COUP-TFII regulates hemoglobin switching by activating the BCL11A-XL repressor LIN28B and directly binding δ and β globin promoters in fetal *versus* adult erythroid cells

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Abstract

The reactivation of fetal globin genes is the most promising treatment for β-hemoglobinopathies. This implies the reversal of the naturally occurring hemoglobin switching. Here, we show that expression of the orphan nuclear receptor COUP-TFII in adult HUDEP2 erythroid precursor cells activates γ-globin (HbF) at the expense of β-adult globin by specific occupation of the 'adult' δ-β-region within the β-locus. Notably, although COUP-TFII and the main γ-globin repressor BCL11A-XL share a similar DNA binding consensus and a large number of chromatin targets, including the locus control region of the β-locus itself, they bind differentially to the γ and β promoters, eliciting an opposite transcriptional outcome. In addition, we find that COUP-TFII activates Lin28B, a known post-transcriptional repressor of BCL11A-XL. Our work identifies a molecular mechanism that could be leveraged to increase γ-globin levels in patients affected by β-hemoglobinopathies.

Introduction

The switching from fetal (HbF) to adult (HbB) globin expression is a crucial event during human development and the possibility of reverting it represents a concrete therapeutic option for patients suffering from β-hemoglobinopathies.[1,2] In this perspective, efforts to reactivate fetal γ-globin to cure β-hemoglobinopathies have primarily focused on targeting HbF repressors, with BCL11A-XL being one of the most important.[3-7]
Erythroid-specific inactivation of BCL11A-XL has been proven to be an effective therapeutic strategy,[1] emphasizing the importance of uncovering mechanisms and players that influence developmental globin gene expression. On the other hand, the molecular understanding of HbF activation is still largely unknown.
In this direction, we recently demonstrated that the orphan nuclear receptor COUP-TFII/NR2F2, which is physiologically expressed during mouse development in cells of yolk sac (YS) origin,[8] is capable of reactivating fetal globin when re-expressed in adult cells (including human Sardinian β^{039}-thalassemic cells), thus overcoming the repressive environment dictated by the presence of the full set of fetal globin repressors. However, its molecular mechanism of action remains largely unexplored.
Here, to understand the mechanistic basis of the ability of COUP-TFII to promote embryo/fetal erythropoiesis and to partially revert globins expression in adult cells, we undertook a genome-wide analysis of COUP-TFII binding in HUDEP cells.[9] These cells, immortalized from human cord blood, offer the unique advantage to model globin gene regulation in fetal *versus* adult erythropoietic environments: HUDEP1 uniquely express fetal γ-globin, whereas HUDEP2 express adult β-globin and the full set of γ-globin repressors, including BCL11A-XL. Since COUP-TFII recognizes on DNA the GGTCA motif of nuclear receptors,[10-12] which is virtually identical to that of BCL11A-XL,[13,14] HUDEP2 cells allow us to test whether the ability of COUP-TFII to

overcome γ-globin repression relies on its direct competition with BCL11A-XL for the occupancy of the same DNA sequences within the β-locus. In addition, the comparison of the CUT&RUN profile in HUDEP1 and HUDEP2 offers the possibility of assessing chromatin genomic redistribution of COUP-TFII in fetal-like *versus* adult-like cells and to identify targets, other than globin loci, relevant for controlling hemoglobin switching.
We discovered that COUP-TFII reactivates γ-globin in HUDEP2, where it occupies the adult δ and β promoters. Moreover, COUP-TFII likely contributes to the maintenance of the fetal-like globins' expression profile by directly activating the γ-globin inducer LIN28B, a post-transcriptional BCL11A-XL repressor.[10,11]
The discovery of these novel mechanisms of COUP-TFII-mediated γ activation offers a new perspective for developing strategies to treat β-hemoglobinopathies.

Methods

Single cell analysis

Single-cell RNA sequencing data were obtained from Popescu *et al.*[12] (E-MTAB-7407) via the Human Developmental Cell Atlas and from Ranzoni *et al.*[13] (E-MTAB-9067). For fetal livers, further data processing and analysis were performed using Scanpy (version 1.10.4) and Numpy (version 2.0.2). Cells with fewer than 200 detected genes were excluded, and cells with mitochondrial DNA content exceeding 5% were also removed to reduce potential biases from stressed or dying cells. After normalization, fetal liver datasets were annotated with Celltypist (v1.6.3), applying the pre-trained "Pan_Fetal_Human.pkl" model to ensure consistency across analyses. For YS data, no additional pre-processing steps were applied and cell type annotation followed the original authors' classification. The results were visualized using UMAP embeddings.

Cells lines

HUDEP1 and HUDEP2 cells were grown according to standard protocols.[9] Details of media and conditions are provided in Table 1.

Constructs

The Nr2f2 murine cDNA was cloned into the IRES-EmeraldGFP (eGFP) pHR SIN BX IR/EMW lentiviral vector.[8] psPAX2 and pMD2.GVSVG packaging plasmids were used to produce lentiviral pseudo-particles in HEK293T cells (lentiweb.com).

Lentiviral transduction

Seventy-two hours post HEK293T cell transfection, recombinant virus particles were collected and titrated on K562 cells. A MOI of 20-30 was used to transduce HUDEP1 and HUDEP2 cells. The percentage of infected cells (scored as eGFP^{+} cells) used in the experiments was ≥75% (*Online Supplementary Figure S1*).

RNA isolation and real-time quantitative polymerase chain reaction

Total RNA was purified with TRIzol Reagent (Euroclone) and retrotranscribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem). Real-time analysis was performed on a StepOne™ instrument (Applied Biosystems) by using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). Primers are listed in Table 2.

Table 1. HUDEP cell growing media.

Cell line	Medium	Supplements
HUDEP proliferation	IMDM	0.2% HSA 0.1% lipid mixture 300 μg/mL transferrin 10 μg/mL insulin 100 μM sodium pyruvate 2 U/mL EPO 100 ng/mL SCF 1 μM dexamethasone 2 μg/mL doxycycline
HUDEP 72-hr differentiation	IMDM	3% HSA 0.1% lipid mixture 1000 μg/mL transferrin 10 μg/mL insulin 100 μM sodium pyruvate 10 U/mL EPO 2% FBS

EPO: erythropoietin; FBS: fetal bovine serum; hr: hour; HSA: human serum albumin; IMDM: Iscove's Modified Dulbecco's Medium; SCF: stem cell factor.

Table 2. List of real-time quantitative polymerase chain reaction primers.

Primers used for RT-PCR		
Gene	**F/R**	**Sequence (5'-3')**
GAPDH	F R	ACGGATTTGGTCGTATTGGG TGATTTTGGAGGGATCTCGC
HBA	F R	GAGGCCCTGGAGAGGATGTTCC ACAGCGCGTTGGGCATGTCGTC
HBB	F R	TACATTTGCTTCTGACACAAC ACAGATCCCCAAAGGAC
HBG	F R	CTTCAAGCTCCTGGGAAATGT GCAGAATAAAGCCTATC TTGAAAG
HBE	F R	GCCTGTGGAGCAAGATGAAT GCGGGCTTGAGGTTGT
LIN28B	F R	GCGCATGGGATTTGGATTCA ACTTCCTAAACAGGGGCTCC
HBZ	F R	TCTGACCAAGACTGAGAGA TTGAAGTTGACCGGGTCCAC

F: forward; R: reverse; RT-PCR: real-time polymerase chain reaction.

Western blot

Total and nuclear extracts were prepared according to standard procedures. Protein extracts (5-10 μg/lane) were resolved by SDS/PAGE in a 10-15% acrylamide gel and blotted onto Hybond-ECL nitrocellulose membrane (GE healthcare). Membranes were blocked, incubated with the appropriate antibodies and, after washing, ECL reagent (Millipore) was used for detection. Antibodies are listed in Table 3.

CUT&RUN

CUT&RUN-LoV-U was performed as described in Zambanini *et al.*[14] A total of 500,000 cells were harvested per each sample (HUDEP1/2 untransduced or transduced with the COUPT-FII vector). Cells were washed three times with nuclear extraction buffer and the nuclei were bound to Magnetic ConA Agarose beads. Samples were incubated with antibodies overnight. Antibody in excess was washed and the samples where incubated with pAG-MNase for 30 minutes (min) at 4°C. pAG-MNase in excess was washed and digestion was initiated on ice using CaCl2 (2 mM) and stopped in STOP buffer after precisely 30 min. Digestion buffer was collected and the beads were resuspended in Urea STOP buffer for DNA elution for 1 hour at 4°C. Digestion buffer of each sample was mixed with the corresponding DNA elution. DNA was bound to beads, the supernatant was discarded, and beads were washed two times in Ethanol 80%. DNA was eluted in Tris-HCl and the procedure was repeated twice, adding new beads. Library preparation and data processing are detailed in the *Online Supplementary Methods*.

Electrophoretic mobility shift assay

Nuclear extracts from COS7 cells transfected either with a BCL11A-XL-myc or a COUP-TFII-Flag vector were prepared according to previously published procedures.[15] The oligonucleotide sequence, from the ApoA1 enhancer: FW: 5' ACTGAACCCTTGACCCTGCCCT, REV: 5' AGGGCAGGGTCAAGGGTTCAGT. ^{32}P.labeled DNA oligonucleotide probe was incubated for binding with 1-3 μg of nuclear extracts (NE) for 20 min at room temperature in a buffer containing 5% glycerol, 50 mM NaCl, 20 mM Tris pH7.9, 0.5 mM EDTA, 5 mM MgCl, 1 mM DTT, 100 ng/mL poly(dI-dC), 50 ng/mL BSA and 0.05% PFA in a 15 μL final reaction mixture. Protein-DNA complexes were then separated on a 5% polyacrylamide gel (29:1 acrylamide / bisacrylamide ratio) and visualized by autoradiography. BCL11A-XL and COUP-TFII bands were univocally identified by using anti-myc (Ab32 Abcam) and anti-Flag (F7425 Sigma-Aldrich) antibodies.

Results

COUP-TFII expression in early human erythropoiesis

In E11.5 mouse embryonic liver, *Coup-TFII* is expressed in erythroid-myeloid-progenitors (EMP) and decreases as these cells undergo erythroid maturation.[8] To understand whether this expression pattern is conserved in human erythropoiesis, we analyzed three single-cell RNAseq datasets, representative of three different timepoints: 4-7 post conception week (PCW) YS,[12] 7-17 PCW fetal liver,[12] and 17-22 PCW fetal liver[13] (Figure 1). The 4-7 PCW YS dataset by Popescu *et al.*[12] identifies two discrete cell populations expressing Coup-TFII (left panel). The first population is annotated as endothelium and expresses high levels of COUP-TFII and low levels of globins. The second population, annotated as megakaryocyte-erythroid-mast cell progenitors (MEMP), co-expresses COUP-TFII and globins (predominantly HBG2).

Discrete cell populations co-expressing COUP-TFII and globin genes (COUP-TFII$^+$globins$^+$) are also identified in fetal liver by two independent datasets, representative of

Table 3. List of antibodies and reagents.

Antibodies and reagents	Cat N	Manufacturer
Anti-FLAG	F7425	Sigma-Aldrich
Anti-Coup CUTandRUN A	ABIN6928040	Antibodies-online
Anti-Coup CUTandRUN B and WB	ab41859	Abcam
Anti Lin28B	4196	Cell Signaling
Anti U2AF	U4758	Sigma-Aldrich
Anti Vinculin	AB129002	Abcam
Anti HbG	AB283313	Abcam
rIgG	PP64	Millipore
Phosphate-buffered saline	ECB4004L	Euroclone
Fetal bovine serum	F7524	Sigma-Aldrich
L-glutamine	ECB3000D	Euroclone
Penicillin-streptomycin	ECB3001D	Euroclone
High capacity cDNA RT Kit	4368814	Applied Biosystems
SsoAdvanced Universal SYBR^w Green	1725274	Bio-Rad
IMDM medium	P04-20250	PAN
Human serum albumin	P06-27100	PAN
rh SCF	11343325	ImmunoTools
rh EPO	11344795	ImmunoTools
Dexamethasone	D4902	Sigma-Aldrich
Doxycicline	D9891	Sigma-Aldrich
Lipid mixture	L0288	Sigma-Aldrich
Sodium pyruvate	P5280	Sigma-Aldrich
Insulin	I6634	Sigma-Aldrich
Transferrin	T1147	Sigma-Aldrich

Cat N: catalog number. EPO: erythropoietin; IMDM: Iscove's Modified Dulbecco's Medium; rh: human recombinant; SCF: stem cell factor.

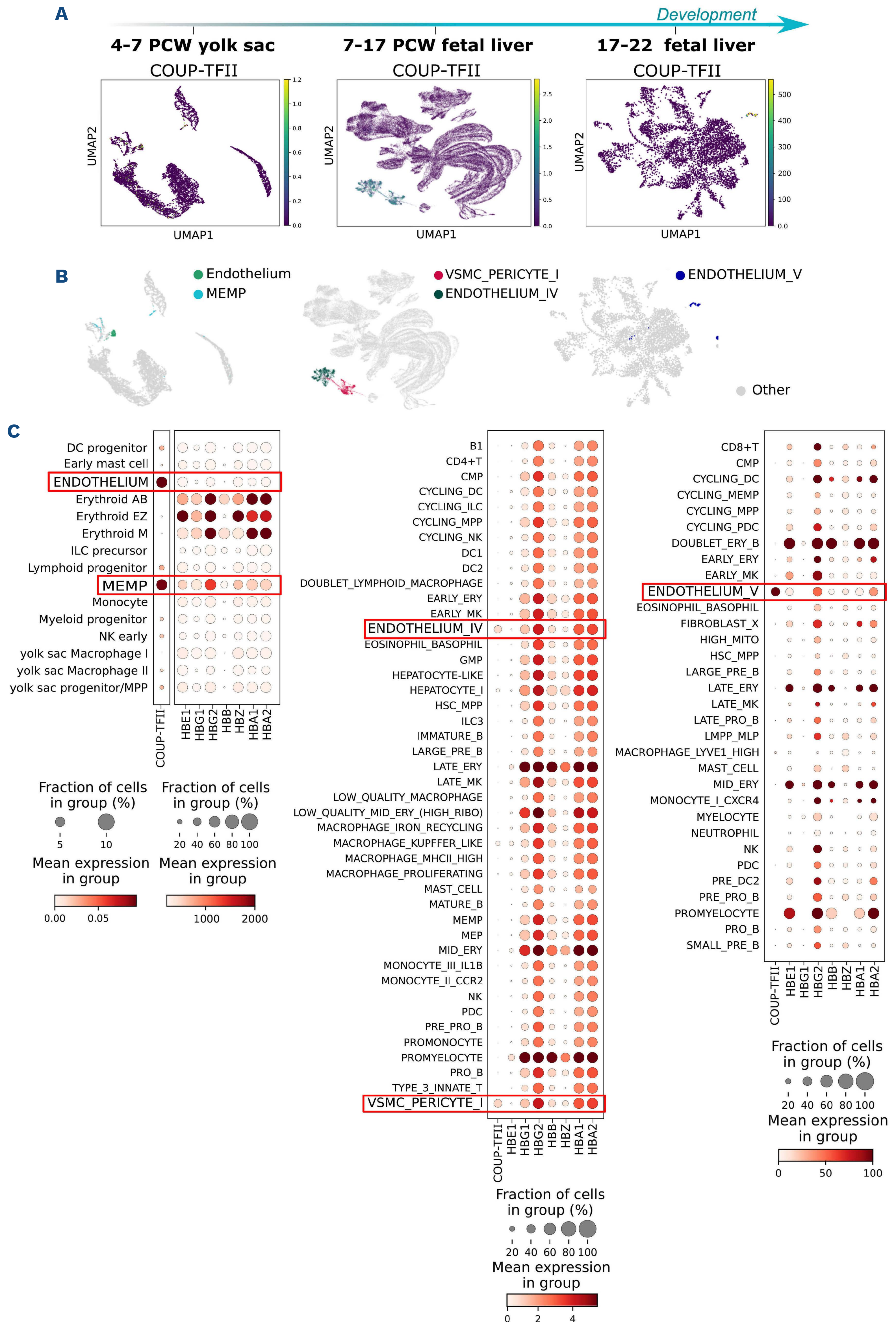

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Figure 1. Co-expression of COUP-TFII and γ-globin in human early embryonic yolk sac and fetal liver. (A) UMAP from single-cell RNA-seq data of the 4-7 post conception week (PCW) yolk sac[12] (left), 7-17 PCW fetal liver[17] (middle), and 17-22 PCW fetal liver[13] (right). COUP-TFII-expressing cells are highlighted according to the color key scale flanking each panel. (B) Cell type annotations for COUP-TFII positive clusters are shown for each dataset. (C) Dot plots displaying co-expression of COUP-TFII and globin genes across identified cell types in each dataset (rectangles). Circle size represents the fraction of cells expressing each gene, and color intensity reflects gene expression levels. MEMP: megakaryocyte-erythroid-mast cell progenitor; VSMC: vascular smooth muscle cell.

consecutive developmental stages (Figure 1, middle and right panels). In both fetal liver datasets, a COUP-TFII^{+-} globin^{+} population is identified as endothelium. At 7-17 PWC, an additional population of 'vascular smooth muscle cell (VSMC) pericytes' is identified. The genes commonly expressed at this stage by these two COUP-TFII^{+}globin^{+} populations, are associated with Diamond-Blackfan anemia (DBA) by the Orphanet database of rare diseases (https://maayanlab.cloud/Enrichr/enrich?dataset=608a66f9e8a7bd75e308bd91e8ed9024). DBA is a rare congenital aplastic anemia with erythroblastopenia affecting very early erythroid progenitors. This observation is consistent with early erythroid features.

In all three datasets, COUP-TFII expression becomes undetectable in highly hemoglobinized cells, compatible with COUP-TFII decline during erythroid differentiation, as we previously observed in mouse.[8] The "endothelium", "vascular smooth muscle cell (VSMC) pericytes", and "MEMP" annotations are in line with the proposed common ontogenic origin of these cell types.[16-18]

COUP-TFII and BCL11A-XL interplay

In adult cells, including thalassemic β^{039} cells, COUP-TFII re-expression induces γ-globin at the expense of adult β-globin.[8] Here, to study the molecular mechanism of COUP-TFII-mediated γ activation in adult cells, we re-expressed it in adult-like HUDEP2 (expressing adult β-globin) and in fetal-like HUDEP1 (expressing fetal γ-globin) cord blood-derived progenitors. In HUDEP2, COUP-TFII induces γ-globin both at the RNA (Figure 2A) and protein level (Figure 2B), as expected. The BCL11A-XL consensus site on DNA is identical to the canonical COUP-TFII binding motif (GGTCA) (Figure 2C). On this basis, we hypothesized that COUP-TFII could directly compete with BCL11A-XL for its genomic occupancy at the γ-globin promoters.[19] To test this hypothesis, we first checked for the ability of BCL11A-XL to bind *in vitro* in EMSA assays to a well-known COUP-TFII DNA consensus on the ApoAI enhancer.[20] We observed that this probe is indeed bound by both proteins, each univocally identified by supershift with specific antibodies (Figure 2D). We then mapped the genome-wide occupancy of COUP-TFII in HUDEP2 by CUT&RUN and we compared it with that of BCL11A-XL, published by Liu *et al.*[19] As a control, we also performed COUP-TFII CUT&RUN in fetal-like HUDEP1 cells, which do not express BCL11A-XL and express high levels of γ-globin. To this end, we generated both HUDEP1 and HUDEP2 cells expressing a tagged version of COUP-TFII (*Online Supplementary Figures S1, S2*).

The comparison of CUT&RUN datasets for BCL11A-XL and COUP-TFII in HUDEP2 cells revealed a substantial number of common peaks (Figure 2E, F), as well as protein-specific genomic binding sites. Of interest in this context, the top differentially expressed genes (DEG) associated with peaks generated by COUP-TFII (HUDEP1 and HUDEP2 results merged) are similar to the genes perturbed by Bcl11a knockout in mice (https://maayanlab.cloud/Enrichr/enrich?dataset=bfd73c13b56ba7bbecd7a75c3e725f5c), supporting the idea that these two proteins could regulate the same genes in opposite directions by competing for DNA occupancy of the same sequences (Figure 2G).

To specifically address COUP-TFII-BCL11A-XL interplay in globin regulation, we mapped their binding within the β-locus in HUDEP2 (Figure 2H), taking advantage of data published by Liu *et al.*[19] This analysis revealed that the two proteins share common peaks at the locus control region (LCR). In particular, COUP-TFII is recruited to the DNAseI hypersensitive sites (DHS), where it prominently occupies the β-HS3 (Figure 2H). Surprisingly, COUP-TFII does not bind to the GGTCA sequences within the γ-promoters, which are the targets of BCL11A-XL.[19] Instead, COUP-TFII occupies the 'adult' δ- and β-globin promoters (Figure 2H), likely interfering with their interaction with the LCR.

COUP-TFII binding to the globin loci

To deepen our analysis, we generated two additional replicates of the COUP-TFII CUT&RUN experiment using an additional COUP-TFII antibody and we considered only the peaks present in at least three out of the four replicates (*Online Supplementary Figures S2*). This analysis returns a COUP-TFII binding profile at the β-locus, shown in Figure 3A, which is superimposable to the one in Figure 2H. Further footprinting mapping confirmed COUP-TFII binding at the δ and β promoters (Figure 3B). This region is a known target of natural deletional hereditary persistence of fetal hemoglobin (HPFH)[21] and of artificial deletions created to mimic HPFH phenotype (Figure 3C).

In HUDEP1 cells, which are devoid of BCL11A-XL and express γ-globin levels about 40 times higher than in HUDEP2 cells (calculated as ratio on GAPDH), COUP-TFII still binds to the LCR region (Figure 3D). However, COUP-TFII is not detected at the 'adult' promoters, revealing its ability to rearrange its occupancy within the locus during development. In these same cells, COUP-TFII expression increases ε-globin but not the already high and likely saturated

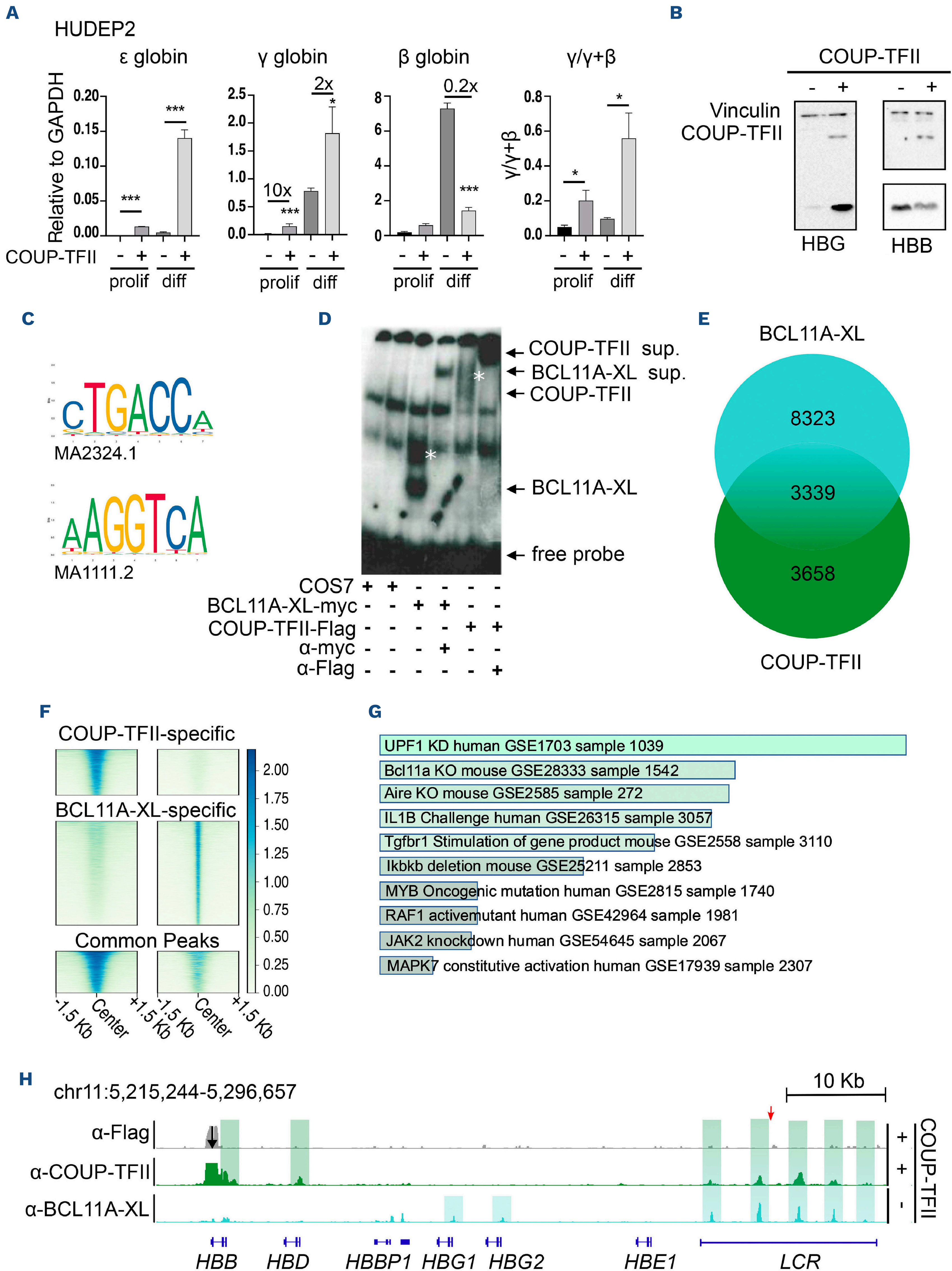

Figure 2. COUP-TFII reactivates embryo/fetal globin genes in HUDEP2 cells. (A) Real-time quantitative polymerase chain reaction analysis of mRNA levels for the indicated globin genes in HUDEP2 upon transduction with the empty vector (-) or with the COUP-

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TFII expressing vector (+) in proliferation and differentiation conditions. Expression levels are expressed relative to GAPDH and in relative ratios (N ≥3, *P≤0.05, ***P≤0.001). (B) Representative western blots showing the increase in γ-globin and the parallel decrease in β-globin at the protein level in HUDEP2 cells expressing COUP-TFII (total protein extracts [TE] 48 hours of differentiation). Given the abundance of β-globin, different exposures are shown for optimal visualization of the bands. (C) Jaspar matrix of DNA binding sites recognized by COUP-TFII and BCL11A-XL. (D) Electrophoretic mobility shift assay (EMSA): nuclear extracts from COS7 cells transfected either with a BCL11A-XL-myc or a COUP-TFII-Flag vector were incubated with a probe corresponding to the COUP-TFII target region on the ApoAI enhancer,[20] containing a double COUP-TFII consensus site. BCL11A-XL and COUP-TFII were univocally identified by using α-myc (Ab32 Abcam) and α-Flag (F7425 Sigma-Aldrich) antibodies. Asterisks indicate bands that are likely due to the binding of two molecules of the same protein. (E) Venn diagram showing overlapping BCL11A-XL and COUP-TFII peaks in HUDEP2 cells. (F) Signal intensity plot summarizing the replicate average signal intensities across different genomic regions for common and specific COUP-TFII- and BCL11A-XL-specific peaks in HUDEP2. COUP-TFII (left) and BCL11A-XL (right). Two replicates per each factor were considered for the analysis. (G) List of differentially expressed genes (DEG) following TF perturbation that are the most similar to the list of genes associated with COUP-TFII peaks (HUDEP1 and HUDEP2 merged) (https://maayanlab.cloud/Enrichr/enrich?dataset=bfd73c13b56ba7bbecd7a75c3e725f5c). (H) IGV visualization for COUP-TFII and BCL11A-XL (data from Liu *et al.*[19]) CUT&RUN profiles at the β-globin locus. The arrow marks an unspecific CUT&RUN artifact, also generated by the anti-Flag control antibody, likely caused by the extreme accessibility of the region in erythroid cells.[30,31] See Methods section for data processing for the comparison of the two data sets.

γ-globin expression (Figure 3E).

Notably, COUP-TFII, while activating γ-globin, in parallel, it reduces β-globin transcription (Figure 2A). This occurs in both HUDEP2 and HUDEP1 cells, where β-globin is expressed at marginal levels (Figure 3E).

Finally, at the α-globin locus, COUP-TFII shows a similar occupancy pattern in HUDEP1 and HUDEP2 and favors embryonic ζ-globin expression when compared to α-globin (*Online Supplementary Figure S3*).

COUP-TFII contributes to the maintenance of the fetal-like environment by activating LIN28B

Our CUT&RUN dataset allowed us to search for potential direct regulation by COUP-TFII of additional genes involved in hemoglobin switching. We focused on BCL11A-XL and LIN28B, selectively expressed in adult-like HUDEP2 and in fetal-like HUDEP1 cells, respectively. In HUDEP2 cells, COUP-TFII occupies the +55Kb DNAseI hypersensitive site within the known *BCL11A* erythroid enhancer[22] and weakly represses its transcription in differentiation conditions (Figure 4A). In HUDEP1, we could not detect COUP-TFII peaks within the *BCL11A* locus, which has a closed chromatin conformation (see Huang *et al*. for ATACseq data[23]). HUDEP1 cells express *LIN28B*, an oncofetal gene[24] encoding for an RNA binding protein capable of activating γ-globin in adult cells[10] by interfering with BCL11A-XL translation.[11] In these cells, we show that COUP-TFII activates LIN28B, increasing both its RNA and protein levels (Figure 4B). In HUDEP2 cells, where the LIN28B locus is not accessible, COUP-TFII does not bind to the LIN28B locus (see Huang *et al*. for data[23]). Together, these data suggest that, depending on chromatin accessibility, COUP-TFII can contribute to the fetal-to-adult transition through cell-type selective occupancy at *BCL11A* and *LIN28B* loci (as proposed in Figure 4C).

Discussion

Research aiming at reactivating fetal globin in patients suffering from β-hemoglobinopathies has so far focused exclusively on targeting γ-repressors. How γ-globin is activated in embryo/fetal cells remains largely unclear. We recently reported for the first time that COUP-TFII activates γ-globin at the expense of the adult β-globin gene in human normal and β-thalassemic cells.[8]

In the present work, to understand how COUP-TFII overrides fetal globin repression in adult cells, partially restoring a fetal-like environment, we profiled its expression in early human development in scRNA dataset (Figure1) and we mapped its genomic occupancy by CUT&RUN in HUDEP1 (fetal-like, γ-expressing) and in HUDEP2 (adult-like, β-expressing) cells. HUDEP1 γ-expressing cells allow us to model human fetal-like erythropoietic cells, which are not readily accessible for both ethical and technical reasons. The comparison of COUP-TFII genomic occupancy in HUDEP1 and HUDEP2 cells revealed a significant genomic redistribution of COUP-TFII at the globin loci. COUP-TFII, when expressed in adult-like HUDEP2 cells, activates γ-globin both at the RNA and protein level (Figure 2A, B), as expected on the basis of our previous results.[8] Since COUP-TFII shares the same core DNA consensus with BCL11A-XL, the major γ-globin repressor in adult cells (Figure 2C), and both proteins are able to recognize the same sequence *in vitro* (electrophoretic mobility shift assay in Figure 2D), we compared their occupancy profile at the β-locus in detail (Figure 2H). Although the two proteins share a large number of genomic targets (Figure 2E, F), they also show significant differences. In both HUDEP1 and HUDEP2, COUP-TFII binds to the LCR, suggesting that it could participate in the transcription complexes assembled at the LCR that co-ordinate the sequential activation of globin gene transcription during development.[25] However, surprisingly, COUP-TFII does not bind to the GGTCA sequences within the γ-promoters, which are the targets of BCL11A-XL.[19] Instead, COUP-TFII occupies the 'adult' δ- and β-globin promoters (Figure 2H). In this scenario, we hypothesize that the presence of COUP-TFII at δ and β promoters could hinder or weaken their interac-

Figure 3. In depth analysis of COUP-TFII binding to the adult δ and β promoters, within a region target of deletional HPFH. (A) IGV visualization for COUP-TFII CUT&RUN profile at the β-globin locus in HUDEP2 cells considering 3 out of 4 peaks, obtained with two independent antibodies. The arrow marks an unspecific CUT&RUN artifact. The plot is superimposable to that obtained with the previous analysis in Figure 2H. (B) Footprints at the δ and β promoters. (C) Alignment of COUP-TFII peaks with some overlapping, naturally occurring deletional HPFH within the β-locus. Deletions involving both δ and β COUP-TFII-bound promoters (in red) are amongst the ones associated with the highest γ-globin levels. HbF levels are indicated beside each type of deletion.[32] (D and E) HUDEP1 cell analysis: COUP-TFII occupancy mapped by CUT&RUN (D) and real-time quantitative polymerase chain reaction on globin genes. (E) Expression levels are expressed relative to GAPDH and in relative ratios (N ≥3, *P≤0.05, ***P≤0.001).

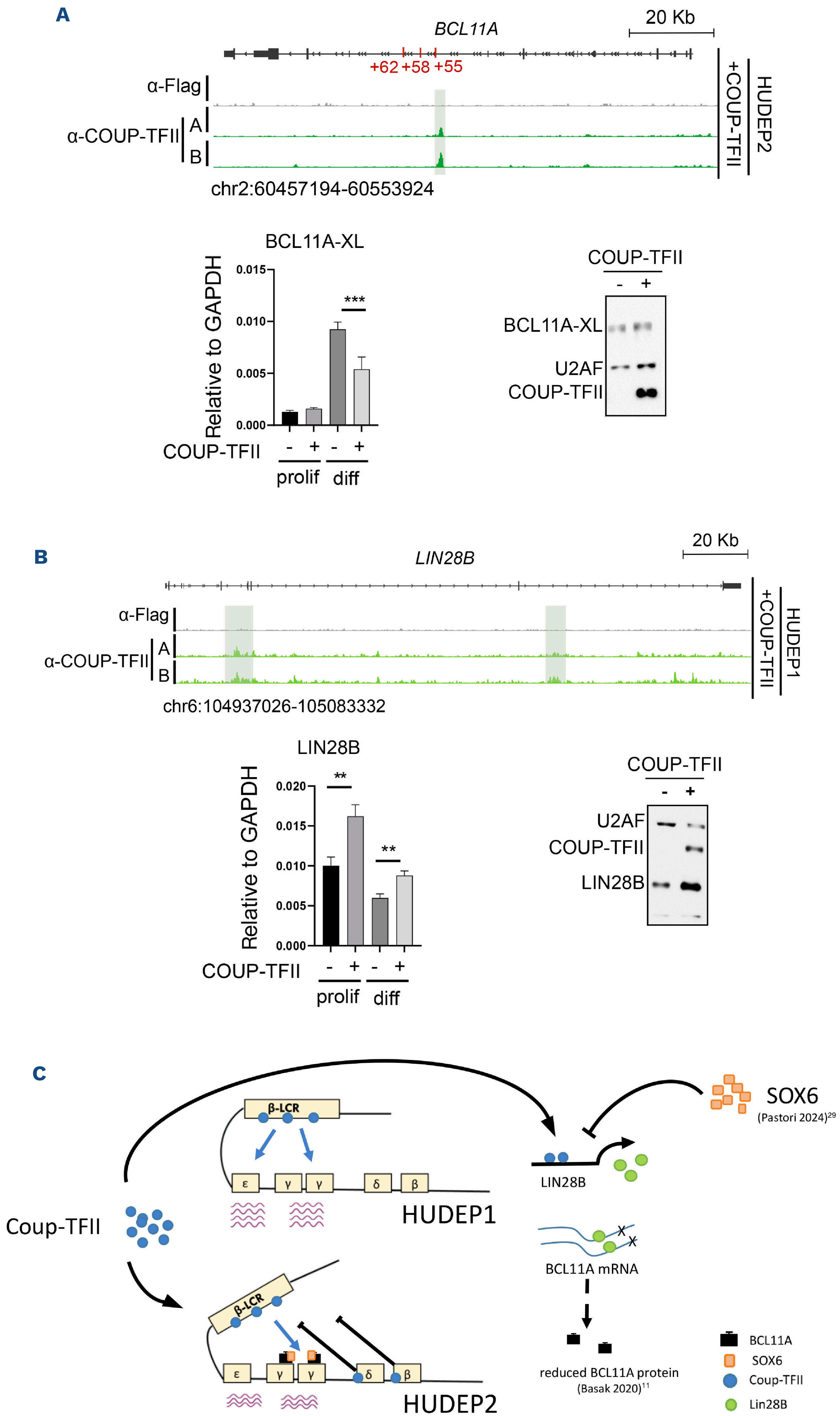

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Figure 4. COUP-TFII binds to the *BCL11A* locus and binds to and activates *LIN28B*. (A) In HUDEP2 cells, COUP-TFII binds to the *BCL11A* locus. (Left) IGV visualization of CUT&RUN tracks at the *BCL11A* +55 region. (Middle) Real-time quantitative polymerase chain reaction for BCL11A-XL in HUDEP2 cells upon transduction with the empty vector (-) or with the COUP-TFII expressing vector (+) in proliferation and differentiation conditions. Expression levels are relative to GAPDH (N ≥3, ***P*≤0.01, ****P*≤0.001). (Right) Western blot analysis showing BCL11A-XL protein upon COUP-TFII expression in differentiation conditions. (B) In HUDEP1 cells, COUP-TFII binds to and activates *LIN28B*. The same analysis as in (A) was applied to LIN28B. Western blot was performed in cells under proliferation conditions. (C) Proposed model for the COUP-TFII role in controling the hemoglobin fetal-to-adult transition. In fetal-like HUDEP1 cells, γ-globin is expressed at very high levels. In these cells, COUP-TFII binds to the LCR, increasing ε but not detectably γ transcription levels. In addition to interacting with the β-locus, COUP-TFII binds to and activates the transcription of *LIN28B*, a post-transcriptional repressor of BCL11A-XL.[11] Instead, SOX6 contributes to the silencing of LIN28B[29] and co-operates with BCL11A-XL.[28] In HUDEP2, where the full set of γ-globin inhibitors (including BCL11A-XL) is present, both γ-globin and LIN28B expression are switched off and β-globin is actively transcribed. In these cells, COUP-TFII occupies the δ and β promoters. Overall, COUP-TFII expression results in activation of embryo/fetal genes and in reduction of β. Our working model highlights COUP-TFII multiple contributions to establishing an environment favorable to the expression of γ-globin genes and proposes a molecular mechanism to explain its ability to relieve γ-globin repression in adult cells.

tion with the LCR. This mechanism would lead to γ-globin activation at the expense of β-globin (Figure 2A), thereby overcoming the γ-globin silencing imposed by the concerted action of fetal repressors, including BCL11A-XL.[26-28]
The observation that COUP-TFII, while activating embryo/fetal globins, also reduces β expression (Figure 2A) is consistent with our previous data obtained in β^{039} thalassemia erythroid cultures from $CD34^+$ cells.[8] This finding suggests that reactivating COUP-TFII in adult cells may be a viable strategy to increase γ-globin also in sickle cell disease, while simultaneously inhibiting the intracellular accumulation of defective β^S chains. The potential clinical relevance of this observation in quantitative terms will require further investigation in appropriate cellular and *in vivo* HbS models.
Why COUP-TFII occupancy at the adult region is not detected in HUDEP1 cells is not clear. We think it may reflect the different accessibility of this region, which is open only in HUDEP2,[23] allowing COUP-TFII recruitment to these positions. In HUDEP1, COUP-TFII expression increases ε-globin but not the already high and likely saturated γ-globin expression (Figure 3E).
By profiling COUP-TFII genome occupancy in fetal *versus* adult cells, we uncover an unexpected, multi-layered role of COUP-TFII in the differential regulation of globin gene expression. In HUDEP2 cells, COUP-TFII, beside binding to the δ and β promoters, it transcriptionally represses BCL11A-XLby binding to its +55Kb enhancer (Figure 4A). However, in these cells, upon COUP-TFII expression, we failed to demonstrate a significant decrease in the BCL11A-XL protein. This result is unclear. We may speculate the existence during the fetal-to-adult switching, of some transient cell population not captured by HUDEP1 and HUDEP2, in which COUP-TFII expression concurs to BCL11A-XL downregulation and thereby to the progressive establishment of an adult environment.
We could not detect COUP-TFII peaks within the closed *BCL11A* locus in HUDEP1 cells (see Huang *et al*. for ATACseq data[23]). In HUDEP1 cells, COUP-TFII promotes a fetal-like phenotype by transcriptionally activating LIN28B, a post-transcriptional repressor of BCL11A-XL[11] (Figure 4B).
Interestingly, we recently identified *LIN28B* as a target repressed by SOX6,[29] a known γ-globin repressor that cooperates with BCL11A-XL through protein-protein interaction to the silencing of γ-globin.[28]
Together, our data add a new layer to the complex regulatory network that controls the differential expression of globin genes. We suggest that the decline of COUP-TFII, together with the parallel increase in SOX6, during the fetal to adult transition state, could contribute to hemoglobin switching through multiple mechanisms: direct regulation of the globin loci and modulation of the translation of BCL11A-XL through its post-transcriptional repressor LIN28B (as proposed in Figure 4C). As a note of caution, we wish to point out that this molecular model, derived from HUDEP cells, may not fully capture human hemoglobin switching *in vivo* and requires further validation in primary human cells around the switching time. Further *in vivo* studies will clarify whether changes in the fetal/adult globin ratio induced by Coup-TFII are clinically significant.
Overall, our present work contributes to understanding the molecular basis of COUP-TFII-mediated reactivation of γ-globin in adult cells within the β-locus and provides novel insights into how COUP-TFII integrates in the complex network sustaining the embryo/fetal transcriptional milieu, favorable to γ-globin expression.

Disclosures
No conflicts of interest to disclose.

Contributions
CF, VP and GZ performed experiments, and analyzed and interpreted data; MF and SA helped with the experimental work; EC interpreted data and wrote the manuscript; CC and AER conceived and designed the experiments, interpreted data, and wrote the manuscript.

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Data-sharing statement
Please address all correspondence to the corresponding authors. Data are available at Array express E-MTAB-14720, under private access.

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