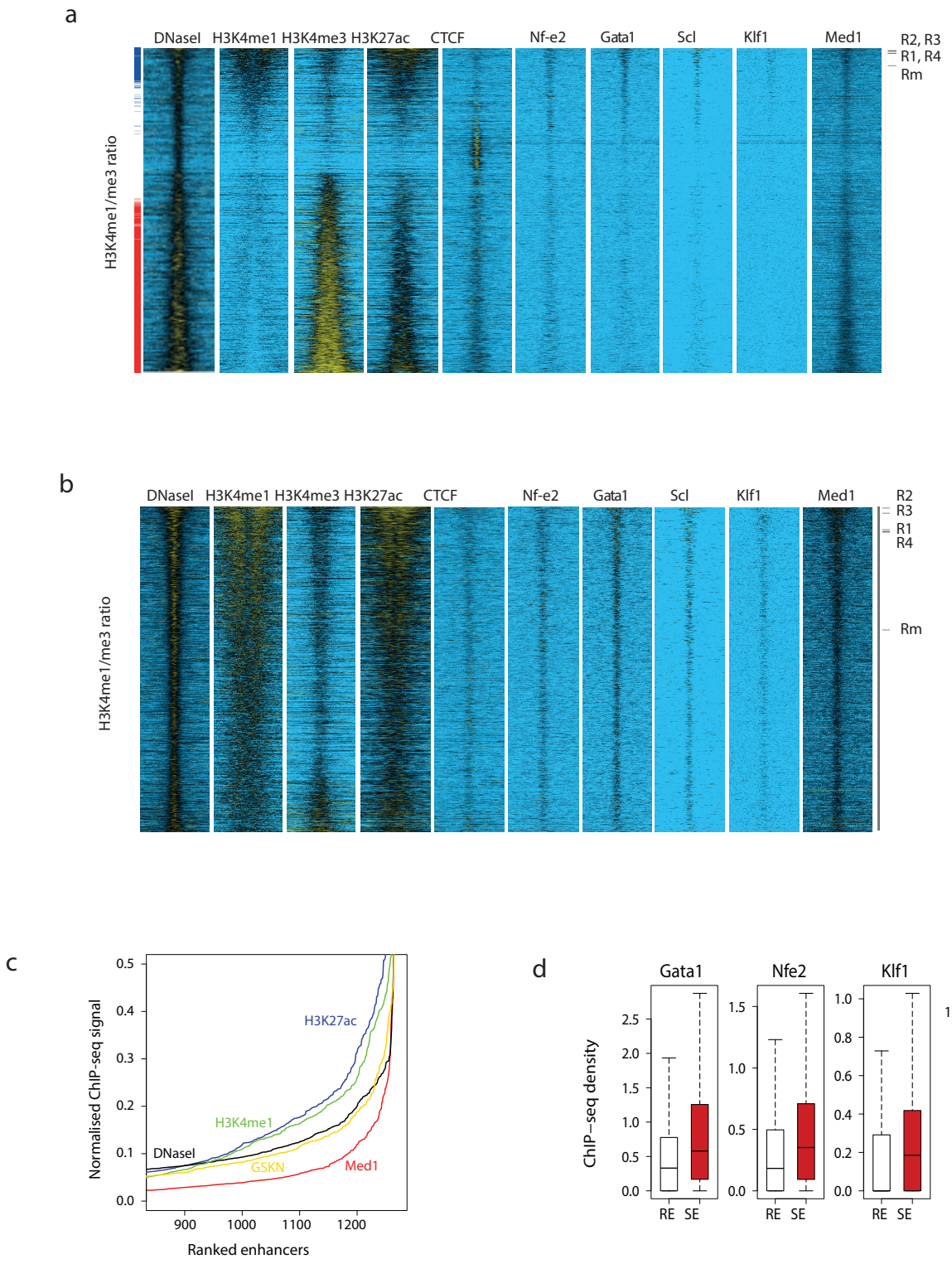
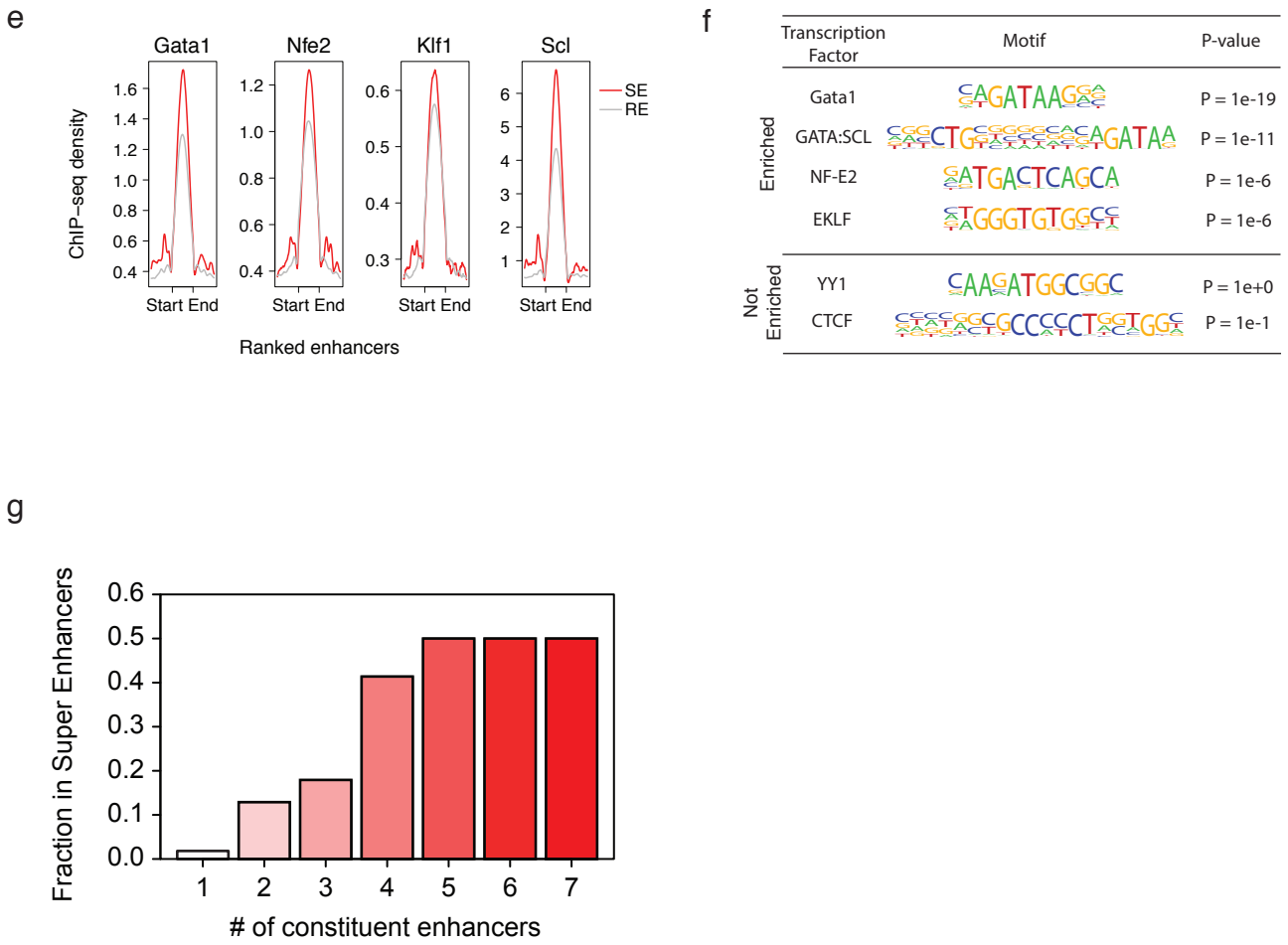
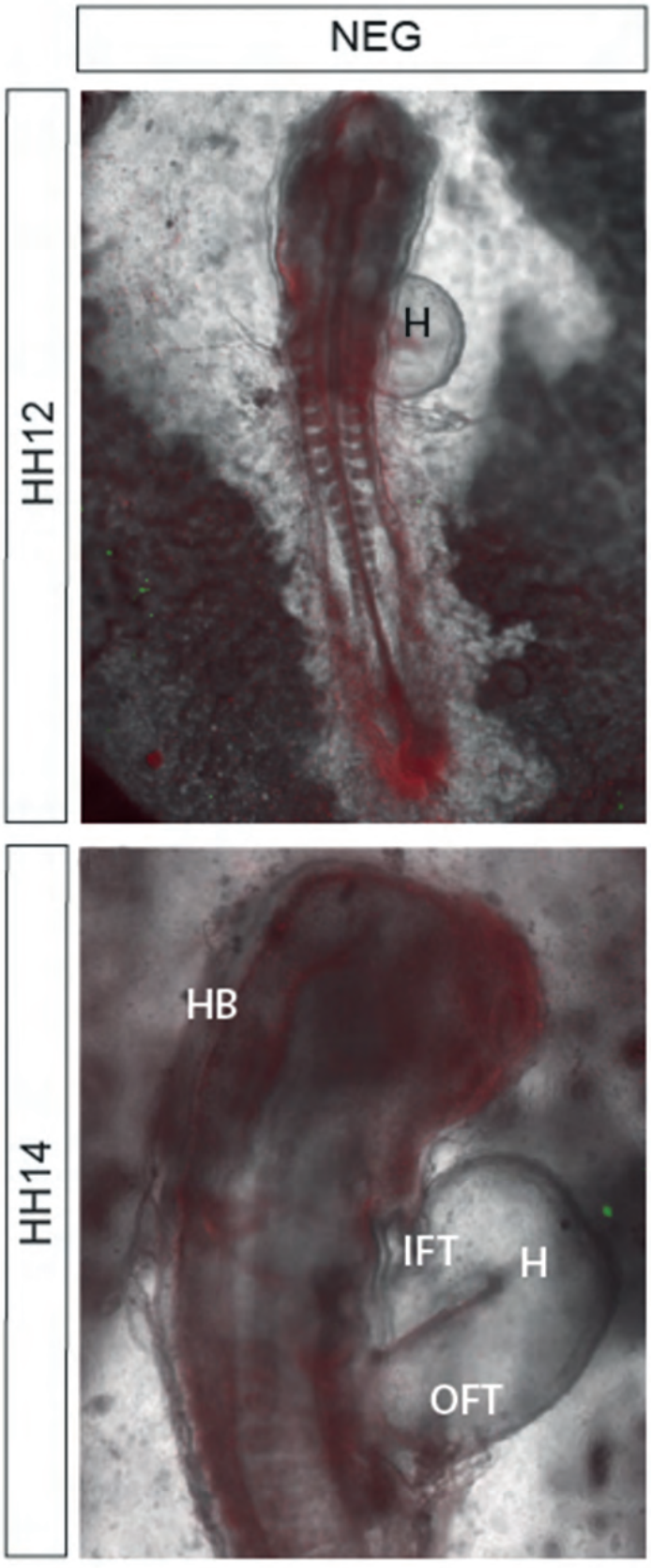
Supplementary Figure 1





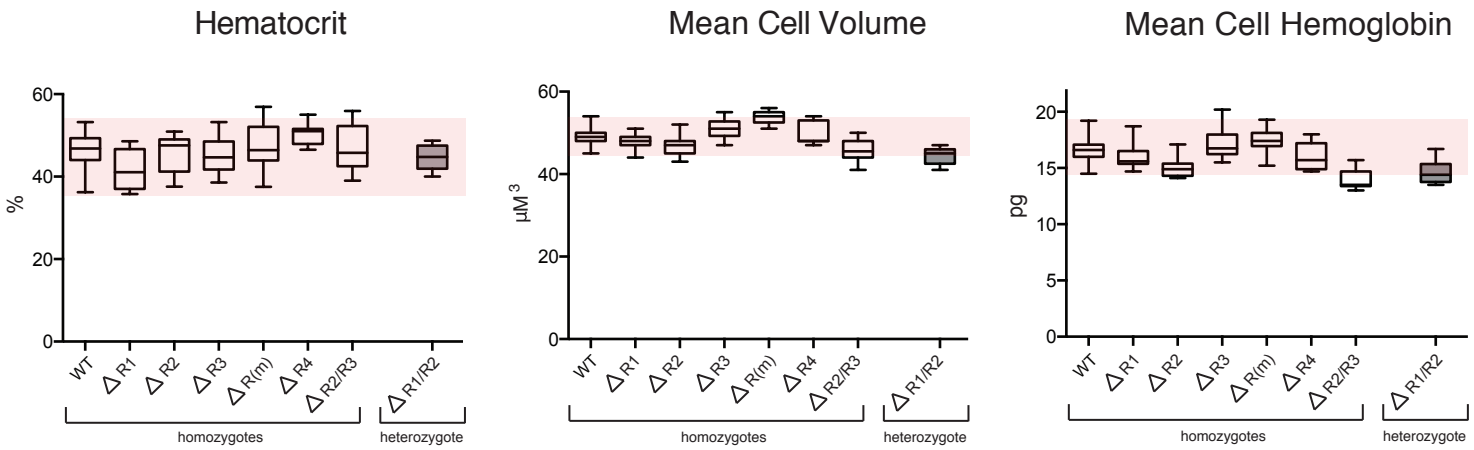
Supplementary Figure 1

a. Heatmap showing log-scaled DNase I-seq and ChIP-seq signal +/- 2kb around the DNaseI peak call regions (15849 peaks), sorted by ratio of H3K4me1 to H3K4me3. In the side panes the annotated "putative enhancers" are marked blue, and "putative promoters" marked red. 3665 of the peaks show strong signal in the CTCF ChIP (annotated as CTCF sites for the PAM clustering analysis). The positions of α-globin regulatory elements are indicated. b. As panel a, but focused +/- 2kb around the enhancer regions only (1963 peaks). c. Normalized distribution of DNaseI hypersensitivity, Med1, H3K27ac, H3K4me1, and GSKN (Gata1, Scl, Klf1, Nf-e2) ChIP-seq signal across 1268 stitched erythroid enhancers. Enhancers are ranked for the plotted feature and divided by the maximum detected input-subtracted ChIP-seq signal. The axes are scaled so that differences between different features can be observed. d. Box plots for the normalized ChIP-seq density (input-subtracted reads per base-pair per million) of Gata1, Scl, Klf1 and Nf-e2 at constituent enhancers of regular and super-enhancers. Transcription factor occupancy was significantly different between constituents of regular and super-enhancers by two-tailed t-test (Gata1: p = 4.378772e-07, Scl: p = 5.972195e-4, Klf1: p = 9.76816e-4, Nf-e2: p = 4.649954e-03) e. Average distribution of transcription factor (Gata1, Scl, Klf1 and Nf-e2) ChIP-seq signal (reads per base-pair per million) across constituents of regular and super-enhancers and a flanking region of 2kb. f. The binding motifs for key erythroid transcription factor are enriched at erythroid super-enhancers. g. The fraction of stitched enhancers that fall into the super-enhancer category as a function of the number of constituents present in the stitched enhancer region.



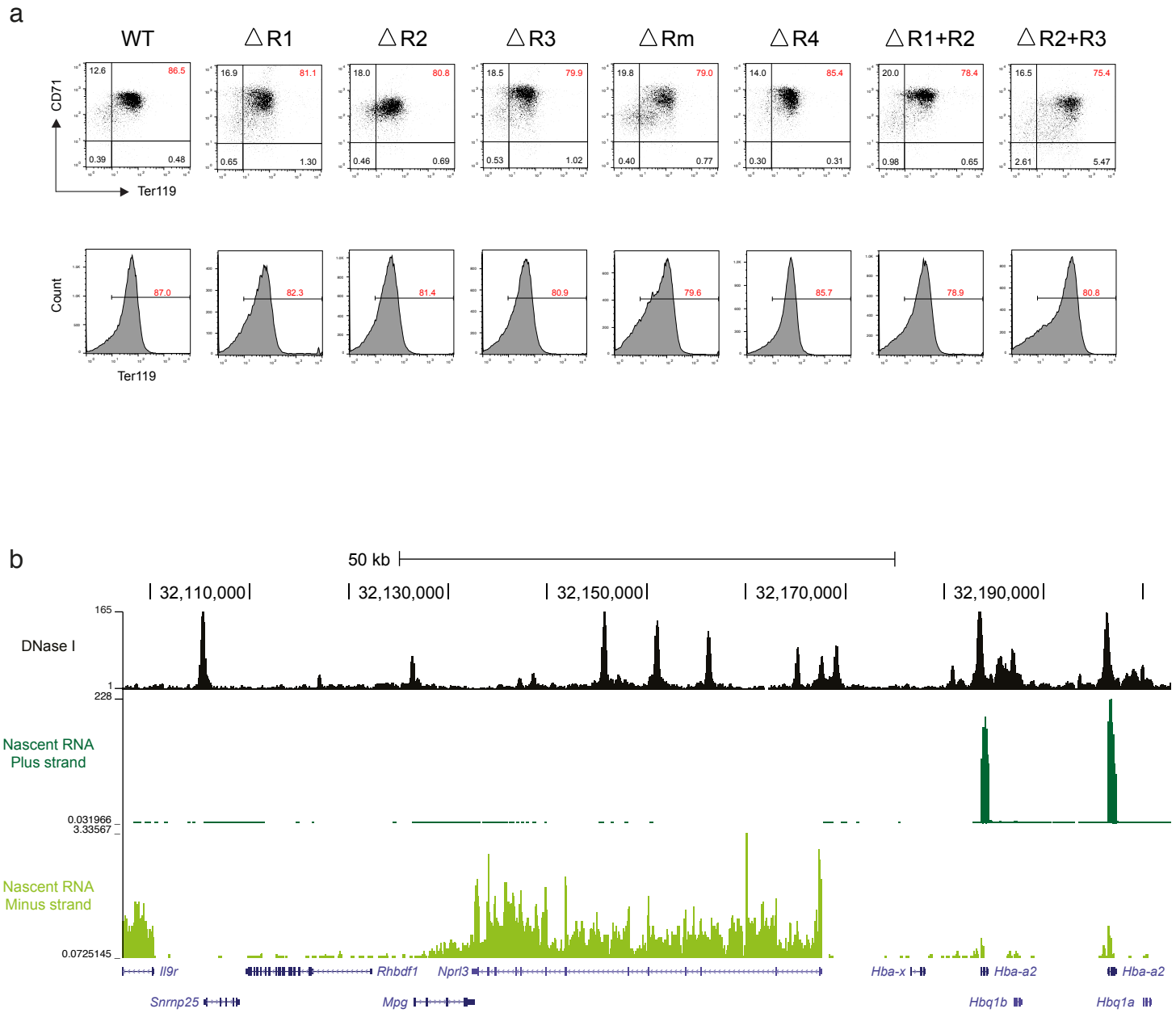
Supplementary Figure 2.
Negative controls (construct without enhancer sequence) for the enhancer assay in chick embryonic development, taken at HH12 and HH14. (IFT inflow tract; OFT, outflow tract; H, heart; HB, hind-brain).

Supplementary Figure 2b (movie)
R1 enhancer activity in circulating blood cells, captured using a Red Epic camera attched to Olympus MVX10 microscope, recorded at 240 frames per second at 1280 iso, 20X magnification.



Supplementary Figure 3

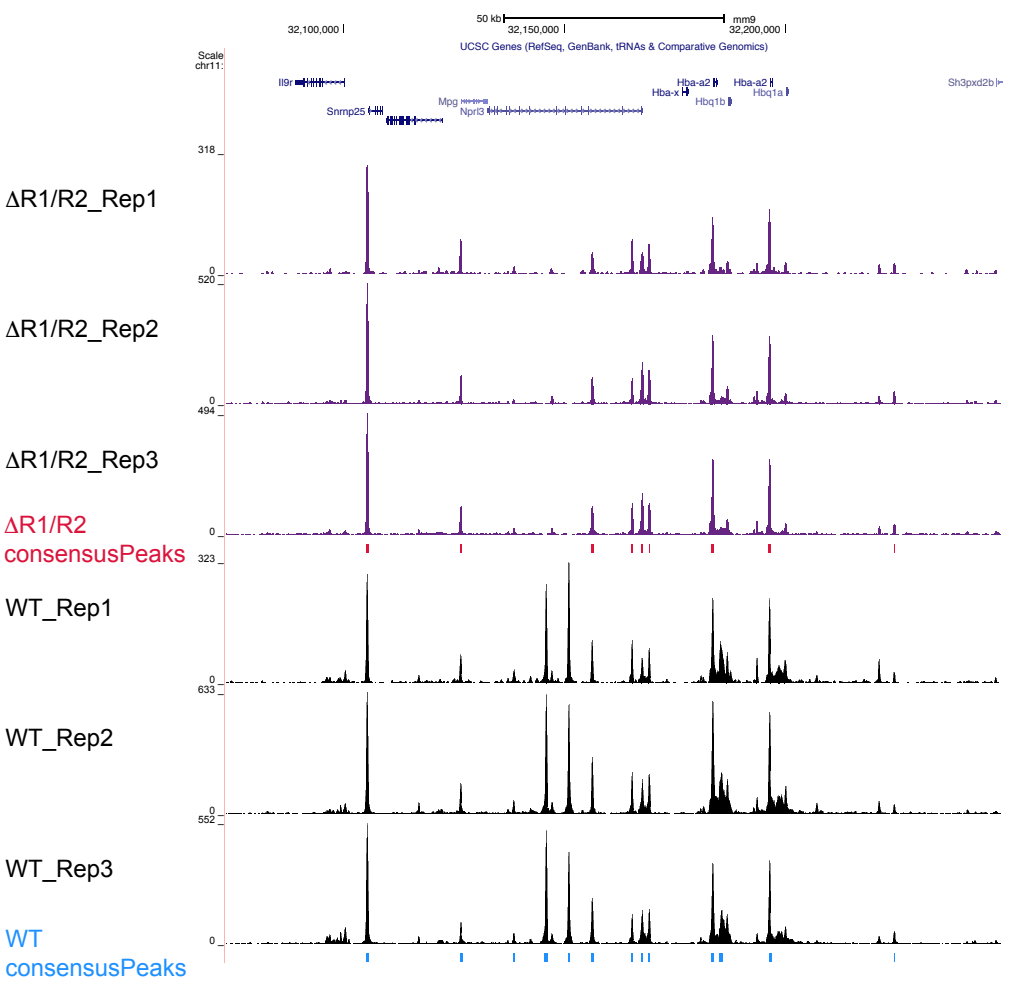
Hematological parameters for mice homozygous for single enhancer deletions and the R2/R3 deletion. Mean values for hematocrit, mean red cell volume and mean cell hemoglobin remain within normal limits for all homozygotes, though with a non-significant trend towards hypochromic red cells for the ΔR2 homozygotes as previously described (Anguita et al. 2002), and for the ΔR2/R3 homozygotes.



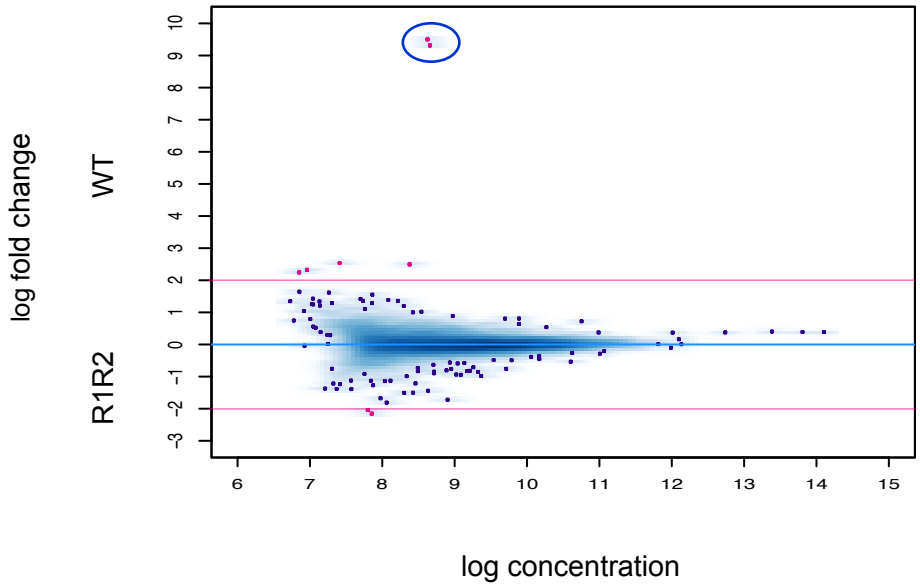
Supplementary Figure 4

a. FACS analysis of fetal liver cultures from wild type and enhancer knockout mice at E12.5. Cells from WT and knockout mice show a similar pattern of expression of the surface markers CD71 and Ter119, used to define late stage erythroblasts.
b. Strand specific Nascent RNA-seq at the α globin locus, used to validate the nascent RNA collection protocol. Data are as for figure 5D, but now auto-scaled to expression of the α globin genes

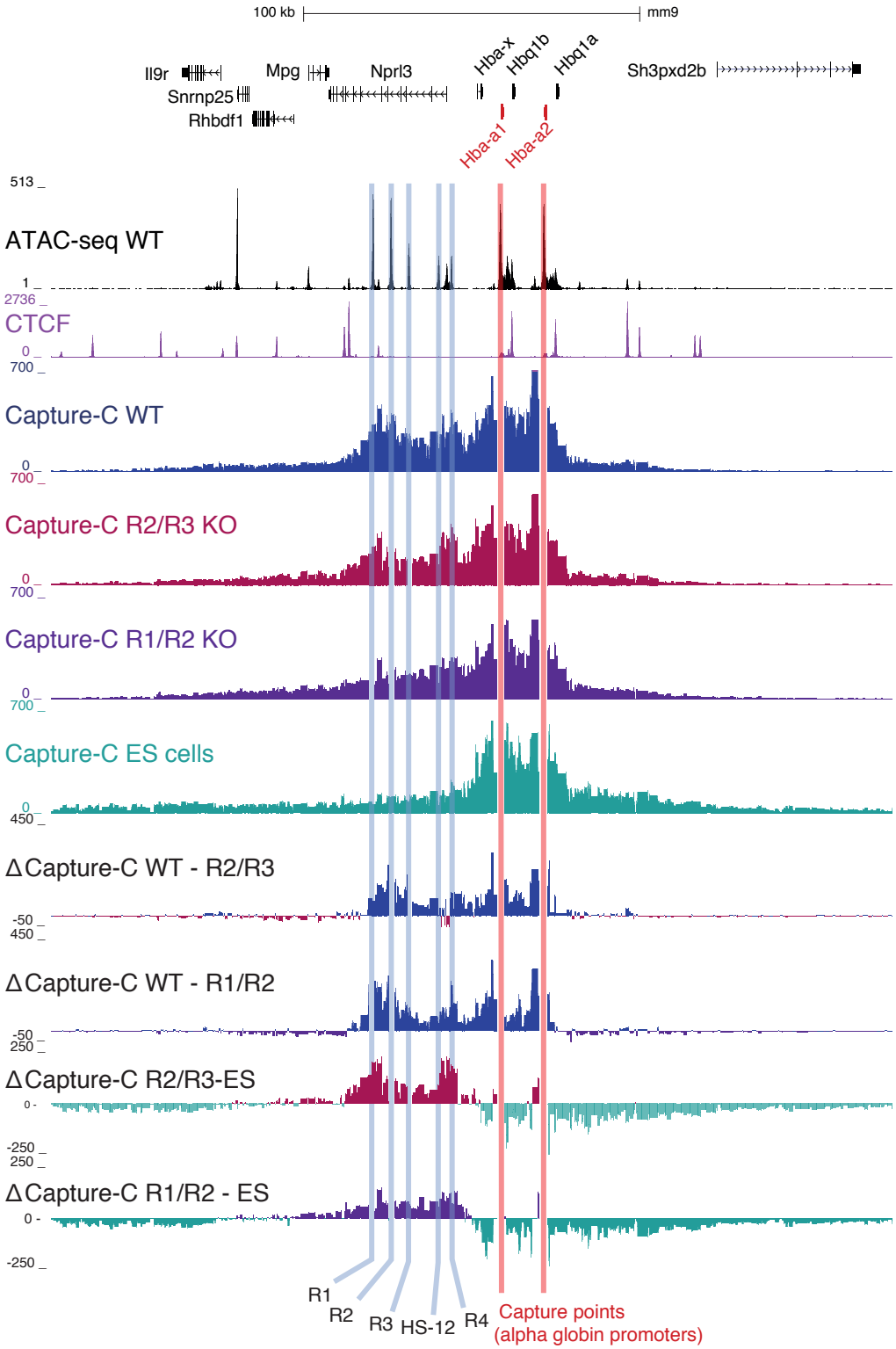
a



b

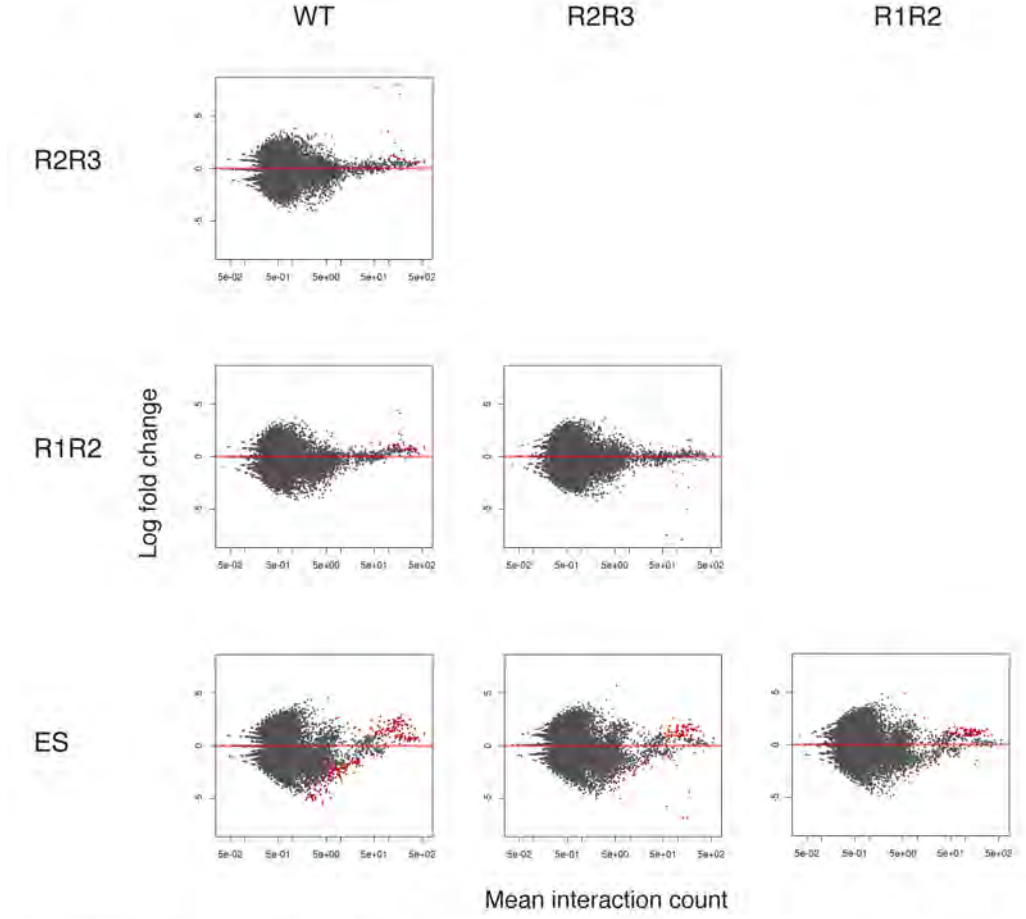


Supplementary Figure 5.
a. ATAC-seq in ΔR1/R2 and WT erythroid cells visualized in the UCSC browser, the consensus peak call from wild type cells used for signal quantification is shown below as blue boxes.
b. MAplot showing significant differential sites genome-wide (FDR <0.01 and fold change >2) highlighted in red. The signals corresponding to the R1 and R2 elements, absent from the knockout models are circled in blue.

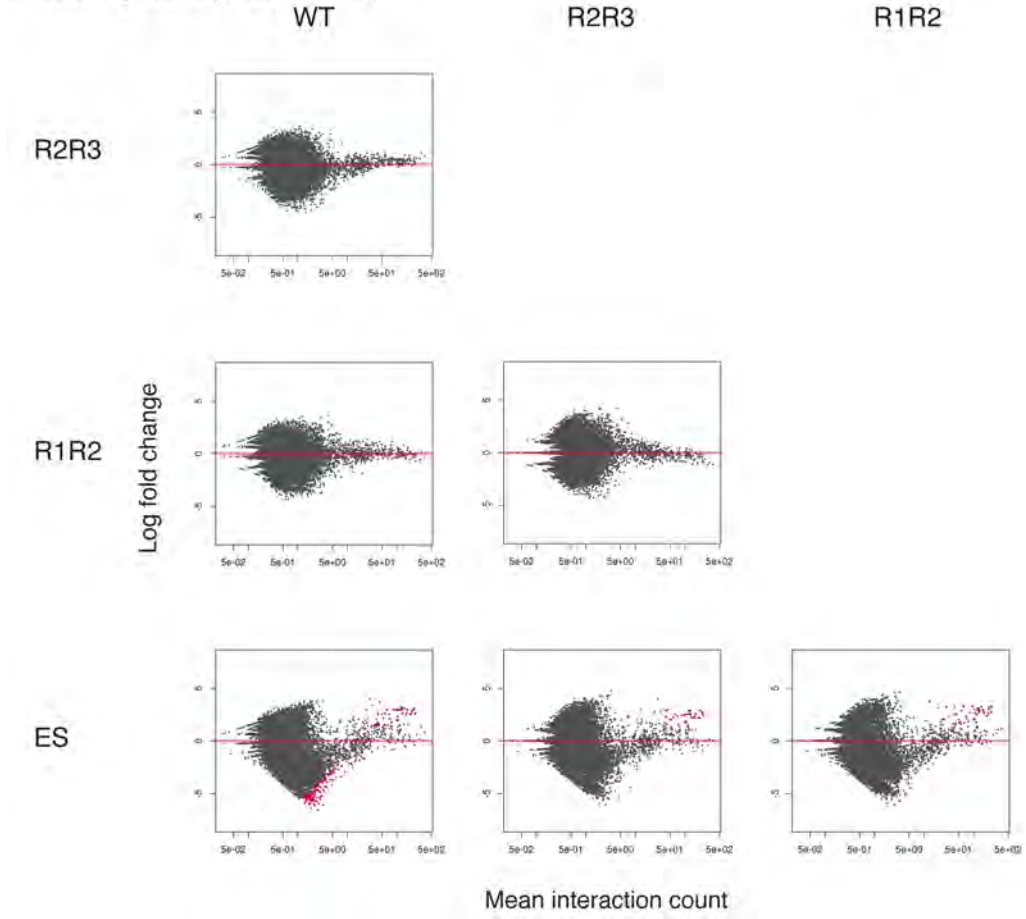


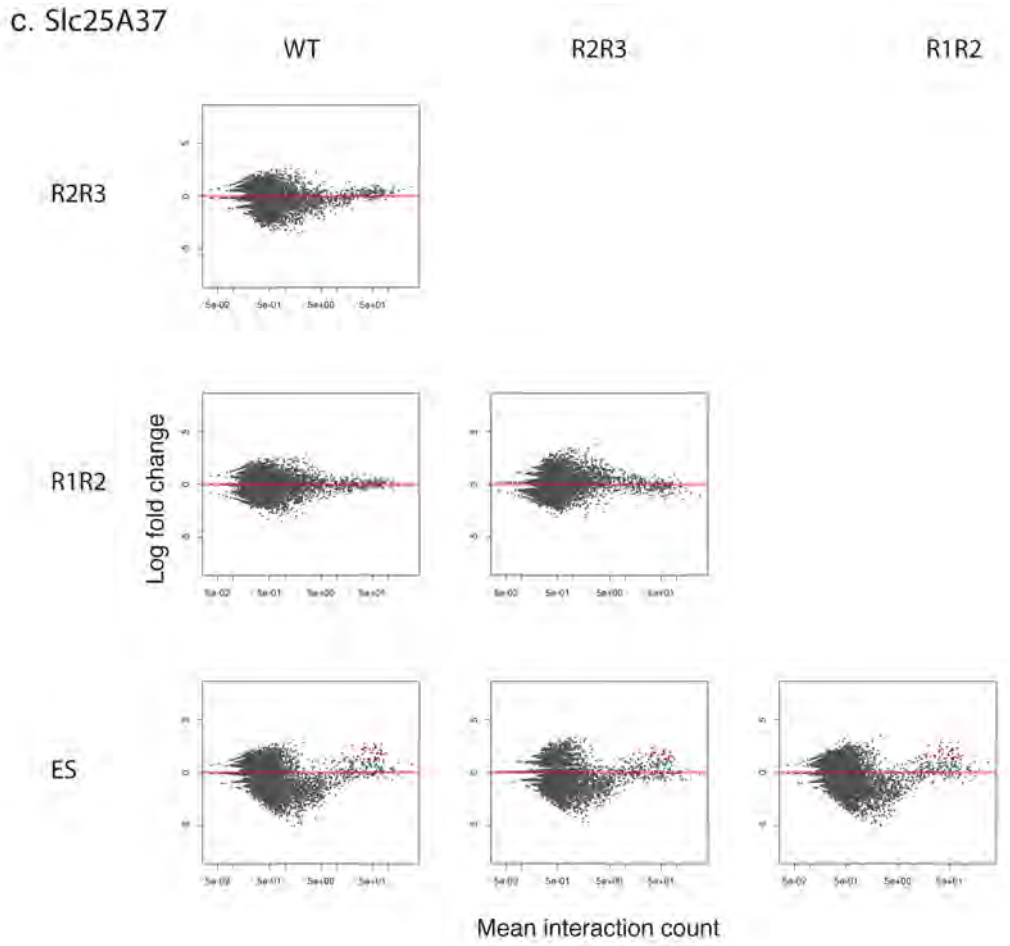
Supplementary Figure 6.
NG Capture-C performed using the *Hba-a1&2* promoters as a viewpoint in primary erythroid cells from ΔR1/ΔR2 and ΔR2/ΔR3 knockout mouse models. The Δ Capture-C profiles show the subtraction of the one profile from another. These show that enhancer KO results in diminished interactions between the enhancers and the promoters of *Hba-a1&2* compared to WT. The interaction profile is clearly still present in the ΔR2/ΔR3 KO. In the ΔR1/ΔR2 KO, interactions are reduced but are clearly present between the promoter and enhancers, relative to ES cells. In particular, there appear to be conserved interactions with the CTCF site between the R1 and R2 enhancers

a. Alpha globin (Hba-a1&2)



b. Beta globin (Hbb-a1&2)





Supplementary Figure 7
Next generation Capture-C was performed from the promoters of *Hba-a1&2*, *Hbb-b1&2* and *Slc25a37* in WT fetal liver, ΔR1/R2 and ΔR2/R3 double knockout cells from three biological replicate of each cell type. DESeq2 was used to compare the number of interactions with the capture points (promoters) per Dpn II restriction fragment genome-wide for the ΔR1/R2 and ΔR2/R3 models in erythroid cells and in WT ES cell non-erythroid controls (Davies et al Nature Methods doi: 10.1038/nmeth.3664). The results are presented as comparison matrices for the α globin, the β globin and the *Slc25a37* loci captured in the same reaction as internal controls for comparability. Statistically different interactions are shown in red with above the midline representing an increase in interactions and below a decrease. Panel a, shows the comparison matrix of these analyses for the α globin locus. The top line of the matrix shows the comparison of the NG Capture-C analysis of the interactions of the *Hba-a1&2* promoters in the double deletion of the R2 and R3 elements (ΔR2/R3) against wild type (WT). The second line shows the comparison between the double deletion of the R1 and R2 elements (ΔR1/R2) against WT and ΔR2/R3. The third line shows the comparison of the non-erythroid control cell type (mES) against WT, ΔR2/R3 and ΔR1/R2. The same comparisons are performed from the point of view of the promoters of the two control loci in panels b and c, *Hbb-b1&2* and *Slc25a37* respectively.

Supplementary Table 1: α- and β-globin rank as strong super-enhancers in erythroid cells

Factor	α-globin regulatory region total reads	α-globin regulatory region relative signal	α-globin regulatory region rank	Classified as Super-enhancer?
Med1	2812	0.87	**2**	**YES**
DNaseI	20309	0.67	**2**	**YES**
H3K4me1	83839	1	**1**	**YES**
H3K27ac	56746	0.81	**8**	**YES**
GSKN	6186	1	**1**	**YES**

Factor	β-globin LCR total reads	β-globin LCR relative signal	β-globin LCR rank	Classified as Super-enhancer?
Med1	3219	1	**1**	**YES**
DNaseI	12991	0.43	**8**	**YES**
H3K4me1	42850	0.51	**11**	**YES**
H3K27ac	31676	0.45	**31**	**YES**
GSKN	3626	0.59	**4**	**YES**

Supplementary Table 2: transcription factors present in individual enhancers

Cluster no.	MED1	NFE2	GATA1	SCL	KLF	CTCF	# factors
1	+++	X	X	X	X	X	5
2	+++	X	X	X	X		4
3	++	X	X		X		3
4	++		X	X	X		3
5	++	X	X	X			3
6	+	X	X				2
7	+		X			X	2
8	+	X				X	2
9	+		X		X		2
10	+	X			X		2
11	+		X	X			2
12	+			X	X		2
13	(+)		X				1
14	(+)				X		1
15	-	X					1
16	-			X			1
17	-					X	1
0	-						0

Supplementary Table 3. Summary of transgenic enhancer testing in mouse and chick

		Mouse embryos		Chick embryos	
Element	**Genomic coordinates in Mm9**	**Total number of embryos with LacZ activity[(1)]**	**Staining consistent with enhancer activity during hematopoiesis[(2)]**	**Total number of embryos showing Citrine activity**	**Staining consistent with enhancer activity during hematopoiesis**
R1	chr11:32145540-32146033 bp	5	Yes	16 (100%)	Yes
R2	chr11:32150804-32151369 bp	7	Yes	21 (100%)	Yes
R3	chr11:32156068-32156646 bp	5	No	18 (100%)	Yes
Rm	chr11:32165216-32165550 bp	4	No	11 (100%)	Yes
R4	chr11:32168944-32169344 bp	6	No	20 (100%)	Yes

1. Figures in this column refer to the number of embryos obtained showing any LacZ staining.
2. Enhancer elements were scored as having activity during hematopoiesis if LacZ staining was visualized in hematopoietic cells following tissue sectioning.

Supplementary Table 4

a. Litter size from Heterozygote x Heterozygote crosses

Model	ΔR1	ΔR2	ΔR3	ΔR(m)	ΔR4	ΔR1+ΔR2	ΔR2+ΔR3
Number of pups	7.0	7.5	8.0	11	7.5	5.3	6.5

b. Percentage Homozygotes, Heterozygotes and WT mice in litters from Heterozygote x Heterozygote crosses

	ΔR1	ΔR2	ΔR3	ΔR(m)	ΔR4	ΔR1+ΔR2	ΔR2+ΔR3
WT	28	18	25	18	14	23	31
Heterozygotes	43	53	25	55	33	77	31
Homozygotes	28	29	50	27	53	0	38
χ^2	*0.143*	*0.857*	*6.000*	*0.273*	*6.467*	*10.700*	*2.077*
P	*0.9311*	*0.6514*	*0.0498*	*0.8725*	*0.0394*	*0.0047*	*0.3540*

c. Percentage Homozygotes and Heterozygotes in litters from Heterozygote x Homozygote crosses

	ΔR1	ΔR3	ΔR(m)	ΔR4
Heterozygotes	61	62	50	46
Homozygotes	39	38	50	54
χ^2	0.692	0.500	0.000	0.077
P	0.4054	0.4795	1.000	0.7815

d. Percentage Homozygotes, Heterozygotes and WT mice in litters from ΔR1/ΔR2 Heterozygote x Heterozygote crosses

	ΔR1/ΔR2 – term litters	ΔR1/ΔR2 – in litters at 12.5 dpc
WT	23	30
Heterozygotes	77	48
Homozygotes	0	22
χ^2	10.700	0.333
P	0.0047	0.8465

Supplementary Table 5: Hematologic data for enhancer knockout mice and WT controls							
		RBC	**HGB**	**HCT**	**MCV**	**MCH**	***Retics %**
KO-8 het KO	n=10	9.5±1.4	16.3±1.3	48.1±5.8	51±4.1	17.4±1.9	2.53±0.8
KO-8 hom KO	n=12	10.1±0.7	16.4±0.7	50.1±2.3	50.±2.5	16.3±1.1	2.0±0.9
KO-12 het KO	n=17	8.9±0.86	15.9±0.9	46.3±3.7	52±1.7	17.9±1.3	1.7±0.6
KO-12 hom KO	n=8	8.6±1.1	15.3±2.1	45.8±6.1	53±1.3	17.8±1.0	1.3±0.9
KO-21 het KO	n=4	9.4±0.12	15.5±0.33	46.5±0.83	49.5±0.58	16.4±0.24	1.5±0.47
KO-21 hom KO	n=9	8.5±0.75	14.7±0.70	42.6±2.6	50.5±2.4	17.5±1.6	2.7±1.1.7
KO-26 het KO	n=4	9.8±0.6	15.6±0.6	47.6±0.3	49±2.6	16±1.0	2.6±0.3
KO-26 hom KO	n=6	10.3±1.2	15.0±1.4	46.9±4.9	45.8±1.7	14.6±0.5	2.2±0.56
KO-31 het KO	n=8	8.4±1.5	14.6±1.6	41.4±7.0	49.3±1.6	17.6±1.7	2.3±0.84
KO-31 hom KO	n=9	8.4±1.0	13.7±1.1	40.6±5.3	48.4±1.5	16.5±1.3	4.1±0.9
KO-21/-26 het KO	n=10	9.5±0.9	15.1±0.9	45.1±3.6	47.7±1.6	16.0+0.8	2.3±1.1
KO-21/-26 hom KO	n=10	10.4±0.9	14.3±1.26	46.9±5.4	45.7±2.5	14.0±0.9	2.5±1.1
KO-26/-31 het KO	n=10	9.9±0.9	14.5±1.4	43.8±4.0	44.5±1.9	14.7±1.0	3.3±1.1
all WT controls	n=68	8.6±0.78	14.2±1.03	42.3±3.57	44.7±1.76	15.0±0.89	1.9±0.81

* retic counts were performed on at least 3 biological replicates from each knockout model, and from 24 WT mice

Legends to Supplementary Tables

Supplementary Table 1.
Ranks of the α- and β-globin regulatory regions in the super-enhancer ranking for various erythroid master factors associated with enhancer activity (Med1, H3K4me1, H3K27ac, GSKN (Gata1, Scl, Klf1 and Nf-e2), and DNase I). Both input-subtracted read-count over the stitched regions and the relative value (divided by the maximum read-count observed for the factor) are displayed.

Supplementary Table 2.
Table depicting the enrichment of transcription factor binding motifs in super-enhancers over genomic background regions. CTCF motifs are not enriched in super-enhancers.

Supplementary Table 3.
Summary of transgenic enhancer testing, showing coordinates of the enhancer region tested, numbers of embryos assessed and conclusions for both mouse and chick models.

Supplementary Table 4.
a. Litter sizes from heterozygote breedings for each model.
b. Observed distribution of wild type, heterozygote and homozygote pups from heterozygote crosses and heterozygote x homozygote crosses of enhancer knockout mice, relative to expected Mendelian ratios. All genotyped by PCR.
c. Observed genotypes from litters of heterozygote x homozygote crosses for each single enhancer knockout
d. Observed distribution of wild type, heterozygous and homozygous fetuses from heterozygous crosses of ΔR1/ΔR2 knockout mice, when the litter was observed at E12.5.

Supplementary Table 5
Hematologic parameters for adult mice from each knockout model: WT, heterozygote and homozygote littermates were assessed where possible. Blood was collected into heparinized capillary tubes and blood counts were assayed using the Horiba Medical Scil Vet abc Plus+ instrument. Reticulocyte counts were performed manually with Brilliant Cresyl blue staining.