A mammalian acetate switch regulates stress erythropoiesis

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Supplemental Figure 1. Acss2 regulates coupled Cbp/HIF-2 recruitment to chromatin.
Recruitment of (**a**) HIF-2α, HIF-1α, CBP, or p300; (**b**) HIF-2α with CBP or p300; (**c**) CBP or p300 with HIF-2α, or (**d**) HIF-1α with CBP or p300 to the *EPO* enhancer during normoxia or hypoxia. (**e)** Recruitment of HIF-1α as well as of HIF-1α with CBP or p300 to the *PGK1* promoter during normoxia or hypoxia. (**f**) Recruitment of HIF-2α or HIF-1α to the *RPL13A* promoter during normoxia or hypoxia. All experiments were performed in Hep3B cells in the presence or absence of ACSS2 (single pool generated from triplicate biological samples/manipulation; duplicate measurements/pool; mean). Key aspects of the above findings have been confirmed by at least one additional independent experiment.

Supplemental Figure 2. Acss2 acts selectively on HIF-2 signaling in cells.

Contribution of HIF-1α and HIF-2α to hypoxic induction of (**a**) a preferential HIF-2 target gene (*EPO*), (**b**) co-regulated HIF-1/HIF-2 *(VEGFA, PAI1, GLUT1)* or preferential HIF-2 *(MMP9)* target genes, or (**c**) a preferential HIF-1 target gene (*PGK1*). Contribution of ACSS1, ACSS2, and ACLY to hypoxic induction of (**d**) co-regulated HIF-1/HIF-2 *(VEGFA, PAI1, GLUT1)* or preferential HIF-2 *(MMP9)* target genes as well as (**e**) a preferential HIF-1 target gene (*PGK1*). All data are derived from a single pool of triplicate biological replicates/manipulation, triplicate measurements/pool, mean/SD. Separate analyses of 0, 2, and 8 h hypoxia samples was performed by two-way ANOVA with Dunnett's multiple comparison post-hoc test using control siRNA as reference within each group; only results for 8 h hypoxia are indicated (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$). All experiments were performed with Hep3B cells. Key aspects of the above findings have been confirmed by at least one additional independent experiment.

Supplemental Figure 3. Acss2 is expressed in endocrine Epo synthesis sites in mice.

(**a**) Targeting strategy for generating a mouse Acss2 null allele that lacks exons 3 through 16. Acss2 protein immunodetection in (**b**) kidneys and (**c**) livers from mixed strain Acss2 wild-type (WT) and knockout (KO) mice (scale bar = 500 microns). (**d**) Hematocrits of Acss2 WT and KO mice after exposure to hypoxia (*n*=12 biological replicates/group with 6 male/6 female mice for 0 and 2 h time-points, *n*=3 biological replicates/group with 2 male/1 female mice for 0.5 and 16 h time-points, n=3 biological replicates/group with all male mice for 8 h time-point; single measurements/replicate; mean/SD).

Supplemental Figure 4. Acetate is required for Cbp/HIF-2α interactions in cells.

(**a**) Acetylation of endogenous HIF-2α following exposure to normoxia/normal medium (N), hypoxia (H), or 2 h hypoxia-conditioned medium under normoxia (CM). (**b**) Endogenous CBP/HIF-2α complexes formed after incubation with N or CM. (**c**) Final cellular acetate levels and medium pH for cells following exposure to N, H, CM, or acetate-supplemented medium (A) (n=3 biological replicates/group; single measurement/replicate; mean/SD). Comparison by one-way ANOVA with Dunnett's multiple comparison post-hoc test using normoxia as reference (****$P \leq 0.0001$). (**d**) Endogenous CBP/HIF-2α or p300/HIF-2α complexes induced by acetyl CoA addition to whole cell extracts prepared following Acss2 knockdown and 2 h hypoxia exposure. (**e**) Endogenous CBP/HIF-2α or p300/HIF-2α complexes induced by acetyl CoA, acetate, ATP, or acetate plus ATP addition to whole cell extracts prepared after 8 h hypoxia exposure. All experiments were performed with Hep3B cells. Key aspects of the above findings have been confirmed by at least one additional independent experiment.

Supplemental Figure 5. Acetate facilitates recovery from acute anemia.

(**a**) Hematocrits and (**b**) reticulocytes of CD1 wild-type female mice after treatment with phenylhydrazine (PHZ), followed by once daily oral vehicle (water) or acetate (glycerol triacetate, GTA) supplementation (*n*=5 biological replicates/group; single measurements/replicate; mean/SD). The dotted line indicates mean for all control CD1 female mice (*n*=40 biological replicates). Acetylated HIF-2α present in (**c**) kidneys and (**d**) livers of same mice. Key aspects of the above findings have been confirmed by at least one additional independent experiment.

Supplemental Figure 6. Acetate augments HIF-2 signaling during acute anemia.

Epo and *Pgk1* time-course in (**a**) kidneys and (**b**) livers of CD1 wild-type mice with phenylhydrazine (PHZ)-induced acute anemia and treated with once daily oral vehicle (water) or acetate (glycerol triacetate, GTA) supplementation (duplicate measurements/replicate; mean/SEM). Key aspects of the above findings have been confirmed by at least one additional independent experiment.

Supplemental Figure 7. Acetate increases erythropoiesis in chronic anemia.

(**a**) Hematocrits (single measurement/replicate; mean/SD) and (**b**) reticulocytes (triplicate field measurements/replicate; mean/SD) of CD1 wild-type female mice before (Pre) or after (Post) 5/6 partial nephrectomy to induce chronic renal failure (CRF), followed by thrice-weekly (Monday, M/Wednesday, W/Friday, F) intra-peritoneal vehicle (Veh) or sodium acetate/PBS (Ac) injections (n=3 biological replicates/group except for basal n=5 biological replicates/group). (**c**) *Epo* gene expression (triplicate measurements/replicate; mean/SEM) and (**d**) acetate levels (single measurement/replicate; mean/SD) in kidneys of a subset of mice in (**a**) (n=3 biological replicates/group). (**e**) Hematocrits (single measurement/replicate; mean/SD), (**f**) reticulocytes (triplicate field measurements/replicate; mean/SD), (**g**) *Epo* gene expression in kidneys (triplicate measurements/replicate; mean/SEM), and (**h**) acetate levels in kidneys (single measurement/replicate; mean/SD) of WBB6 F1 hybrid wild-type (WT) or double heterozygous (DH) cKit mutant mice supplemented thrice-weekly (M/W/F) with intra-peritoneal Veh or Ac with n=4 biological replicates/group. All comparisons by one-way ANOVA with Tukey's post-hoc multiple comparison test using pre-nephrectomy/no treatment for CRF control and WT cKit/vehicle treatment as control for cKit mutant mice control (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

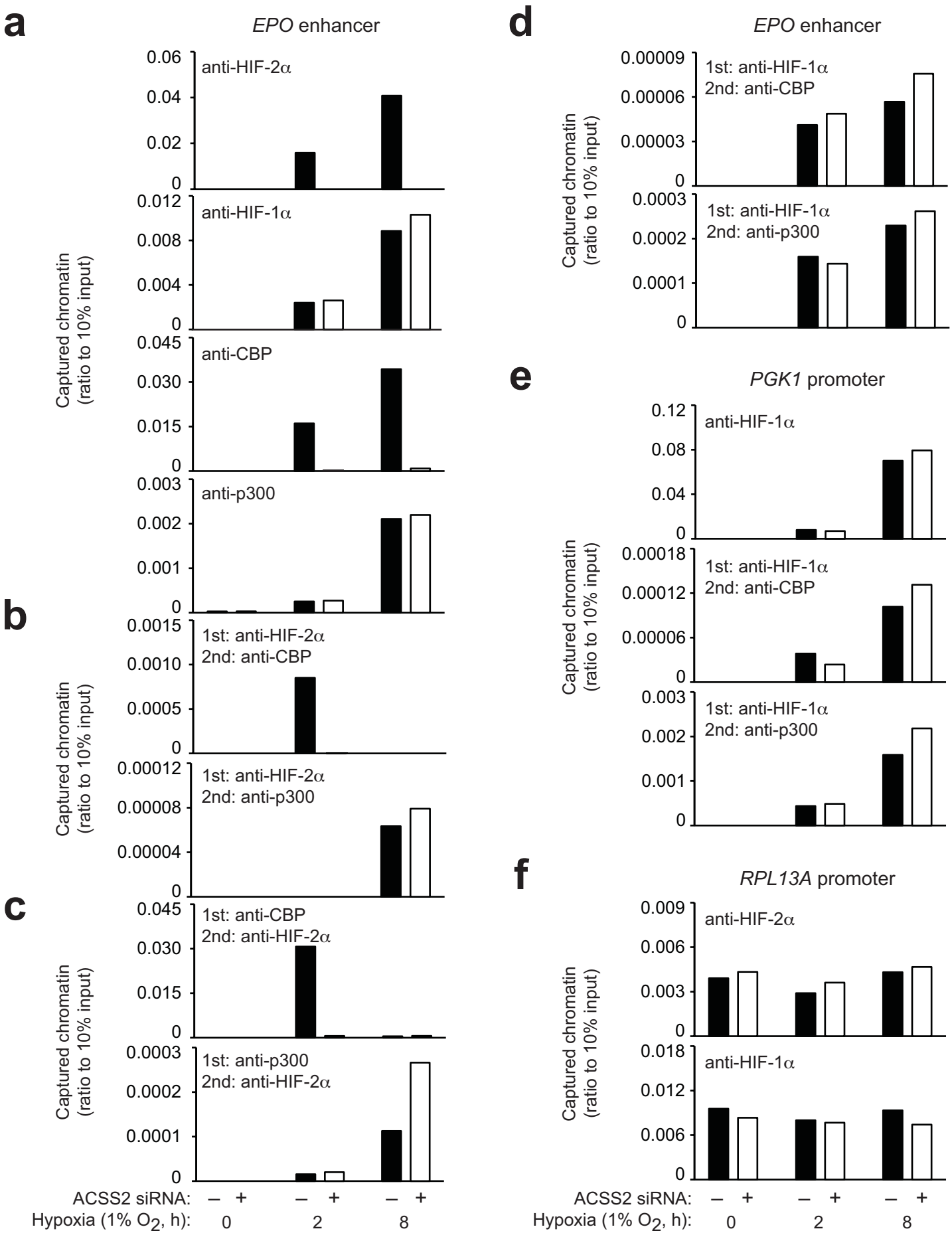
Supplemental Figure 8. Acetate alters HIF-2α acetylation patterns in anemic mice.

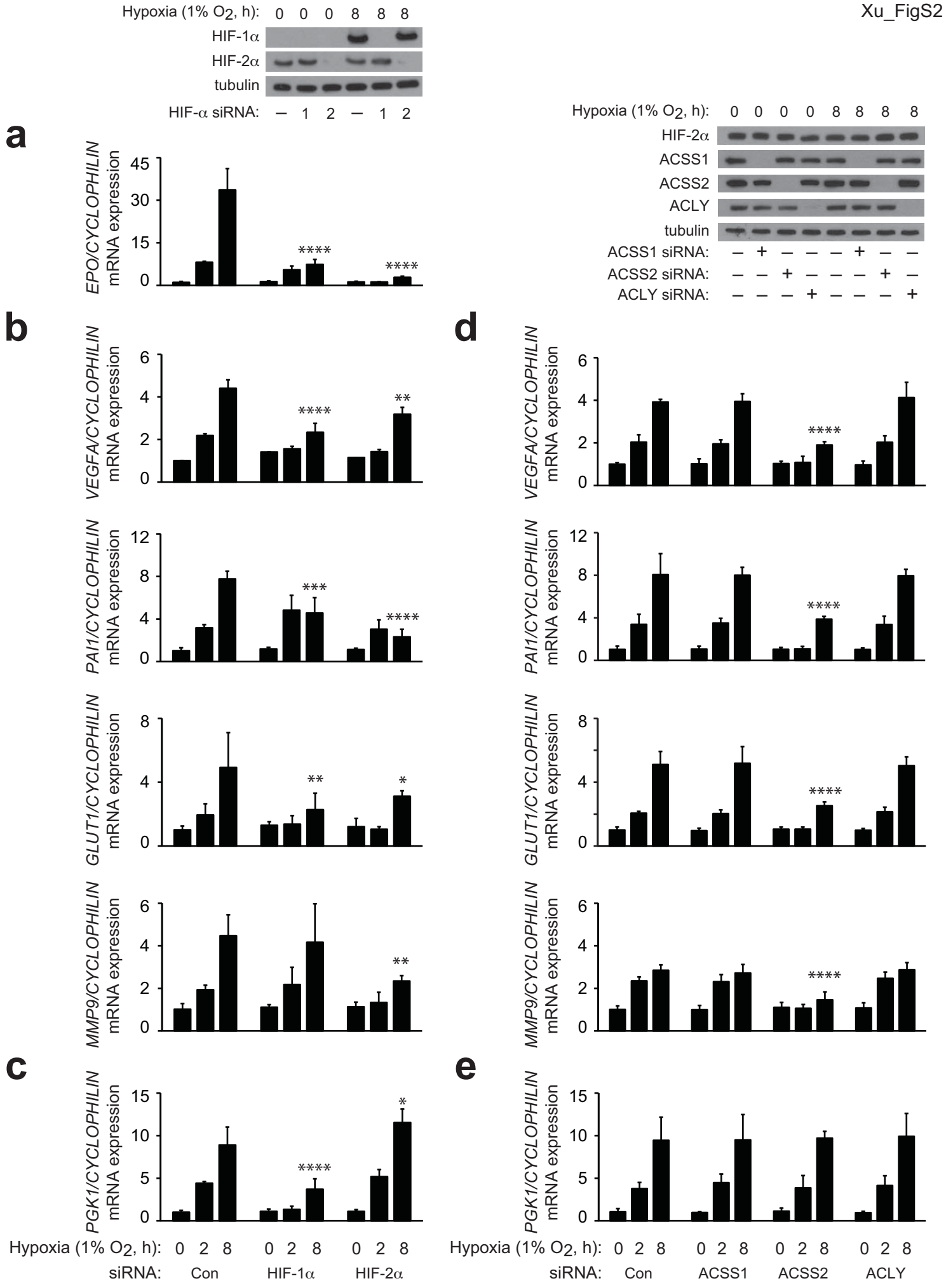
(**a**) Serial plasma Epo protein measurements before (Pre) or after (Post) partial 5/6 nephrectomy to induce chronic renal failure (CRF) and treated with thrice-weekly (Monday, M/Wednesday, W/Friday, F) intra-peritoneal PBS vehicle (Veh) or sodium acetate/PBS (Ac) (*n*=6 biological replicates/group; duplicate measurements; mean/SEM). (**b**) Plasma Epo protein measurements obtained from WBB6 F1 hybrid double heterozygous (DH) cKit mutant mice without or with thrice-weekly (M/W/F) intra-peritoneal Ac supplements (*n*=4 biological replicates/group; duplicate measurements/replicate; mean/SEM). All comparisons by two-way ANOVA with Dunnett's multiple comparison post-hoc test with week 0 as reference within each group (****$P \leq 0.0001$). (**c**) Immunoblot of kidney extracts from three CD1 wild-type female mice with CRF either not treated (None), treated for one week with three (M/W/F) supplements of intra-peritoneal Ac (On), or treated for one week with three (M/W/F) supplements of intra-peritoneal Ac followed by cessation of treatment for one week (Off). (**d**) Immunoblot of kidney extracts from three WBB6 F1 hybrid wild-type (WT) or DH cKit mutant mice either not treated (None) or treated for one week with three (M/W/F) supplements of intra-peritoneal Ac (On).

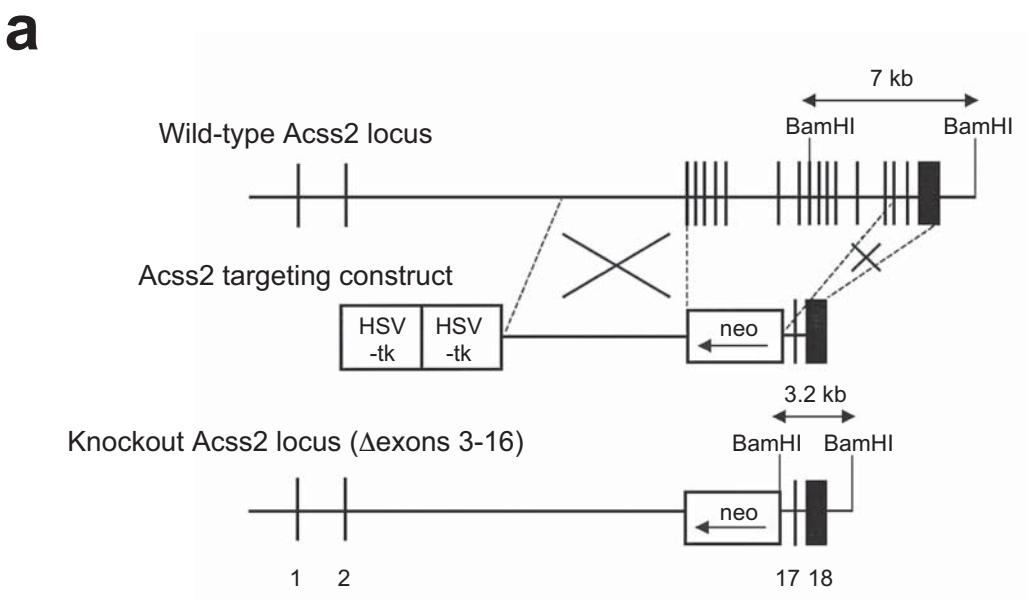
Supplemental Table 1. Renal and hematocrit assessment of chronic renal failure mice.

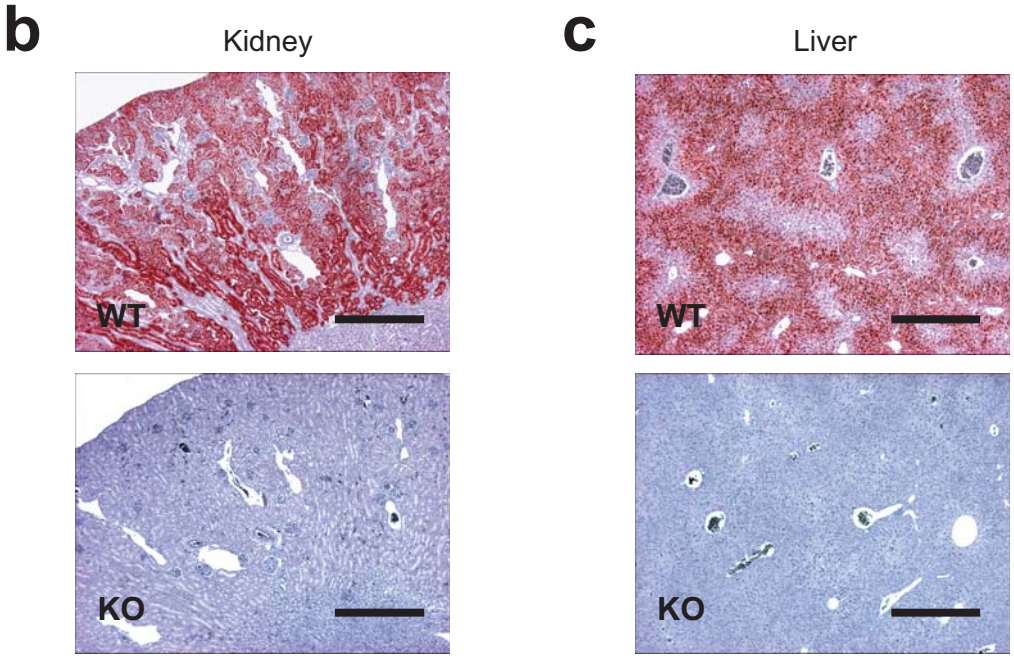
Wild-type CD1 female mice were housed under standard conditions in the UTSWMC animal facility. Prior to nephrectomy, blood was collected for baseline blood urea nitrogen (BUN), plasma creatinine (CREAT), and hematocrit (Hct) measurements. Three weeks following 5/6 partial nephrectomy, repeat measurements were obtained and mice were then separated into three groups with comparable post-nephrectomy BUN, plasma CREAT, and Hct values.

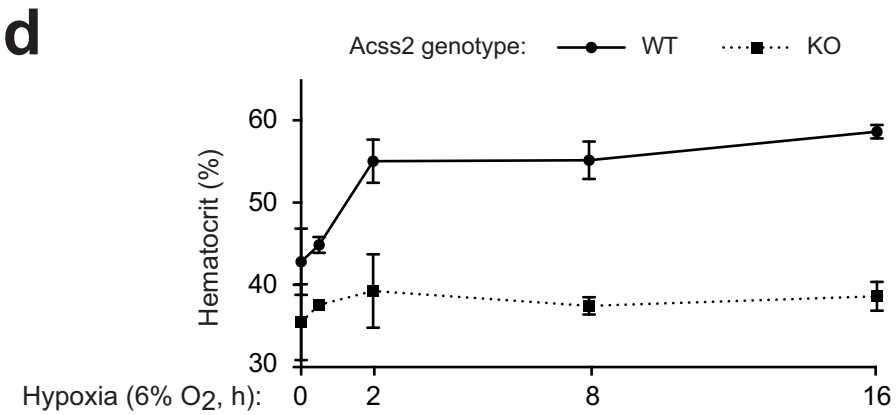
Mouse Number	*BUN (mg/dL)*	*CREAT (mg/dL)*	*Tail Hct (%)*	*BUN (mg/dL)*	*CREAT (mg/dL)*	*Tail Hct (%)*	*Group*
	Pre-nephrectomy			*Post-nephrectomy*			
26162	15	0.15	50.00	108	0.56	30.43	A
26194	14	0.16	50.00	55	0.34	30.00	A
26161	15	0.10	50.00	59	0.33	32.00	A
26150	12	0.10	60.00	54	0.38	31.25	A
26151	16	0.08	44.44	134	0.61	33.33	A
26191	13	0.21	50.00	53	0.32	32.65	A
26188	19	0.12	48.00	60	0.27	30.51	A
26152	13	0.10	50.00	58	0.31	33.33	A
26181	14	0.21	51.85	55	0.32	32.50	A
26155	15	0.10	47.50	49	0.27	35.38	A
26195	16	0.14	50.00	48	0.20	39.29	A
Average	**14.73**	**0.13**	**50.16**	**66.64**	**0.36**	**32.79**	
SD	**1.90**	**0.05**	**3.80**	**27.75**	**0.12**	**2.67**	
26175	15	0.16	44.44	44	0.26	30.30	B
26165	13	0.18	50.00	73	0.40	32.50	B
26154	17	0.15	53.33	31	0.39	32.26	B
26179	16	0.16	50.00	80	0.39	30.77	B
26185	18	0.08	50.00	46	0.26	32.26	B
26186	14	0.10	50.00	90	0.54	32.50	B
26183	14	0.14	53.33	79	0.41	32.76	B
26167	17	0.14	50.00	165	0.81	32.00	B
26149	11	0.13	52.17	29	0.16	34.29	B
26178	17	0.11	42.86	41	0.26	36.07	B
26192	13	0.08	62.50	34	0.33	36.67	B
Average	**15.00**	**0.13**	**50.79**	**64.73**	**0.38**	**32.94**	
SD	**2.19**	**0.03**	**5.08**	**39.88**	**0.17**	**1.98**	
26158	15	0.13	55.56	42	0.37	30.77	C
26164	13	0.10	50.00	120	0.61	26.79	C
26171	16	0.08	50.00	32	0.22	31.25	C
26176	10	0.14	50.00	42	0.23	32.26	C
26156	14	0.15	55.56	133	0.74	32.76	C
26163	15	0.08	50.00	75	0.40	32.50	C
26189	18	0.11	53.57	70	0.41	35.56	C
26197	14	0.22	52.38	59	0.30	34.15	C
26180	15	0.15	50.00	67	0.25	34.62	C
26190	18	0.16	50.00	56	0.24	35.48	C
26187	20	0.15	56.25	49	0.27	36.59	C
Average	**15.27**	**0.13**	**52.12**	**67.73**	**0.37**	**32.97**	
SD	**2.72**	**0.04**	**2.64**	**31.96**	**0.17**	**2.77**	

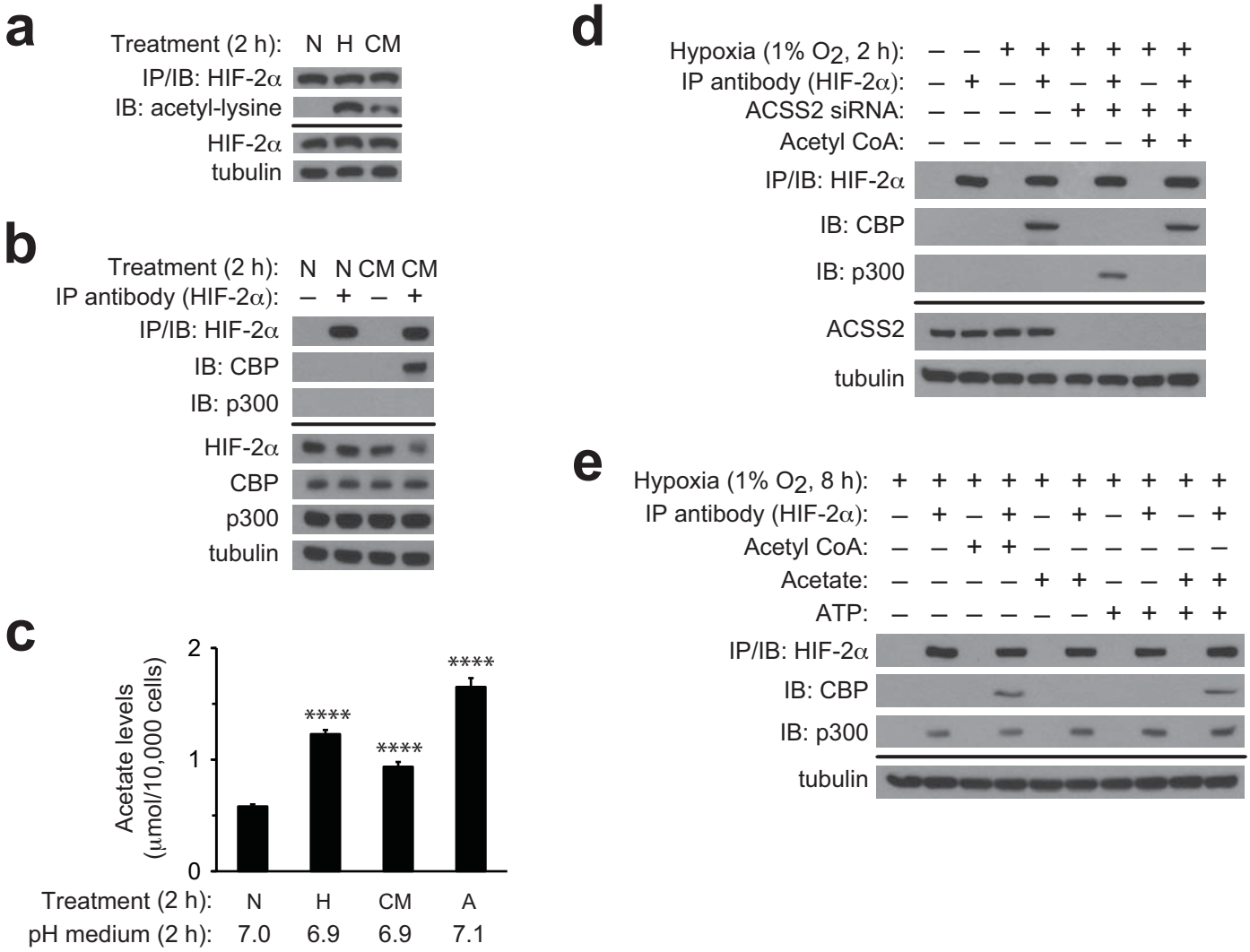

a
EPO enhancer
anti-HIF-2α
anti-HIF-1α
anti-CBP
anti-p300
Captured chromatin (ratio to 10% input)
b
1st: anti-HIF-2α
2nd: anti-CBP
1st: anti-HIF-2α
2nd: anti-p300
c
1st: anti-CBP
2nd: anti-HIF-2α
1st: anti-p300
2nd: anti-HIF-2α
d
EPO enhancer
1st: anti-HIF-1α
2nd: anti-CBP
1st: anti-HIF-1α
2nd: anti-p300
e
PGK1 promoter
anti-HIF-1α
1st: anti-HIF-1α
2nd: anti-CBP
1st: anti-HIF-1α
2nd: anti-p300
f
RPL13A promoter
anti-HIF-2α
anti-HIF-1α
ACSS2 siRNA: – + – + – +
Hypoxia (1% O2, h): 0 2 8


Hypoxia (1% O2, h): 0 0 0 8 8 8
HIF-1α
HIF-2α
tubulin
HIF-α siRNA: – 1 2 – 1 2
Hypoxia (1% O2, h): 0 0 0 0 8 8 8 8
HIF-2α
ACSS1
ACSS2
ACLY
tubulin
ACSS1 siRNA: – + – – – + – –
ACSS2 siRNA: – – + – – – + –
ACLY siRNA: – – – + – – – +
a
EPO/CYCLOPHILIN mRNA expression
b
VEGFA/CYCLOPHILIN mRNA expression
PAI1/CYCLOPHILIN mRNA expression
GLUT1/CYCLOPHILIN mRNA expression
MMP9/CYCLOPHILIN mRNA expression
c
PGK1/CYCLOPHILIN mRNA expression
d
VEGFA/CYCLOPHILIN mRNA expression
PAI1/CYCLOPHILIN mRNA expression
GLUT1/CYCLOPHILIN mRNA expression
MMP9/CYCLOPHILIN mRNA expression
e
PGK1/CYCLOPHILIN mRNA expression
Hypoxia (1% O2, h): 0 2 8 0 2 8 0 2 8
siRNA: Con HIF-1α HIF-2α
Hypoxia (1% O2, h): 0 2 8 0 2 8 0 2 8 0 2 8
siRNA: Con ACSS1 ACSS2 ACLY

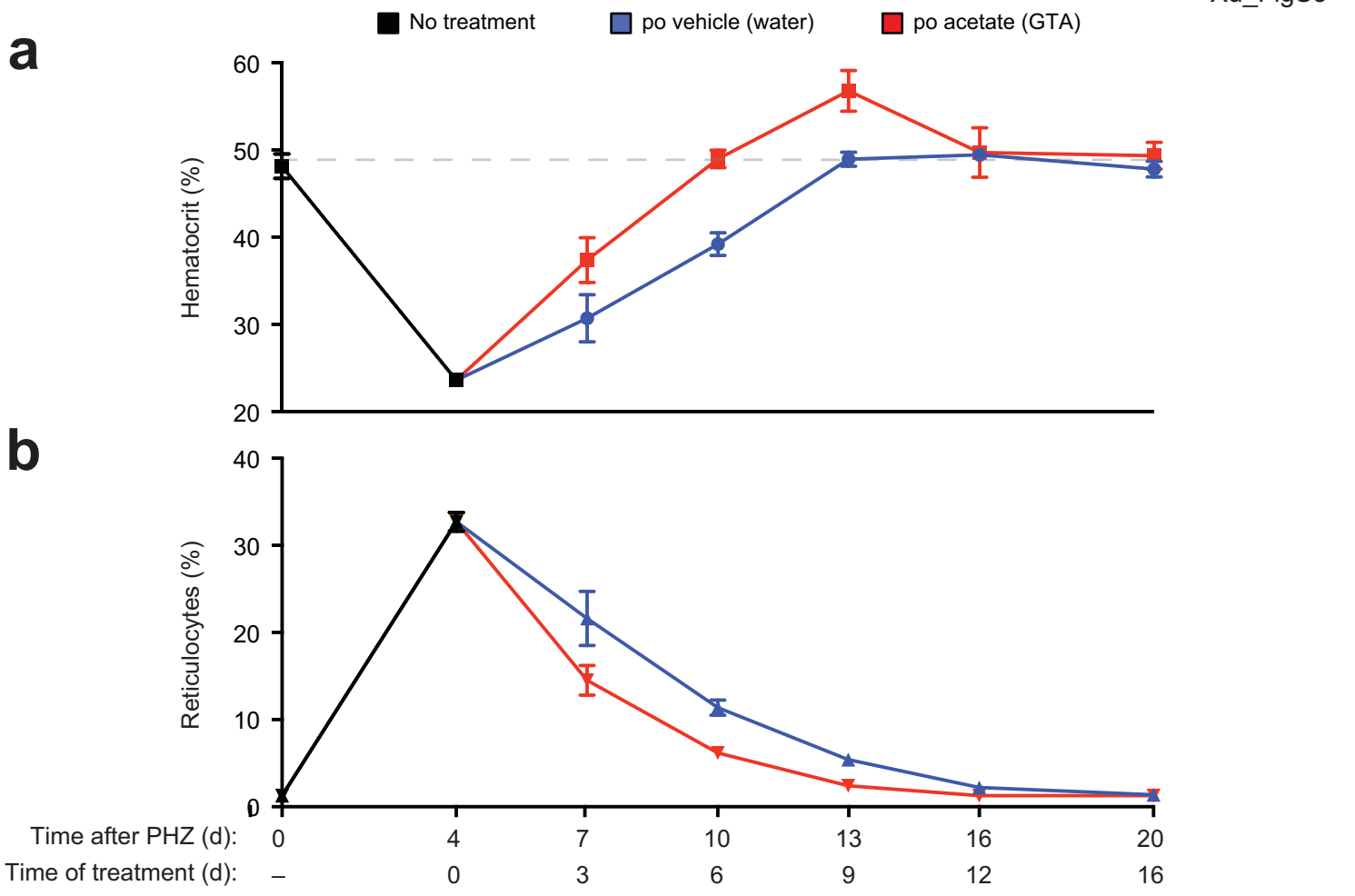

a
7 kb
BamHI
BamHI
Wild-type Acss2 locus
Acss2 targeting construct
HSV -tk
HSV -tk
neo
3.2 kb
Knockout Acss2 locus (Δexons 3-16)
BamHI
BamHI
neo
1
2
17
18

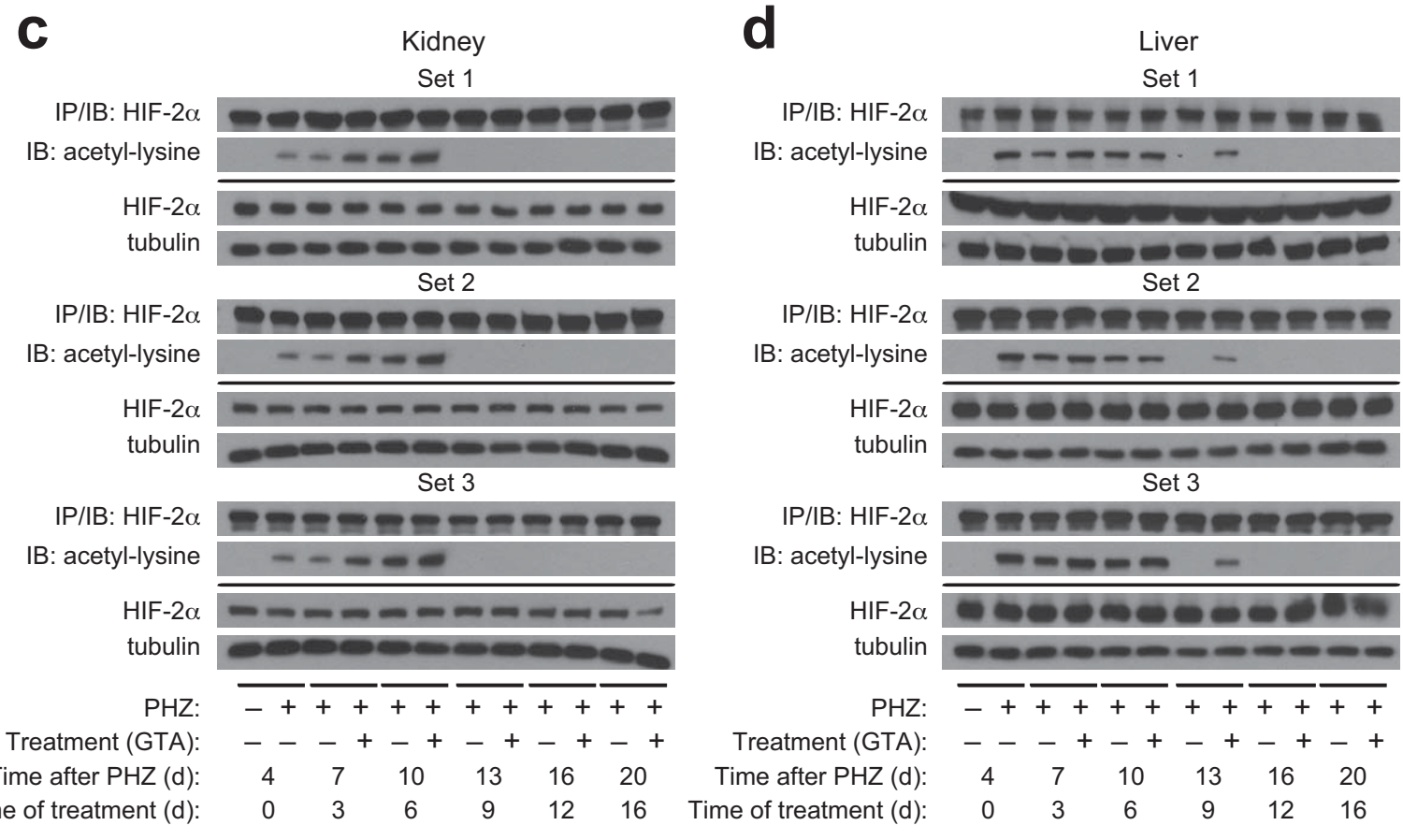

b
Kidney
WT
KO
c
Liver
WT
KO

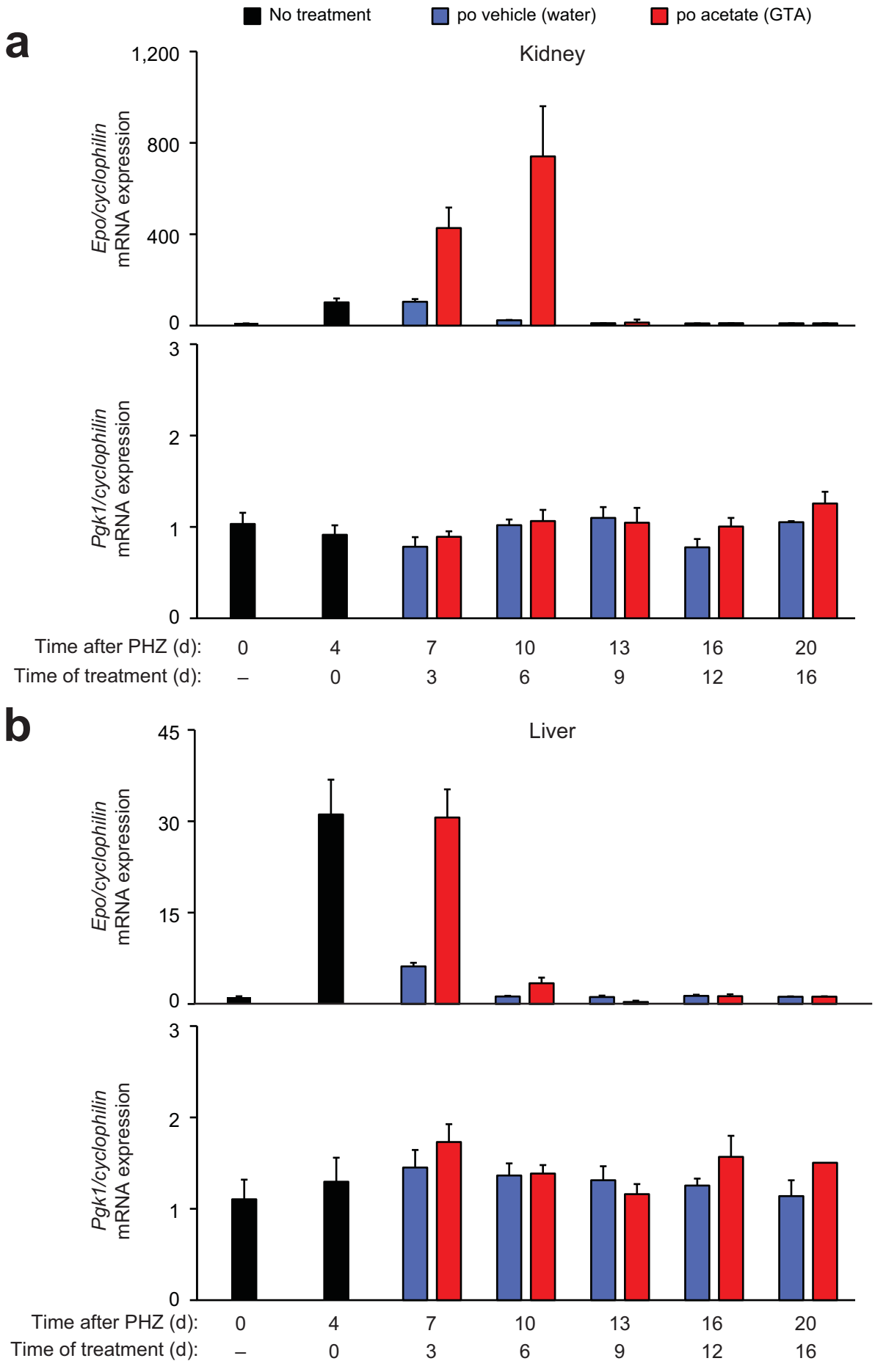

d
Acss2 genotype:
WT
KO
Hematocrit (%)
60
50
40
30
Hypoxia (6% O2, h):
0
2
8
16

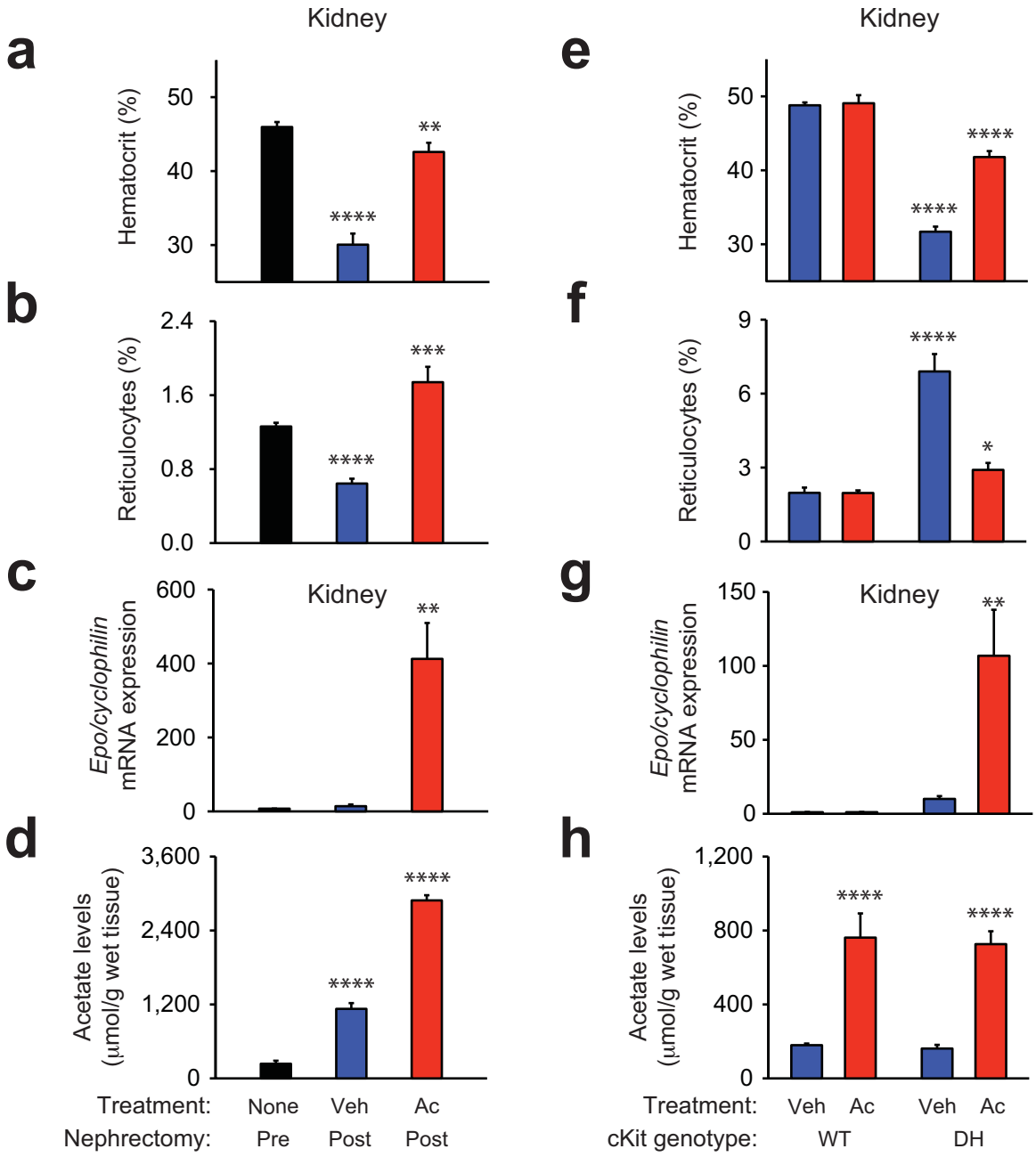

a
Treatment (2 h): N H CM
IP/IB: HIF-2α
IB: acetyl-lysine
HIF-2α
tubulin
b
Treatment (2 h): N N CM CM
IP antibody (HIF-2α): – + – +
IP/IB: HIF-2α
IB: CBP
IB: p300
HIF-2α
CBP
p300
tubulin
c
Acetate levels (μmol/10,000 cells)
2
1
0
****
****
****
Treatment (2 h): N H CM A
pH medium (2 h): 7.0 6.9 6.9 7.1
d
Hypoxia (1% O2, 2 h): – – + + + + + +
IP antibody (HIF-2α): – + – + – + – +
ACSS2 siRNA: – – – – + + + +
Acetyl CoA: – – – – – – + +
IP/IB: HIF-2α
IB: CBP
IB: p300
ACSS2
tubulin
e
Hypoxia (1% O2, 8 h): + + + + + + + + + +
IP antibody (HIF-2α): – + – + – + – + – +
Acetyl CoA: – – + + – – – – – –
Acetate: – – – – + + – – + +
ATP: – – – – – – + + + +
IP/IB: HIF-2α
IB: CBP
IB: p300
tubulin

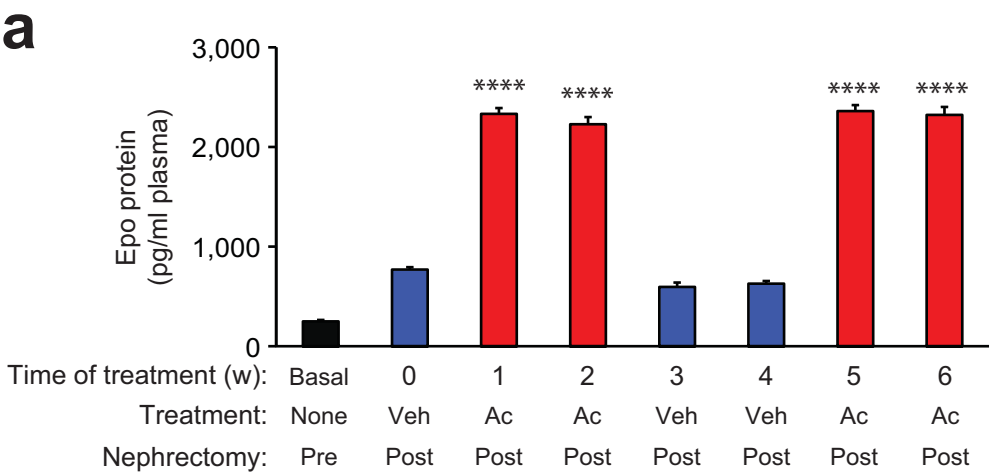
Xu_FigS5

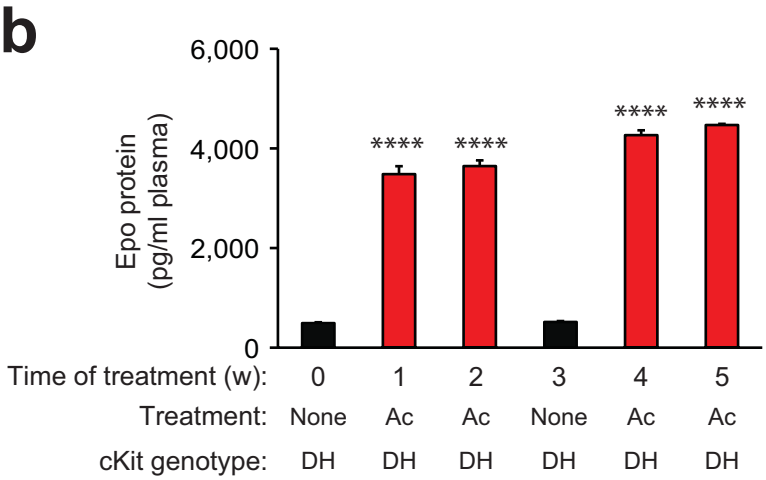

a
No treatment
po vehicle (water)
po acetate (GTA)
Hematocrit (%)
60
50
40
30
20
b
Reticulocytes (%)
40
30
20
10
0
Time after PHZ (d): 0 4 7 10 13 16 20
Time of treatment (d): – 0 3 6 9 12 16

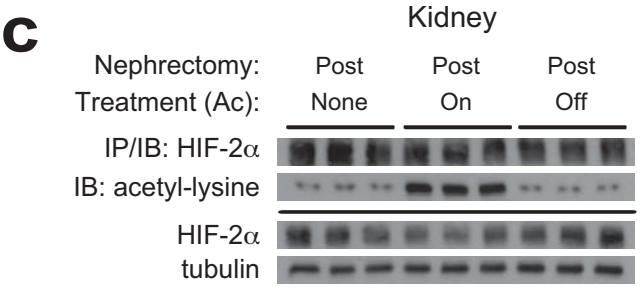

c
Kidney
Set 1
IP/IB: HIF-2α
IB: acetyl-lysine
HIF-2α
tubulin
Set 2
Set 3
d
Liver
PHZ: – + + + + + + + + + + +
Treatment (GTA): – – – + – + – + – + – +
Time after PHZ (d): 4 7 10 13 16 20
Time of treatment (d): 0 3 6 9 12 16

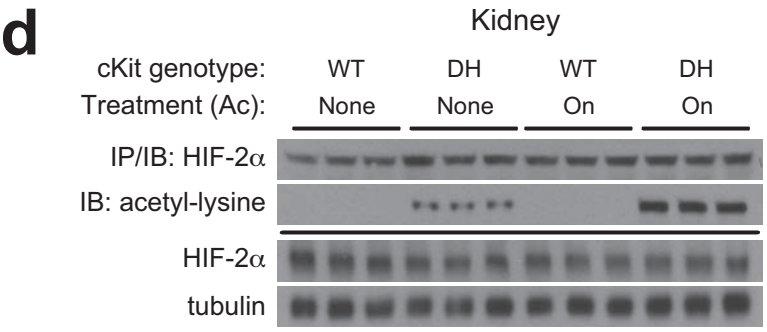

No treatment
po vehicle (water)
po acetate (GTA)
a
Kidney
Epo/cyclophilin mRNA expression
1,200
800
400
0
Pgk1/cyclophilin mRNA expression
3
2
1
0
Time after PHZ (d): 0 4 7 10 13 16 20
Time of treatment (d): – 0 3 6 9 12 16
b
Liver
Epo/cyclophilin mRNA expression
45
30
15
0
Pgk1/cyclophilin mRNA expression
3
2
1
0
Time after PHZ (d): 0 4 7 10 13 16 20
Time of treatment (d): – 0 3 6 9 12 16


a
Kidney
Hematocrit (%)
50
40
30
****
**
b
Reticulocytes (%)
2.4
1.6
0.8
0.0
****
***
c
Kidney
Epo/cyclophilin mRNA expression
600
400
200
0
**
d
Acetate levels (µmol/g wet tissue)
3,600
2,400
1,200
0
****
****
Treatment: None Veh Ac
Nephrectomy: Pre Post Post
e
Kidney
Hematocrit (%)
50
40
30
****
****
f
Reticulocytes (%)
9
6
3
0
****
*
g
Kidney
Epo/cyclophilin mRNA expression
150
100
50
0
**
h
Acetate levels (µmol/g wet tissue)
1,200
800
400
0
****
****
Treatment: Veh Ac Veh Ac
cKit genotype: WT DH


a
3,000
2,000
1,000
0
Epo protein (pg/ml plasma)
****
****
****
****
Time of treatment (w): Basal 0 1 2 3 4 5 6
Treatment: None Veh Ac Ac Veh Veh Ac Ac
Nephrectomy: Pre Post Post Post Post Post Post Post


b
6,000
4,000
2,000
0
Epo protein (pg/ml plasma)
****
****
****
****
Time of treatment (w): 0 1 2 3 4 5
Treatment: None Ac Ac None Ac Ac
cKit genotype: DH DH DH DH DH DH


c
Kidney
Nephrectomy: Post Post Post
Treatment (Ac): None On Off
IP/IB: HIF-2α
IB: acetyl-lysine
HIF-2α
tubulin


d
Kidney
cKit genotype: WT DH WT DH
Treatment (Ac): None None On On
IP/IB: HIF-2α
IB: acetyl-lysine
HIF-2α
tubulin