The Coup-TFII orphan nuclear receptor is an activator of the γ-globin gene

Cristina Fugazza,[1*] Gloria Barbarani,[1*] Sudharshan Elangovan,[1*°] Maria Giuseppina Marini,[2] Serena Giolitto,[1] Isaura Font-Monclus,[1] Maria Franca Marongiu,[2] Laura Manunza,[3] John Strouboulis,[4] Claudio Cantù,[5] Fabio Gasparri,[6] Silvia M.L. Barabino,[1] Yukio Nakamura,[7] Sergio Ottolenghi,[1] Paolo Moi[3] and Antonella Ellena Ronchi[1]

[1]Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Milano, Italy; [2]Istituto di Ricerca Genetica e Biomedica del Consiglio Nazionale delle Ricerche, Cagliari, Italy; [3]Dipartimento di Sanità Pubblica, Medicina Clinica e Molecolare, Università degli Studi di Cagliari, Cagliari, Italy; [4]School of Cancer and Pharmaceutical Sciences, King's College London, London, UK; [5]Wallenberg Center for Molecular Medicine, Department of Clinical and Experimental Medicine (IKE), Linköping University, Linköping, Sweden; [6]Department of Biology, Nerviano Medical Sciences S.r.l., Nerviano, Milano, Italy and [7]University of Tsukuba, Tsukuba, Ibaraki, Japan

**CF, GB and SE contributed equally as co-first authors.*

°Present address: Genomics Division, Wipro Life Sciences Laboratory, WIPRO Limited, Bengaluru, Karnataka, India

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Correspondence: *ANTONELLA ELLENA RONCHI1* - antonella.ronchi@unimib.it

Supplemental Material

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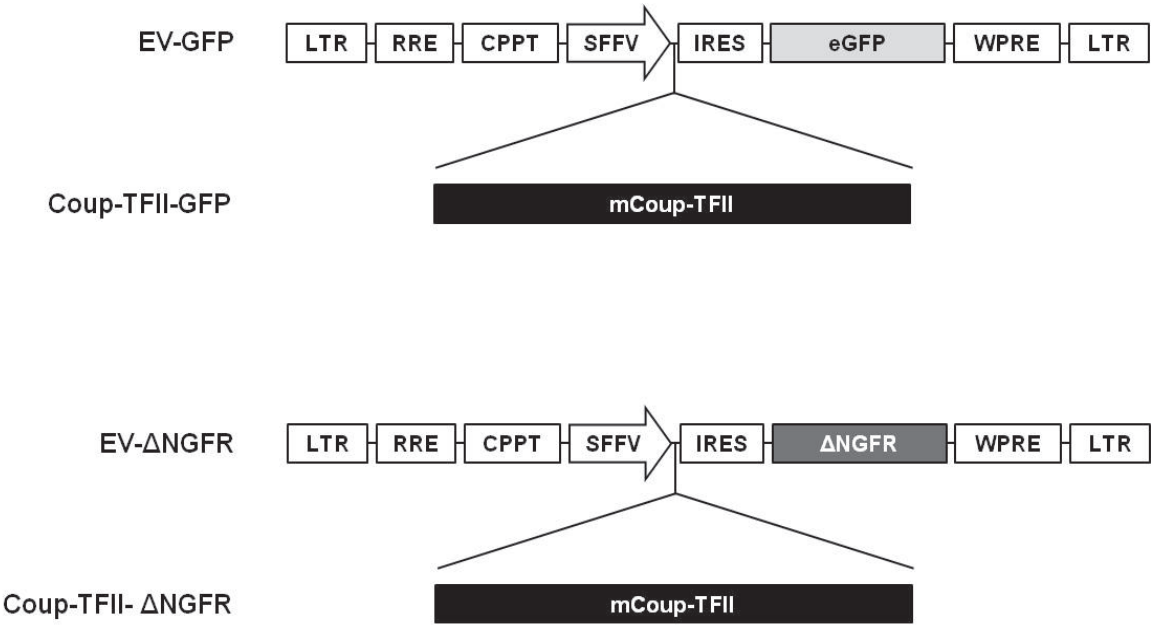
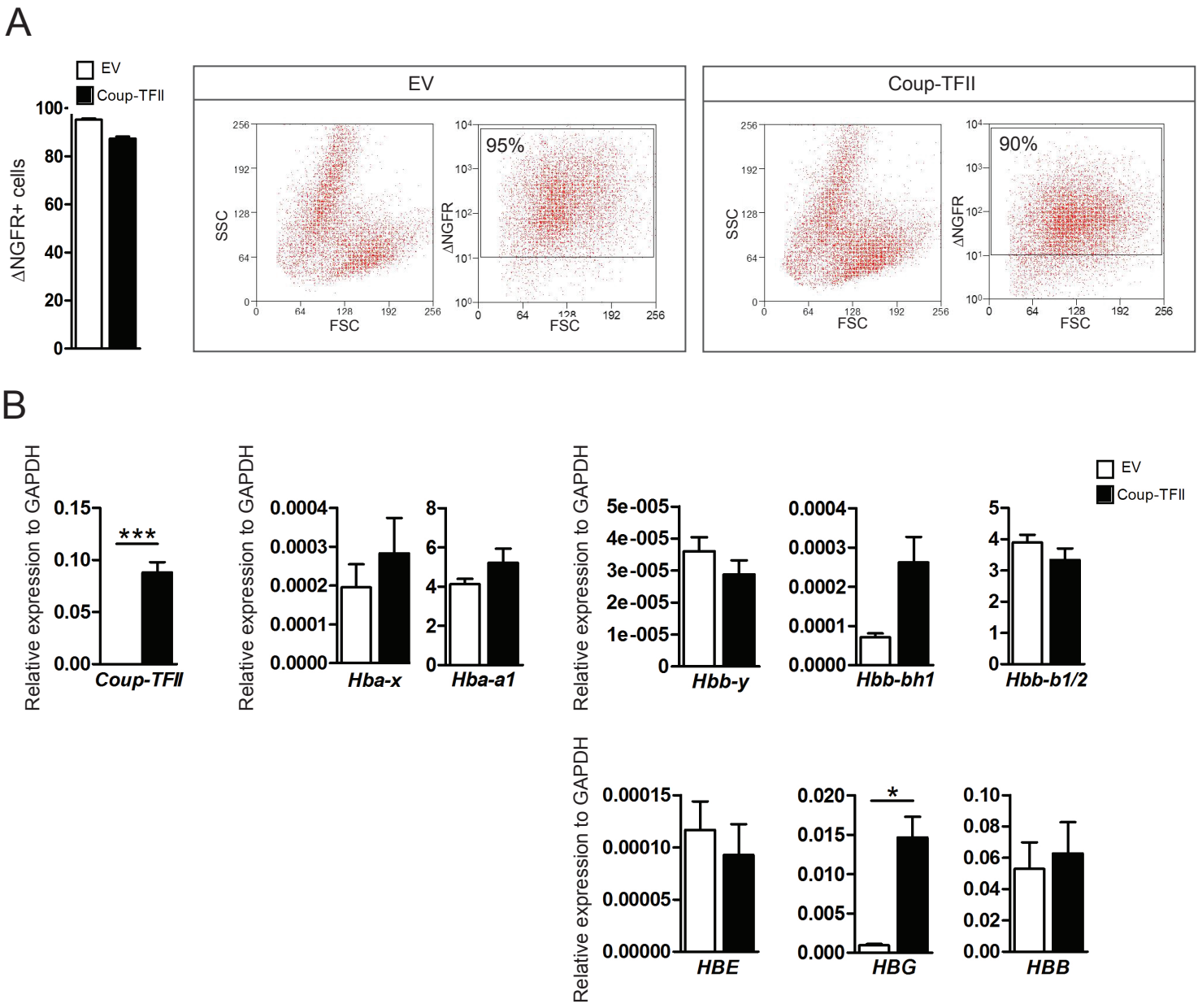
Supplementary Figure1



Supplementary Figure 2



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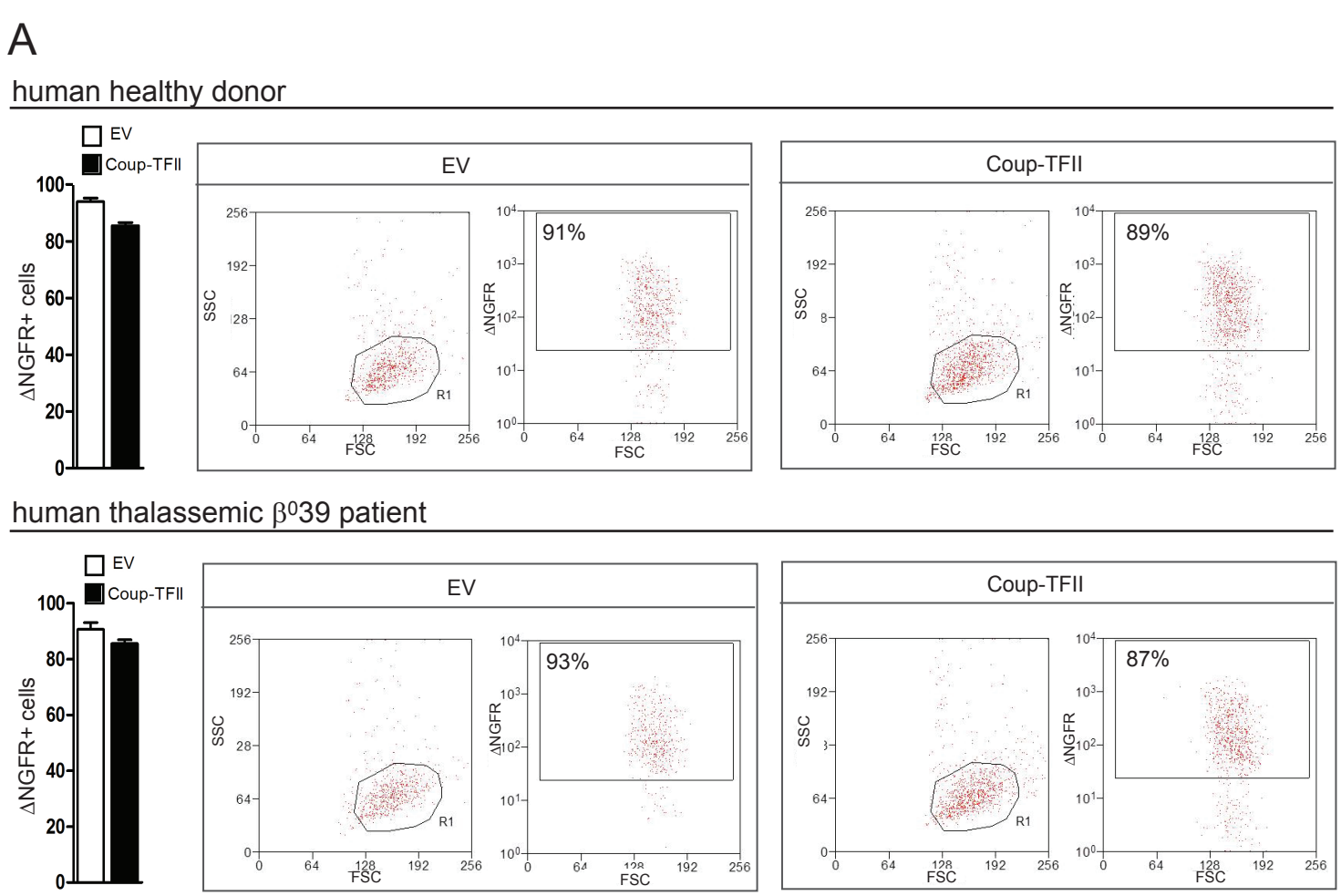

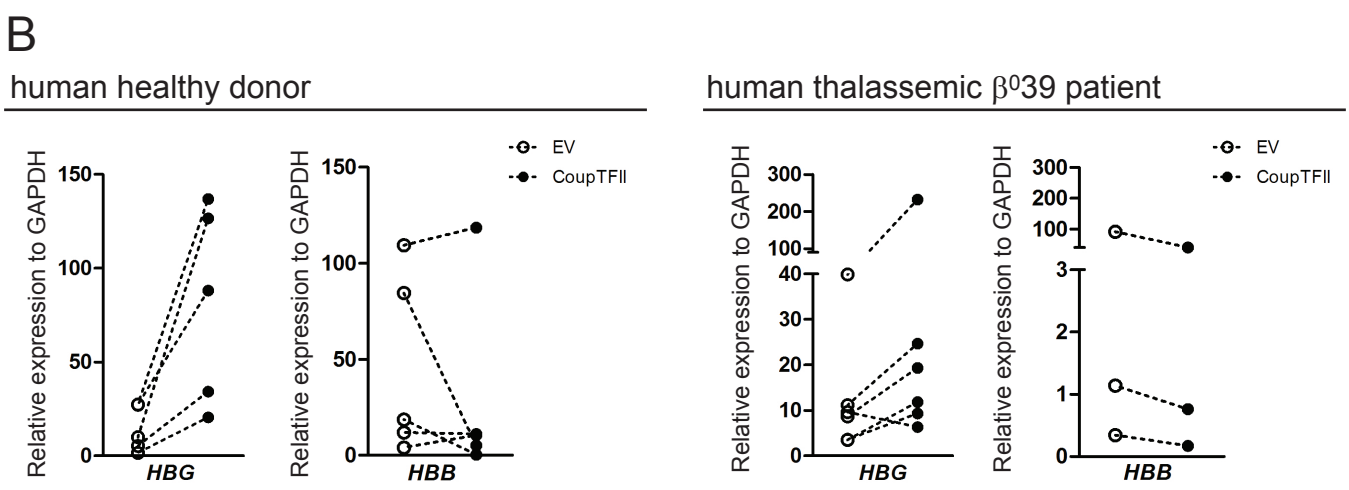

Supplementary Figure 4

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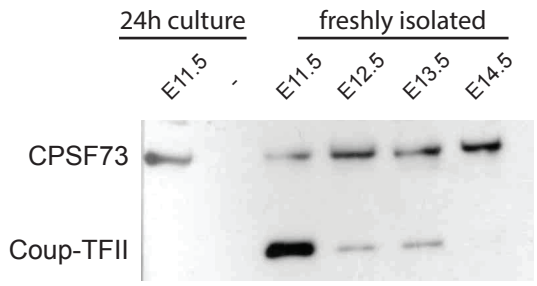

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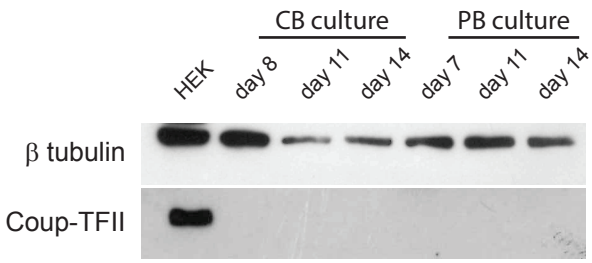

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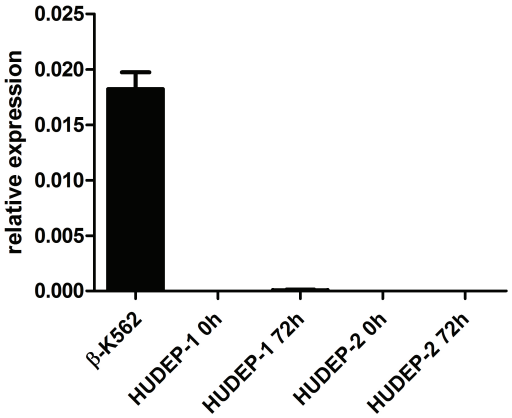
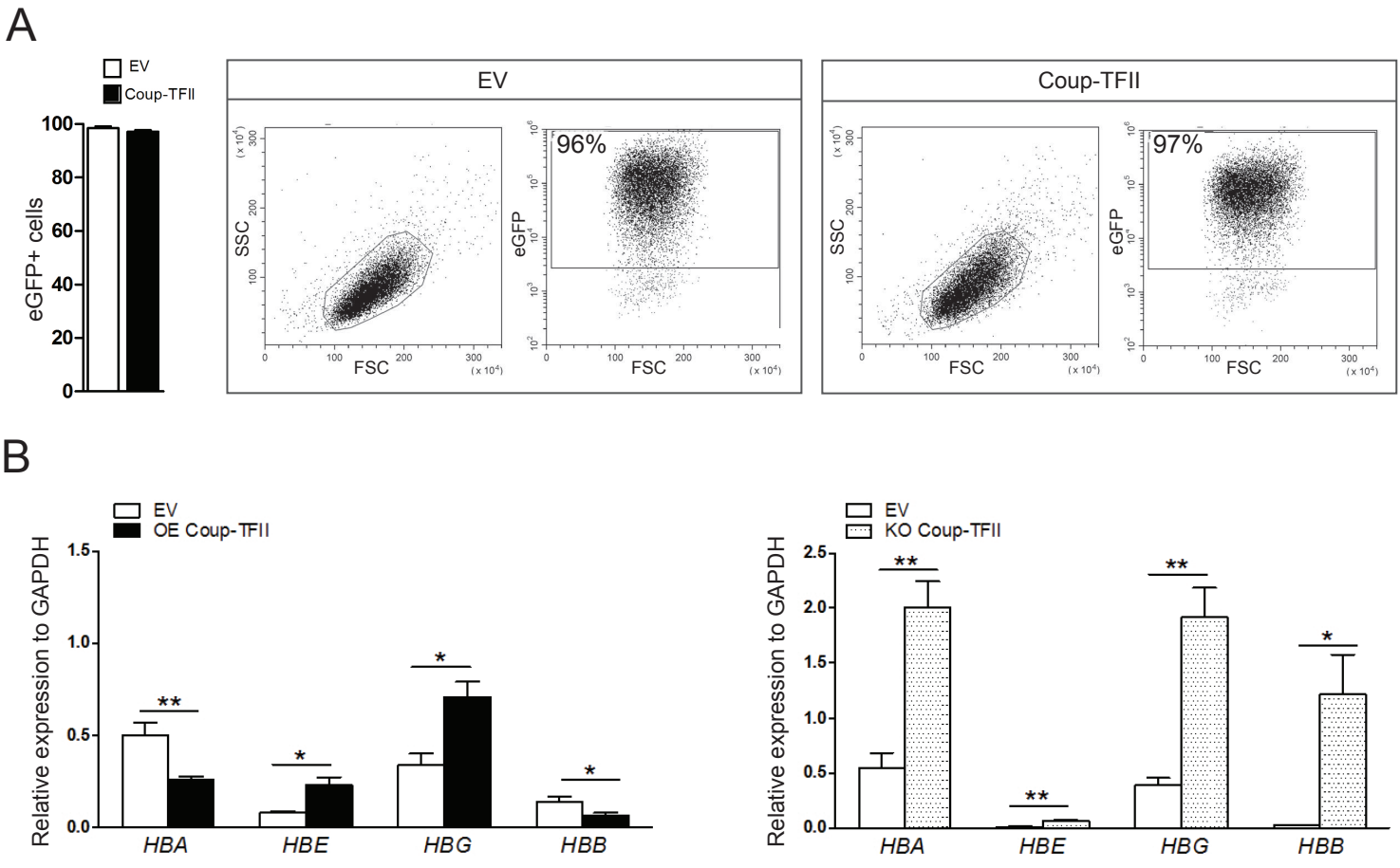
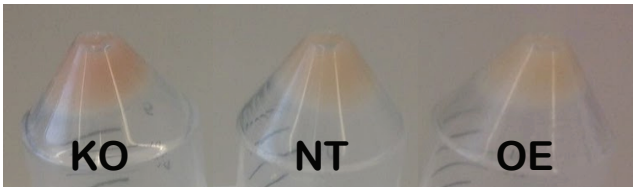


Supplementary Figure 5



Supplementary Figure 6

A



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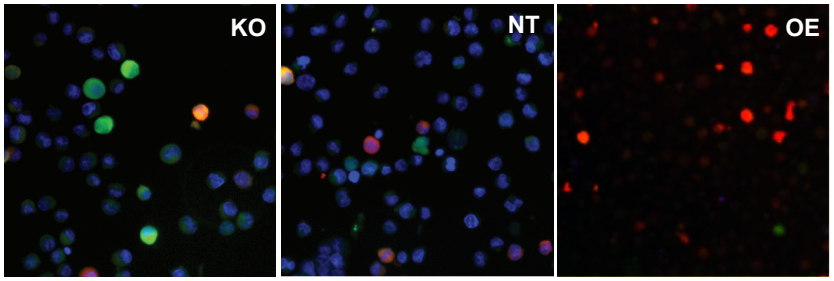
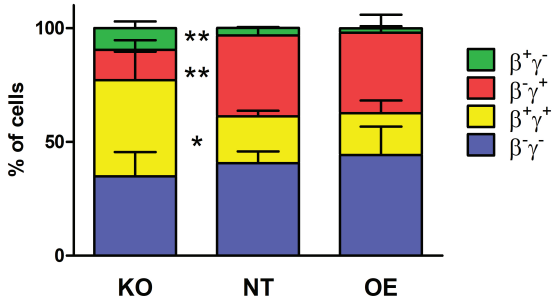
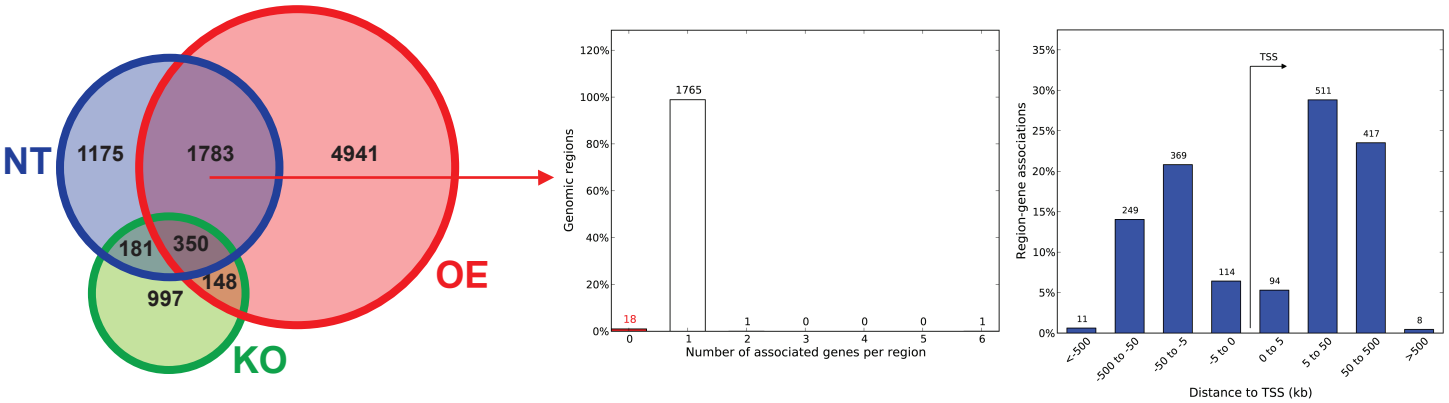




Supplementary Figure 7



Supplementary Figure 8

5′ GCACTGACCCCGA **HS2**
5′ TTAGTGACCTCCC **HS2**

5′ CTAGGGGCAAGTG **HS3**
5′ GTTAGGTCAGGTT **HS3**
5′ GTGGGGTCAGGGC **HS3**

5′ CTCAGGTCATGGC **HS4**

Figure Legends

Supplementary Figure 1

Schematic representation of the CSI-based bi-cistronic lentiviral vectors used for overexpression experiments. LTR: long-terminal repeat; RRE: Rev Response Element; CPPT: central polypurine track; IRES: internal ribosomal entry site; eGFP: emerald green fluorescent protein; ΔNGFR: truncated low-affinity nerve growth factor receptor protein; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

Supplementary Figure 2

***ex vivo* mouse cultures. (A) ΔNGFR expression in cells infected with the EV and Coup-TFII vectors at day three upon infection**. Left panel: Histograms showing the percentage of $\Delta NGFR^+$ cells (n≥3). Right panel: representative plots for EV- and Coup-TFII vector-infected cells. **(B) RTqPCR showing Coup-TFII overexpression and globins expression levels.** Histograms show expression as relative to Gapdh (n≥3, error bars: SEM; *=*P*<0.05; **=*P*<0.01; ***=*P*<0.001)**.**

Supplementary Figure 3

Human *ex vivo* cultures from $CD34^+$ cells isolated from peripheral blood. (A) ΔNGFR expression in cells infected with the EV and Coup-TFII vectors after their immunomagnetic isolation. Left panel: Histograms showing the percentage of $\Delta NGFR^+$ cells (n≥3). Right panel: representative plot. **(B) Globins expression levels as relative to Gapdh** in cells infected with the EV (empty circles) or the Coup-TFII vector (filled circles). Each line corresponds to a donor (healthy or thalassemic).

Supplementary Figure 4

Only the erythroleukemic β-K562 cell line expresses Coup-TFII in culture

(A) Western Blot showing that Coup-TFII expression is present only in freshly isolated fetal liver cells, where it declines starting from E11.5. FL E11.5 cells cultured for 24 hours lose Coup-TFII expression. CPSF73: loading control. **(B)** Western Blot showing that both human Cord Blood (CB) and Peripheral Blood (PB) cultures do not express Coup-TFII. HEK (human embryonic kidney cells 293) extracts: positive control; β tubulin: loading control. **(C)** RT-qPCR analysis shows the absence of Coup-TFII expression in both HUDEP-1 and HUDEP-2 cells (1) grown in expansion condition or after 72h in erythroid differentiation medium, according to Dr. Nakamura protocols and suggestions. β-K562: positive control. The relative expression on *GAPDH* is shown.

Supplementary Figure 5

β-K562 experiments. (A) Efficiency of infection. Left panel: Histograms showing the percentage of $eGFP^+$ cells (n≥3). Right panel: representative plots for EV and Coup-TFII infected cells. **(B) RTqPCR showing expression levels of globins genes.** Histograms show expression levels as relative to *GAPDH* (n≥3, error bars: SEM; *=*P*<0.05; **=*P*<0.01; ***=*P*<0.001)**.**

Supplementary Figure 6

Depletion of Coup-TFII in β-K562.

(A) Cells phenotype: Knock-out cells (KO) show a reddish pellet when compared to overexpressing (OE) and not transduced (NT) cells. **(B)** Single cell analysis of globins at the protein level. The same cells analyzed by RT-qPCR in Fig. 3 were stained with anti-γ and anti-β antibodies and analyzed by using an automatic ArrayScan VTI reader. The relative percentage of $\gamma^-\beta^-$, $\gamma^+\beta^-$, $\gamma^+\beta^+$, $\gamma^-\beta^+$ cells is shown in the right panel (n≥3, error bars: SEM; *= *P* <0.05). At the protein level, we confirmed the increase of β in KO cells, whereas in OE cells we failed to detect a significant increase of γ, possibly because of the limit in the method sensitivity.

Supplementary Figure 7

Features of the Coup-TFII genomic interactions

Almost 100% of the bona fide Coup-TFII peaks have an associated gene. The peaks distribution relative to the Transcription Start Site (TSS) is shown the right panel. Peaks were assigned to the nearest transcription starting site by using GREAT "Genomic Regions Enrichment of Annotations Tool" (2). Graphs represent the number of associated genes per region and their distance from the Transcription Start Site, TSS (single nearest gene -1000kb max extension-curated regulatory domains included).

Supplementary Figure 8

Putative Coup-TFII consensus sequences within the LCR

Coup-TFII consensus found within the HS2, HS3 and HS4 core regions as identified by JASPAR software (3).

Supplementary Tables

Supplementary Table 1: Reagents and antibodies

Reagents / Antibodies	Cat No:	Manufacturer	Note
RPMI Medium 1640	BE12-167F	Lonza	
Phosphate-buffered saline (PBS)	ECB4004L	Euroclone	
Fetal Bovine Serum (FBS)	F7524	Sigma-Aldrich	
L-glutamine	ECB3000D	Euroclone	
Penicillin-Streptomycin	ECB3001D	Euroclone	
Puromycin	P8833	Sigma	
Formaldehyde	F8775	Sigma	
Luminata Western HRP substrate	WBLUR0500	Millipore	
TRI REAGENT	AM9738	Applied Biosystems	
DNaseI (Rnase free)	M6101	Promega	
High Capacity cDNA RT Kit	4368814	Applied Biosystems	
Anti-Sox6 (Rabbit)	ab30455	Abcam	
Anti-Coup-TFII (Mouse)	H7147	Abcam	
Anti-human γ globin	sc-21756	SantaCruz	
Anti-human β globin	sc-21757	SantaCruz	
Anti-BCL11A-XL	NB-600-261	Novus Biologicals	
Anti-U2AF	U4758	Sigma	
Anti-β-actin	sc-130656	SantaCruz	
Anti-IgG (Rabbit)	sc-2027X	SantaCruz	for ChIP
Anti-IgG (Mouse)	sc-2025	SantaCruz	for ChIP
Anti-Coup-TFII	sc-271940 X	SantaCruz	for ChIP
Anti-Sox6	AB5805	Millipore	for ChIP
StemPro®-34 SFM	10639-011	GIBCO	
Erythropoietin (Epo)	PR-402	Jena Bioscience	
Dexamethasone	D4902	Sigma-Aldrich	
Stem Cell Factor	250-03	Peprotech	
Anti-mouse (PE) conjugated CD71	113807	Biolegend	
Anti-mouse (FITC) conjugated Ter119	116205	Biolegend	
Anti-mouse (APC) conjugated CD117	105811	Biolegend	
Anti-mouse (PE Cy5) conjugated Ter119	116210	Biolegend	
Anti-mouse(PE Cy7) CD41	133915	Biolegend	
Anti-mouse (APC Cy7) CD16/32	101327	Biolegend	
Anti-human (APC) conjugated CD271 (NGFR)	345108	Biolegend	
Anti human (PE) conjugated CD271 (NGFR)	345106	Biolegend	
Anti-human (APC) conjugated CD235ab (GpA)	306607	Biolegend	
Anti human (PE) conjugated CD71	334105	Biolegend	
Mouse PE selection kit	18514	Stem Cells Technology	
Anti mouse globins antibodies were a kind gift from Dr. J. Palis			

Supplementary Table 2: List of Primers

Primers used for RTqPCR (human genes)		
Gene	**F/R**	**Sequence (5'-3')**
GAPDH	F	ACGGATTTGGTCGTATTGGG
	R	TGATTTTGGAGGGATCTCGC
α-globin	F	GAGGCCCTGGAGAGGATGTTCC
	R	ACAGCGCGTTGGGCATGTCGTC
β-globin	F	TACATTTGCTTCTGACACAAC
	R	ACAGATCCCCAAAGGAC
γ-globin	F	CTTCAAGCTCCTGGGAAATGT
	R	GCAGAATAAAGCCTATCCTTGAAAG
ε-globin	F	GCCTGTGGAGCAAGATGAAT
	R	GCGGGCTTGAGGTTGT
COUP-TFII	F	TTGACTCAGCCCGAGTACAGC
	R	AAAGCTTTCCGAATCTCGTC
Primers used for RTqPCR (mouse genes)		
Gene	**F/R**	**Sequence (5'-3')**
Gapdh	F	TGTGTCCGTCGTGGATCTGA
	R	CCTGCTTCACCACCTTCTTGA
Hba-x	F	ACCATGGGTCTCAGCAGTTG
	R	ACAGGAGCTTGAAGTTGACC
Hbb-y	F	GGAGAGTCCATTAAGAATCTAGACAA
	R	CTGTGAATTCATTGCCGAAGTGAC
Hba-a1	F	CTACCCCCAGACGAAGACCTA
	R	CTTAACCGCATCCCCTACGG
Hbb-bh1	F	TGGACAACCTCAAGGAGACC
	R	ACCTCTGGGGTGAATTCCTT
Hbb-b1/b2	F	ATGGCCTGAATCACTTGGAC
	R	ACGATCATATTGCCCAGGAG
Coup-TFII	F	TCCAAGAGCAAGTGGAGAAG
	R	CTTCCAAAGCACACTGGGAC
Primers used for Chromatin Immuno Precipitation (human sequences)		
Gene	**F/R**	**Sequence (5'-3')**
HS2 LCR	F	CCATAGTCCAAGCATGAGCA
	R	CTGGGGACCCAGATAGGAGT
HS3 LCR	F	GGCAAGTGCCTTGACTCCTA
	R	TCTTCTGGAACTTGCCTGCT
HS4 LCR	F	TGGCATCTAGCGCAATGACTT
	R	GGGCAAGCCATCTCATAGCTG
γ-promoter	F	AAACGGTCCCTGGCTAAACT
	R	GCTGAAGGGTGCTTCCTTTT

sgRNAs sequences used for Coup-TFII CRISPR/Cas9-mediated knock-out

sgRNA	Sequence (5'-3')
Addgene HG GeCKOv2 libA_39900	CCCAACCAGCCGACGAGATT
Addgene HG GeCKOv2 libA_39901	TATATCCGGACAGGTACGAG

Supplementary methods

Murine Fetal liver erythroid cultures

Cells isolated from E13.5 fetal liver were grown in StemPro®-34 SFM medium supplemented with Stem Pro Supplement (GIBCO), 4mM L-glutamine and 100ug/ml Penicillin-Streptomycin, Stem Cell Factor (10 ng/ml, Peprotech), Epo 0.5 U/ml, Dexhamethasone (10^{-6} M, Sigma D4902).

Ex vivo human cell cultures

Human BFU-E cultures from peripheral blood were obtained using a two-phase method, originally published by Fibach (4) and further developed by Migliaccio (5), with minor modifications. Briefly, peripheral blood mononuclear cells were cultured in expansion medium made of Dulbecco's minimal essential medium (Sigma M8042) supplemented with 10% FBS (Sigma, 12003C), Interleukin 3 (10 ng/ml, R&D Systems), recombinant human Stem Cell Factor (10 ng/ml, Preprotech), Cyclosporin A (1 mg/ml, Novartis Farma), Dexamethasone (10^{-6} M, Sigma D4902), Holo-human transferrin (300 mg/ml, Sigma), l-Glutamine (1.5 mM), 100 U/ml Penicillin, 100 mg/ml Streptomycin. On day six after expansion, the non-adherent cells were harvested, washed and re-cultured in fresh differentiation medium supplemented with 30% FBS (Sigma, 12003C), 10^{-5} M beta-mercaptoethanol (Sigma), 1% deionized BSA (Sigma) and Erythropoietin (3U/ml, EPREX Janssen-Cilag SPA). Cells were then transduced with either the Coup-TFII-overexpressing or with the corresponding empty vector at MOI of 30 and incubated for four more days until final harvest.

RNA isolation and RTqPCR

Total RNA was extracted with TRI Reagent (Applied Biosystems AM9738) according to the manufacterer's protocol. RNA quantity and purity were assessed by using Nanodrop (Thermo Fisher Scientific Inc.) and RNA integrity was checked on 2% agarose gel. ≈ 2 µg. of RNA were treated with RQ1 DNase (Promega) in 10µl and subsequently retro-transcribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Negative control reactions (RT-) gave no signal. RTqPCR analysis was performed using ABI Prism 7500, (PE

Applied Biosystems). For each experiment, ≥3 biological replicates (and 3 technical replicates for each sample) were analyzed.

Coup-TFII CRISPR/Cas9-mediated knock-out

The sequences of two sgRNAs against Coup-TFII (Suppl. table 2) were obtained from the human GeCKOv2 library (https://www.addgene.org/crispr/libraries/geckov2). The sgRNA oligos were cloned into the LentiCrisprV2 plasmid (Addgene #52961). Experiments were performed with the sgRNA libA_39900, which gave the best knock-out efficiency. Seven days after transduction and selection in puromycin, we isolated and seeded single KO cells by limiting dilution (to a theoretical concentration of 0.5 cells/well) in a 96 well plate, in order to grow single clones, which we analyzed 60 days after transduction.

3C chromosome conformation capture

3C experiments was performed as described in refs (6,7). Briefly, crosslinking reaction was performed at RT for 10 minutes with 2% formaldehyde and blocked with 0.125M Glycin. Pelleted cells were then lysed in ice-cold lysis buffer (10mM Tris-HCl at pH 8, 10mM NaCl, 0.2% NP40, 1 mM DTT). Permeabilisation was performed by resuspending the nuclei in 0.3% SDS+NEBuffer 2.1 (New England BioLabs cat.nr. #B7202S) at 37°C for 1h while shaking. SDS was subsequently sequestered by adding TritonX-100 to a final concentration of 2%. Samples were then digested with EcoRI overnight at 37°C. EcoRI was heat inactivated and DNA ligation was performed in T4 DNA Ligase buffer by adding T4 DNA Ligase at 16°C for 4h and at RT for 30 minutes. Crosslinks were reversed and DNA was purified by multiple phenol extractions and subsequent ethanol precipitation. Crosslinking frequency and ligation efficiency were normalized to *ERCC3* locus according to ref (6). Quantification of the digestion efficiencies was performed by qPCR.

Supplemental Reference list

1. Kurita R, Suda N, Sudo K, Miharada K, Hiroyama T, Miyoshi H, et al. Establishment of immortalized human erythroid progenitor cell lines able to produce enucleated red blood cells. PLoS One. 2013;8(3):e59890

2. McLean CY, Bristor D, Hiller M, Clarke SL, Schaar BT, Lowe CB, et al. GREAT improves functional interpretation of cis-regulatory regions. Nat Biotechnol. 2010 May;28(5):495-501.

3. Mathelier A, Fornes O, Arenillas DJ, Chen CY, Denay G, Lee J, et al. JASPAR 2016: a major expansion and update of the open-access database of transcription factor binding profiles. Nucleic Acids Res. 2016 Jan 04;44(D1):D110-5.

4. Fibach E, Manor D, Oppenheim A, Rachmilewitz EA. Proliferation and maturation of human erythroid progenitors in liquid culture. Blood. 1989 Jan;73(1):100-3.

5. Migliaccio G, Di Pietro R, Di Giacomo V, Di Baldassarre A, Migliaccio AR, Maccioni L, Galanello R, Papayannopoulos T. *In vitro* mass production of human erythroid cells from the blood of normal donors and thalassemic patients Blood Cells, Molecules and Diseases. 2002, 28(2) 169-180.

6. Palstra RJ, Tolhuis B, Splinter E, Nijmeijer R, Grosveld F, de Laat W. The beta-globin nuclear compartment in development and erythroid differentiation. Nat Genet. 2003 Oct;35(2):190-4.

7. Xu J, Sankaran VG, Ni M, Menne TF, Puram RV, Kim W, et al. Transcriptional silencing of {gamma}-globin by BCL11A involves long-range interactions and cooperation with SOX6. Genes Dev. 2010 Apr 15;24(8):783-98.