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Supplemental Information

Cryptochromes Suppress HIF1α in Muscles

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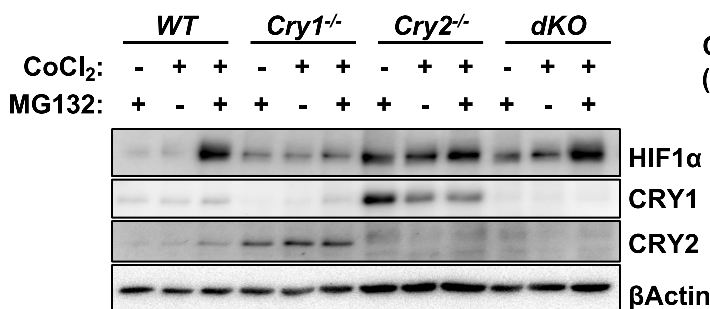

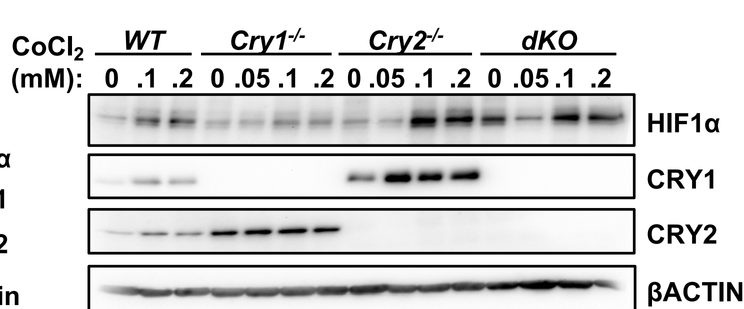

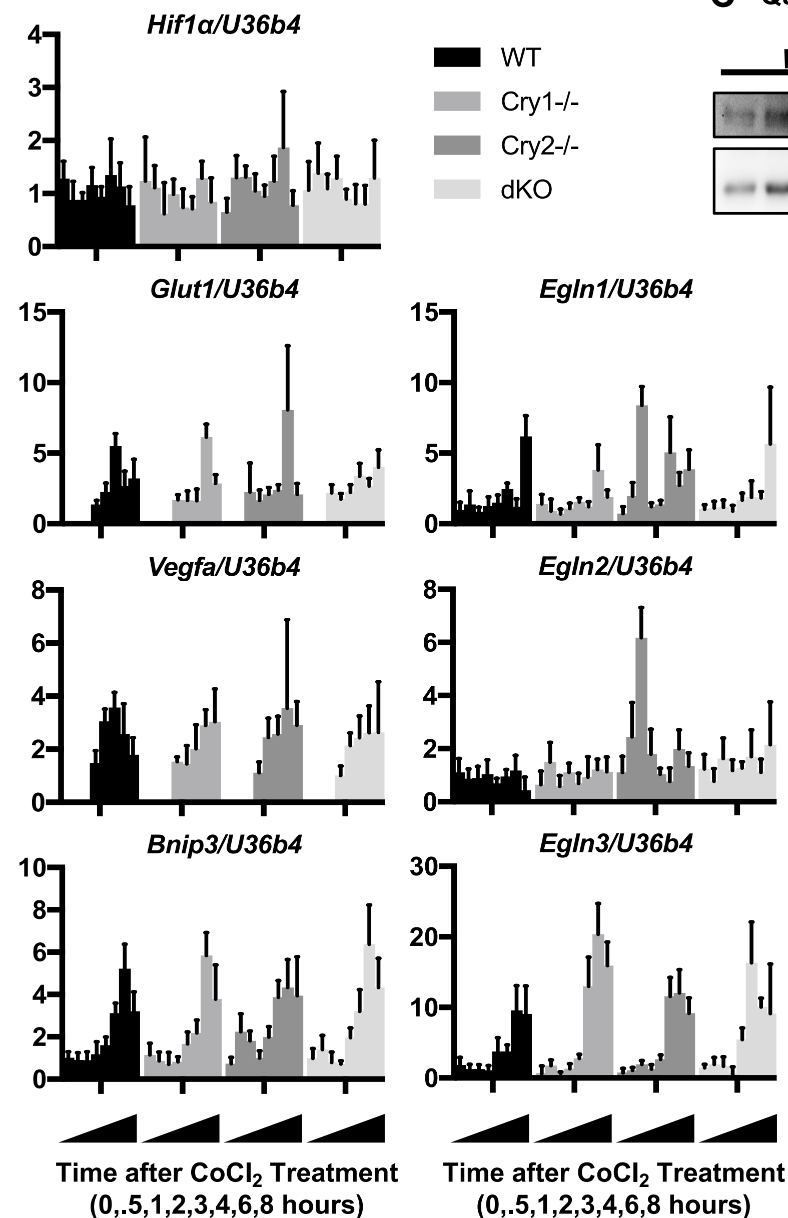

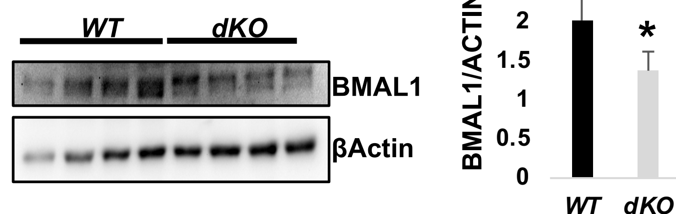

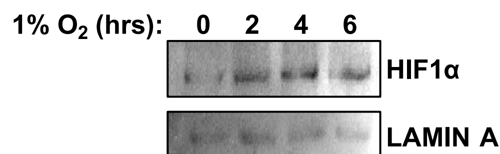

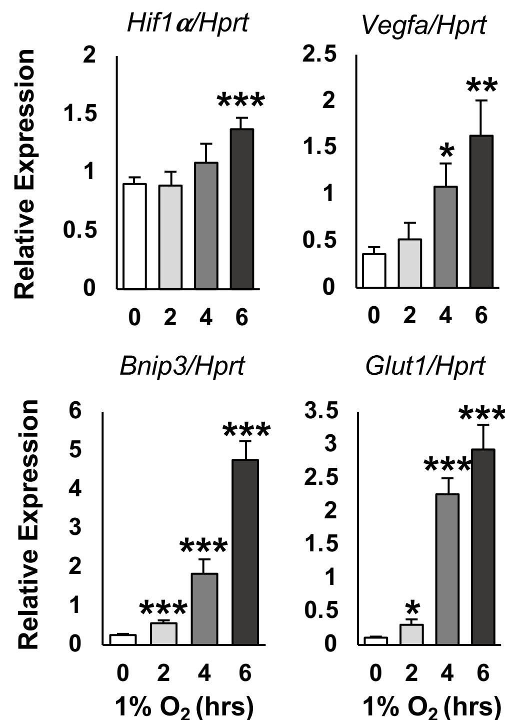

Figure S1: CRYs Suppress HIF1α. Related to Figure 1.
(A,B) HIF1α, CRY1, CRY2, and βACTIN detected by IB in EFs isolated from mice of the indicated genotypes and treated with vehicle (-) or 100μM $CoCl_2$ (+, or as indicated) in the presence (+) or absence (-) of 20 μM MG132. (C) Endogenous BMAL1 detected by IB in nuclear extracts of quadriceps muscles of female mice dissected at ZT14. Quantification, right. * $P < 0.05$ by t-test. (D) HIF1α and LAMIN A detected by IB in *WT* 1°MTs exposed to 1% O_2 for 0-6 hours. (E) Expression of the indicated transcripts measured by quantitative PCR (qPCR) in 1°MTs plated and treated as in (D), normalized to *Hprt*. (F) Expression of the indicated transcripts measured by qPCR in EFs treated with 100μM $CoCl_2$ for 0-8 hours, normalized to *U36b4*.

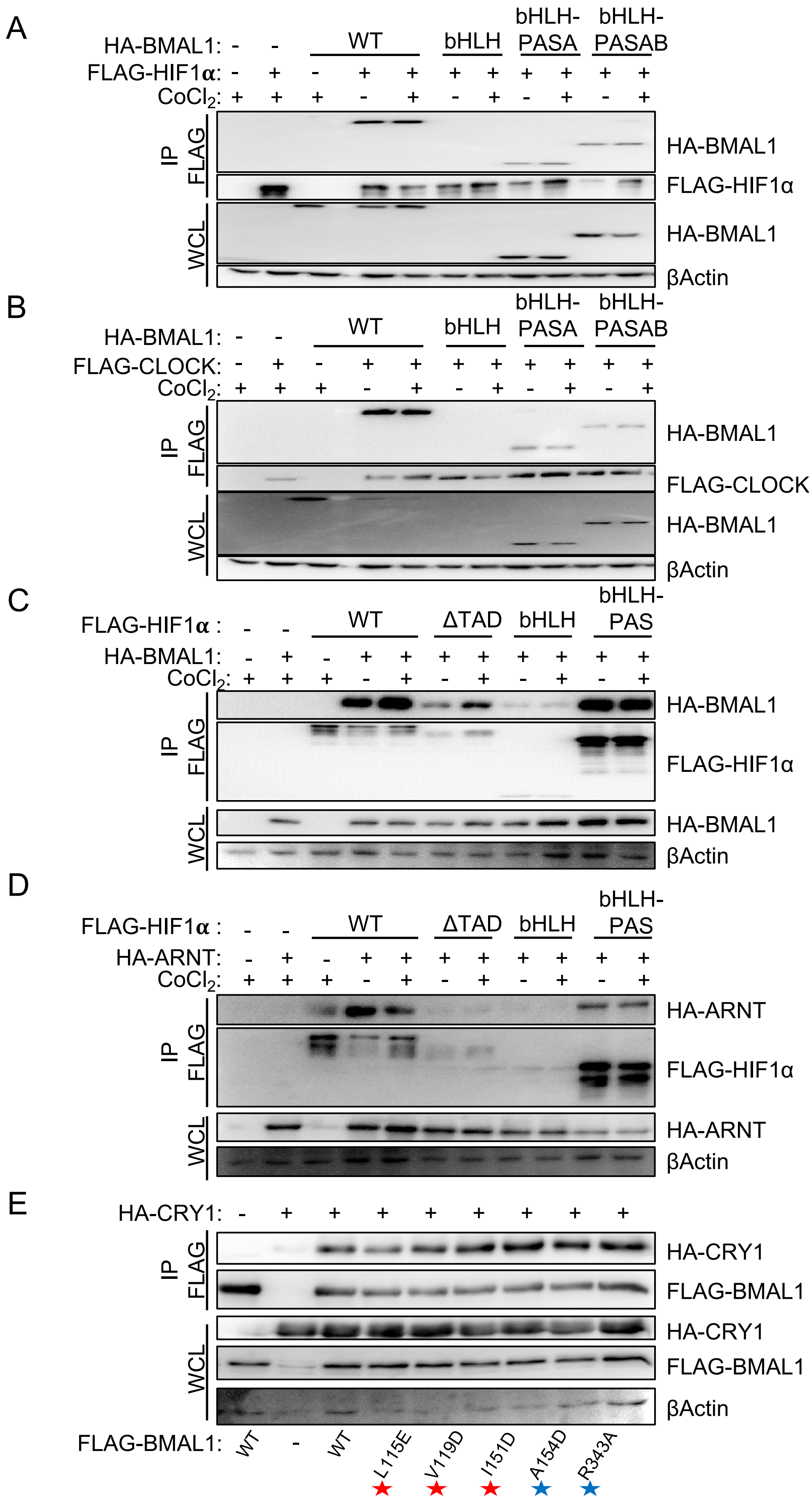

Figure S2: HIF1α Interacts with Clock Proteins via Unique Domains. Related to Figure 3.
(A-E) Proteins detected by immunoblot (IB) following FLAG IP from lysates of HEK293T cells expressing the indicated plasmids and treated with either vehicle control or 100μM $CoCl_2$. Note that bHLH domains cannot be evaluated because we cannot detect them due to their small size.

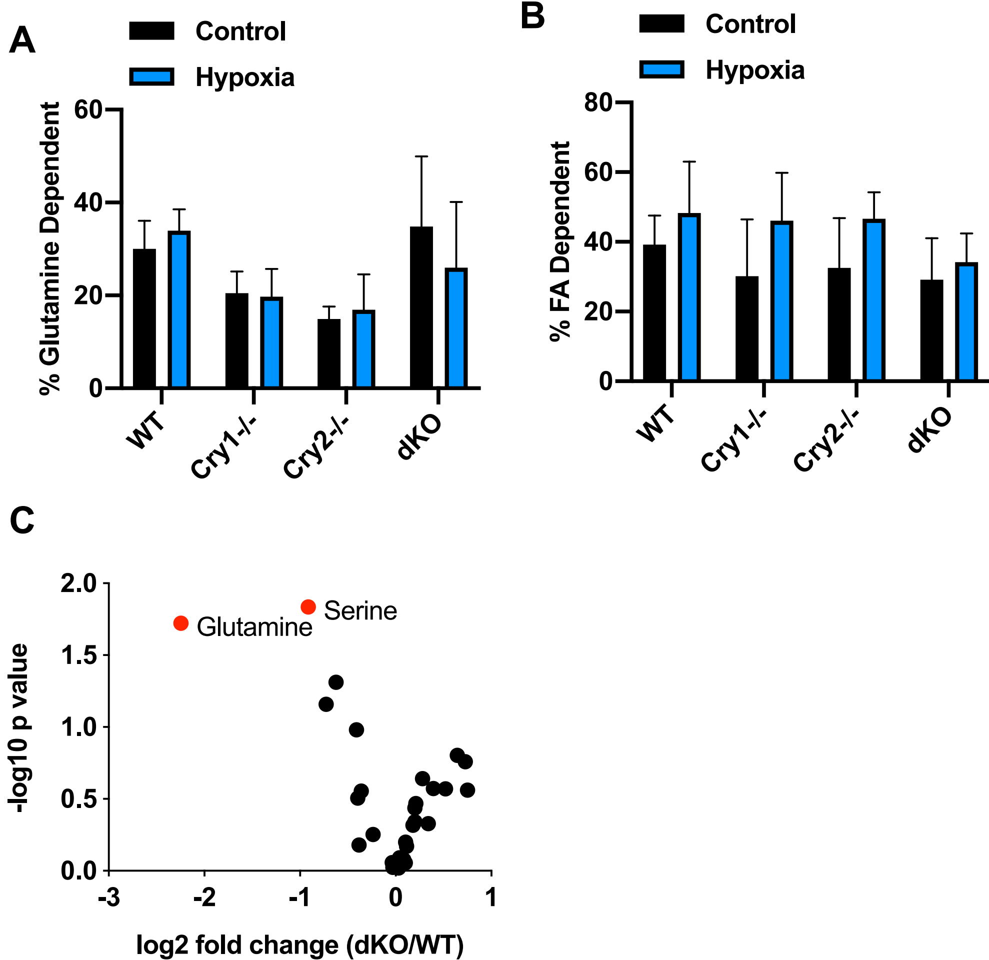

Figure S3: CRYs alter Muscle Metabolic Profile. Related to Figure 5.
(A,B) Glutamine (A) and fatty acid (B) dependency measured in 1°MTs isolated from mice of the indicated genotype and treated with either vehicle control or 100μM $CoCl_2$. (C) Metabolite abundance measured in quadriceps muscles from mice of the indicated genotypes.

TRANSPARENT METHODS

Contact for reagent and resource sharing

Further information and requests for reagents or resources should be directed to the Lead Contact, Katja Lamia (klamia@scripps.edu)

Mouse models: *dKO* mice were from Dr. Aziz Sancar (Thresher *et al.*, 1998). They were backcrossed ≥10 generations to c57Bl6/J prior to transfer to us and we performed an additional 4 backcrosses to c57Bl6/J mice from the TSRI breeding colony. Mice were maintained in standard 12:12 light:dark conditions and were given ad libitum access to normal chow and water. All animal care and treatments were in accordance with Scripps Research guidelines for the care and use of animals.

Cell lines: Primary myoblasts were isolated from six-week-old male *WT,* $Cry1^{-/-}$, $Cry2^{-/-}$, and $Cry1^{-/-};Cry2^{-/-}$ (*dKO)* littermates. Isolation and culture conditions for myoblasts and myotubes are as previously described (Vaughan and Lamia, 2019). 293T (ATCC® CRL3216™) cells were purchased from the American Type Culture Collection (ATCC) and are derived from female human embryonic kidney cells. U2OS (ATCC® HTB-96™) cells were purchased from the American Type Culture Collection (ATCC) and are derived from female osteosarcoma cells. HEK293T cells were grown in complete Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen #10569) supplemented with 10% fetal bovine serum, and 1% penicillin and streptomycin. Ear fibroblasts (EFs) were isolated from six-week-old male *WT, Cry1KO, Cry2KO,* and *DKO* littermates. EFs were grown in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum, and 1% penicillin and streptomycin. Cells were grown in a 37°C incubator maintained at 5% CO_2.

Drug treatment: All $CoCl_2$ treatments were performed by dissolving Cobalt(II) chloride anhydrous crystals (Sigma #60818) in warm culture media at a concentration of 100 μM. All treatments were performed for 4 hours prior to cell collection. DMOG and MG132 (Sigma C2211) were used at concentrations of 200 μM and 20 μM, respectively.

Cell culture and transfection: Transfections in HEK293T cells were performed using polyethylenimine (PEI; Polysciences Inc #23966-2) following standard protocols. pcDNA3-2xFlag-mCRY1 and pcDNA3-2xFlag-mCRY2 are described in (Lamia *et al.*, 2009). pBABE-mCRY2, pcDNA3-Myc-mCRY1, pcDNA3-Myc-mCRY2, pcDNA3-HA-mCRY1, pcDNA3-HA-mCRY2, and pcDNA3-2xFlag-Fbxl3 are described in (Huber *et al.*, 2016). pcDNA3.1-HIF1α-FLAG and pcDNA3.1-HIF1β-HA were a gift from Dr. Carrie Partch. pLX304-HIF1α-V5 was a gift from Dr. Enrique Saez. pcDNA3-2x-FLAG-BMAL1 and pcDNA3-2x-FLAG-CLOCK were a gift from Dr. Charles Weitz. All point mutations and truncations were generated using Q5 Site-Directed Mutagenesis kit and protocol (NEB #E0554S). pBABE-Puro was a gift from Dr. Tyler Jacks (MI, Boston, MA).

Co-immunoprecipitation and Western blotting: HEK293T whole cell extracts were prepared as previously described (Lamia *et al.*, 2009). Immunoprecipitation was performed using anti-Flag M2 agarose beads (Sigma #A2220) and anti-HA agarose beads (Sigma #A2095). Antibodies for Western Blots were anti-Flag polyclonal (Sigma #F7425), anti-βActin (Sigma #A1978), anti-Cry1-CT and anti-Cry2-CT as described (Lamia *et al.*, 2011), anti-HIF1α polyclonal (Novus Biologicals #NB100-449), anti-HIF1β (Santa Cruz Biotechnology #sc-17811), anti-HA polyclonal (Sigma #H6908), anti-Myc Tag (Sigma #SAB1305535), and anti-Lamin A (Sigma #L1293).

Quantitative RT-PCR: RNA was extracted from EFs with Qiazol reagent using standard protocols (Qiagen #799306). cDNA was prepared using QScript cDNA Supermix (VWR #101414-106) and analyzed for gene expression using qPCR with iQ SYBR Green Supermix (Biorad #1708885). Primers used are listed in Table S1.

Nuclear Fractionation of Cells: Cells were washed with 5 mL cold PBS. 5 mL of fresh PBS was added before cells were scraped into falcon tubes and centrifuged for 5 minutes (2000 rpm). Cell pellets were transferred to 1.5-mL Eppendorf tubes and centrifuged 5 minutes (2000 rpm). Cell pellets were resuspended in Solution A (10 mM Hepes pH 8, 1.5 mM $MgCl_2$, 10 mM KCL, protease inhibitors, phosphatase inhibitors), and incubated for 15 minutes at 4 °C. An equal volume of Solution B was added (Solution A + 1% NP40) and incubated for an additional 5

minutes at 4 °C. Tubes were centrifuged for 5 minutes (3000 rpm). Cell pellets were washed twice with cold PBS and lysed in RIPA buffer.

Muscle Nuclear Fractionation: Quadriceps muscles were dissected from 8-week-old mice and then rinsed in cold PBS. Fractionation was performed as described (Dimauro *et al.*, 2012).

Luciferase Assays: U2OS cells were seeded at a density of 12,000 cells per 96-well. Cells were transfected after 24 hours with 35 ng reporter construct Per2Luc as described (Kriebs *et al.*, 2017), HRELuc (Addgene #26731, deposited by Dr. Navdeep Chandel), or pHIF1αLuc (Addgene #40172, deposited by Dr. Alex Minella); 5 ng BMAL1; 15 ng CLOCK or HIF1α; 5 ng for ARNT; 2 ng Renilla Luciferase (a gift from Dr. Ian MacRae); 1-5 ng CRY1 or CRY2 or mCherry (as described in Kriebs *et. al.* 2017). All plasmid dilutions were prepared fresh immediately before transfection. A media change was performed on the day following transfection. The following day luciferase activity was measured using the Dual-Glo® Luciferase Assay System (Promega #E2920).

Substrate Dependency Experiments: For Seahorse experiments (XF96, Seahorse Biosciences), XF96 plates were prepared as described (Vaughan *et. al.* 2019). $3x10^4$ primary myoblasts were seeded in 40 µL of differentiation media in each well. Plated cells were grown in a chamber at 37 °C, 5% CO_2 for four days, during which time 80% of the media was replaced every day. OCR and ECAR were measured on day 5 following manufacturer's instructions in the Agilent Seahorse XF Mito Fuel Flex Test Kit (Agilent Technologies #103260-100). Data shown is a representative assay following three independent repeats of the fuel dependency test. Plate setup was varied between plates and wells on the perimeter of the plate were excluded from all experiments.

Hypoxia Experiments: For hypoxic chamber and hypoxia-mimicking drug treatment experiments, fibroblasts were plated at a density of 70-80% (~250,000 cells) on a 10-cm plate and left undisturbed for 24 hours after plating to allow attachment before exposure to hypoxia or $CoCl_2$. Myoblasts were plated in differentiation media and allowed to differentiate for 4 days, during which time the media was changed daily, prior to exposure to hypoxia or $CoCl_2$. Cell

plates were placed inside a 37°C incubator maintained at 1% O_2 for 0-6 hours prior to collection for 1% O_2 treatments. For experiments in which hypoxia was mimicked with drug treatments, the hypoxia-related exposure was induced by replacing the media with media containing 100 µM $CoCl_2$. For protein collection, nuclear fractionation was performed on 4-5 plates of cells in order to measure HIF1α levels. For qPCR analysis, RNA was collected from one plate of cells; all conditions were performed in triplicate.

Metabolic Tracing Experiments and Tissue Metabolite Analysis: Myotubes were seeded in differentiation media (Vaughan *et. al.* 2019) at a density of 300,000 cells per well in six-well plates. Cells were allowed to attach to plates and differentiate for five days prior to isotope tracing when cells were incubated in media where the metabolite specified was replaced with the ^{13}C labeled version for 24 hours. For cell culture and tissues, polar metabolites were extracted and analysed using GCMS as previously described (Wallace et al. 2018). The % isotopologue distribution of each metabolite was determined and corrected for natural abundance using in-house algorithms adapted from (Fernandez *et al.*, 1996). Mole percent enrichment (MPE) was calculated via the following equation:

$$\sum_{i=1}^{n} \frac{M_i \cdot i}{n}$$

where n is the number of carbon atoms in the metabolite and M_i is the relative abundance of the ith mass isotopologue.

Quantification and Statistical Analysis: Detailed descriptions of sample numbers and statistical tests are provided in the Figure Legends. In general, statistical analyses were done using two-tailed Student's t-test or with two-way ANOVA. Results presented are either a representative experiment or an average of three replicates ± SEM.

Data and Code Availability: This study did not generate or analyze any datasets or code.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-FLAG M2 agarose beads	Sigma	A2220
Anti-HA agarose beads	Sigma	A2095
Rabbit Anti-FLAG polyclonal	Sigma	F7425
Mouse Anti-Bactin	Sigma	A1978
Guinea pig Anti-Cry1-CT	Lamia et. al. 2011	N/A
Guinea pig Anti-Cry2-CT	Lamia et. al. 2011	N/A
Rabbit Anti-HIF1a polyclonal	Novus	NB100-449
Rabbit Anti-HIF1b	Santa Cruz Biotechnology	Sc-17811
Rabbit Anti-HA polyclonal	Sigma	H6908
Rabbit Anti-Myc tag	Sigma	SAB1305535
Rabbit Anti-PHD2	Novus Biologicals	NB100-2219
Rabbit Anti-LaminA	Sigma	L1293
Bacterial and Virus Strains		
psPAX	Addgene	12260
pMD2.G	Addgene	12259
pLKO.1 sh_Scramble	Addgene	1864
pBABE-Puro	Laboratory of Dr. Tyler Jacks	
Chemicals, Peptides, and Recombinant Proteins		
Cobalt Chloride Anhydrous	Sigma	60818
Polyethylenimine	Polysciences Inc	23966-2
MG132	Sigma	C2211
Critical Commercial Assays		
Q5 Site-Directed Mutagenesis Kit	NEB	E0554S
Qiazol	Qiagen	799306
Qscript cDNA Supermix	VWR	101414-106
iQ SYBR Green Supermix	Biorad	1708885
Dual-Glo Luciferase Assay System	Promega	E2920
Agilent Seahorse XF Mito Fuel Flex Test Kit	Agilent Technologies	103260-100
DMOG	Sigma	D3695
Experimental Models: Cell Lines		
Primary mouse ear fibroblasts and myotubes	This study	N/A
Human Embryonic Kidney 293T cells	ATCC	CRL3216
U-2 OS cells	ATCC	HTB-96
Experimental Models: Organisms/Strains		
Cry1;Cry2 dKO mice	Thresher et. al. 1998	N/A
Oligonucleotides		
See Table S1	Table S1	Table S1
Recombinant DNA		
pcDNA3-2Xflag-mCRY1	Lamia et. al. 2009	N/A
pcDNA3-2xFlag-mCRY2	Lamia et. al. 2009	N/A
pBABE-mCRY2	Huber et. al. 2016	N/A

pcDNA3-Myc-mCRY1	Huber et. al. 2016	N/A
pcDNA3-Myc-mCRY2	Huber et. al. 2016	N/A
pcDNA3-HA-mCRY1	Huber et. al. 2016	N/A
pcDNA3-HA-mCRY2	Huber et. al. 2016	N/A
pcDNA3-2xFlag-Fbxl3	Huber et. al. 2016	N/A
pcDNA3.1-HIF1a-FLAG	Laboratory of Dr. Carrie Partch	N/A
pcDNA3.1-HIF1b-HA	Laboratory of Dr. Carrie Partch	N/A
pLX304-HIF1a-V5	Laboratory of Dr. Enrique Saez	N/A
pcDNA3-2x-FLAG-BMAL1	Laboratory of Dr. Charles Weitz	N/A
pcDNA3-2x-FLAG-CLOCK	Laboratory of Dr. Charles Weitz	N/A
pLKO.1 shRNA Fbxl3 #1	Sigma	TRCN0000126944
pLKO.1 shRNA VHL	Sigma	TRCN0000009737
Per2Luc	Kriebs et. al. 2017	N/A
pHIF1aLuc	Addgene	40172
HRELuc	Addgene	26731
pcDNA3-Renilla Luciferase	Laboratory of Dr. Ian MacRae	N/A
pcDNA3-mCherry	Kriebs et. al. 2017	N/A
Other		
Single Flow Meter	Stemcell Technologies	27311
Microplate Reader	Versamax	N/A