HIF2α is an essential molecular brake for postprandial hepatic glucagon response independent of insulin signaling

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Supplement Table 1 (Related to Experimental procedure- Real-time PCR)

Primers	Sequence
G6pase F	GTGTCCAGGACCCACCAATA
G6pase R	ACTGTGGGCATCAATCTCCT
Pepck F	CTGGATGAAGTTTGATGCCC
Pepck R	TGTCTTCACTGAGGTGCCAG
Epo F	CATCTGCGACAGTCGAGTTCTG
Epo R	CACAACCCATCGTGACATTTTC
Hif-2α F	TGAGTTGGCTCATGAGTTGC
Hif-2α R	TATGTGTCCGAAGGAAGCTG
Irs-1 F	GGAGGATTTGCTGAGGTCAT
Irs-1 R	CTATGCCAGCATCAGCTTCC
Irs-2 F	CACAATTCCAAGCGCCACAA
Irs-2 R	CATCACCTCCTCCCAGGGTA
Pgc-1 F	TGAGGACCGCTAGCAAGTTT
Pgc-1 R	TGTAGCGACCAATCGGAAAT
Pkar1α F	CCCTCGAGTCAGTACGGATG
Pkar1α R	GCATTCCTTCGGGAATACTTT
Pkar2α F	CCTCCTCTTCTTCATCAGGG
Pkar2α R	GAGTGACTCGGACTCGGAAG
Pkar2β F	GATCATCGGTTTTGGGATGT
Pkar2β R	ATAAACCGGTTCACAAGGCG
Pkacα F	ATTCTGAGAAGGGGTCTCCC
Pkacα R	AAGAAGGGCAGCGAGCAG
Pkacβ F	TCCTCAAGCCCAGCATTACT
Pkacβ R	CAAGAAAGGCAGCGAAGTG
PDE1B F	TGCAGTAGAGTATGTGGGGC
PDE1B R	AGAAACCCAAGTTCCGAAGC
PDE2A F	CCCCATCACTCCTCATCATC
PDE2A R	AGAACCAGCCATTGCTCTTG
PDE3A F	CGAGGACCAAGGAAGAGATTC
PDE3A R	ATGAGCTGCTCCCTGGGTAT
PDE3B F	ATCGCCTCTTGGTCTGCCAG
PDE3B R	GCCTTCTGTCCATCTCAAATGTAGG
PDE4A F	CAGAACCTCAGCAAGCGCCA
PDE4A R	CCTGAGGACCTGGATACGG
PDE4C F	CGGACTGAGCGTCTAGGAGA
PDE4C R	GGGACACAGAGAAACCTTGG
PDE4D F	TGCTCAGGTCTTGGCTAGCCTGC
PDE4D R	TCCTCCAGGGTCTCGCTGGC
PDE7A F	GTGTTAATCAGCCGTTTCTTATT
PDE7A R	CAAAGCACCTATCTGAGCCT
PDE7B F	TCAATGAATGGGTAGGAGCC
PDE7B R	GAGCCCTGAACAGAGTGTCA
PDE10A F	AAATTATATGGTTTGACGGATG
PDE10A R	ACAGTCTCTGCACTAACAC

Supplement figure legends

Supplement Figure 1 *Progressive improvement in glucose and glucagon tolerance is independent of IRS-2 levels at 1-week following VHL disruption (Related to Figure 1).* (A) Glucose tolerance test in mice at two week following disruption of VHL in liver (Vhl^{LivKO}). *p < 0.05 compared to $Vhl^{F/F}$. Five mice were assessed per group. (B) qPCR analysis of *Irs-1* and *Irs-2* in the livers of $Vhl^{F/F}$ and Vhl^{LivKO} at one and two week following disruption of VHL in liver (Vhl^{LivKO}). Expression was normalized to β-actin mRNA. Each bar represents the mean value ± S.E.M. *p < 0.05 compared to $Vhl^{F/F}$. (C) Western blot analysis of IRS-2 in primary hepatocytes extracted at 1-week following VHL disruption or (D) in livers at 7-, 14- and 30-days following VHL disruption. Three mice assessed per group. (E) Western blot analysis of pFOXO1 and FOXO1 in cytosolic and nuclear fraction from Vhl^{LivKO} at 1-week following VHL disruption. Three mice assessed per group. (F) Glucagon tolerance test in mice at two week following disruption of VHL in liver (Vhl^{LivKO}). Four to five mice were assessed per group. *p < 0.001 compared to $Vhl^{F/F}$. Four to five mice were assessed per group.

Supplement Figure 2 *Decreased gluconeogenesis in Vhl^{LivKO} mice could not be restored by PGC-1 overexpression or STAT-3 inhibition (Related to Figure 2).* (A) Pyruvate tolerance test in mice at two weeks following disruption of VHL in liver (Vhl^{LivKO}). *p < 0.05, **p < 0.01, ***p < 0.001 compared to $Vhl^{F/F}$. Four to five mice were assessed per group. (B) Western blot analysis of insulin stimulated AKT phosphorylation in primary hepatocytes isolated from $Vhl^{F/F}$ and Vhl^{LivKO} mice that were pretreated for 1-hour with 50 nM LY294002 or 50 nM MK-2206. (C) qPCR analysis of PGC-1 in the primary

hepatocytes of *Vhl*$^{F/F}$ and *Vhl*^{LivKO} mice (D) *G6pase* in the primary hepatocytes of *Vhl*$^{F/F}$ and *Vhl*^{LivKO} mice infected with recombinant adenovirus expressing PGC-1α (Ad-PGC-1α). (E) Western blot analysis for pSTAT3 and STAT3 in primary hepatocytes of *Vhl*$^{F/F}$ and *Vhl*^{LivKO} mice. (F) Western blot analysis for pSTAT3 and STAT3 in primary hepatocytes of *Vhl*^{LivKO} mice treated with the STAT3 inhibitor (STAT3i), 100 μM S3i-201 (Apexbio, Houston, TZ) for 2-hours. (G) qPCR analysis of *G6pase* and *Pepck* in the primary hepatocytes of *Vhl*$^{F/F}$ and *Vhl*^{LivKO} treated with STAT3i for 2-hours. Gene expression studies are done in triplicates and expression was normalized to β-actin mRNA. Each bar represents the mean value ± S.E.M. ***$p < 0.001$ compared to *Vhl*$^{F/F}$. ###$p < 0.05$ compared to untreated.

Supplement Figure 3 *Improved glucose homeostasis and glucagon resistance in Vhl*^{LivKO} *mice is HIF-2α dependent (Related to Figure 3).* (A) Fed and fasting glucose monitored in WT (*Vhl/Hif-1α*$^{F/F}$) and mice with compound disruption of VHL in liver (*Vhl/Hif-1α*^{LivKO}). (B) Glucose, (C) glucagon and (D) pyruvate tolerance test performed in WT and *Vhl/Hif-1α*^{LivKO} mice. **$p < 0.01$, ***$p < 0.001$ compared to *Vhl/Hif-1α*$^{F/F}$. Seven to eight mice were assessed per group.

Supplement Figure 4 *Decrease in gluconeogenic genes after refeeding is associated with decreased CREB phosphorylation (Related to Figure 4).* (A) Serum insulin levels measured in mice fed, overnight fasted or overnight fasted and refed for 30 minutes to 2-hours. (B) Western blot analysis in whole cell lysates of mice that were either fasted overnight or refed for 30 minutes to 2-hours. (C) qPCR analysis of *G6pase* and *Pepck*

mRNA in mice that were either fed, overnight fasted or refed for 30 minutes to 2-hours. Expression was normalized to β-actin mRNA. Each bar represents the mean value ± S.E.M. *p < 0.05, ***p < 0.001 compared to fasted mice. Five mice were assessed per group.

Supplement figure 5 *Abrogation of glucagon signaling in Vhl^{LivKO} mice is due to increased hydrolysis of cAMP (Related to Figure 5).* (A) G6pase luciferase assays in primary hepatocytes of $Vhl^{F/F}$ and Vhl^{LivKO} mice that were infected with Ad-G6pase-luciferase virus and then pre-treated with 0.2 μM Okadaic acid for 2-hours, followed by treatment with 50 nM glucagon. Luciferase assays were performed in triplicates and activities normalized to protein content. Each bar represents the mean value ± S.E.M. #p < 0.05, ###p < 0.001 compared to untreated. ***p < 0.001 compared to $Vhl^{F/F}$. (B) Western blot analysis of pCREB in primary hepatocyte lysate incubated invitro with recombinant PKA (2500U/ml; NewEngland Biolabs) for 30 minutes at 30C. (C) qPCR analysis of PKA subunit mRNA in the primary hepatocytes of $Vhl^{F/F}$ and Vhl^{LivKO} mice. (D) Western blot analysis of PKA subunits in the primary hepatocyte lysates from $Vhl^{F/F}$ and Vhl^{LivKO} mice. (E) Immunostaining for PKA-C in the primary hepatocytes from $Vhl^{F/F}$ and Vhl^{LivKO} mice that were treated with 50 nM glucagon for 30 minutes. (F) Western blot analysis for PKA-C in the nuclear lysates of $Vhl^{F/F}$ and Vhl^{LivKO} mice. (G) Co-immunoprecipitation with PKA-C antibody in the primary hepatocytes from $Vhl^{F/F}$ and Vhl^{LivKO} mice that were treated with 50 nM glucagon for 30 minutes and then probed for PKA-C and CREB. (H) qPCR analysis of AKAP-12 and Syk in the primary hepatocytes of $Vhl^{F/F}$ and Vhl^{LivKO} mice. (I) Western blot analysis for AKAP-12 in the primary

hepatocytes from *Vhl*$^{F/F}$ and *Vhl*^{LivKO} mice. (J) qPCR analysis to assess glucagon induced *G6pase* and *Pepck* in the primary hepatocytes of *Vhl*$^{F/F}$ and *Vhl*^{LivKO} mice that were pre-treated with Piceatannol (Syk inhibitor) or PMA (AKAP inhibitor) for 2-hours. qPCR analysis for *Pepck* in the primary hepatocytes of *Vhl*$^{F/F}$ and *Vhl*^{LivKO} mice that were treated with (K) forskolin or (L) 200 μM 8-br-cAMP for 2-hours. (M) Western blot analysis for p-PKA substrate antibody in the primary hepatocytes from *Vhl*$^{F/F}$ and *Vhl*^{LivKO} mice that were treated with 200 μM 8-br-cAMP for 30 minutes. Gene expression studies are done in triplicated and expression was normalized to β-actin. Each bar represents the mean value ± S.E.M. #$p < 0.05$, ###$p < 0.001$ compared to untreated. *$p < 0.05$, ***$p < 0.001$ compared to *Vhl*$^{F/F}$. (N) Glucagon stimulated *Pepck* mRNA in *Vhl*$^{F/F}$ and *Vhl*^{LivKO} primary hepatocytes pre-treated with 1 mM IBMX for one hour. (O) qPCR analysis for glucagon induced *G6pase* expression in the primary hepatocytes of *Vhl*^{LivKO} mice that were pre-treated with 20 μM Bay-60-7550 (PDE2), 20 μM Milrinone (PDE3), and 250 μM Papaverine (PDE10) for 2-hours. qPCR analysis of PDEs in the primary hepatocytes of (P) *Vhl*$^{F/F}$ and *Vhl*^{LivKO} mice or (Q) *Vhl/Hif2α*$^{F/F}$ and *Vhl/Hif2α*^{LivKO} mice. *$p < 0.05$ compared to *Vhl*$^{F/F}$ or *Vhl/Hif2α*$^{F/F}$. Gene expression studies are done in triplicates and expression was normalized to β-actin mRNA. Each bar represents the mean value ± S.E.M. *$p < 0.05$, ***$p < 0.001$ compared to *Vhl*$^{F/F}$. # $p < 0.05$, ###$p < 0.001$ compared to Vehicle, \$$p < 0.05$, \$\$\$$p < 0.001$ are IBMX treated compared to their untreated controls. (R) Western blot analysis of PDE4D in liver lysates of *Vhl*$^{F/F}$ and *Vhl*^{LivKO} mice. Experiments using primary hepatocytes were done in triplicates and repeated at least three times.

Supplement Figure 6 *Global change in PDEs results in blunted glucagon signaling in Vhl^{LivKO} mice (Related to Figure 6).* (A) Glucagon stimulated *G6pase* expression in primary hepatocytes of $Vhl^{F/F}$ and Vhl^{LivKO} mice infected with 100 MOI Adeno-GFP or Adeno-MEK1DN virus for 48 hours. ####, $p < 0.0001$ compared to no glucagon; ****, $p < 0.0001$ compared to $Vhl^{F/F}$; $, $p < 0.05$ compared to Ad-GFP; $$, $p < 0.01$ compared to Ad-GFP. (B) Glucose production in hepatocytes pretreated with 10 μM SU6656 for 16-hours and then treated with glucagon for 4-hours. Each bar represents the mean value ± S.E.M. *, $p < 0.01$ compared to $Vhl^{F/F}$; ##, $p < 0.001$ compared to no glucagon; ^, $p < 0.05$ compared to substrate. (C) Western blot analysis of p42/p44 ERK phosphorylation in liver lysates from mice with compound disruption of VHL/ARNT ($Vhl/Arnt^{LivKO}$). (D) Blood glucose at fed state and (E) qPCR analysis for *G6pase* and *Pepck* in the livers of $Vhl/Arnt^{LivKO}$ mice. Four mice were used per group and experiments with primary hepatocytes were done in triplicates and repeated at least three times.

Supplement Figure 7 *Insulin signaling in STZ treated mice (Related to Figure 7).* (A) Serum insulin levels assessed in STZ treated mice. *$p < 0.05$ compared to untreated mice. (B) Co-immunostaining for insulin and glucagon performed in the pancreas of Vehicle or STZ treated mice. (C) Serum glucagon level and (D) Insulin:glucagon ratio assessed in STZ treated mice. Five to seven mice were assessed per group. *$p < 0.05$, ***$p < 0.001$ compared to Vhl^{LivKO} mice.

Supplement Figures

Figure S1

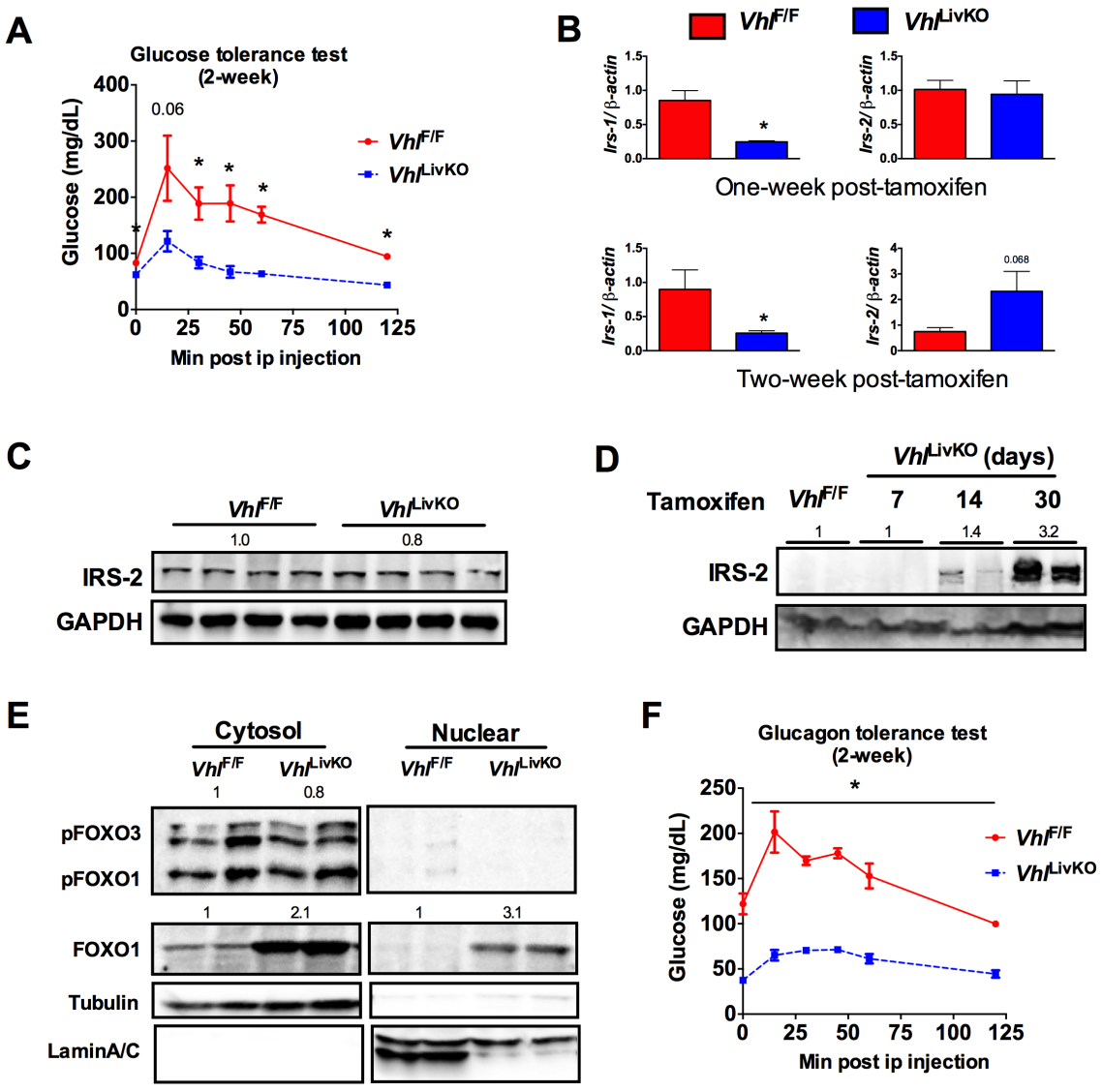
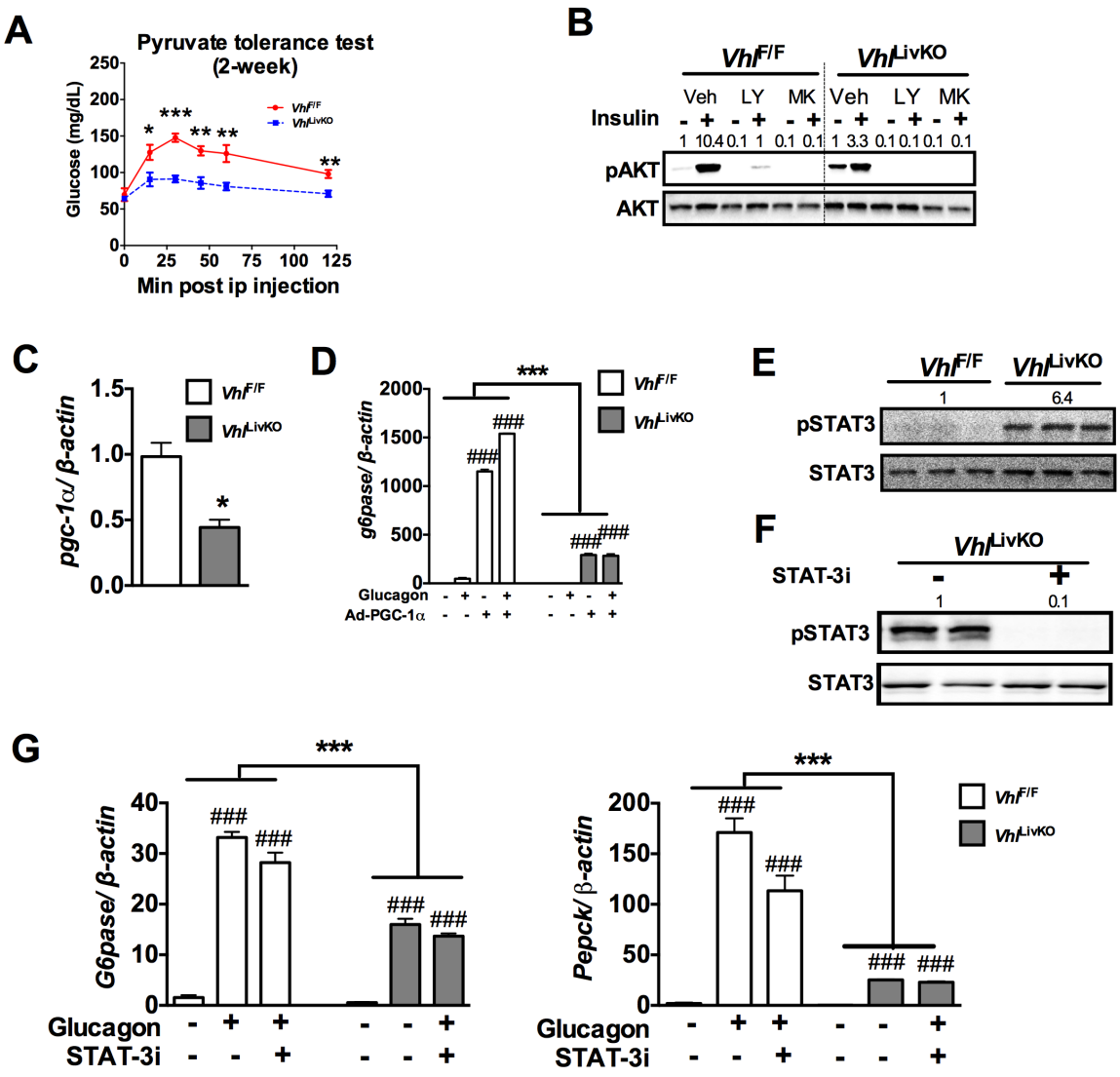


Figure S2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Pyruvate tolerance test
(2-week)
Glucose (mg/dL)
Min post ip injection
B
Insulin
Veh LY MK Veh LY MK
pAKT
AKT
C
pgc-1α/ β-actin
D
g6pase/ β-actin
Glucagon
Ad-PGC-1α
E
pSTAT3
STAT3
F
STAT-3i
G
G6pase/ β-actin
Pepck/ β-actin
Glucagon
STAT-3i

Figure S3

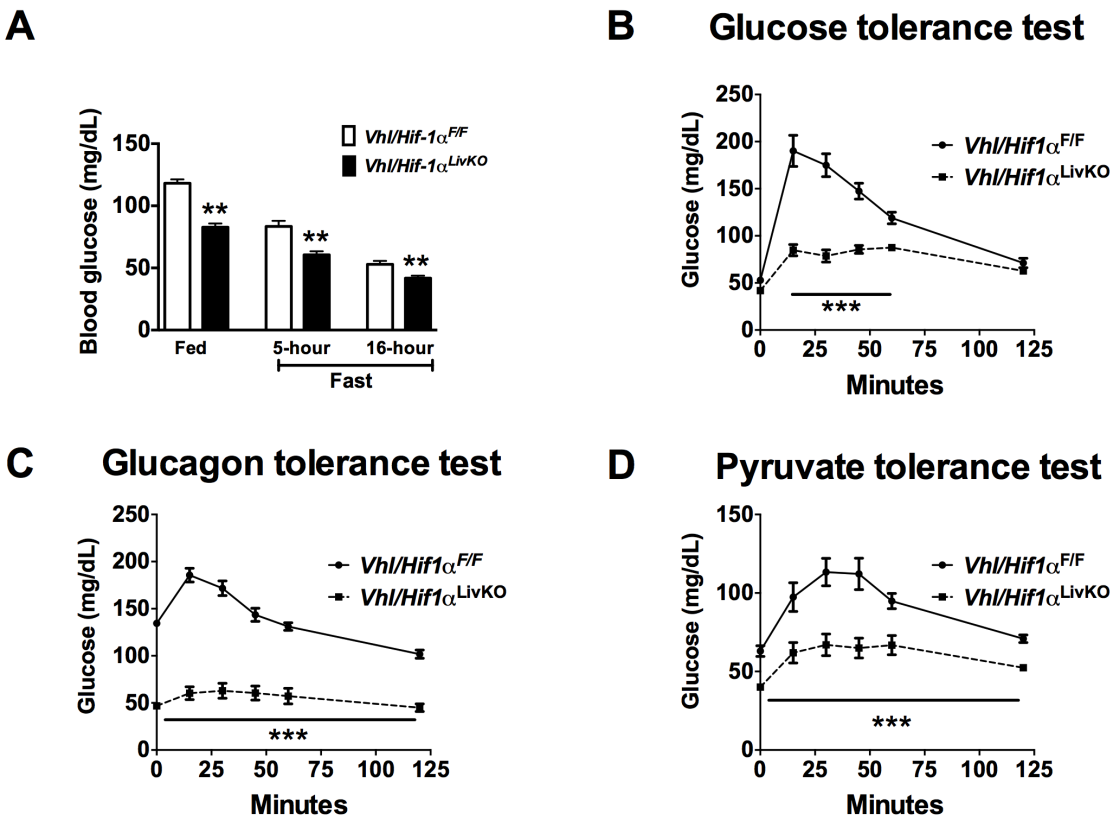

A
Blood glucose (mg/dL)
150
100
50
0
Vhl/Hif-1α F/F
Vhl/Hif-1α LivKO
**
**
**
Fed
5-hour
16-hour
Fast
B
Glucose tolerance test
Glucose (mg/dL)
250
200
150
100
50
0
Vhl/Hif1α F/F
Vhl/Hif1α LivKO
***
0
25
50
75
100
125
Minutes
C
Glucagon tolerance test
Glucose (mg/dL)
250
200
150
100
50
0
Vhl/Hif1α F/F
Vhl/Hif1α LivKO
***
0
25
50
75
100
125
Minutes
D
Pyruvate tolerance test
Glucose (mg/dL)
150
100
50
0
Vhl/Hif1α F/F
Vhl/Hif1α LivKO
***
0
25
50
75
100
125
Minutes

Figure S4

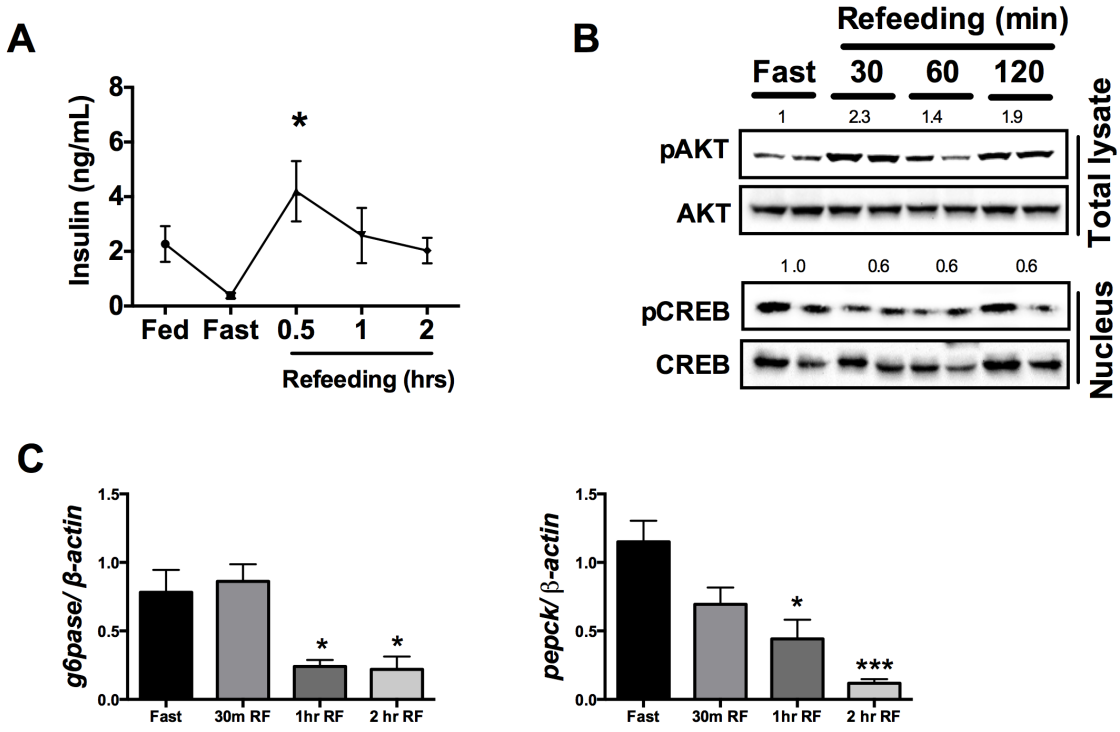

A
Insulin (ng/mL)
8
6
4
2
0
*
Fed
Fast
0.5
1
2
Refeeding (hrs)
B
Refeeding (min)
Fast
30
60
120
1
2.3
1.4
1.9
pAKT
AKT
Total lysate
1.0
0.6
0.6
0.6
pCREB
CREB
Nucleus
C
g6pase/ β-actin
1.5
1.0
0.5
0.0
*
*
Fast
30m RF
1hr RF
2 hr RF
pepck/ β-actin
1.5
1.0
0.5
0.0
*
***
Fast
30m RF
1hr RF
2 hr RF

Figure S5

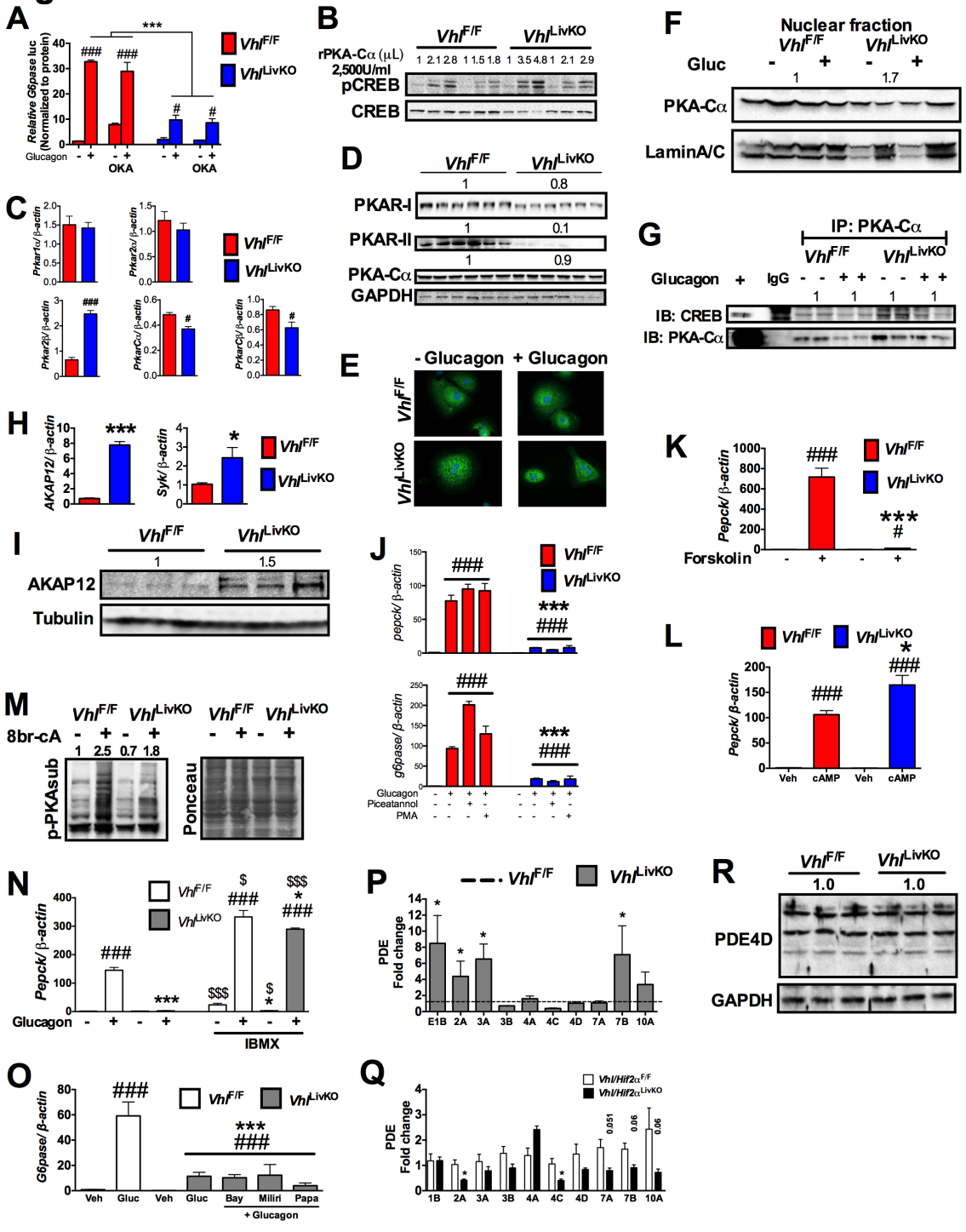

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
Vhl F/F
Vhl LivKO
Glucagon
OKA
rPKA-Cα (μL)
2,500U/ml
pCREB
CREB
PKAR-I
PKAR-II
PKA-Cα
GAPDH
- Glucagon
+ Glucagon
Nuclear fraction
Gluc
LaminA/C
IP: PKA-Cα
IgG
IB: CREB
IB: PKA-Cα
AKAP12
Tubulin
8br-cA
p-PKAsub
Ponceau
Forskolin
Veh
cAMP
Piceatannol
PMA
IBMX
Bay
Miliri
Papa
+ Glucagon
PDE4D
PDE Fold change
Vhl/Hif2α F/F
Vhl/Hif2α LivKO

Figure S6

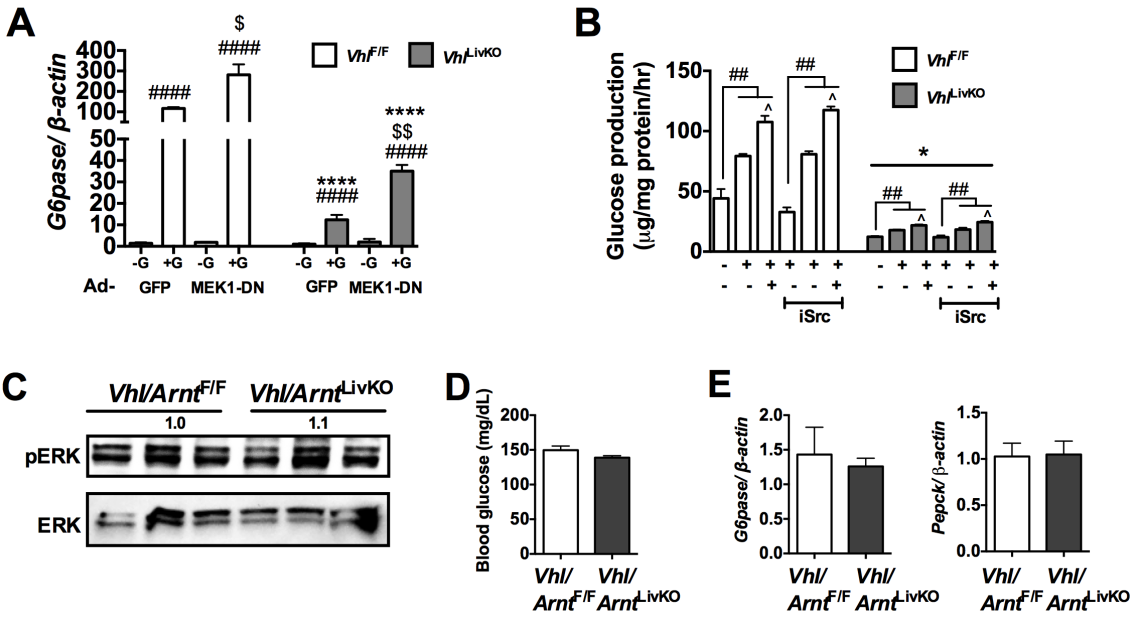
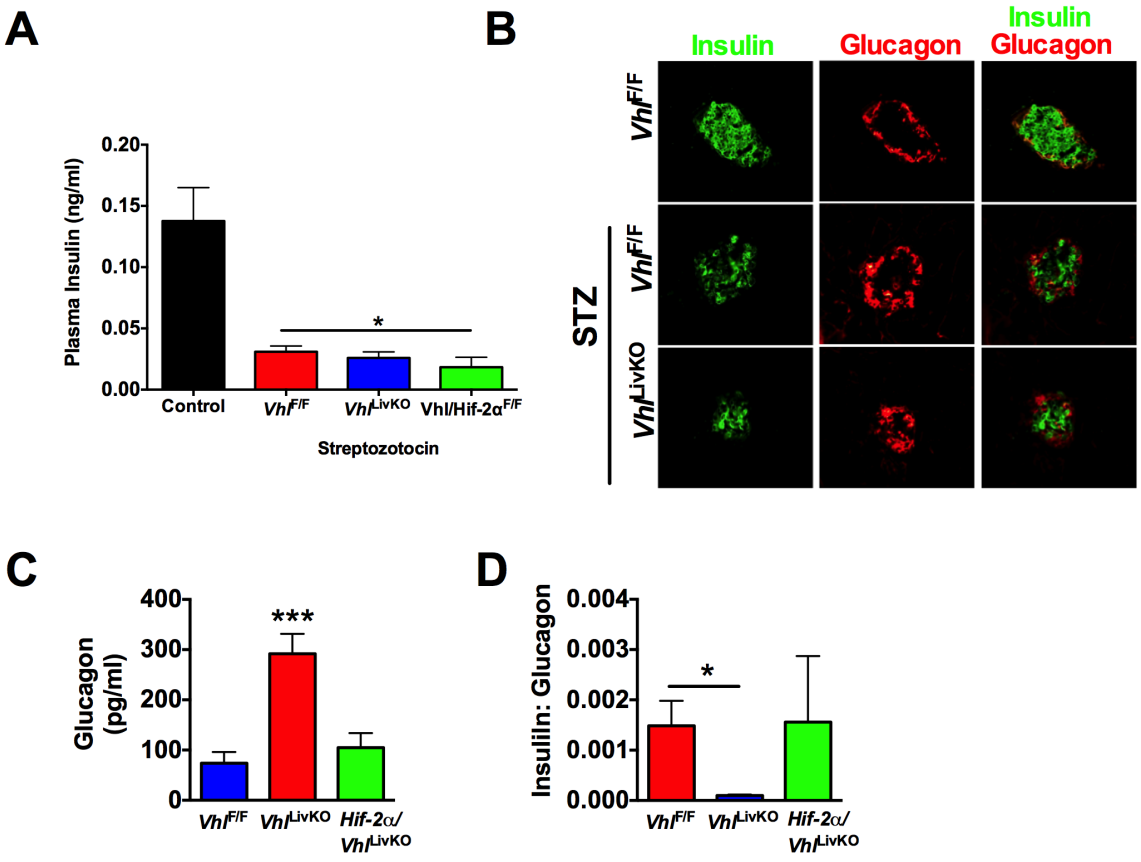

A
G6pase/ β-actin
VhlF/F
VhlLivKO
Ad-
GFP
MEK1-DN
GFP
MEK1-DN
B
Glucose production (μg/mg protein/hr)
VhlF/F
VhlLivKO
iSrc
iSrc
C
Vhl/ArntF/F
Vhl/ArntLivKO
1.0
1.1
pERK
ERK
D
Blood glucose (mg/dL)
Vhl/ ArntF/F
Vhl/ ArntLivKO
E
G6pase/ β-actin
Pepck/ β-actin
Vhl/ ArntF/F
Vhl/ ArntLivKO
Vhl/ ArntF/F
Vhl/ ArntLivKO

Figure S7


A
Plasma Insulin (ng/ml)
0.20
0.15
0.10
0.05
0.00
*
Control
VhlF/F
VhlLivKO
Vhl/Hif-2αF/F
Streptozotocin
B
Insulin
Glucagon
Insulin
Glucagon
VhlF/F
STZ
VhlF/F
VhlLivKO
C
Glucagon (pg/ml)
400
300
200
100
0
***
VhlF/F
VhlLivKO
Hif-2α/ VhlLivKO
D
Insulin: Glucagon
0.004
0.003
0.002
0.001
0.000
*
VhlF/F
VhlLivKO
Hif-2α/ VhlLivKO