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Integrated RNA-seq and ChIP-seq analysis reveals a feed-forward loop regulating H3K9ac and key labor drivers in human placenta

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ABSTRACT

Background: Chromatin alterations are important mediators of gene expression changes. We have recently shown that activated non-canonical NF-κB signaling (RelB/p52) recruits histone acetyltransferase CBP and deacetylase HDAC1 to selectively acetylate H3K9 (H3K9ac) to induce expression of corticotropin-releasing hormone (*CRH*) and prostaglandin-endoperoxide synthase-2 (*PTGS2*) in the human placenta. Both of these genes play a role in initiating parturition in human pregnancy.

Methods: We performed chromatin immunoprecipitation followed by gene sequencing (ChIP-seq) in primary term human cytotrophoblast (CTB) with use of antibodies to RelB, CBP, HDAC1 and H3K9ac. We further associated these chromatin alterations with gene expression changes from mid-trimester to term in CTB by RNA sequencing (RNA-seq).

Results: We detected a genome-wide differential gene enrichment between mid-trimester and term human placenta. Pathway analysis identified that cytokine-cytokine receptor interaction, NF-κB, and TNF are the leading pathways enriched in term placenta and associated with these chromatin alterations.

Discussions: Our analysis has provided the first-time characterization of the key players of human placental origin with molecular changes resulting from chromatin modifications, which could drive human labor.

1. Introduction

Human parturition involves many events including cervical ripening and dilation, uterine contractions, fetal membrane rupture, and placental detachment. Onset of such events is a consequence of activation of a series of endocrine and immune responses. A number of studies have highlighted a major role of inflammatory processes in the initiation of human labor, which are exemplified by increased activities of cytokines, chemokines, and growth factors in human myometrium, cervix, amniochorion, and decidua [1–3]. Despite a recent study using single-cell transcriptomics of the human placenta to define cell communication network of the maternal-fetal interface [4], investigations of genome-wide gene expression changes associated with the gestational length and characterization of molecular mechanisms responsible for these changes in this important endocrine organ have remained scarce.

The human placenta synthesizes a large and diverse number of hormones and cytokines that exert major influences on ovarian, uterine, mammary and fetal physiology. Moreover, there is good evidence that the clock that governs the length of human pregnancy resides in the placenta and corticotropin-releasing hormone (CRH) is considered to be part of this clock [5,6]. To this end, we have developed a plausible model that explains how the placenta contributes to initiation of labor, and we have ascribed the non-canonical NF-κB [RelB/NF-κB2 (p100/p52)] pathway a central regulatory role in regulation of the placental clock [7,8]. Apart from CRH, other well-characterized mechanisms of placental origin contributing to pregnancy advancing and parturition include oxytocin (OXT), urocotins (UCNs), and prostaglandins (PGs) [9]. Mechanistically, these molecules are known to modulate uterine contractility, blood vessels tone, and immune function.

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Abbreviations

HDAC1	Histone deacetylase-1
H3K9ac	H3 lysine 9 acetylation
CRH	Corticotropin-releasing hormone
PTGS2	Prostaglandin-endoperoxide synthase-2
ECM	Extracellular matrix. MMP: Matrix metalloproteinase
C-section	Cesarean-section
M-T:	Mid-trimester
CTB	Cytotrophoblast
ChIP-seq	ChIP-sequencing
RNA-seq	RNA-sequencing
EGA	Estimated gestational age
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
siRNA	Short-interfering RNA
qPCR	Quantitative PCR; RT-qPCR: Reverse transcription-qPCR
GSEA	Gene Set Enrichment Analysis
DEGs	Differentially-expressed genes
KEGG	Kyoto Encyclopedia of Genes and Genomes
ES	Enrichment score; S2N: Signal-to-noise
NIK	NF-κB inducing kinase (MAP3K14)
TRAF	TNF receptor associated factor

Mammalian gene expression is tightly regulated at multiple epigenetic levels and four major determinants are chromatin conformation characteristics, non-coding RNAs, DNA methylation patterns, and histone modifications [10]. While DNA or histone methylation is closely associated with transcriptional silencing of target genes, in the eukaryotic chromatin structure, acetylation of lysines at the N termini of histones H3 or H4 enables the transcription machinery to access nucleosomal DNA and in turn to activate gene transcription [11]. Further studies have advanced our understanding of this mechanism and found that histone acetylation is highly dynamic, which means that histone lysines are subjected to constant acetylation and deacetylation and dynamic interplay between these two opposing activities play a critical role in enhancing relaxation of the nucleosomal DNA and gene transcription [12,13]. In mammals, acetylation and deacetylation of histone lysines are mediated by histone acetyltransferases (HATs) and deacetylases (HDACs), respectively. Recent work from our laboratory has demonstrated that glucocorticoid drives non-canonical NF-κB pathway, which induces RelB/p52 heterodimers to bind to gene promoter of *CRH* and prostaglandin-endoperoxide synthase-2 (*PTGS2* or *COX-2*) [8]. We have further shown that RelB/p52 heterodimers associate with the histone acetyltransferase CBP and the histone deacetylase HDAC1 at the *CRH* and *PTGS2* promoters to selectively and dynamically acetylate histone 3 lysine 9 (H3K9), thereby causing an epigenetic induction of these genes.

In this study, we performed a genome-wide investigation for those genes associated with RelB/p52, CBP, HDAC1, and acetylated H3K9 (H3K9ac) in the human placenta. We integrated ChIP-seq and RNA-seq analysis in primary cultures of human villous cytotrophoblast (CTB) and identified multiple subsets of genes constituting key signaling pathways involved in the initiation of human parturition.

2. Materials and methods

2.1. Study approval

This study was approved by the Institutional Review Boards of Rutgers University (Pro20150001445 and Pro20120002052). All patients signed a written informed consent for their specimen to be used for this study. All methods were performed in accordance with the relevant guidelines and regulations.

2.2. Purification of CTB

Placental specimens from full-term pregnancies were collected from healthy women with estimated gestational age (EGA) of 38 and 40 weeks who were delivered by Cesarean section (C-section). Mid-trimester placentas were collected from healthy individuals with EGA between 18 to 22 weeks following elective termination of pregnancy. Women with complications of pregnancies, including diabetes, hypertension, autoimmune diseases, infection, fetal growth restriction, and preeclampsia, were excluded from the study.

Purification of placental CTB was performed as previously described [7]. CTB were maintained at 37 °C and 5% CO_2 at least for 48 h for spontaneous syncytialization prior to further analysis.

2.3. ChIP-sequencing (ChIP-seq)

We collected 3 individual placentas at full-term pregnancy for ChIP-seq analysis. Chromatin and DNAs were prepared and recovered as detailed in our previous and recent studies [7,14,15]. Briefly, a total of approximately 1×10^7 of CTB cells were cross-linked with 1% formaldehyde for 5 min at room temperature. Cells were lysed in ChIP lysis buffer (50 mM HEPES–KCl, pH 7.5; 140 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% sodium deoxycholate; 0.1% SDS) with freshly added 1 × protease inhibitor cocktail (Roche Applied Science) and then sonicated to shear chromatin into 150- to 200-bp fragments. Chromatin was then immunoprecipitated with individual ChIP-grade antibody and DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Concentrations of DNA were determined by Qubit Fluorometric (Invitrogen, CA) and at least 10 ng/per sample were submitted for ChIP-seq with Illumina HiSeq platform and 1 × 50 bp configuration (GENEWIZ, NJ). All ChIP-seq raw and processed data have been deposited into public database Gene Expression Omnibus (GEO) with accession number of GSE124080.

2.4. RNA-sequencing (RNA-seq)

Total RNAs were extracted from the placenta from the subject at either mid-trimester (n = 4) or term (n = 4) pregnancy with use of Trizol method (Invitrogen, CA) and re-suspended in DEPC-treated H2O. At least 1 μg/per sample were submitted for RNA-seq with Ion Proton System (RMANJ, NJ). All RNA-seq raw and processed data have been deposited into GEO with accession number of GSE124282.

2.5. GSEA and KEGG pathway enrichment analysis

To identify the functions of genes that are differentially expressed (DEGs) between term and mid-trimester groups, we first performed a standard Gene Set Enrichment Analysis (GSEA) on the RNA-seq data. The gene matrix transposed file used in this analysis is known as curated KEGG (2016) gene sets. Then we performed the analysis with default parameters. For each pathway involved in the analysis, some typical plots are generated, including a running enrichment score (ES) plot, an ES distribution plot and an expression heatmap of leading edge genes. The running enrichment score plot shows how the ES is accumulated when the genes hit or miss the target pathway across all genes ranked by signal-to-noise (S2N) ratio, which is measured by the difference of means scaled by the standard deviation. Once the running ES reaches its maximum value, all of the genes contributing to the maximum ES that hit the pathway will be the leading-edge genes, whose expression values will be shown in the heatmap for all samples. A null distribution of ES was then generated by the permutation of gene labels,

which was used to evaluate the significance of the observed ES. Besides a standard GSEA, we conducted a simple KEGG pathway enrichment analysis for DEGs from term against mid-trimester. The target lists, with targets (overlap of DEGs and binding targets) of each factor, are uploaded to Enrichr [16], and KEGG_2016 category was then chosen.

2.6. Integrated RNA-seq and ChIP-seq analysis

DEGs were obtained from samples of term or mid-trimester by EdgeR (version: 3.16.5) [17]. Besides the DEG list generated by EdgeR, we also drew volcano and MA plots, indicating proper data transformation and normalization had been performed, and a heatmap of gene expression values of all DEGs to intuitively show the differential expression of those genes from different groups. For ChIP-seq data, we had 3 individual specimens for RelB, CBP, HDAC1 and H3K9ac. The peaks are called by MACS [18] and each peak was assigned a p-value according to its significance. A gene is regarded as a target if it has significant peaks in its promoter/gene body. To obtain a final target list of a factor, we used Fisher's method to combine the p-values from 3 individual specimens for each factor. Genes with meta p-value < 0.05 are then remained as the final targets of the factor. The gene names of both data types are transformed to official symbols using MyGene.info APIs [19]. To integrate these two types of data, DEGs and the target list of a factor are uploaded to ChIP-Array [20], a web tool to construct regulatory networks using expression and regulator binding profiles. Then co-occupancy analysis was selected to investigate the common targets of those factors.

2.7. Gene silencing

siRNA transfection was performed as previously detailed using transfection reagent Lipofectamine2000 (Invitrogen) with use of FlexiTube siRNAs target RelB or p100 (QIAGEN, CA) [7,21]. Total RNAs were isolated from cells and analyzed by RT-qPCR. Each experiment was repeated in 3 individual specimens.

2.8. Reverse transcription quantitative PCR (RT-qPCR)

Total RNAs were extracted by means of Trizol (Invitrogen). Total cDNA synthesis was prepared by the oligo-dT primer method using the Superscript II Reverse Transcription kit (ThermoFischer Scientific). PCR was performed using a StepOne Plus Real Time PCR System (Applied Biosystems) and power SYBR green PCR master (ThermoFisher Scientific). PCR primers (forward/reverse) included: CRH, 5′-GCAGTT AGCACAGCAAGCTCAC-3′/5′-CAAATGGCATAAGAGCAGCG-3′; PTGS2, 5′-TGAGCATCTACGGTTTGCTG-3′/5′-TGCTTGTCTGGAACAACTGC-3′; IL1B, 5′-AAGGCGGCCAGGATATAACT-3′/5′- CTGGCTGATGGACAGGA GAT-3′; MMP9, 5′-AGGTGGACCGGATGTTCC-3′/5′-GGCACTGCAGGAT GTCATAG-3′; MMP1, 5′-TGGATCCAGGTTATCCCAAA-3′/5′-TGGAGAG TCAAAATTCTCTTCG-3′;

IL-8 (CXCL8), 5′-CTGCGCCAACACAGAAATTA-3′/5′-ATTGCATCTG GCAACCCTAC-3′; TNF, 5′-CCCCAGGGACCTCTCTCTAA-3′/5′-TCTCAG CTCCACGCCATT-3′; UCN2, 5′-CAAACCTGCATGAAGCAAGA-3′/ 5′-GCGTGAAGTGGGACAGAGTT-3′; OSM, 5′- TGGGTGACCTCACTTTA GGC-3′/5′-TTACGTCAGGGAATCCAAGC-3′; MMP14, 5′- CAGAGAAGG CACACAAACGA-3′/5′-CACTGGTGAGACAGGCTTGA-3′; MMP19, 5′- CCAGGGGAGGGATATGTCTT-3′/5′-CAAAGGAGCCTGGTCTTCAG-3′, GAPDH, 5′-CTCCCGCTTCGCTCTCTG-3′/5′-CTGGCGACGCAAAAG AAG-3′, RelB, 5′-TCCCAACCAGGATGTCTAGC-3′/5′-AGCCATGTCCCT TTTCCTCT-3′, and NFKB2, 5′-GAACAGCCTTGCATCTAGCC-3′/5′-TCC CAGTCGCTATCAGAGG-3′.

2.9. Statistical analysis

Each experiment was repeated in a minimum of three times. Data (bars) are presented as mean ± standard deviation. Student *t*-test (2-tailed) and one-way ANOVA with Dunnett's test was used to compare two groups and ≥ three groups. $p < 0.05$ was considered statistically significant. False discovery rate (FDR)-based multiple comparisons were applied. In this study, a FDR of 0.25 or smaller was set as the criterion for reporting significant differences between mid-trimester

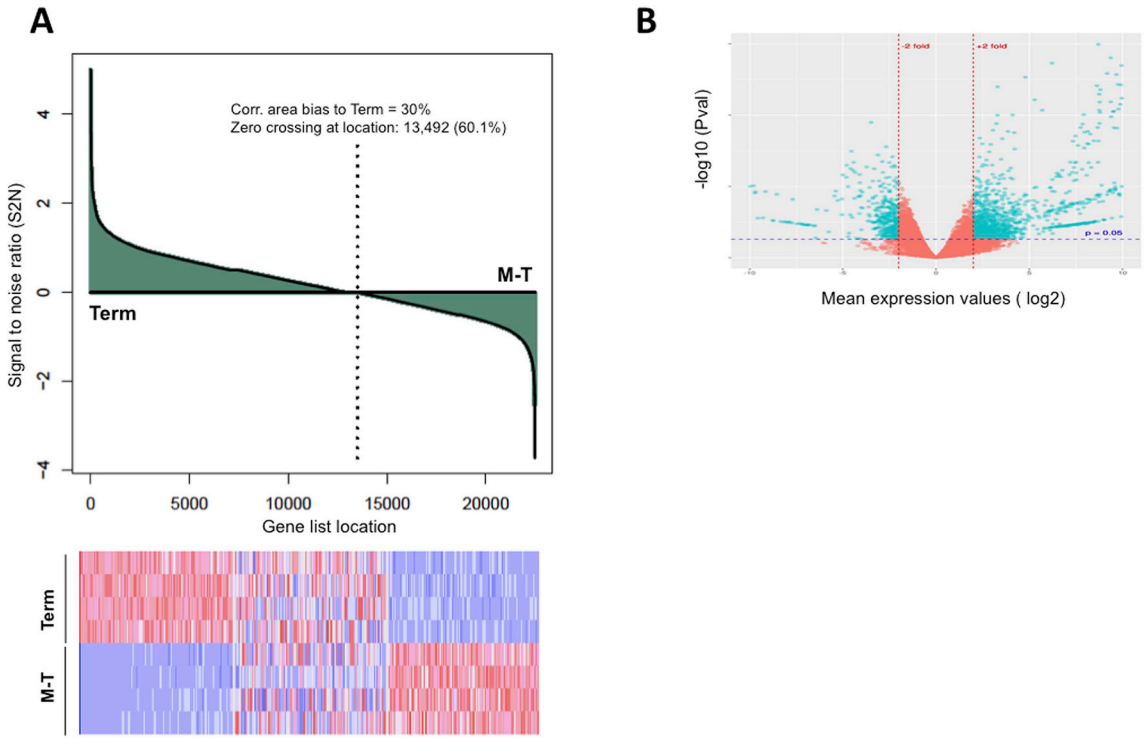

Fig. 1. The gene expression landscape of human CTB. (A) The S2N plot (upper panel) and expression values of corresponding genes from both groups (lower panel). Genes are ranked by S2N ratio, which was measured by the difference of means scaled by the standard deviation. (B) The volcano plot generated by DEG analysis. A zero-centered log2 fold change across the mean expression values indicates good data normalization. M-T, mid-trimester.

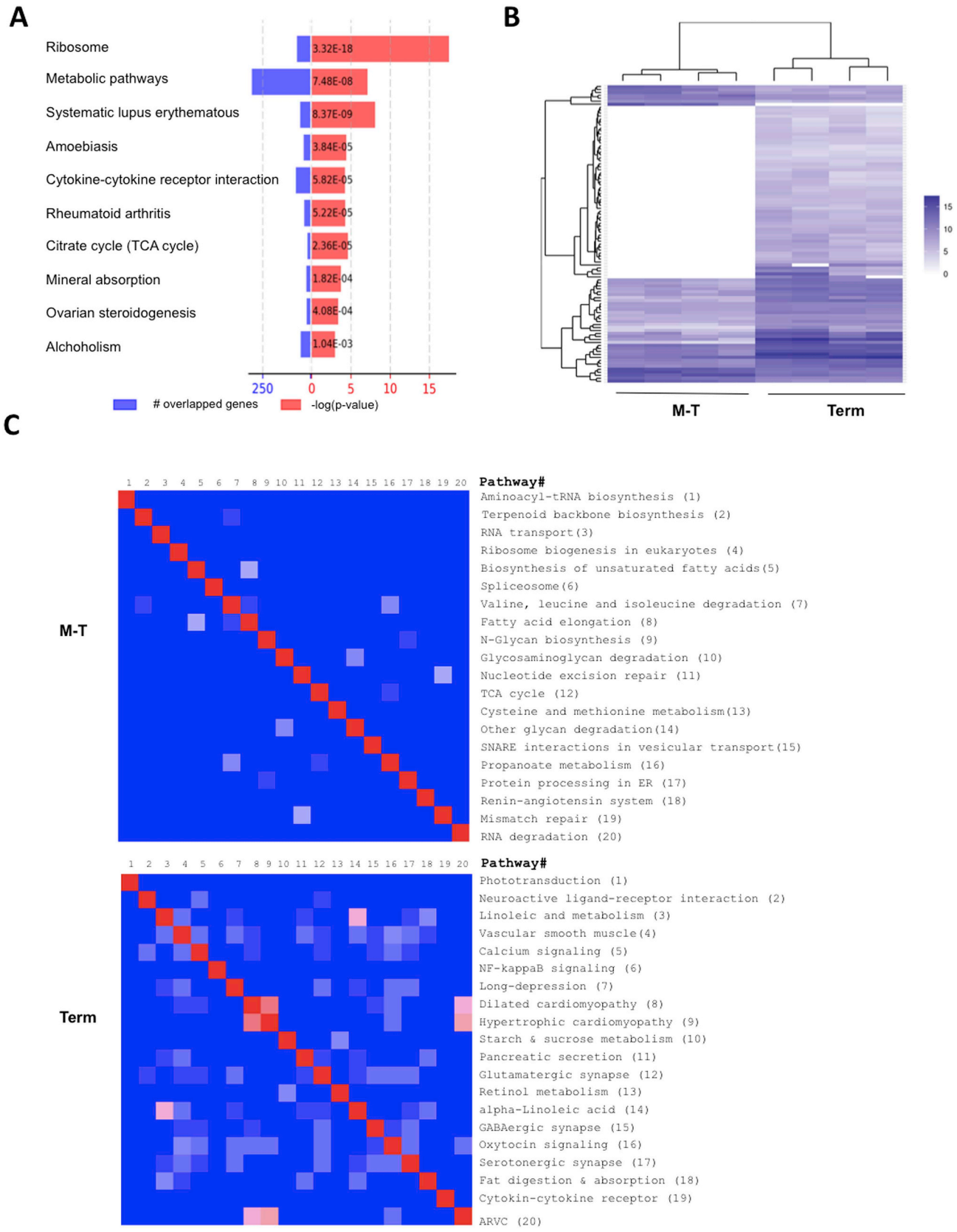

Fig. 2. The landscape of signaling pathways differentially enriched in mid-trimester and term CTB. (A) The pathways from KEGG_2016 enriched in the differentially-expressed genes. Blue bars indicate the number of overlapped genes of the pathway and differentially-expressed genes, and red bars are –log10(p-value) from the hypergeometric test of the genes from the pathway against the differentially-expressed genes. (B) The heatmap of expression values of top 100 differentially-expressed genes. (C) The set-to-set heatmap of leading edge genes. A dark red means set A and B have the same leading edge genes and a dark blue indicates no common leading edge genes between sets A and B. The color corresponds to the ratio of the number of common leading edge genes and the number of union leading edge genes in set A and B.

and term pregnancy.

3. Results

3.1. Transcriptome profiling of human villous CTB by RNA-seq

Across the whole genome, we found large differences in the relative expression of each transcript between term and mid-trimester (Fig. 1). Of 22,463 transcripts detected in human CTB, about 60.1% (13,492) had single to noise ratio (S2N) above 0 in term CTB. There were 19,880 transcripts detected in all 4 individual specimens for either group with a magnitude of change of 2-fold or greater.

3.2. Mid-trimester and term CTB are enriched in differential signaling pathways

With a cutoff of $p < 0.05$ and FDR < 0.25 reported by EdgeR, there were 3927 genes with a significant change in relative abundance with increasing gestational, among which 1896 were upregulated and the other 2013 were down-regulated at term compared to mid-trimester. Pathway analysis showed that the top ten pathways that involve these genes included ribosome, metabolic and cytokine-cytokine receptor (Fig. 2A and B, and Supplementary Table 1).

Further dissecting pathway analysis revealed that mid-trimester CTB cells were enriched in a total of 80 signaling pathways including terpenoid backbone biogenesis and protein translation that includes subgroups of aminoacyl-tRNA biosynthesis, RNA transport, and ribosome biogenesis (Fig. 2C and Supplementary Table 2). Given that steroids and sterols in mammals are biologically produced from terpenoid precursors [22], these results are consistent with the observation that the placenta needs to produce a large amount of steroid and peptide hormones such as progesterone, estradiol, and human chorionic gonadotropin for placentation and maintenance of pregnancy during early gestation [23]. In contrast, there was a total of 197 signaling pathways particularly enriched in the human placenta at term (Fig. 2C and Supplementary Table 3). Among the top 20 were neuroactive ligand-receptor interaction/oxytocin signaling, NF-κB signaling, and cytokine-cytokine receptor interaction, which have all been implicated in the onset of labor [9,24]. These results suggest a shift in human placental function, from maintaining pregnancy towards parturition.

3.3. Term human placenta is enriched with signaling pathways involved in the initiation of human labor

An increasing body of evidence has demonstrated that NF-κB transcription factor family plays an important role in the process of human labor and delivery [24,25]. The canonical NF-κB signaling (RelA/p50) has long been considered a prototypical pro-inflammatory pathway because it can activate varied types of pro-inflammatory genes including cytokines, chemokines, adhesion molecules, and ECM enzymes. A number of studies have shown that non-canonical NF-κB pathway (RelB/p52) is essential for lymphoid organogenesis and B-lymphocyte function. However, our recent studies have shown that both *CRH* and *PTGS2* are also regulated by this signaling pathway [7,14]. Indeed, pathway and gene enrichment analysis has revealed that expression of key genes constituting both canonical and non-canonical pathways including NFKB2 (p100/p52), NFKB1 (p105/p50), RELB (RelB), and RELA (RelA) were significantly elevated in term CTB (Fig. 3). Consistent with this increase, a variety of types of pro-inflammatory cytokines and chemokines regulated by NF-κB signaling including IL1B (IL-1β), TNF (TNF-α), CXCL8 (IL-8), CXCL1, CXCL2, and CXCL3 significantly increased with advancing gestation (Supplementary Fig. 1).

Because we have shown that RelB/p52, but not RelA/p50, is constitutively activated in villous trophoblast of normal term human placenta following C-section [7], these data suggest that upregulation of pro-labor mediators in term placenta could be a consequence of activation of non-canonical NF-κB signaling. Combined with increased expression of multiple ECM enzymes such as matrix metalloproteinase (MMP) in term placenta, these results further suggest that the signal molecules of human placental origin may play a critical role in pregnancy progression and the initiation of labor.

3.4. Epigenetic mechanisms regulating pro-labor genes in the human placenta

Indeed, our recent study has demonstrated that in term placenta, constitutively activated RelB/p52 heterodimers bind NF-κB response element of *CRH or PTGS2* gene promoter, which in turn stimulates recruitment of the lysine acetyltransferase CBP and histone deacetylase HDAC1 to selectively acetylate histone 3 lysine 9 (H3K9ac) and trigger epigenetic induction (Fig. 4A) [8]. Of note, in this study we had also profiled acetylation levels of H3K4, H3K9, H3K14, H3K18, H3K27, H4K5 H4K8, H4K12, and H4K16 in human CTB at both mid-trimester and term pregnancy. We showed that markedly increased acetylation only occurred at H3K9 in term placenta cells compared with mid-trimester cells. H3K27, acetylation of which is widely known as a transcriptional enhancer [26], is not acetylated in either mid-trimester or term CTB.

Here we sought to determine other genes regulated similarly by performing a genome-wide association study. ChIP was performed in normal term placenta with use of antibodies to ReB, CBP, HDAC1, and H3K9ac. We did not assess these ChIP assays in mid-trimester CTB because our recent study has unequivocally shown that non-canonical NF-κB signaling (RelB/p52) is not activated in early human pregnancy [27]. We chose RelB other than both of RelB and p52 because RelB always heterodimerizes with p100/p52 in villous trophoblast and only RelB contains a transactivation domain. With use of ChIP-seq, we found that there was a total of 563, 335, 319, and 901 genomic loci associated with ReB, CBP, HDAC1, and H3K9ac (Supplementary Table 4), respectively, and further overlapping analysis showed that 315 genomic coordinates were concurrently associated with RelB, CBP, HDAC1, and H3K9ac in term human placenta (Fig. 4Band C and Supplementary Fig. 2).

Taken together, these results suggest a difference of RelB-chromatin accessibility among different tissues. These results further suggest that there are at least 563 genomic coordinates allowing access of RelB followed by recruitment of CBP and/or HDAC1 to acetylate H3K9, and induce transcription of genes in the human placenta, which may contribute to onset of parturition with advancing pregnancy.

3.5. Integrated ChIP-seq and RNA-seq analysis

Dynamic acetylation of N-termini of histone H3 or H4 is generally associated with on-going gene transcription. We employed the combination of RNA-seq and ChIP-seq on a genome-wide level to assess which genes might be regulated similarly to *CRH* and *PTGS*2. To this end, we were particularly interested in genes that are associated with RelB, CBP, HDAC1, or H3K9ac as well as upregulated in term CTB as described in our previous study [8].

Integrated ChIP-seq and RNA-seq data analysis uncovered that there were 157 genes associated with RelB, 89 with CBP, 83 with HDAC1, and 299 with H3K9ac, the expression levels of which were significantly increased in term CTB compared to mid-trimester ones (Supplementary Table 5). Interestingly, pathway analysis showed that the leading three signaling pathways enriched across all four sets of ChIP-seq data were consistently NF-κB, TNF-signaling, and cytokine-cytokine receptor interaction (Fig. 5A–D).

Gene overlapping analysis demonstrated that among those genes with expression elevated in term CTB, 81 interacted with RelB, CBP, HDAC1, and H3K9ac simultaneously, 8 with RelB, CBP, and H3K9ac, 1 with RelB, HDAC1, and H3K9ac, and 67 with only RelB and H3K9ac (Fig. 5E). Of note, there was virtually no association of any genes with

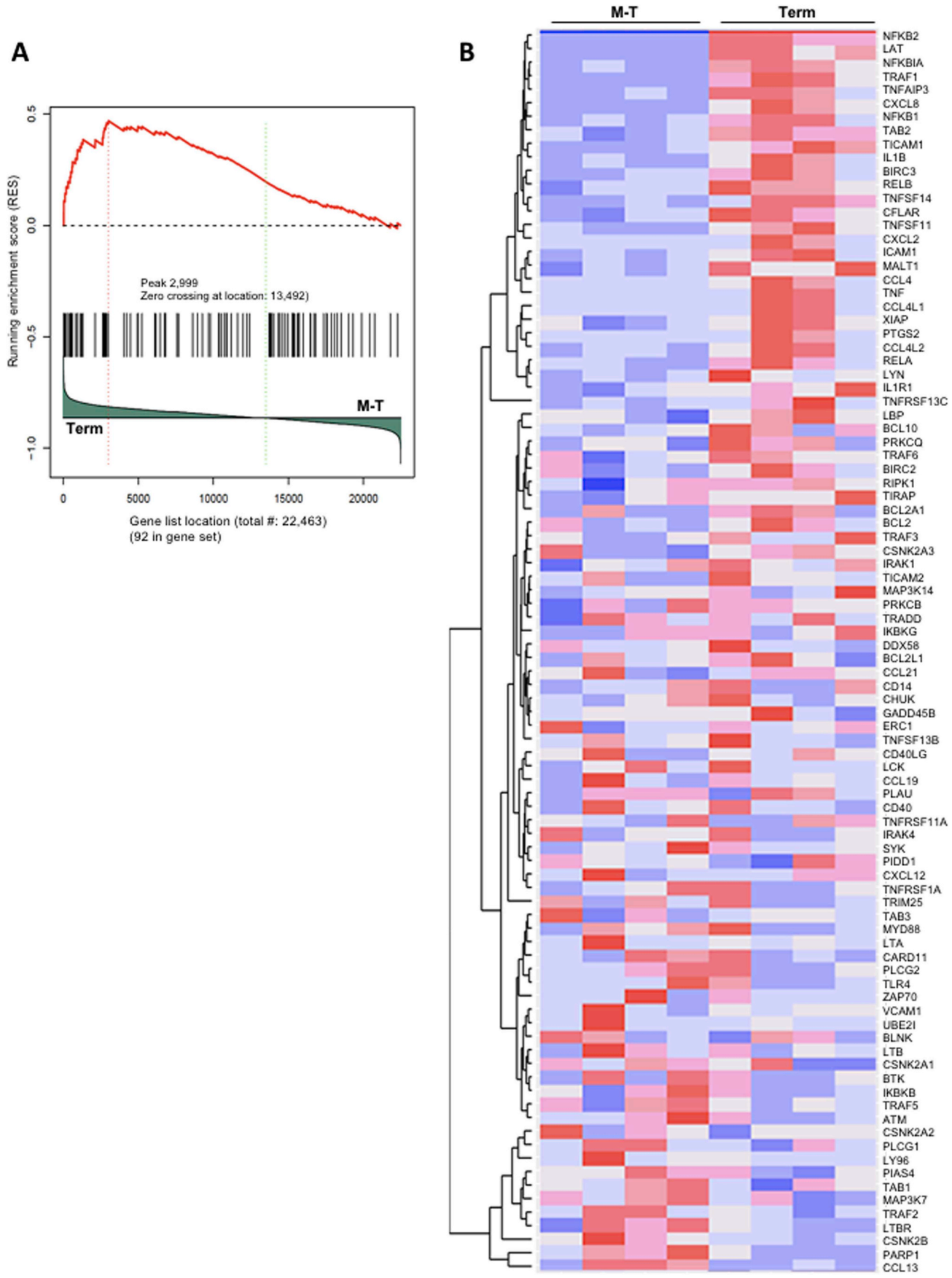

Fig. 3. Upregulation of NF-κB signaling pathway in term CTB. (A) The result plot from typical GSEA analysis. The genes are ranked by the S2N ratio (bottom panel). Across all genes, the running enrichment score is shown in the top panel and the indicators are drawn in the middle panel if the genes are present in the pathway. (B) The heatmap of expression values of leading edge genes.

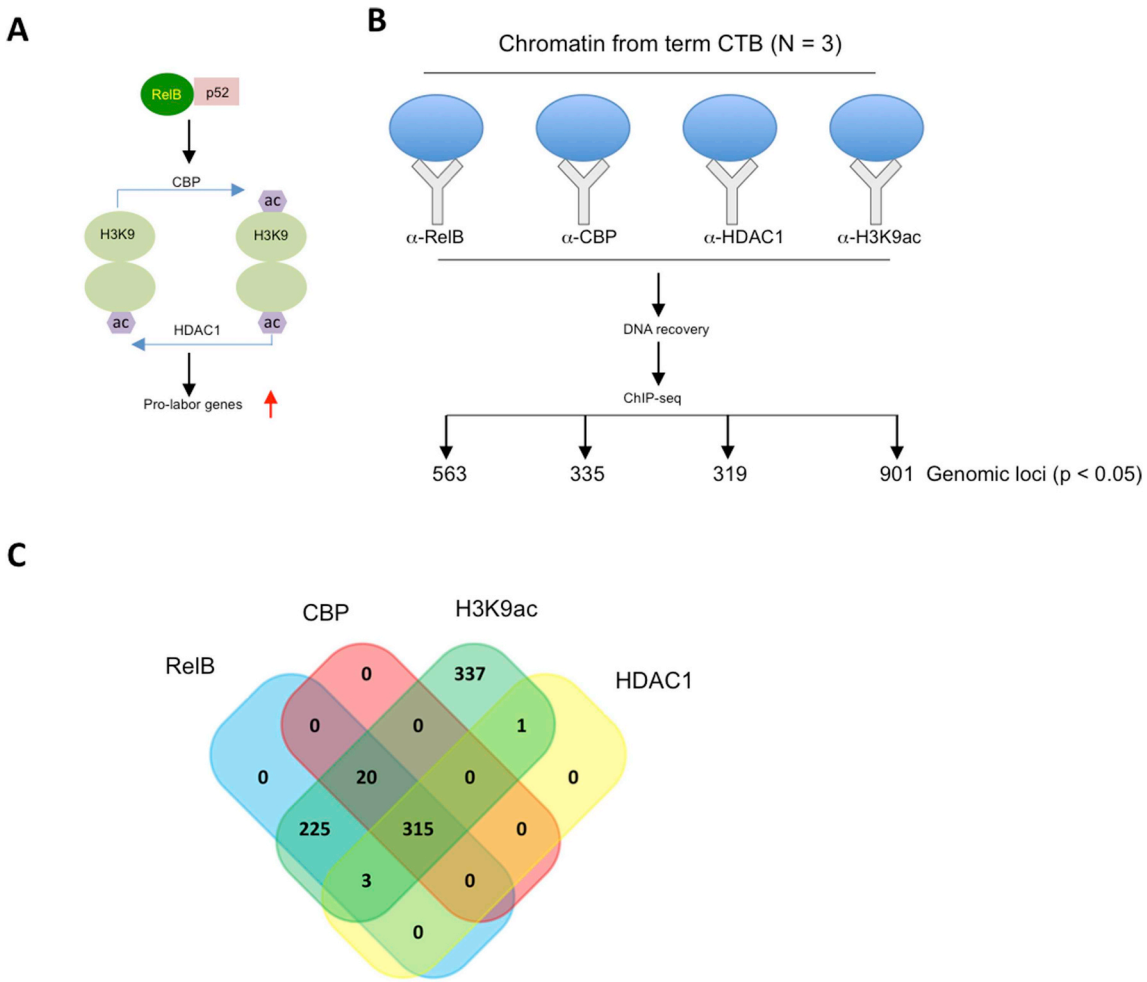

Fig. 4. Genomic loci associated with RelB, CBP, HDAC1, or H3K9ac in term CTB. (A) Proposed model whereby RelB/p52 dimers-induced dynamic acetylation (ac) of H3K9 is mediated by CBP and HDAC1. (B) The ChIP-seq experiments for 4 factors with 3 individual specimens for each of them. The genomic loci will be reported with a p-value if there are significant peaks located in the gene promoter/body. A genomic coordinate is identified as a target if it is differentially expressed and has a meta p-value < 0.05, which was a combined p-value from 3 duplicates using Fisher's method. (C) The Venn plot of overlapped genomic coordinates associated with those 4 factors.

CBP or HDAC1 in the absence of RelB, implying that non-canonical NF-κB pathway almost exclusively recruits these epigenetic regulators to target genes in the term placenta. Also of note is that activated RelB/p52 accounts for elevated expression of more than 50% of H3K9ac-regulated genes in term human placenta. Lastly, the only gene that associated with RelB, HDAC1, and H3K9ac but not CBP was *CRH*, the only gene whose product increases exponentially as gestation advances. Perhaps, transient association of CBP with leads *CRH* to a permanent epigenetic change that permanently activates the gene. This idea will need further study.

3.6. Identification of major pathways of placental origin involved in the initiation of human labor

Among 81 upregulated genes associated with RelB, CBP, HDAC1, and H3K9ac aforementioned (Supplementary Fig. 3), noticeably, included were NFKB2 (p100), COX-2, TNF-α, IL-8, CXCR4, and multiple CXCLs, which have been considered as important players for human parturition [24]. In addition, increased expression of OSM (oncostatin M), MMP14, CDKN1A, CCL3, TNFAI3, or SOCS3 has been observed in other gestational tissues including amnion and choriodecidua from patients following spontaneous labor [2,3]. Eight upregulated genes with RelB, CBP, and H3K9ac included IL-1β and MAP3K8, and CRH is the only one associated with only RelB, HDAC1, and H3K9ac. Among 67 upregulated genes associated with RelB and H3K9ac, we found UCN2, MMP19, ICAM1, and several TNF superfamily members (Fig. 6A). We further validated these results with use of RNAi-mediated gene silencing assay for select genes of different groups (Fig. 6B).

Pathway analyses demonstrated that these genes are involved in 11 signaling pathways including: 1) cytokine-cytokine receptor interaction, 2) TNF, 3) NF-κB, 4) MAPK, 5) Toll-like receptor, 6) NOD-like receptor, 7) JAK-STAT, 8) PI3K-AKT-mTOR, 9) Oxytocin, 10) Neuroendocrine, and 11) ECM remodeling. All these pathways have been confirmed/implicated as key drivers of human term and preterm labor.

4. Discussion

Combined with our previous studies [7,8,14], we propose that the following feed-forward mechanism could contribute to the initiation of human labor: First, as pregnancy progresses and the fetus grows and causes placental stress [28], free placental cortisol becomes available because fetal production exceeds the ability of placental 11β-hydroxysteroid dehydrogenase to oxidize it to inactive cortisone. Cortisol drives RelB transcription [14], which subsequently dimerizes with p100. Active RelB/p52 translocate into the nucleus to bind NF-κB response element of target gene promoter, and in turn, elicit recruitment of CBP and/or HDAC1 to regulate H3K9ac for epigenetic induction of

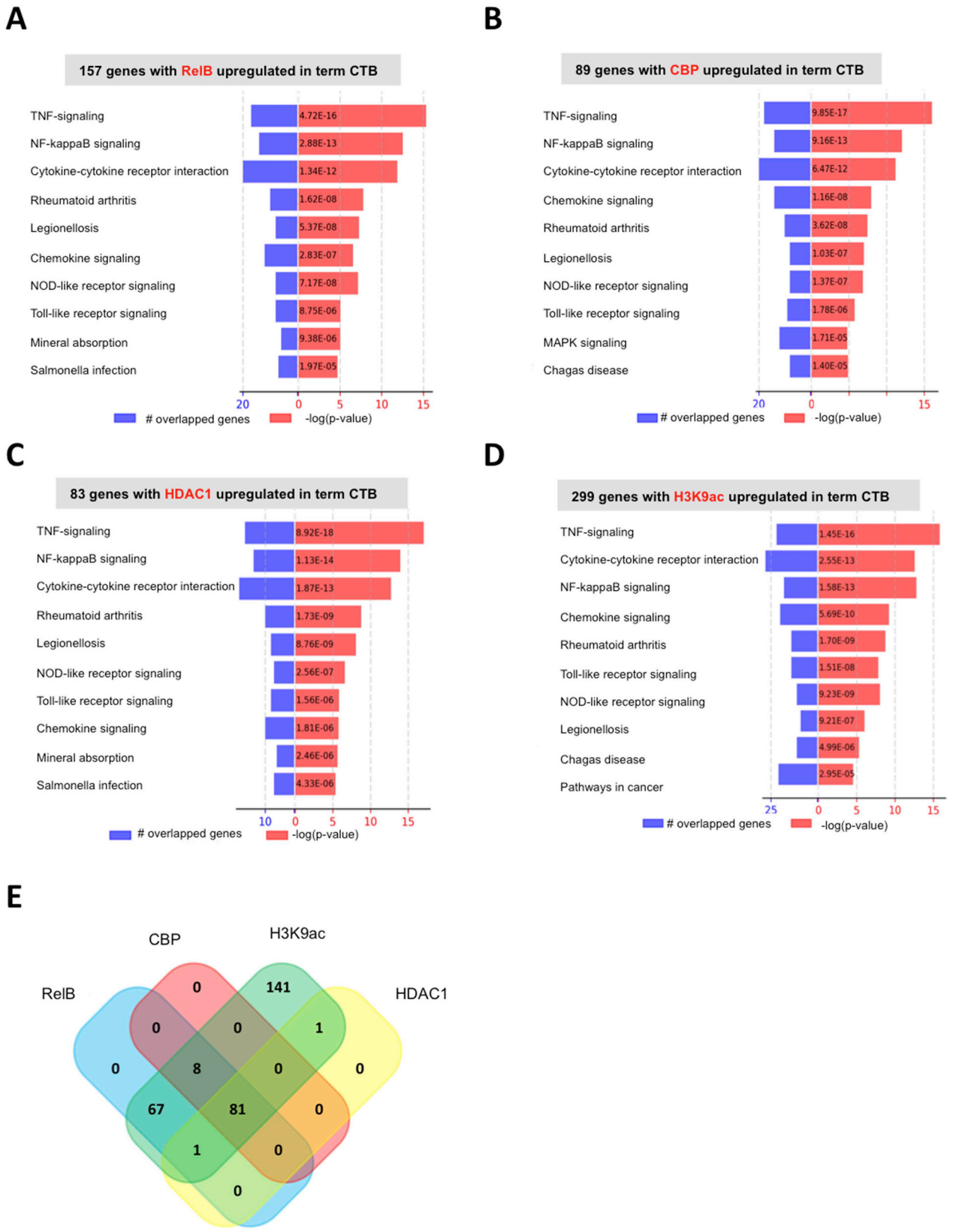

Fig. 5. Signaling pathways enriched in term CTB. (A–D) The pathways from KEGG_2016 enriched in the targets of the 4 factors, respectively. Blue bars indicate the number of overlapped genes of the pathway and target genes, and red bars are –log10(p-value) from the hypergeometric test of the genes from the pathway against the targets. (E) The Venn plot of overlapped upregulated target genes for the 4 factors.

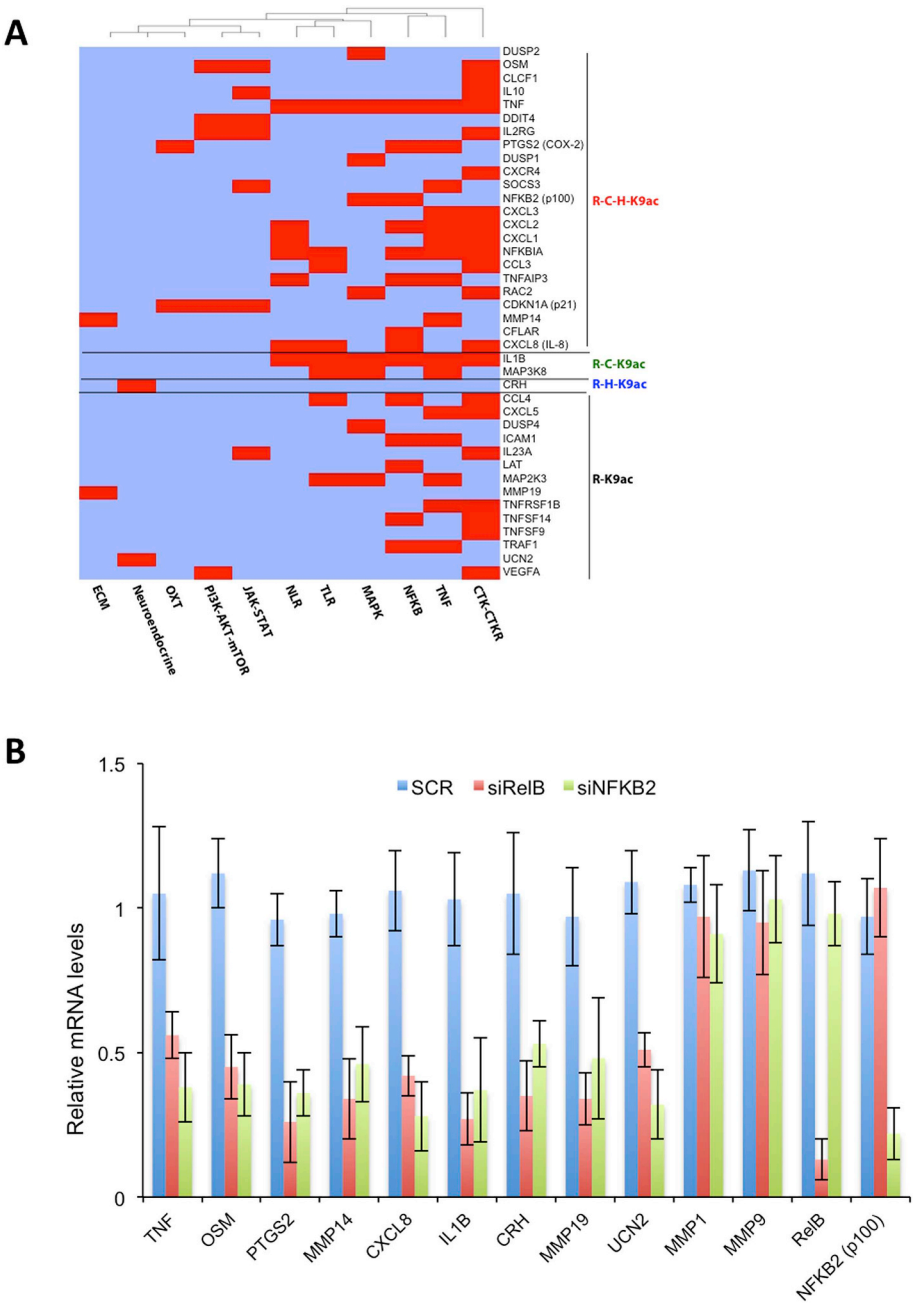

Fig. 6. Genes simultaneously associated with RelB, CBP, HDAC1, and H3K9ac and upregulated in term CTB. (A) Pathway analysis for overlapped upregulated genes for a combination of RelB (R) with CBP (C), HDAC1 (H), or H3K9ac (K9ac). TLR, Toll-like receptor; NLR, NOD-like receptor; OXT, oxytocin; ECM, extracellular matrix modeling, CTK, cytokine, CTKR, cytokine receptor, NFKB, NF-κB. (B) Validation of the role of RelB or p100 in regulation of select target genes from different combinations. Term CTB were transfected with siRNAs to RelB (siRelB) or p100/p52 (siNFKB2) with scramble siRNA (SCR) as the control, and mRNA levels were assessed by RT-qPCR (N = 3 individual experiments). *p < 0.01.

parturition triggers including NFKB2 (p100) *per se* (Fig. 7).

Integrated gene enrichment and pathway analysis based on RNA-seq and ChIP-seq data was performed to identify key functional pathways activated by NF-κB with advancing gestational age. CRH is considered to part of the placental clock that governs of the length of human pregnancy and initiation of labor. In addition, the highly enriched pathways in term placenta were largely pro-inflammatory in nature, and activation of which is closely associated with the onset of human parturition. Genes such as cytokines IL-8 and TNF, chemokines CXCLs, chemokine receptor CXCR4, and PTGS2 control immunomodulation, leukocytes trafficking, and synthesis of prostaglandins. Furthermore, the remarkable overlap identified in the current study with recently profiling studies in other gestational tissues [1–3] provides a compelling evidence for their involvement in human parturition and reassurance of the accuracy of the integrated analysis conducted in this study. Upregulation of SOCS3, OSM, IL10, and CDKN1A provides additional evidence for involvement of JAK-STAT [21,29,30] and mTOR signaling [31,32] in timing human birth, and supports functional consequences of the discerned changes in mRNA levels. Finally, Toll-like receptor, NOD-like receptor, and MAPK signaling have been implicated in preterm labor [33–35], and the current results provide further evidence for their link to human parturition.

There was a total of 234 genes associated with chromatin changes of RelB, CBP, HDAC1, and H3K9ac but with RNA levels unaltered or even down-regulated in normal term human placenta. It is known that H3K9 acetylation is an epigenetic mark of transcriptionally active chromatin, but H3K9 tri-methylation is a mark of transcriptional silencing [36]. Addison and colleagues have shown that in adipocytes, a concurrent loss of methylation and an increase of acetylation of H3K9 at the *Zfp423* promoter are indicative of decreased gene repression [37]. As such, turn-on or turn-off of transcription of H3K9-target genes is dependent on relative levels of methylation and acetylation.

Human parturition is a precisely coordinated process resulting from activation of a series of endocrine and immune responses. Preterm labor

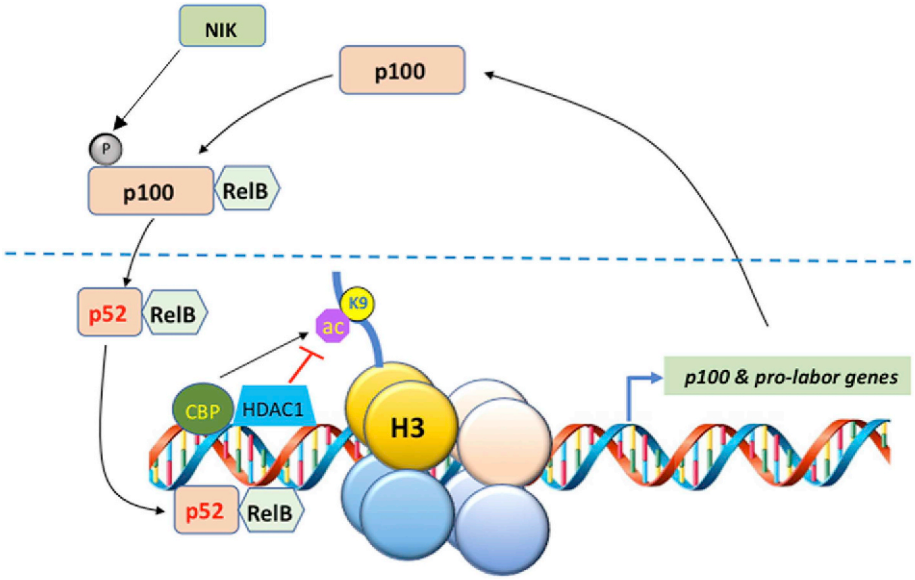

Fig. 7. A feed-forward mechanism regulating expression of key labor drivers in human placenta. NIK, NF-κB inducing kinase; K9, H3K9; ac, acetylation; P, phosphorylation.

is a syndrome initiated by multiple mechanisms including stress, endocrine disorders, and other immunologically mediated processes [38,39]. Overall, our study provides insights into the mechanisms underlying the key players of human placental origin with molecular changes resulting from chromatin modifications, which could drive human labor. As a result, such mechanisms could make highly attractive therapeutic targets in prevention of preterm labor.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.01.010.

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