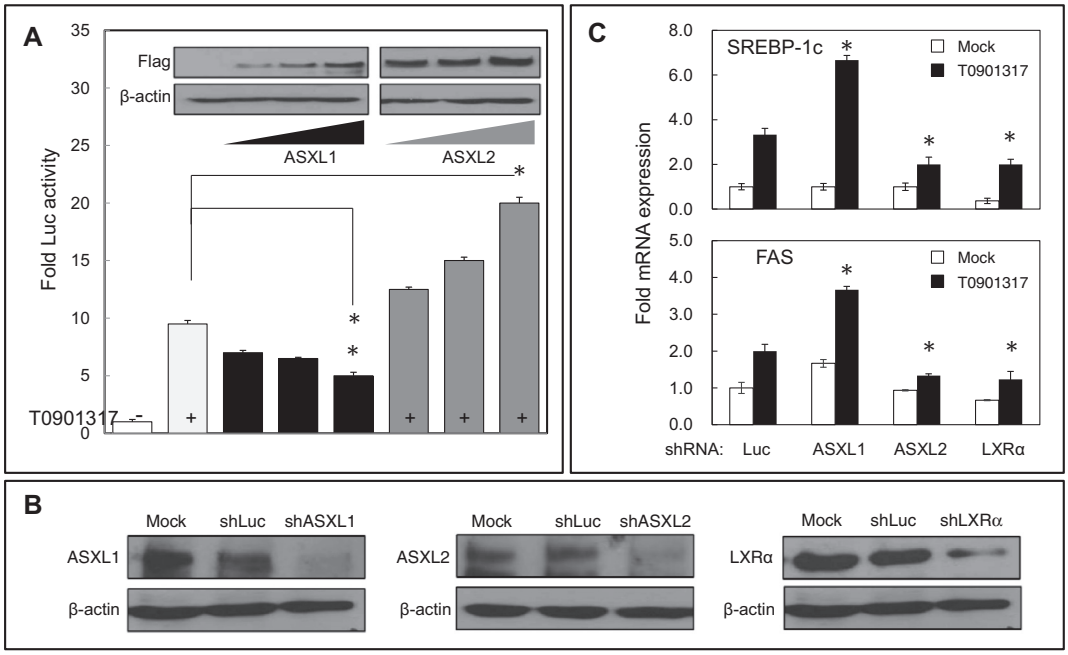

Fig. 2. Opposite regulation of LXRα activity by ASXL1 and ASXL2. (A) Luciferase reporter gene assays. HEK293 cells were co-transfected with Flag-mLXRα, pSG5-mRXRα, LXRE-tk-luciferase, and increasing amounts of Flag-mASXL1 or Flag-hASXL2 (0.1, 0.2, and 0.5 μg) in the presence of 10 μM of T0901317. Error bars represent the mean ± S.D. ($n = 3$, $^*p < 0.01$). (B) Generation of H4IIE cells stably depleted of ASXL1, ASXL2, or LXRα. The protein expression was monitored by Western blotting (WB) using the indicated antibodies. Mock and shluciferase (shLuc) were included as controls. (C) Effect of ASXL1 and ASXL2 knockdown on the expression of LXRα-responsive genes. Stably depleted H4IIE cells were treated with T0901317 for 24 h and subjected to RT-qPCR using primers specific for two LXRα target genes, *SREBP-1c* and *FAS*. Error bars represent the mean ± S.D. ($n = 3$, $^*p < 0.01$).

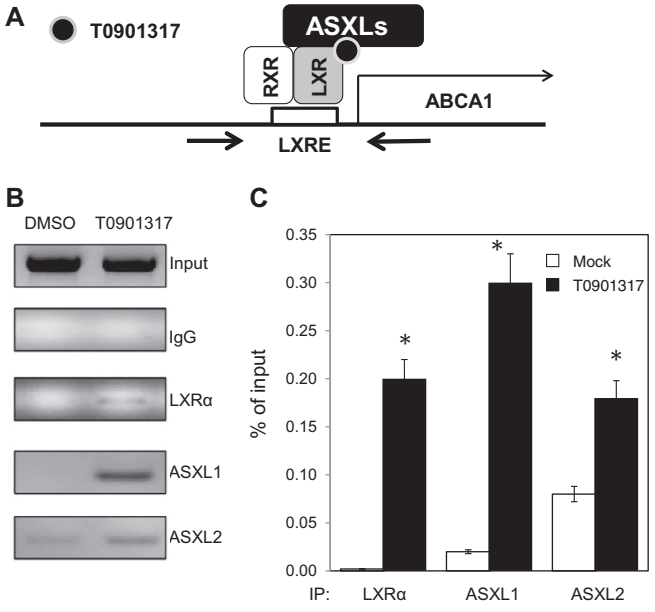

Fig. 3. Ligand-dependent recruitments of ASXL1 and ASXL2 to the LXRα target promoter. (A) Schematic diagram of the *ABCA1* promoter used for ChIP assays. Locations of LXRE and the target primer set are shown by arrows. (B and C) ChIP assays by semiquantitative PCR (B) and by quantitative PCR (C). Sonicated DNA from H4IIE cells treated with T0901317 were precipitated with the indicated antibodies and subjected to different PCRs using primer sets specific for the LXRE of the *ABCA1* promoter. Error bars represent the mean ± S.D. ($n = 3$, $^*p < 0.01$).

ligand-dependent recruitments of LXRα, ASXL1, and ASXL2 to the *ABCA1* promoter (Fig. 3C). Overall, these data suggest that ASXL1 and ASXL2 are recruited to the LXRα-responsive promoter where they oppositely regulate LXRα activity via interaction with LXRα in response to the ligand.

3.4. ASXL1 and ASXL2 oppositely regulate lipogenesis in liver cells

To assess whether the opposite regulation of LXRα by ASXLs is functionally linked to their roles in lipogenesis, H4IIE cells constitutively expressing shASXL1, shASXL2, and shLXRα were generated by stable transfections. Before assessing the knockdown effects, we determined the optimal condition of lipid deposition in H4IIE cells. Upon increasing free fatty acids (such as sodium palmitate) to 0.1 mM, lipid accumulation became evident as shown by Nile Red staining (Fig. 4A). We then monitored the knockdown effects of ASXLs on lipid accumulation by treating cells with 0.1 mM palmitate. Nile Red staining, followed by WB analysis (Fig. 2B), revealed that ASXL1 knockdown increased intracellular lipid accumulation, whereas ASXL2 knockdown significantly blocked it (Fig. 4B). As expected, the depletion of LXRα, a key mediator of lipogenesis in the liver, resulted in great reduction of lipid deposition. In addition, the expression of Asxl1 and Asxl2 under fasting and feeding conditions was measured by RT-qPCR. As shown in Fig. 4C, Asxl1 is up-regulated upon treating H4IIE cells with Fsk and Dex like fasting marker genes, Pepck and G6Pase (fasting: left panel), while Asxl2 is up-regulated in response to insulin like Srebp-1c (feeding: right panel), suggesting the opposite role of Asxl1 and Asxl2 in fasting and feeding. To further investigate the role of Asxl1 and Asxl2 in lipogenesis *in vivo*, we examined the expression of Asxls in the livers of mice fed HFDs for 3, 6, and 9 months. Although the expression of both Asxls gradually increased during HFDs, the level of increase was much higher for Asxl2 than Asxl1 at 6 months (Fig. 4D). Collectively, these data suggest that lipogenesis is differentially regulated by Asxl1 and Asxl2 *in vivo*, presumably because the two proteins oppositely modulate LXRα activity.

In summary, we found that ASXL1 and ASXL2 interact with ligand-bound LXRα and are recruited to the LXRα-responsive promoter where they oppositely regulate LXRα activity in response to the ligand. The biological relevance of the differential regulation

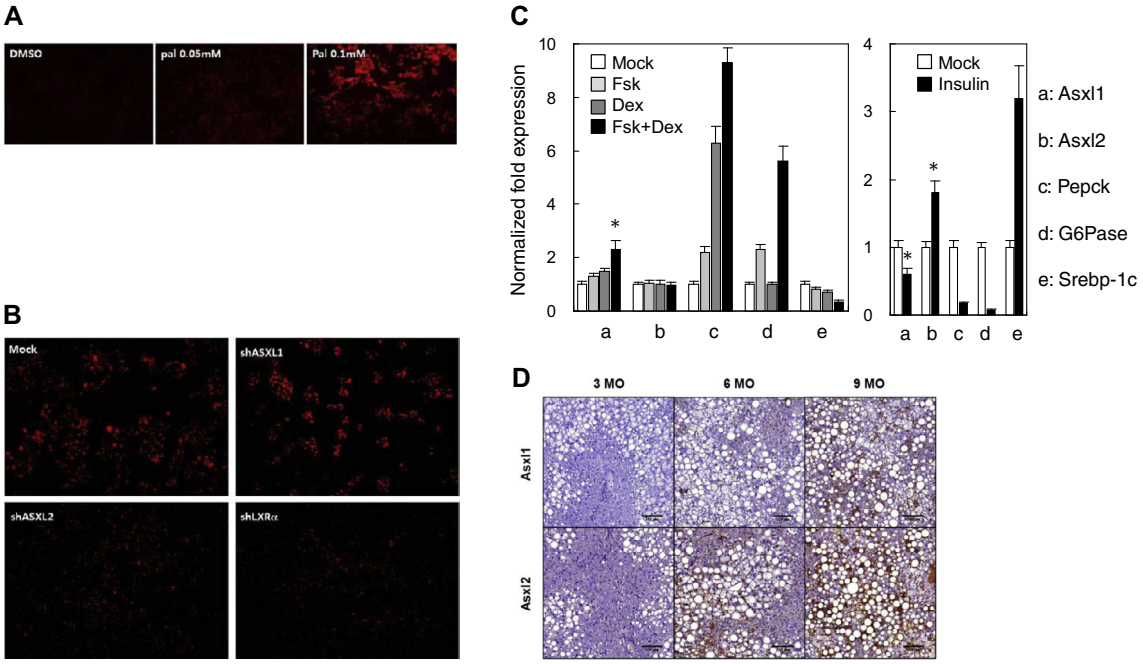

Fig. 4. Opposite roles of ASXL1 and ASXL2 in lipogenesis. (A) Palmitate-induced lipid accumulation in H4IIE cells. H4IIE cells were treated with 0.05 and 0.1 mM sodium palmitate for 24 h. Intracellular lipid accumulation was stained with Nile Red and photographed. (B) Effect of ASXL1 and ASXL2 depletion on palmitate-induced lipogenesis. H4IIE cells stably depleted of ASXL1, ASXL2, and LXRα were treated with 0.1 mM palmitate for 24 h. Mock controls were used. (C) Expression of ASXL1 and ASXL2 under fasting and feeding conditions. For fasting, H4IIE cells were treated with forskolin (Fsk: 10 μM), dexamethasone (Dex: 1 μM), or Fsk and Dex for 2 h. Insulin (100 nM for 24 h) was treated for feeding. Pepck, Phosphoenolpyruvate carboxykinase; G6Pase, Glucose 6-phosphatase; Srebp-1c, Sterol regulatory element-binding protein-1c. Error bars represent the mean ± S.D. ($n = 3$, $^{*}p < 0.01$). (D) Expression of ASXL1 and ASXL2 in high-fat diet (HFD)-fed mice livers. Immunohistochemistry of liver sections from chow- and HFD-fed mice over 9 months was performed by probing with anti-ASXL1 and ASXL2 antibodies and counterstaining with hematoxylin. Original magnification ×200.

of LXRα by ASXLs was determined by Nile Red staining in rat hepatoma H4IIE cells and explored by measuring the expression of ASXLs in the liver of HFD-fed mice. From these data, we speculate that ASXL1 and ASXL2 oppositely regulate LXRα activity to maintain lipid homeostasis in the liver in response to various diet conditions. To further examine the repressive role of ASXL1 in lipid synthesis in the liver, we are currently generating liver-specific conditional ASXL1 knockout mice. These mice will be used in the discovery of new targets for the treatment of liver-associated metabolic diseases.

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