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Histone modifications regulate the developmental expression of human hepatic UGT1A1

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Abbreviations: EZH2, enhancer of zeste homolog 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H3K4me2, histone-3-lysine-4 dimethylation; H3K27me3, histone-3-lysine-27 trimethylation; HNF1A, hepatocyte nuclear factor 1 alpha; HNF4A, hepatocyte nuclear factor 4 alpha; HNF1AE, HNF1A response element; MLL1, mixed lineage leukemia 1; NCOA6, nuclear receptor coactivator 6; PXR, pregnane X receptor; UGT1A1, UDP-glucuronosyltransferase 1A1.

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Abstract

Human UDP-glucuronosyltransferase 1A1 (UGT1A1) is a unique enzyme involved in bilirubin conjugation. We previously characterized the hepatic expression of transcription factors affecting UGT1A1 expression during development. Accordingly, in this study, we characterized the ontogenetic expression of hepatic UGT1A1 from the perspective of epigenetic regulation. We observed significant histone-3-lysine-4 dimethylation (H3K4me₂) enrichment in the adult liver and histone-3-lysine-27 trimethylation (H3K27me₃) enrichment in the fetal liver, indicating that dynamic alterations of histone methylation were associated with ontogenetic UGT1A1 expression. We further showed that the transcription factor hepatocyte nuclear factor 1 alpha (HNF1A) affects histone modifications around the *UGT1A1* locus. In particular, we demonstrated that by recruiting HNF1A, the cofactors mixed-lineage leukemia 1, the transcriptional co-activator p300, and nuclear receptor coactivator 6 aggregate at the *UGT1A1* promoter, thereby regulating histone modifications and subsequent UGT1A1 expression. In this study, we proposed new ideas for the developmental regulation of metabolic enzymes via histone modifications, and our findings will potentially contribute to the development of age-specific therapies.

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Introduction

Human UDP-glucuronosyltransferase 1A1 (UGT1A1) is a unique enzyme involved in bilirubin conjugation, which prevents hyperbilirubinemia and jaundice (Bosma et al., 1994; Kanou et al., 2004). Previous reports showed that hepatic UGT1A1-expression levels exhibit a dynamic pattern during development and maturation. Briefly, UGT1A1 is highly expressed in adult livers, but is present at very low levels, or even absent, in fetal liver (Kawade and Onishi, 1981; Coughtrie et al., 1988; Burchell et al., 1989; Miyagi and Collier, 2011; Nie et al., 2017). Although the DNA sequence of the *UGT1A1* gene in human hepatocytes is identical, there are obvious differences in UGT1A1 expression from the prenatal to adult stages. Thus, variation in UGT1A1 expression during human hepatic development is not entirely explained by the genome, but is closely related to the epigenome.

Epigenetic mechanisms, which mainly include DNA methylation, histone modifications and noncoding RNA molecules, generate reversible, but heritable, changes in gene expression, without alteration of nuclear DNA sequence (Egger et al., 2004; Bird, 2007). Increasing experimental evidence indicates that, in the regulation of gene expression, the methylation and acetylation of histones H3 and H4 are the most common and functionally important modifications (Ivanov et al., 2014). Histone methylation can activate gene expression or inhibit gene transcription (Xiao et al., 2003; Shi et al., 2006; Wysocka et al., 2006; Shi et al., 2007), and histone acetylation promotes gene expression, whereas deacetylation inhibits gene expression (Peng and

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Zhong, 2015; Di Martile et al., 2016).

Furthermore, epigenetic regulation is important for drug-metabolizing enzymes (Ingelman-Sundberg et al., 2013; Kim et al., 2014; Zanger et al., 2014; Peng and Zhong, 2015). As for phase II enzymes, the epigenetic mechanisms contributing to UGT1A1 regulation have been reported. For example, differential DNA methylation within the *UGT1A1* promoter region may in part, be responsible for inter-individual variations in UGT1A1 expression and activity (Yasar et al., 2013). In addition, tissue-specific UGT1A1 levels are associated with DNA hypermethylation. Along with histone H3 hypoacetylation, DNA hypermethylation diminishes hepatocyte nuclear factor 1 alpha (HNF1A) binding, further contributing to inhibition of *UGT1A1* expression in human kidney (Oda et al., 2013). DNA methylation also leads to silencing of *UGT1A1* expression in colon cancer (Gagnon et al., 2006). Additionally, microRNA-491-3p is a key regulatory factor of UGT1A1 expression and activity (Dluzen et al., 2014). However, the role of epigenetic modifications in ontogenetic UGT1A1 regulation remains unclear. This study explored the histone modifications involved in regulating human hepatic *UGT1A1* expression during development.

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Materials and Methods

Liver sample collection and ethics statement

All livers donor (30 fetuses and 30 adults) were from the First Affiliated Hospital of Zhengzhou University in a Han Chinese population (detailed information is presented in Supplemental Table 1). Adult samples were derived from hepatectomy most often because of hepatic hemangioma. Prenatal samples were obtained from legal abortion. Written informed consent was obtained from each participant. Both adult donors and mothers of fetus donors exhibited normal liver function. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committees of the First Affiliated Hospital of Zhengzhou University.

Cell culture and siRNA transfection

HepG2 (a human hepatoma cell line) and LS174T (a human colon cell line) were cultured at 37 °C under 5% CO₂ in Dulbecco's modified Eagle medium with 10% fetal bovine serum. *HNF1A* siRNA was synthesized by Invitrogen (Shanghai, China), with specific interference targets (5' -GCUAGUGGAGGAGUGCAAUTT-3' and 5' -AUUGCACUCCUCCACUAGCTT-3') and negative-control sequences (5' -UUCUCCGAACGUGUCACGUT-3' and 5' -ACGUGACACGUUCAGAGAATT-3'). *HNF1A* siRNA was transfected into both cell lines using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) in

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6-well plates according to manufacturer instructions. To determine knockdown efficiencies, cells were collected at 24-h and 48-h post-transfection for the measurement of mRNA and protein levels, respectively.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. cDNA was prepared from 1 µg of total RNA using a PrimeScript RT reagent kit (Takara, Dalian, China). The relative mRNA levels of genes of interest were measured by qRT-PCR and normalized to those of *GAPDH* mRNA levels using SYBR Premix Ex Taq II (Takara). Sequences of the primers used for qRT-PCR are presented in Supplemental Table 2.

Western blot

Total proteins collected from cell lines at 48-h post-transfection were prepared using radioimmunoprecipitation assay buffer, and those from livers were isolated using total protein-extraction reagent for mammalian tissue (Boster Bio, Wuhan, China). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Proteins (50 µg) were separated by 6% ~12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The relative protein levels of target genes were determined by immunoblotting using the

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anti-UGT1A1 antibody (ab170858; Abcam, Cambridge, UK), anti-HNF1A (ab174653; Abcam) antibody, and anti-enhancer of zeste homolog 2 (EZH2) antibody (21800-1-AP; Proteintech, Wuhan, China). The anti-GAPDH antibody (10494-1-AP; Proteintech) was used as an internal control for protein loading. All experiments were performed in duplicate.

Chromatin immunoprecipitation (ChIP) analysis

ChIP assays were performed using the EZ-Magna ChIP HiSens ChIP kit (17-10461; Millipore). Briefly, liver tissue samples were cut into small pieces and incubated with 1% formaldehyde for 10 min at room temperature. Samples were then incubated in glycine for 5 min at room temperature to quench cross-links. A Bioruptor (Pico; Diagenode, Liège, Belgium) was used for the sonication of nuclear lysates as follows: 45 s on and 30 s off for 25 cycles. The fragment size of sheared chromatin was determined by agarose gel electrophoresis with visualization using ethidium bromide. DNA fragments between 50 and 500 bp were immunoprecipitated using antibodies against demethylated histone-3-lysine-4 (H3K4me2; 17-677; Millipore) and trimethylated histone-3-lysine-27 (H3K27me3; 17-622; Millipore). DNA was purified using a PCR purification kit (Qiagen, Hilden, Germany) and genomic DNA (input DNA) was prepared as a control.

The enriched DNA generated by ChIP from four samples (two fetuses and two adults) randomly selected from 10 cases (detailed information is presented in

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Supplemental Table 3) was sent to the Huada Company (Shenzhen, China) for high-throughput sequencing and bioinformatics analysis. Statistically significant ChIP-enriched peaks were identified by a comparison of the two groups of samples (fetal and adult livers) based on *P*-values. To validate the ChIP-seq results, qPCR was performed using the enriched DNA fragments generated by the ChIP assay as template. Primers are listed in Supplemental Table 4.

HepG2 and LS174T cells were collected 48 h post-transfection for ChIP analysis using specific antibodies targeting H3K4me2, H3 acetylation (06-599; Millipore), nuclear receptor coactivator 6 (NCOA6; 25241-1-AP; Proteintech), and HNF1A, to investigate histone modifications at a site (P1; Fig. 1B) located near the *UGT1A1* promoter. Enriched DNA fragments were collected for qPCR analysis. ChIP-qPCR data are plotted as percentages of input DNA.

Co-immunoprecipitation (Co-IP) assay

Co-IP of mixed lineage leukemia 1 (MLL1) and HNF1A, p300 and HNF1A, as well as NCOA6 and HNF1A, was performed using Pierce Classic IP kits (26146; Thermo Fisher Scientific) according to the manufacturer's protocol. HepG2 and LS174T cells were harvested and lysed in the immunoprecipitation buffer. Using unprecipitated extracts as an input, whole-cell extracts were immunoprecipitated with anti-MLL1 (14689; CST, Danvers, MA, USA), anti-NCOA6 (Proteintech), or anti-p300 (ab14984; Abcam), followed by immunoblotting assays performed with the

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anti-HNF1A antibody. Reciprocal immunoprecipitations were then performed using anti-HNF1A antibody and western blot performed with anti-MLL1, -NCOA6, and -p300 antibodies.

Statistical analysis

Data are expressed as means \pm standard deviation (S.D.). SPSS 19.0 (IBM, Armonk, NY, USA) was used for statistical analysis. The statistical significance of differences between groups was compared using unpaired *t* tests. Spearman's rank correlation analysis was also performed. Additionally, differences among ChIP-seq results were evaluated by comparison of the two groups using *P*-values based on the Poisson distribution. *P* values < 0.05 were considered significant.

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Results

Ontogenetic changes in UGT1A1 expression are associated with dynamic alterations of histone methylation

H3K4me2 and H3K27me3 profiles along the *UGT1A1* locus in fetal and adult livers are summarized in Fig. 1. There was strong enrichment of H3K4me2, along a 5873 bp sequence around the *UGT1A1* promoter in adult liver, and enrichment of H3K27me3 12 kb upstream of the *UGT1A1* locus in fetal liver. Our previous results suggested that *UGT1A1* expression is very low in fetal livers; however, its expression level is significantly higher in adults (Nie et al., 2017). Hence, the observed enrichment of H3K4me2 at *UGT1A1* in adult liver was consistent with the higher *UGT1A1*-expression level at this developmental stage. Similarly, enrichment of H3K27me3 at the *UGT1A1* locus in fetal liver was consistent with the previous observations of low levels of *UGT1A1* expression early in development. These data suggest that the histone-modification marker, H3K4me2, may explain the transcriptional activation of *UGT1A1* in the adult liver, and that H3K27me3, a marker of transcription inhibition, could silence the mRNA expression of *UGT1A1* in the fetal liver.

Dynamic alterations of H3K4me2 and H3K27me3 along the *UGT1A1* locus during liver development were verified by ChIP-qPCR analysis (Fig. 1C and 1D). Relative to fetal liver, H3K4me2 was enriched by an average of 3.2-fold in the adult

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liver, whereas compared with adult liver, the average enrichment of H3K27me3 was 3.7-fold in the fetal liver. These ChIP-qPCR results are consistent with the findings from ChIP-seq analysis and confirm the reliability of the ChIP-seq data.

***HNF1A* siRNA affects H3K4me2 and H3K27me3 histone modifications in the *UGT1A1*-promoter region in HepG2 and LS174T cells**

HNF1A knockdown in HepG2 and LS174T cells using siRNA caused a significant and specific decrease in corresponding mRNA and protein levels. Additionally, siRNA-treated cells exhibited relatively low *UGT1A1* mRNA and protein expression compared with levels observed in controls (Fig. 2A–C), and the differences were statistically significant ($P < 0.01$).

ChIP-seq results showed that the H3K4me2 enriched peak in the *UGT1A1*-promoter region in adult samples involved the HNF1A response element (HNF1AE) (Fig. 1B). To evaluate the influence of HNF1A on histone modification of the region around the *UGT1A1* promoter, HepG2 and LS174T cells were transfected with *HNF1A* siRNA, followed by ChIP analysis (Fig. 2D and 2E). As expected, *HNF1A* knockdown caused a significant decrease in the enrichment of H3K4me2 and H3 acetylation around the HNF1AE in the *UGT1A1*-promoter region. NCOA6 and HNF1A levels were also negatively affected by *HNF1A* knockdown in the same regions. These results suggested that knockdown of *HNF1A* significantly decreased *UGT1A1* expression, which was accompanied by altered levels of H3K4me2 and H3

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acetylation in the *UGT1A1*-promoter region, as well as reduced levels of NCOA6 and HNF1A expression.

MLL1/NCOA6/p300 interact with HNF1A in HepG2 and LS174T cells

The dimethylated state of histone-3-lysine-4 (H3K4me₂) is achieved by self-recruitment of the histone methyltransferase, MLL1 (Rickels et al., 2016; Sun et al., 2016). Additionally, NCOA6, which is a ligand-dependent nuclear-receptor-interacting protein, accounts for a significant proportion of transcriptional coactivation (Mahajan and Samuels, 2008). Moreover, the transcriptional coactivator p300 can also act as a histone-acetyltransferase and contribute to transcriptional activation through its function in chromatin modification. Based on co-IP analysis (Fig. 3A), the histone-methylating enzyme, MLL1, and the transcription factor, HNF1A, coexisted in a complex in LS174T and HepG2 cells, indicating an interaction between these two proteins. Similarly, NCOA6/p300 and HNF1A also coexisted in a complex (Fig. 3B and 3C).

Correlation between EZH2 and UGT1A1 expression in human liver

Generation of the trimethylated state of histone-3-lysine-27 (H3K27me₃) is catalyzed by the histone methyltransferase, EZH2 (Sharma et al., 2016). Our results indicate that EZH2 is expressed at high levels in fetal liver, with lower levels in adult liver samples (Fig. 4A). By contrast, UGT1A1 was expressed at significantly lower

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levels in fetal livers, with high levels observed in adult livers ($P < 0.001$) (Fig. 4B).

According to Spearman's correlation analysis, UGT1A1 protein levels were negatively correlated with those of EZH2 ($r_s = -0.74$; $P < 0.001$), and curve-fitting analysis showed that EZH2 protein levels may represent a power function of UGT1A1 protein levels (Fig. 4C).

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Discussion

Accumulating evidence indicates that the epigenetic regulation of gene expression is essential for cell differentiation and organ development (Jaenisch and Bird, 2003; Sasaki and Matsui, 2008). Histone modifications of drug-metabolizing enzymes have been emphasized in recent years. In particular, H3K4me2 and H3K27me3 are involved in the ontogenetic expression of *Cyp3a* during mouse liver maturation (Li et al., 2009b). Increases in H3K4me2 can promote *Cyp3a16* expression in the neonatal rat liver and *Cyp3a11* expression in the adult mouse liver, whereas decreased H3K4me2 and increased H3K27me3 are associated with inhibition of *Cyp3a16* expression in adult animals. Similarly, we previously characterized dynamic changes in H3K4me2 and H3K27me3 in the developmental switch of human hepatic CYP3A7 and CYP3A4 expression (He et al., 2016). Our present data demonstrate that H3K4me2 enrichment in the adult liver correlates with *UGT1A1* activation, whereas H3K27me3 enrichment in the fetal liver could contribute to *UGT1A1* suppression. Presumably, as a part of a developmental switch, the activation marker H3K4me2 and the inhibitory marker H3K27me3 are involved in the dynamic expression of hepatic UGT1A1. Indeed, UGT1A1 protein levels in early fetal livers (20–30 weeks) are low compared with those of later fetal (> 30 weeks) and neonatal livers, which is in agreement with previous reports. Hence, the results of the present study provide a rationale for the lower levels of UGT1A1 protein and activity in early fetal life; however, at later time points (> 30 weeks), fetal liver UGT1A1 protein levels are

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similar to, or same as, those of neonates, yet there is negligible UGT1A1 enzyme activity. Further experiments are required to decipher the reasons for little-to-no activity of UGT1A1 in the later fetal livers.

Notably, our data confirm the enrichment of H3K4me2 in the *UGT1A1*-promoter region from the transcription initiation site to approximately 5 kb downstream (i.e., the first exon) in the adult liver. Interestingly, the HNF1AE was included in the region of H3K4me2 enrichment. Numerous studies have demonstrated that, as an evolutionarily conserved transcription factor, HNF1A can mediate the transcriptional activation of UGTs, including UGT1A1, 1A3, 1A4, 1A9, and UGT2B7 (Bernard et al., 1999; Ishii et al., 2000; Toide et al., 2002; Gregory et al., 2004; Odom et al., 2004; Gardner-Stephen and Mackenzie, 2007; Ramirez et al., 2008; Li et al., 2009a; Ehmer et al., 2010). Additionally, H3K4me2 is generated by the histone methyltransferase, MLL1; however, due to a lack of intrinsic DNA-binding activity, histone-modifying enzymes need to be recruited to genomic DNA by other factors, such as transcription factors. Histone-modifying enzymes then interact with transcription factors and are targeted to particular genomic loci via site-specific DNA-binding activity (Rosenfeld et al., 2006; Ivanov et al., 2014). Furthermore, co-IP assays provided evidence of an interaction between MLL1 and HNF1A; therefore, we speculate that by binding to the DNA-response element located near the *UGT1A1*-promoter region, HNF1A may recruit the histone-modifying enzyme, MLL1, to promote the transcription of human hepatic *UGT1A1*.

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Our data also indicate a potential interaction between HNF1A and NCOA6/p300. NCOA6 is known to interact with constitutive androstane receptor (CAR) and hepatocyte nuclear factor 4 alpha (HNF4A) as well as pregnane X receptor (PXR) and HNF4A in the activation of CYP2C9 (Surapureddi et al., 2008; Surapureddi et al., 2011). Moreover, HNF1A interacts with p300 on the promoter of the glucose transporter *GLUT2* (Ban et al., 2002). Additionally, our recent study reported that rifampicin enhances interactions between PXR and NCOA6/p300 in the induction of CYP3A4 (Yan et al., 2017). Similarly, in the present study, HNF1A interacted with NCOA6/p300 to activate *UGT1A1* expression. As expected, we demonstrated that HNF1A affected H3 acetylation, as well as H3K4me₂, near the HNF1A-binding site in the *UGT1A1* promoter. Therefore, our results clarified the mechanisms by which histone modifications regulate the expression of human hepatic UGT1A1. Briefly, the recruitment of HNF1A, leads to aggregation of MLL1, NCOA6, and p300 at the *UGT1A1*-promoter region to regulate H3K4me₂, H3 acetylation, and subsequent *UGT1A1* expression (Fig. 5).

EZH2 is an important component of Polycomb repressive complex, a specific methyltransferase of histone H3K27. Hence, EZH2 is crucial for catalyzing the H3K27me₃ epigenetic status and maintaining transcriptional silencing, particularly during embryonic development and cell differentiation (Otte and Kwaks, 2003; Lund and van Lohuizen, 2004). We observed a significant negative correlation between EZH2 and UGT1A1 levels; therefore, we speculate that high levels of EZH2

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expression during embryonic development catalyze the formation of H3K27me₃, leading to its enrichment in the fetal liver and thereby inhibiting *UGT1A1* expression. Conversely, low levels of EZH2 expression in the adult liver release the transcriptional repression of *UGT1A1*. However, similar to MLL1 and as a histone-modifying enzyme, EZH2 lacks the ability to bind to specific DNA sequences; therefore, the roles of transcription factors and other cofactors involved in EZH2 recruitment and subsequent regulation of *UGT1A1* expression require further investigation.

In conclusion, our results indicate that dynamic alterations of histone methylation are associated with ontogenetic *UGT1A1* expression. In particular, our findings suggest that the recruitment of HNF1A leads to aggregation of the cofactors MLL1, NCOA6, and p300 at the *UGT1A1* promoter, which regulates histone modifications and subsequent *UGT1A1* expression. These findings broaden our understanding of *UGT1A1* expression during liver ontogeny, in the context of epigenetic regulation, and could potentially contribute to the development of age-specific therapies in pediatric patients.

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Authorship Contributions

Participated in research design: Zhang, Kan, and Nie.

Conducted experiments: Nie, Liu, Yan, Wang, and Bi.

Performed data analysis: Nie and Meng.

Wrote or contributed to the writing of the manuscript: Nie and Zhang.

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Footnotes:

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Figure legends

Fig. 1. Dynamic alterations of histone methylation were associated with the ontogenetic expression of *UGT1A1*. (A) H3K4me2 and H3K27me3 profiles along the *UGT1A1* locus in fetal and adult liver determined by CHIP-Seq, presented using the UCSC genome browser. Red arrows represent enrichment sites; yellow boxes represent the *UGT1A1* locus. (B) Schematic depiction of the *UGT1A1* promoter (−12000 to +5000). Primer sets for amplification for ChIP-qPCR (P1–P2). TSS: transcription start site. (C) Validation of ChIP-seq results for H3K4me2 by CHIP-qPCR using primer pair 1 (P1). Average enrichment of H3K4me2 was 3.2-fold in adult livers, compared with fetal livers. (D) Validation of ChIP-seq results for H3K27me3 by ChIP-qPCR using primer pair 2 (P2). Average enrichment of H3K27me3 was 3.7-fold in fetal livers as compared with adult livers. Data presented are the means ± S.D. of three independent experiments. * $P < 0.05$, ** $P < 0.01$, versus fetal livers.

Fig. 2. Changes in histone H3 modification at the *UGT1A1* promoter region after siHNF1A transfection in HepG2 and LS174T cells. (A–C) *HNF1A* and *UGT1A1* expression were knocked down by siHNF1A transfection. *HNF1A* and *UGT1A1* mRNA levels were determined by qRT-PCR and normalized with respect to *GAPDH* levels. *HNF1A* and *UGT1A1* protein levels were determined by western blot using *GAPDH* as a loading control. ** $P < 0.01$ versus negative control. (D and E) H3K4me2 and H3-acetylation enrichment and NCOA6/HNF1A-binding at the sites

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(P1) in the *UGT1A1* promoter region in HepG2 and LS174T cells. Data are shown as the means \pm S.D. of three independent experiments, * $P < 0.05$, ** $P < 0.01$ versus the negative control.

Fig. 3. Protein-protein interactions determined by co-IP assay in LS174T and HepG2 cells. (A and D) HNF1A interacts with MLL1; (B and E) HNF1A interacts with NCOA6; (C and F) HNF1A interacts with p300. Cell proteins before (input) and after (IP) immunoprecipitation were separated by SDS-PAGE, transferred onto polyvinylidene fluoride membranes, and analyzed with the indicated antibodies.

Fig. 4. Analyses of correlations between UGT1A1 and EZH2 protein levels in 60 human liver samples. (A) EZH2 expression was determined by western blot using GAPDH as a loading control. (B) UGT1A1 protein expression was measured by western blot. Data are expressed as means \pm S.D., and groups were compared using unpaired t tests. *** $P < 0.001$ versus fetal liver samples. (C) The fitted curve showing the power function correlation between UGT1A1 protein levels and EZH2 expression. Differences were considered significant at $P < 0.05$.

Fig. 5. Diagram of cofactors that interact with HNF1A in the *UGT1A1* promoter.

Figure 1

A

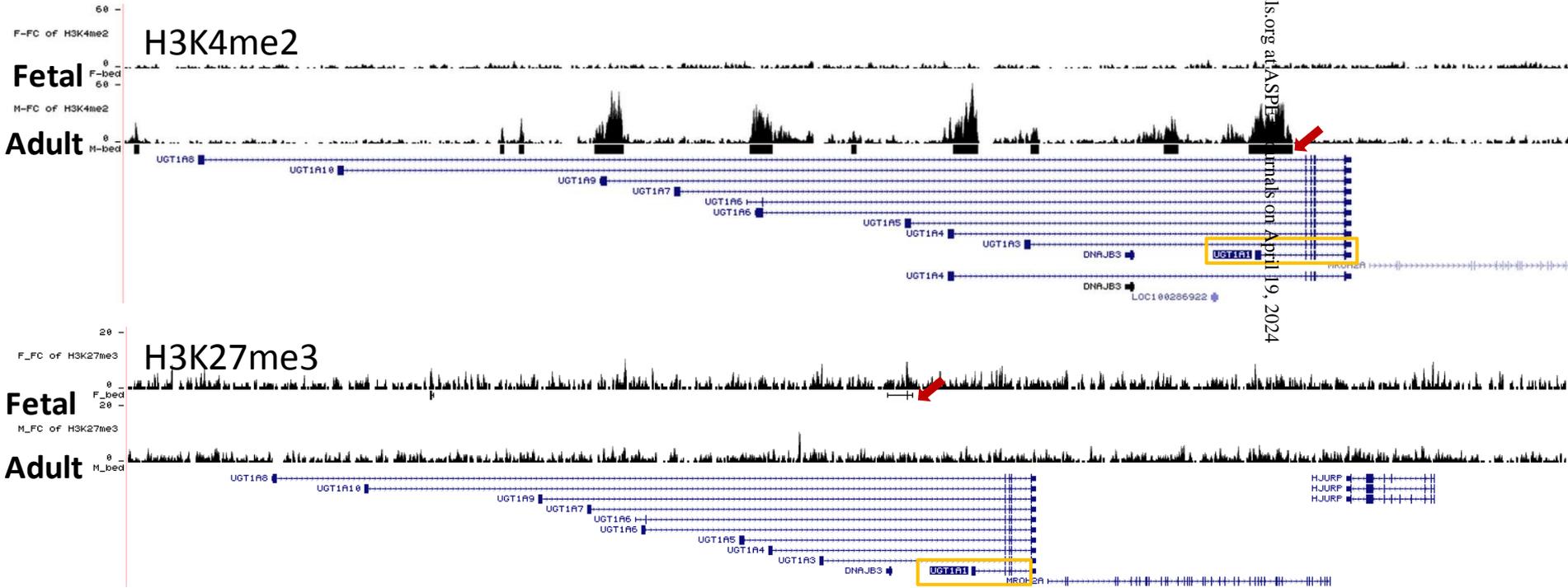


Figure 1

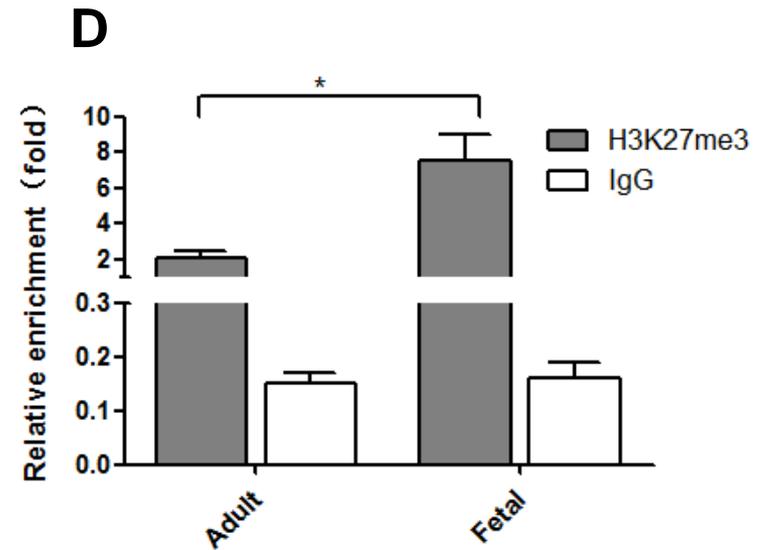
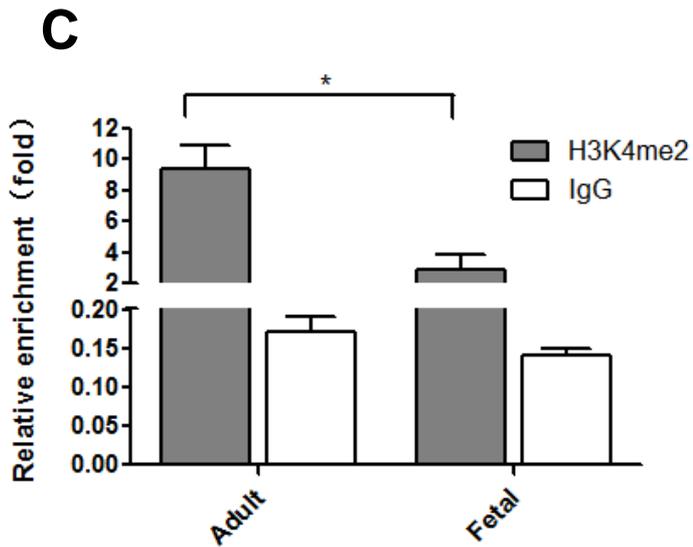
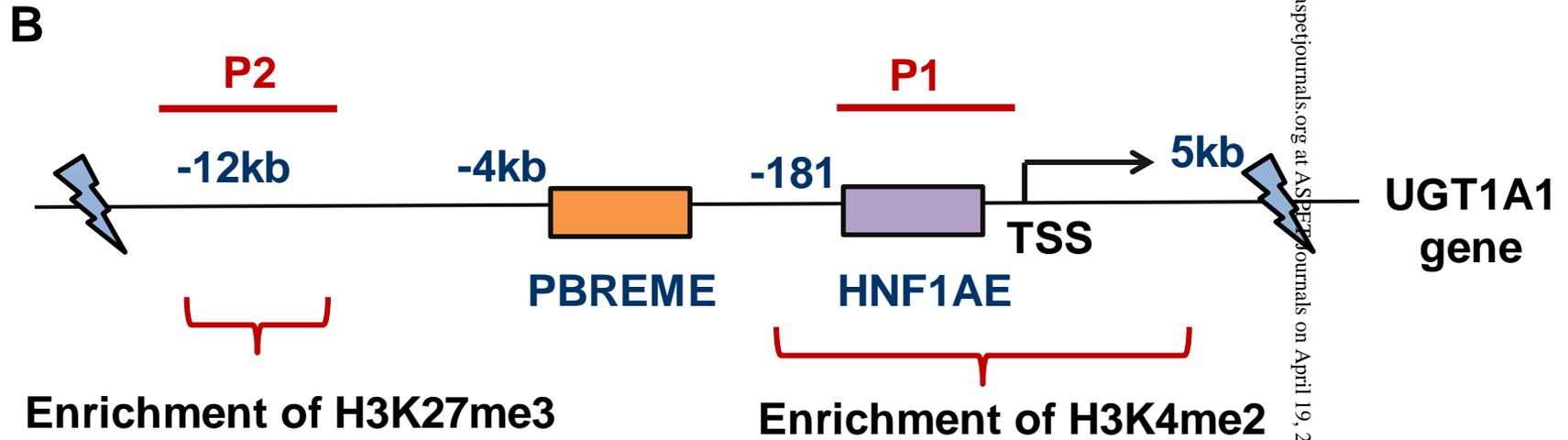
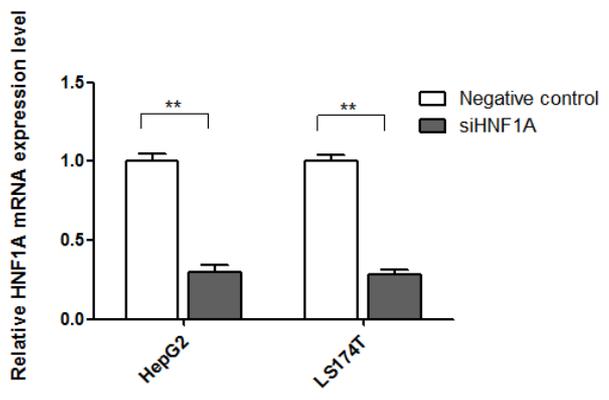
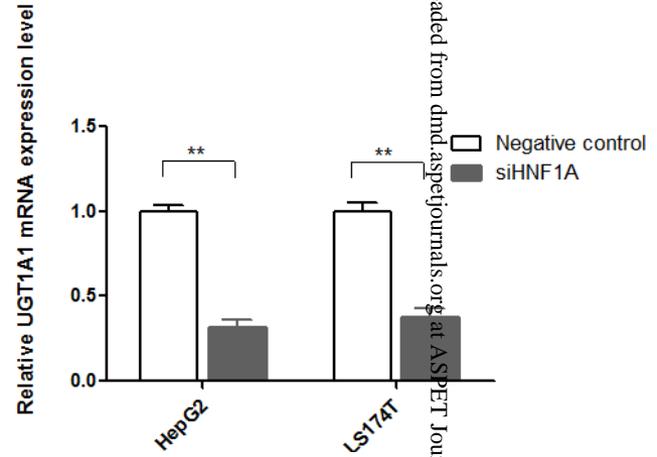


Figure 2

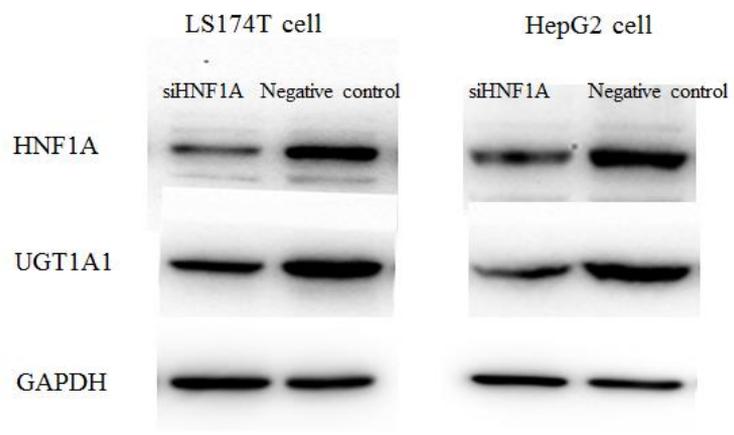
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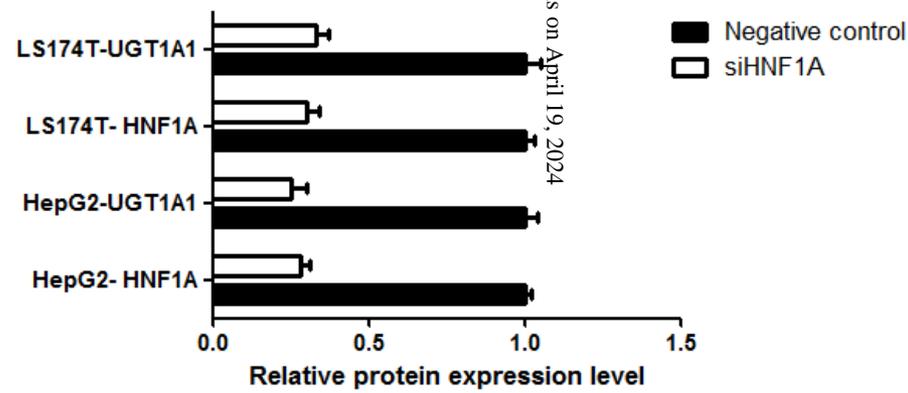
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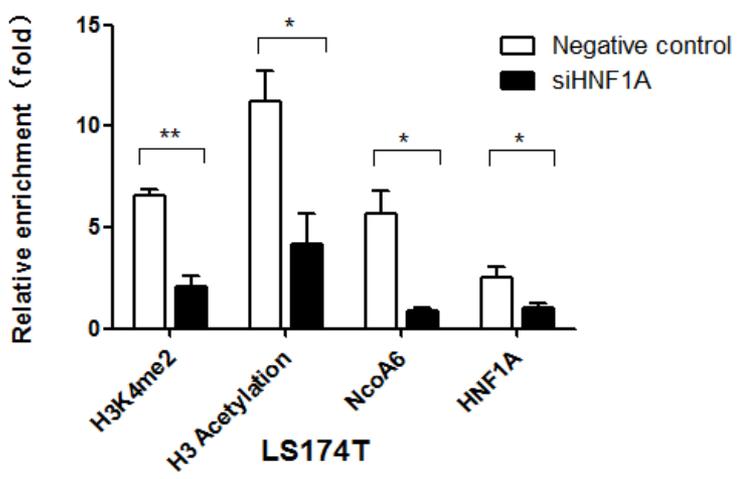
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D



D



E

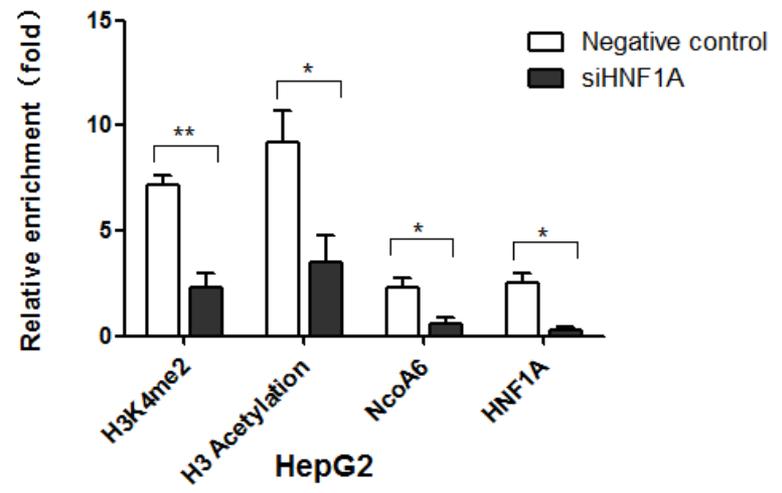
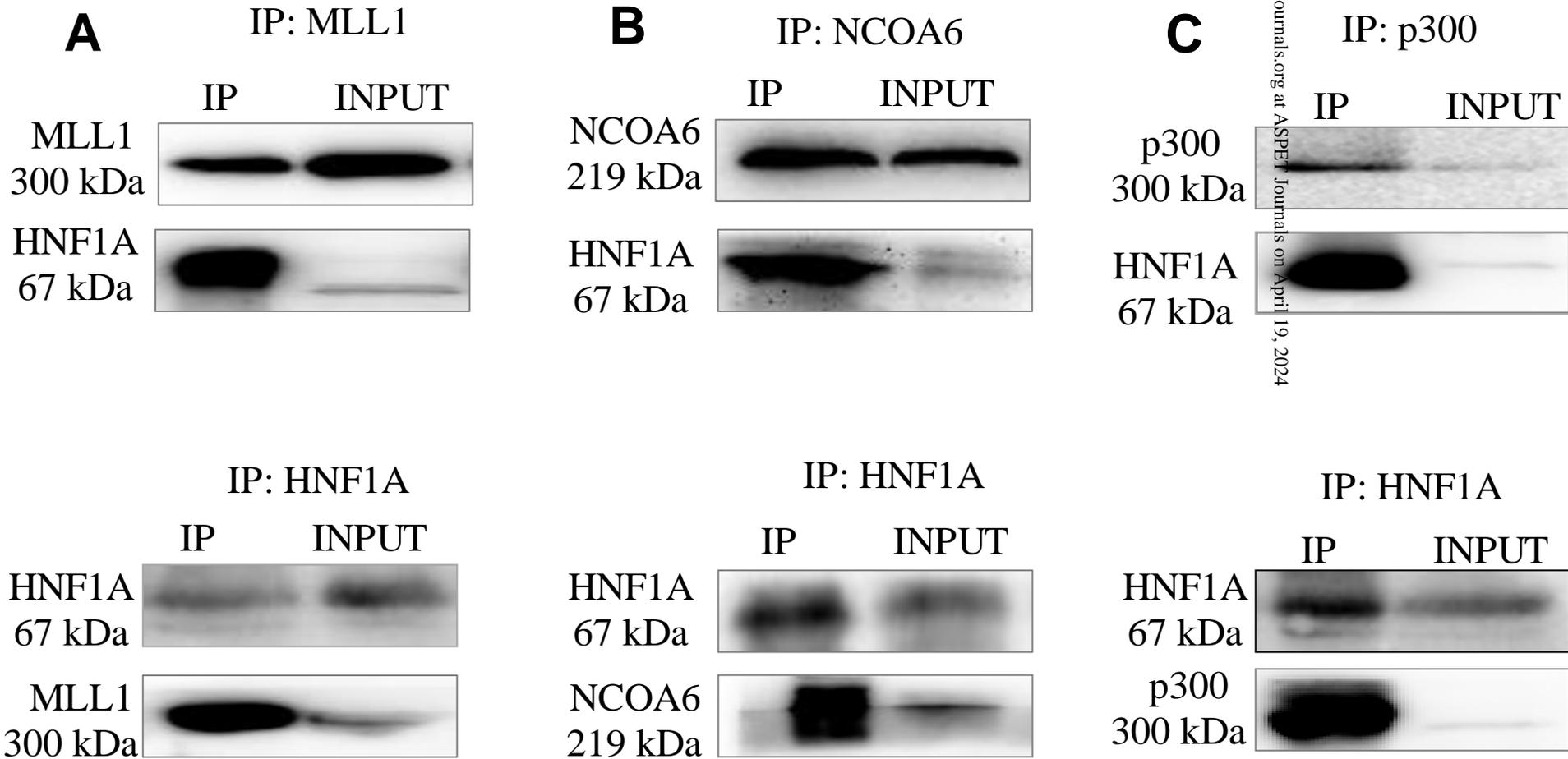


Figure 3



LS174T cell

Figure 3

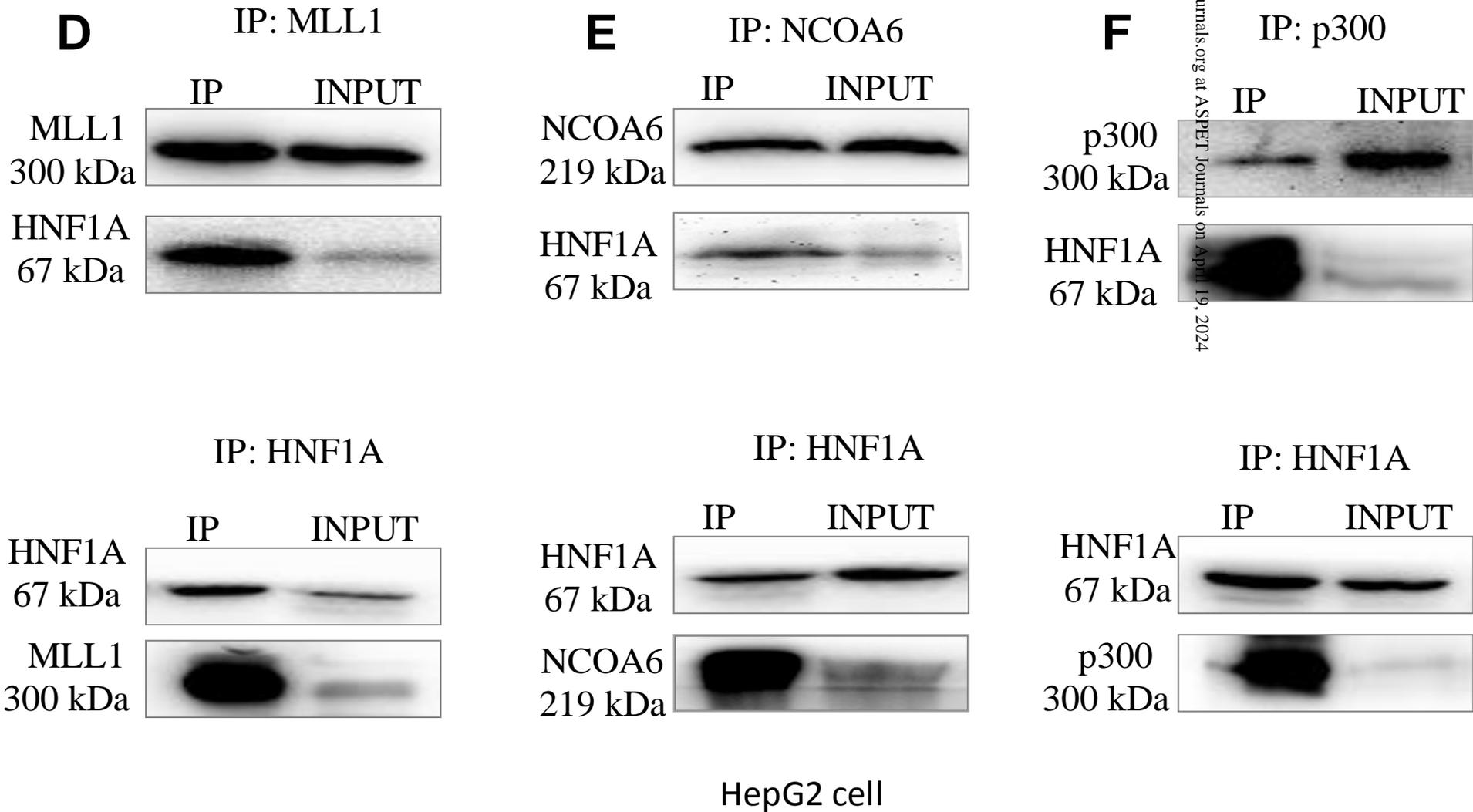
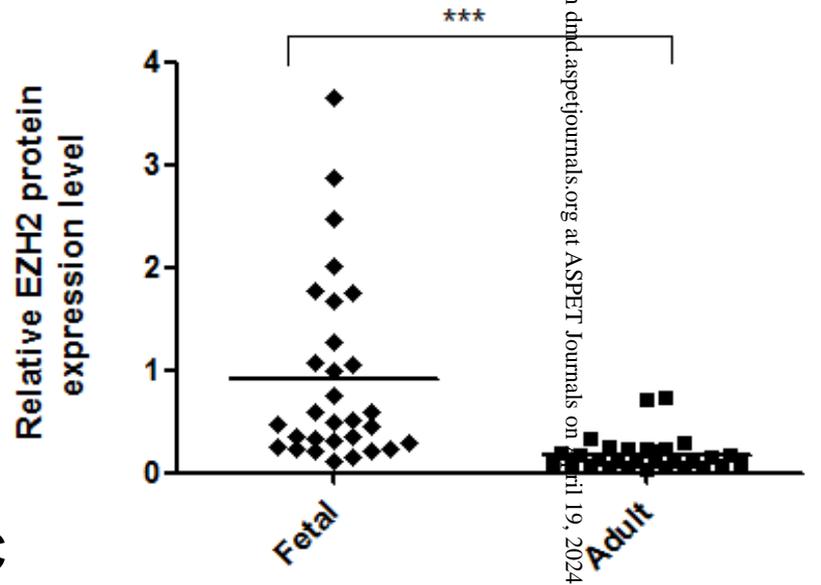
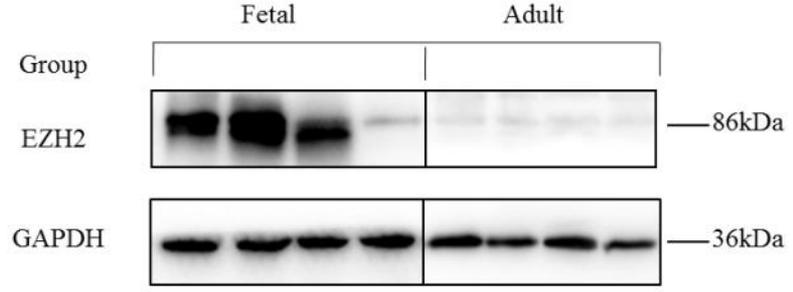
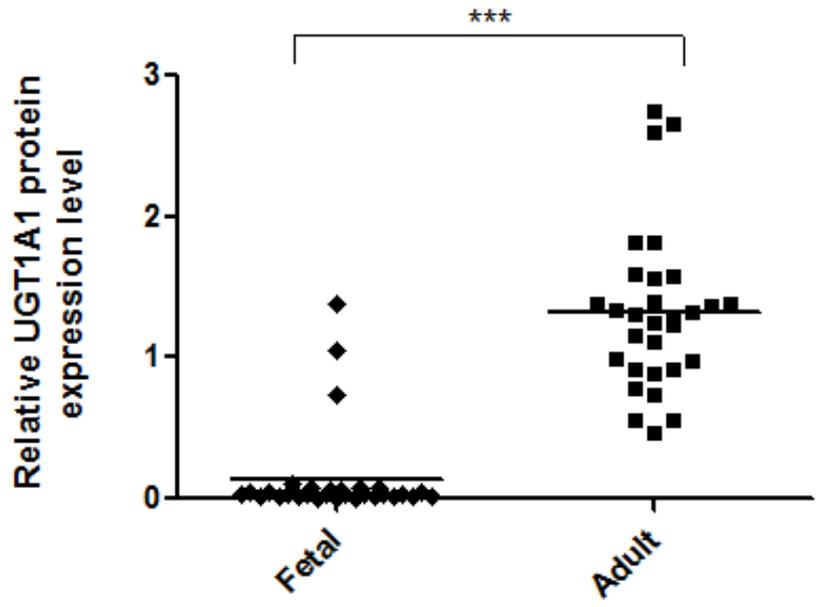


Figure 4

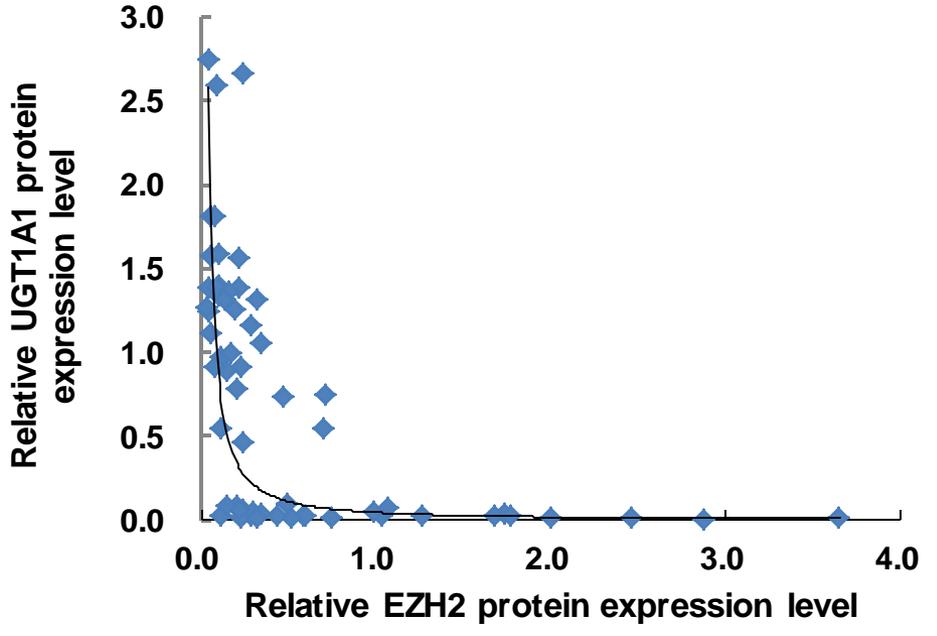
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B



C



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Figure 5

