SUPPLEMENTARY APPENDIX

Distal and proximal hypoxia response elements co-operate to regulate organ-specific erythropoietin gene expression

Ilaria M. C. Orlando,[1,2] Véronique N. Lafleur,[3] Federica Storti,[1,2] Patrick Spielmann,[1,2] Lisa Crowther,[1,2] Sara Santambrogio,[1,2] Johannes Schödel,[4] David Hoogewijs,[2,5] David R. Mole[3] and Roland H. Wenger[1,2]

[1]Institute of Physiology, University of Zürich, Zürich, Switzerland; [2]National Center of Competence in Research "Kidney.CH", Zürich, Switzerland; [3]NDM Research Building, University of Oxford, Oxford, UK; [4]Department of Nephrology and Hypertension, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany and [5]Department of Medicine/Physiology, University of Fribourg, Fribourg, Switzerland

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Correspondence: *ROLAND H. WENGER* - roland.wenger@access.uzh.ch

Supplementary Table 1. Oligonucleotide sequences.

sgRNA construction oligonucleotides (only sense strands shown)	
5'hre-sgrna	5'-ccctgcacgtatgtgctccgggc-3'
3'hre-sgrna	5'-ccctacgtgctgtctcacacagc-3'

EPO HRE PCR amplification primers	
5'hre-fwd	5'-tggctgcggacatttctatc-3'
5'hre-rev	5'-gatgcaggagcctggttcac-3'
5'hre-rev (deep seq.)	5'-ttcagtggcaatgtggaggt-3'
3'hre-fwd	5'-gcacctaccatcagggacag-3'
3'hre-rev	5'-gccctgggcagggttg-3'

EPO HRE PCR amplification/cloning primers	
5'hre-ecorv-fwd	5'-ccccgatatctgcggacatttctatcaggc-3'
5'hre-xhoi-rev	5'-ccccccctcgagttcagtggcaatgtggaggt-3'
3'hre-ecorv-fwd	5'-ccccgatatcgcacctaccatcagggaca-3'
3'hre-xhoi-rev	5'-ccccctcgagctgggcagggttggcag-3'

mRNA RT-qPCR quantification primers	
epo-fwd	5'-tgggggtgcacgaatg-3'
epo-rev	5'-tttggtgtctgggacagtga-3'
caix-fwd	5'-gggtgtcatctggactgtgtt-3'
caix-rev	5'-cttctgtgctgccttctcatc-3'
l28-fwd	5'-gcaattccttccgctacaac-3'
l28-rev	5'-tgttcttgcggatcatgtgt-3'
loxl2-fwd	5'-gagttgcctgctcagaaacc-3'
loxl2-rev	5'-ctgtgacagtcgtgccagat-3'
pai1-fwd	5'-actggaaaggcaacatgacc-3'
pai1-rev	5'-gaggaagggtctgtccatga-3'

ChIP qPCR quantification primers		Used for:
EPO-5'HRE-fwd	5'-gatggagctgtgtctccctg-3'	Figure 4
EPO-5'HRE-rev	5'-agtggcaatgtggaggtctg-3'	Figure 4
EPO-5'HRE-fwd	5'-ggctgcggacatttctatca-3'	Figure 5
EPO-5'HRE-rev	5'-aaggaagccttgcagtcag-3'	Figure 5
EPO-negctrl-fwd	5'-ggtgcttggtcaggagttga-3'	
EPO-negctrl-rev	5'-gacacgttctcgtccaacct-3'	
EPO-promoter-fwd	5'-gtcctgcccctgctctgacc-3'	
EPO-promoter-rev	5'-gctgttatctgcatgtgtgcgt-3'	
EPO-3'HRE-fwd	5'-cagcagtgcagcaggtccaggtcc-3'	Figure 4
EPO-3'HRE-rev	5'-cgagaggtcagacaggctgtgtgag-3'	Figure 4
EPO-3'HRE-fwd	5'-cttcaacctcattgacaagaactg-3'	Figure 5
EPO-3'HRE-rev	5'-ggatttggaggttcccagaaa-3'	Figure 5
NDRG1-HRE-fwd	5'-tccctcccaatctctctcttctt-3'	
NDRG1-HRE-rev	5'-caccatcagcacagcaaactac-3'	
PAI1-HRE-fwd	5'-cagagggcagaaaggtcaag-3'	
PAI1-HRE-rev	5'-ctctgggagtccgtctgaac-3'	

Supplementary Methods

Gene editing

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) gene editing was performed as described.[1] The *EPO* locus was screened for single guide RNA (sgRNA) binding sites using CRISPR Design Tool.[2] Oligonucleotides containing the sgRNA sequences (Supplementary Table 1) were synthesized (Microsynth, Balgach, Switzerland), annealed and ligated into plasmid MLM3636 (#43860; Addgene, Middlesex, UK). Cells were co-transfected with expression vectors for sgRNA, Cas-9 (ToolGen, Seoul, South Korea) and puromycin resistance (linearized pBabe vector; #1764; Addgene) using polyethylenimine (Polysciences, Warrington, PA, USA) for Hep3B and HepG2, and lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for Kelly cells. Puromycin (1.5 μg/ml, Hep3B; 1 μg/ml, HepG2; 0.2 μg/ml, Kelly) was added two days after transfection for up to two weeks. Gene edited cells were cloned by limiting dilution and genotyped by PCR using High Fidelity Phusion Polymerase (Thermo Fisher Scientific) and the primers listed in Supplementary Table 1. Amplicons were analyzed either by TaiI digestion (Thermo Fisher Scientific) followed by 2% agarose gel electrophoresis to check for HRE destruction,[3] or by sub-cloning into pBluescript vector (Agilent, Santa Clara, CA, USA) and plasmid DNA sequencing (Microsynth), or by direct DNA deep sequencing (Functional Genomics Center Zurich).

References

1. Harms DW, Quadros RM, Seruggia D, et al. Mouse genome editing using the CRISPR/Cas system. Current protocols in human genetics. 2014;83:15.17.11-27.
2. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nature protocols. 2013;8(11):2281-2308.
3. Wenger RH, Kvietikova I, Rolfs A, Camenisch G, Gassmann M. Oxygen-regulated erythropoietin gene expression is dependent on a CpG methylation-free hypoxia-inducible factor-1 DNA-binding site. Eur J Biochem. 1998;253(3):771-777.

Kelly

A

5' HRE alleles

```
                                          clone#
WT cat acgtg cagggaga caca gctccatccagc
C4 cat acgtg cagggaga caca gctccatccagc    2
C3 cat ac-tg cagggaga caca gctccatccagc    8
B4 cat ac-tg cagggaga caca gctccatccagc    4
B4 c----------a caca gctccatccagc          3
```

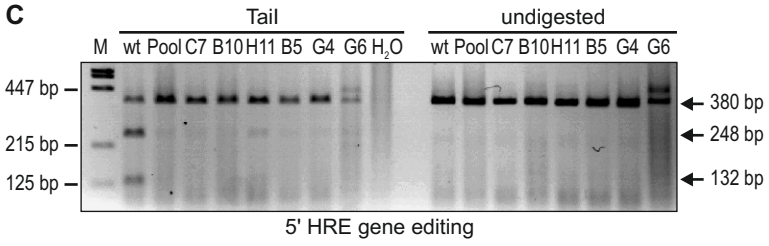
B

3' HRE alleles

```
                                                                                clone#
WT agcaggtccaggtccgggaaacgaggggtggaggggctgggccct acgtg ctgtctca caca gcc
C4 agcaggtccaggtccgggaaacgaggggtggaggggctgggccct acgtg ctgtctca caca gcc    2
T1 ag----------------------------------------------------------tctca caca gcc    9
B2 agcaggtccaggtccgggaaacgaggggtggaggggctgggccct acg---------a caca gcc    1
B2 agcaggtccaggtccgggaaacgaggggtggaggggctgggccct ac-tg ctgtctca caca gcc    2
B2 ag----------------------------------------------------------tctca caca gcc    3
B2 agcaggtccaggtccgggaaacgaggggtggaggg----------g ctgtctca caca gcc    1
B2 agcaggtccaggtccgggaaacgaggggtggaggggctgggccct acgt--tgtctca caca gcc    1
F2 ag----------------------------------------------------------tctca caca gcc    1
F2 agcaggtccaggtccgggaaacgaggggtggaggggctgggccct acg---------a caca gcc    3
A1 agcaggtccaggtccgggaaacgaggggtggaggggctgggccct acgt--tgtctca caca gcc   12
```

Hep3B

C



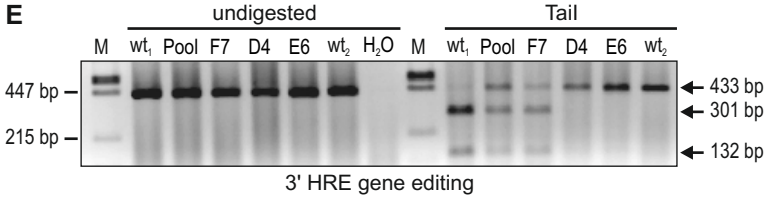
5' HRE gene editing

D

5' HRE alleles

```
                                                    clone# (Seq%)
WT  agcacat acgtg cagggaga caca gctccatccagc
A5  agcacat acgtg cagggaga caca gctccatccagc    2
B5  agc-------tg cagggaga caca gctccatccagc    6
B5  agcacat ac-tg cagggaga caca gctccatccagc    4
H11 agcacat ac-tg cagaaggaga caca gctccatccagc 6   (40%)
H11 agcacat ac-tg cagggaga caca gctccatccagc    9
H11 agcacat at-tg cagggaga caca gctccatccagc    2   (35%)
H11 agcacat acggag cagggaga caca gctccatccagc   0   (20%)
B10 agc-------tg cagggaga caca gctccatccagc     7
B10 agcacat ac-tg cagggaga caca gctccatccagc    7
```

E



3' HRE gene editing

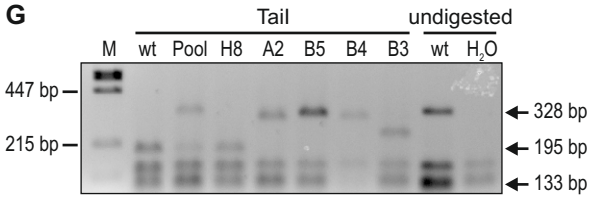
F

3' HRE alleles

```
                                clone#
WT ggccct acgtg ctgtctca caca gcctgt
D4 ggccct acggtg ctgtctca caca gcctgt   2
D4 ggccct acgggg ctgtctca caca gcctgt   2
E6 ggc--------------tca caca gcctgt     2
E6 ggaa ctgca--g ctgtctca caca gcctgt   2
```

HepG2

G



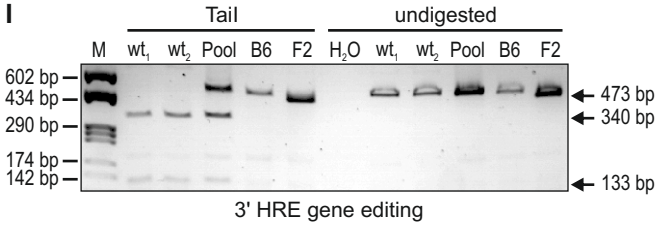
5' HRE gene editing

H

5' HRE alleles

```
                                clone#
WT agcacat acgtg cagggaga caca gctccatccagc
H8 agcacat acgtg cagggaga caca gctccatccagc   2
A2 agc-------tc ca----------tcc-tccagc        6
B5 435 bp deletion                           10
B4 agc-------tc ca----------tcc-tccagc        7
```

I



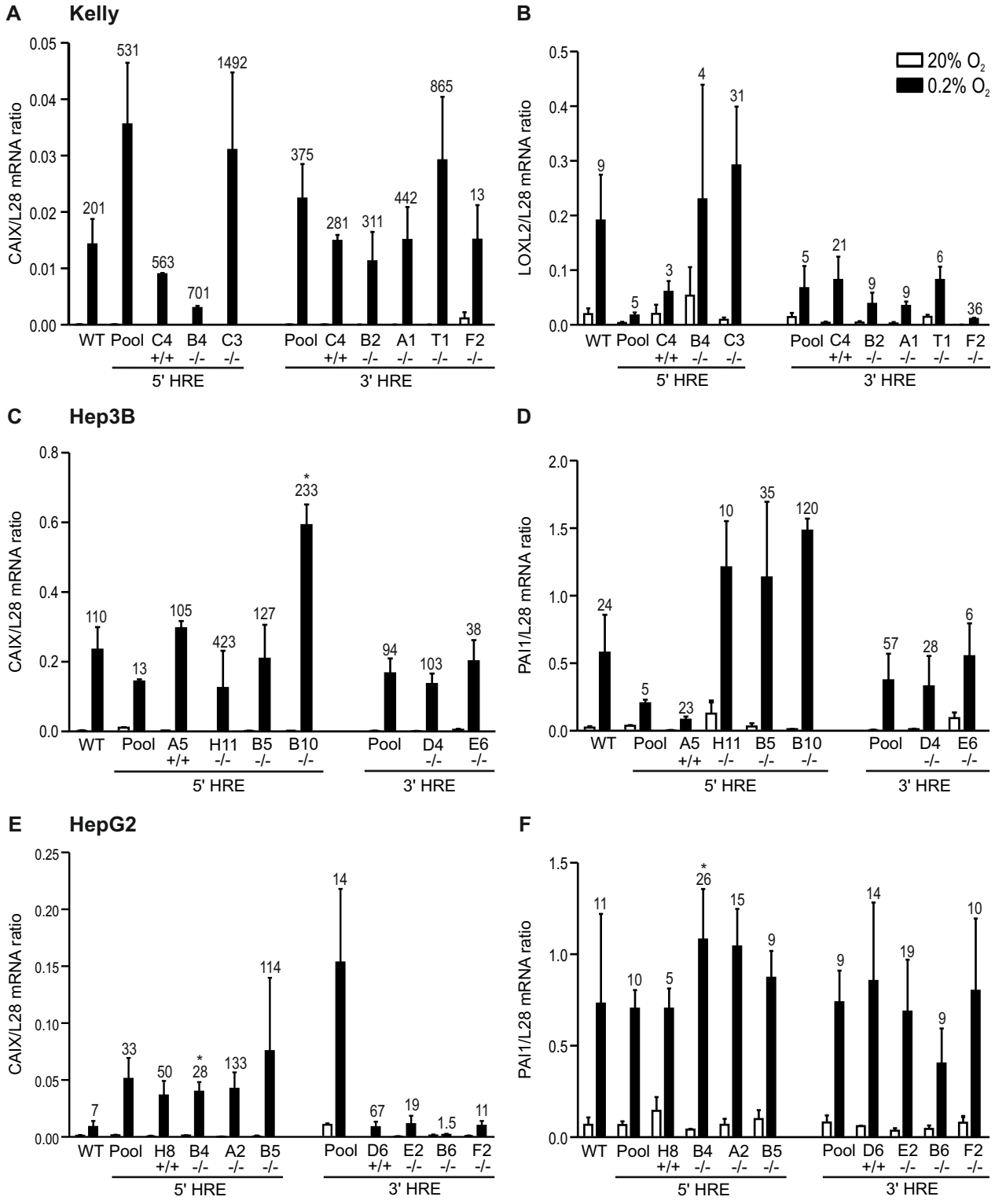
3' HRE gene editing

J

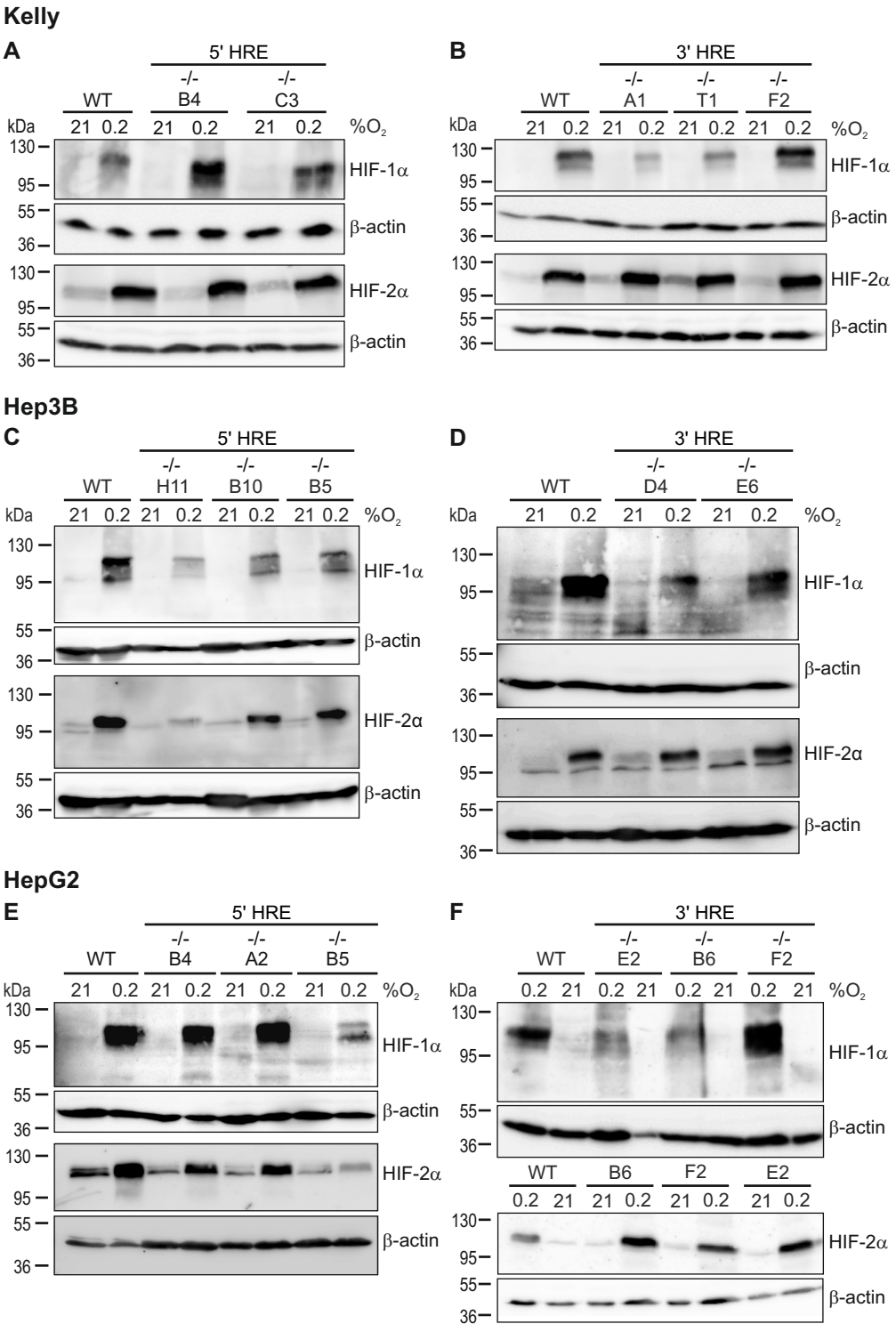
3' HRE alleles

```
                                                clone#
WT acgaggggtggaggggctgggccct acgtg ctgtctca caca gcctgt
D6 acgaggggtggaggggctgggccct acgtg ctgtctca caca gcctgt    2
F2 acgag----------------------------------------gcctgt   10
E2 acgaggggtggaggggctgggccct acggtg ctgtctca caca gcctgt  11
B6 acgaggggtggagggg----------g ctgtctca caca gcctgt        8
```

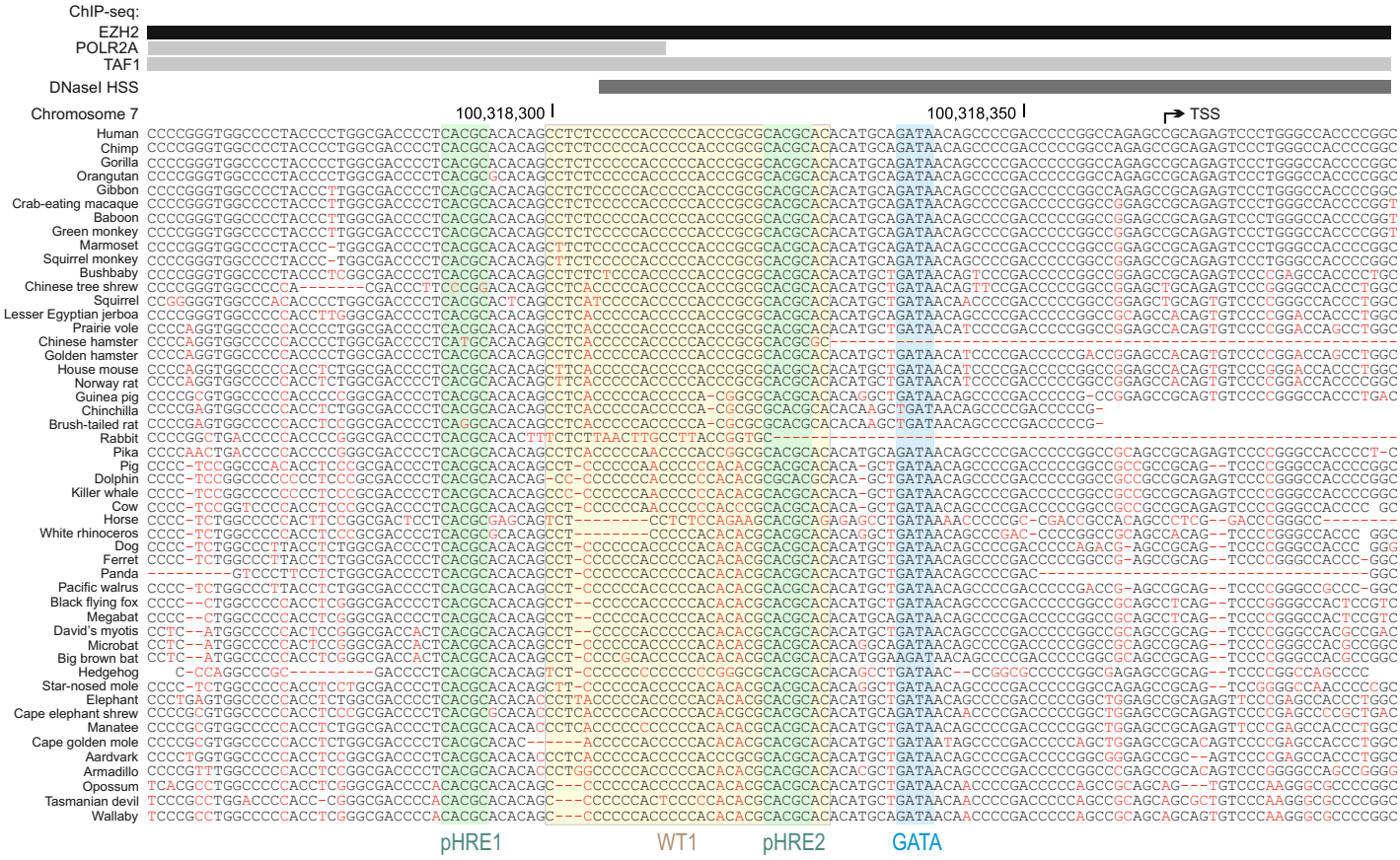
Supplementary Figure 1. Generation and analysis of *EPO* 5' and 3' mutant HRE clonal cell lines. Following mutation of the *EPO* 5' and 3' HREs by CRISPR-Cas9, Kelly (**A**, **B**), Hep3B (**D**, **F**) and HepG2 (**H**, **J**) cells were cloned by limiting dilution, the HRE regions amplified by PCR, and the amplicons either subcloned or directly deep sequenced (bold, consensus 5'-ACGTG-3' and 5'-CACA-3' HRE core and ancillary elements, respectively; WT, wild-type; dashes, nucleotide deletions; highlighted, nucleotide exchanges). The number of independent plasmids harboring the same sequence (clone#) and the percentage of deep sequence reads (Seq%) are indicated on the right. Like outlined for Kelly cells in Figure 1, mutation of the consensus HIF binding site confers resistance to Tail restriction digestion as shown by agarose gel analysis of digested or undigested PCR products using as template genomic DNA isolated from Hep3B (**C**, **E**) and HepG2 (**G**, **I**) cells (M, marker; wt; wild-type; Pool, polyclonal pool of cells; H_2O, water control).



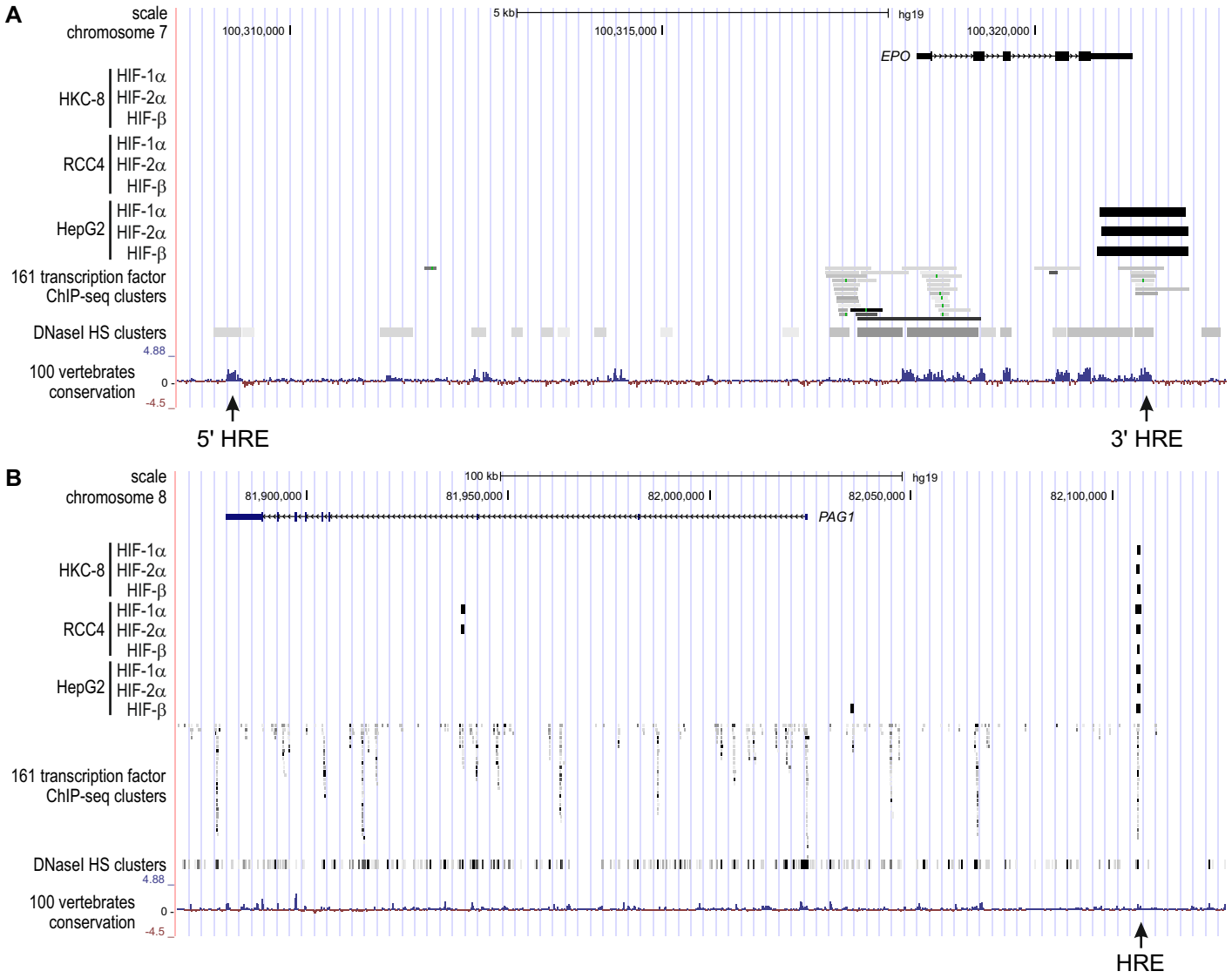
Supplementary Figure 2. Hypoxic induction of HIF target genes in *EPO* 5' and 3' mutant HRE clonal cell lines. Kelly (**A**, **B**), Hep3B (**C**, **D**) and HepG2 (**E**, **F**) samples shown in Figure 2 were analyzed for the mRNA levels of the HIF target genes carbonic anhydrase (CA) IX (**A**, **C**, **E**), lysyl oxidase like (LOXL) 2 (**B**) and plasminogen activator inhibitor (PAI) 1 (**D**, **F**) by RT-qPCR. Transcript levels were normalized to ribosomal protein L28 mRNA and shown as mean + SEM of 3 independent experiments. Numbers above the columns indicate hypoxic induction factors. Student's t-tests were used to statistically evaluate the difference to hypoxic wild-type (WT) cells (*, $p<0.05$; Pool, polyclonal pool of cells; +/+, subclones containing two wild-type alleles; -/-, subclones with biallelic HRE mutation). Note that neither the hypoxic CAIX nor LOXL2 or PAI1 mRNA levels were significantly reduced by *EPO* HRE mutations.



Supplementary Figure 3. Hypoxic stabilization of HIF-1α and HIF-2α in *EPO* 5' and 3' mutant HRE clonal cell lines. Kelly (**A**, **B**), Hep3B (**C**, **D**) and HepG2 (**E**, **F**) wild-type (WT) cells and 5' HRE (**A**, **C**, **E**) or 3' HRE (**B**, **D**, **F**) mutant subclones were exposed to hypoxia (0.2% O_2) for 24 hours followed by immunoblotting for HIF-1α and HIF-2α. β-Actin was used as loading and blotting control. Note that HIFα protein stabilization was not affected by *EPO* HRE mutations.



Supplementary Figure 4. Putative *EPO* promoter HREs. Location of two conserved potential promoter HREs (pHRE1 and pHRE2; green) close to the GATA (blue) and WT1 (yellow) sites in the *EPO* proximal 5' region. Shown is a UCSC Genome Browser output (version *hg19*), including 161 transcription factor ChIP-sequencing (ChIP-seq) tracks derived from the ENCODE database (version 3), clusters of DNaseI hypersensitivity sites (HSS) from 125 cell types, and the transcriptional start site (TSS), with a closer view of the region in 50 vertebrates extracted using the 100-MULTIZ whole-genome multiple sequence alignment algorithm.



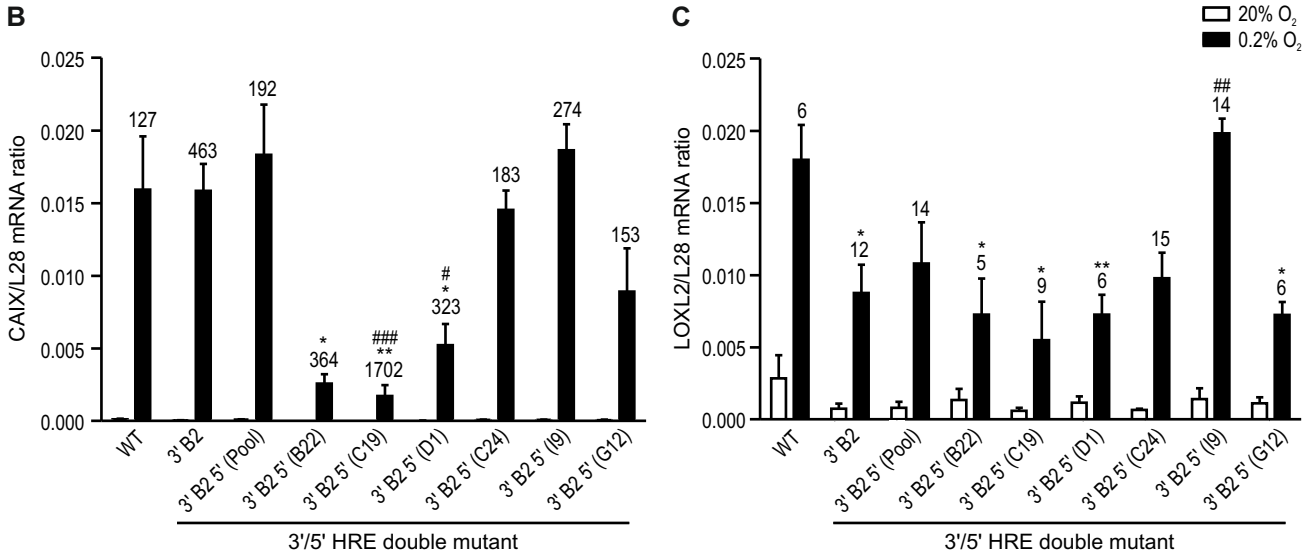
Supplementary Figure 5. UCSC Genome Browser output (*hg19*) of the *EPO* (**A**) and *PAG1* (**B**) genomic regions. HIF-1α, HIF-2α and HIF-β ChIP-seq data of hypoxic non-Epo-producing renal proximal tubule HKC-8 and VHL-deficient clear cell renal cell carcinoma RCC4 as well as of Epo-producing hepatoma HepG2 cell lines are indicated. The *PAG1* locus confirms HRE binding by all three factors in all cell lines analyzed. In addition, the ENCODE integrated regulation tracks containing ChIP-seq data of 161 transcription factors and DNaseI hypersensitivity (HS) clusters are displayed, aligned to the conservation scores of 100 different vertebrate species. Arrows indicate the 5' and 3' HREs of the *EPO* locus and the distal upstream HRE of the *PAG1* locus, respectively.

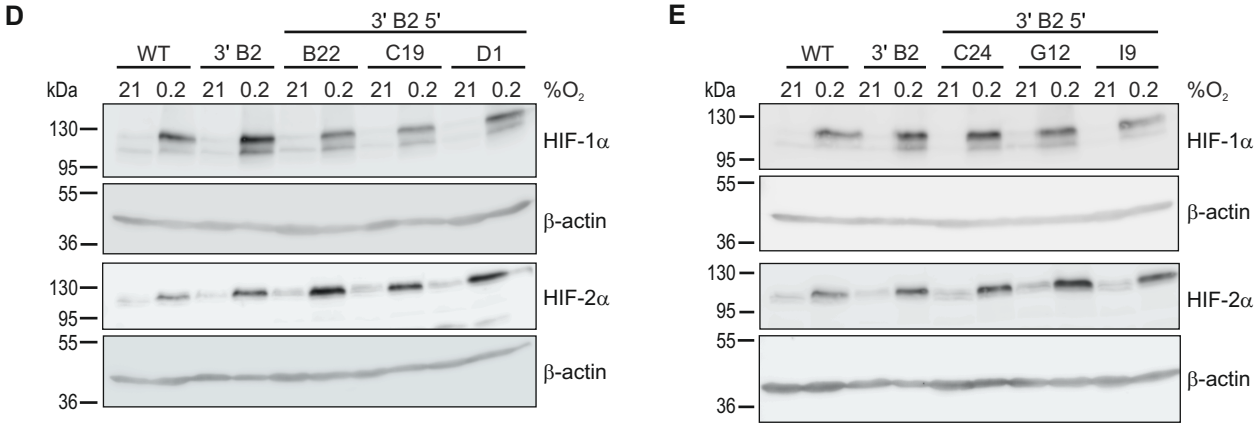
Kelly

A

5' HRE alleles in 3' HRE mutant B2 background

		deletion	(Seq%)
WT	ctcaggcccggagcacat**acgtg**cagggaga**caca**gctccatccagcttc		
B22	ctcaggccc----------atccagcttc	31 bp	(76%)
B22	ctcaggcccggagcaca----------gctccatccagcttc	18 bp	(22%)
C19	ctcaggccc----------atccagcttc	31 bp	(72%)
C19	ctcaggcccggagcaca----------gctccatccagcttc	18 bp	(24%)
D1	ctcaggccc----------atccagcttc	31 bp	(85%)
C24	ctcaggcccggagcacat**ac-tg**cagggaga**caca**gctccatccagcttc	1 bp	(94%)
I9	ctcaggcccggagcacat**ac--g**cagggaga**caca**gctccatccagcttc	2 bp	(99%)
G12	ctcaggcccggagcacat**---tg**cagggagacacagctccatccagcttc	3 bp	(92%)





Supplementary Figure 6. Generation and analysis of *EPO* 5'/3' HRE double-mutant cell lines. (**A**) Following 5' HRE mutation of the Kelly 3' HRE mutant B2 subclone by CRISPR-Cas9 mediated gene editing, the resulting 5'/3' HRE double-mutant subclones were analyzed by PCR amplification and direct deep sequencing (bold, consensus 5'-ACGTG-3' and 5'-CACA-3' HRE core and ancillary elements, respectively; WT, wild-type; dashes, nucleotide deletions). The size of the HRE deletion and the percentage of deep sequence reads (Seq%) are indicated on the right. (**B**, **C**) Kelly samples shown in Figure 5D were analyzed for the mRNA levels of the HIF target genes carbonic anhydrase (CA) IX (**B**) and lysyl oxidase like (LOXL) 2 (**C**) by RT-qPCR. Transcript levels were normalized to ribosomal protein L28 mRNA and shown as mean + SEM of 3 independent experiments. Numbers above the columns indicate hypoxic induction factors. Student's t-tests were used to statistically evaluate the difference to hypoxic WT (*, $p<0.05$; **, $p<0.01$,) or 3' B2 cells ($^{\#}$, $p<0.05$; $^{\#\#}$, $p<0.01$; $^{\#\#\#}$, $p<0.001$). (**D, E**) Kelly samples shown in Figure 5C were analyzed for the protein levels of HIF-1α and HIF-2α by immunoblotting. β-Actin was used as loading and blotting control.