Cell Reports, Volume *22*

Supplemental Information

Cry2 Is Critical for Circadian Regulation of Myogenic Differentiation by Bclaf1-Mediated mRNA Stabilization of Cyclin D1 and Tmem176b

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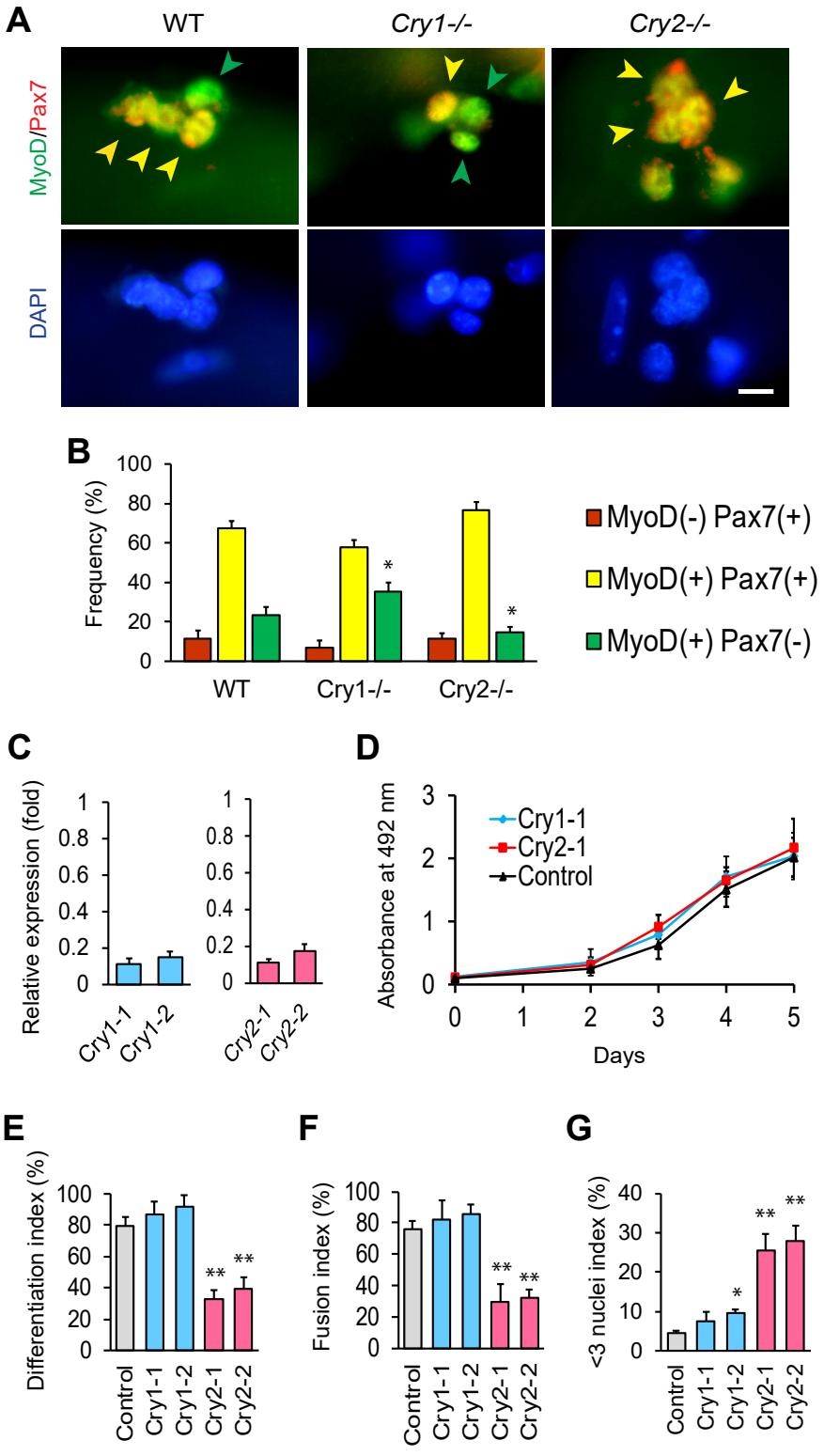

A
WT
Cry1-/-
Cry2-/-
MyoD/Pax7
DAPI
B
Frequency (%)
100
80
60
40
20
0
WT
Cry1-/-
Cry2-/-
MyoD(-) Pax7(+)
MyoD(+) Pax7(+)
MyoD(+) Pax7(-)
C
Relative expression (fold)
1
0.8
0.6
0.4
0.2
0
Cry1-1
Cry1-2
Cry2-1
Cry2-2
D
Absorbance at 492 nm
3
2
1
0
Cry1-1
Cry2-1
Control
0
1
2
3
4
5
Days
E
Differentiation index (%)
100
80
60
40
20
0
**
**
Control
Cry1-1
Cry1-2
Cry2-1
Cry2-2
F
Fusion index (%)
100
80
60
40
20
0
**
**
Control
Cry1-1
Cry1-2
Cry2-1
Cry2-2
G
<3 nuclei index (%)
40
30
20
10
0
*
**
**
Control
Cry1-1
Cry1-2
Cry2-1
Cry2-2

Figure S1. Single muscle culture and KD of *Cry1* and *Cry2* in C2C12 cells, Related to Figures 2 and 3.

(A) Immunofluorescence staining of single muscle fibers with antibodies against MyoD (green) and Pax7 (red) after culture for 72 hr. MyoD(+)Pax7(+) myoblasts and MyoD(+)Pax7(-) differentiating muscle cells are indicated by yellow and green arrow heads, respectively. Satellite cells differentiate from MyoD(-)Pax7(+) to MyoD(+)Pax7(+) and subsequently to MyoD(+)Pax7(-). Scale bars, 10 μm.

(B) Frequency of MyoD(-)Pax7(+), MyoD(+)Pax7(+), and MyoD(+)Pax7(-) cells per cluster in muscle fibers after 72 hr of culture. Nuclei were counterstained with DAPI. * $p < 0.05$ vs WT in each experiment.

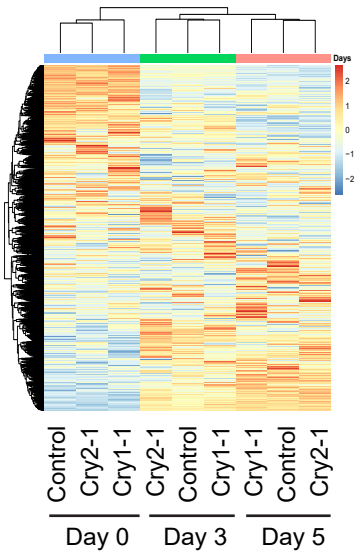
(C) Verification of *Cry1* and *Cry2* KD with two shRNA clones each, as determined by qRT-PCR. Values obtained with scrambled control shRNA were defined as 1.0 for each gene.

(D) MTS assay reflecting the proliferation of undifferentiated C2C12 cells after KD. Cell number and absorbance at 492 nm were proportional in this range.

(E-G) Differentiation index (C), fusion index (D), and <3 nuclei index (E) of KD cells on differentiation day 5 after seeding 50% more cells than in the experiments shown in Figure 3.

* $p < 0.05$ and ** $p < 0.01$ with Student's t-test in comparison to control cells in (D) – (F). Data are presented as mean + or ± SD.

A



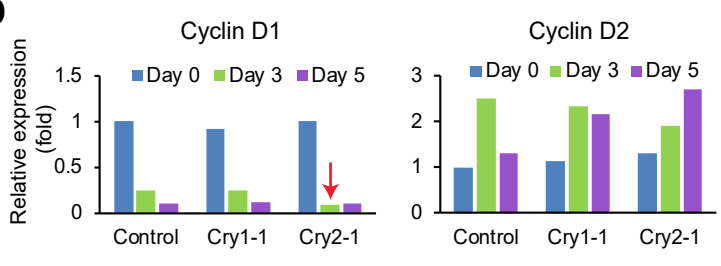
B

Day	GO.biological.process.complete	GO Term	Q-value
Common to Cry1 knockdown and Cry2 knockdown			
Day 0	positive regulation of gene expression	GO:0010628	0.158
Day 3	negative regulation of cellular process	GO:0048523	0.0291
	negative regulation of biological process	GO:0048519	0.0294
	sensory perception of smell	GO:0007608	0.0513
	regulation of response to stimulus	GO:0048583	0.0925
Day 5	biological process	GO:0008150	0.00291
	sensory perception of smell	GO:0007608	0.0662
Unique to Cry1 knockdown			
Day 0	muscle contraction	GO:0006936	1.39E-10
	muscle system process	GO:0003012	2.43E-10
	striated muscle contraction	GO:0006941	1.14E-09
	muscle structure development	GO:0061061	1.69E-08
	single-multicellular organism process	GO:0044707	2.11E-08
Day 3	single-multicellular organism process	GO:0044707	2.09E-07
	single-organism process	GO:0044699	6.72E-07
	single-organism developmental process	GO:0044767	1.03E-06
	sensory perception of smell	GO:0007608	1.69E-06
	developmental process	GO:0032502	2.18E-06
Day 5	sensory perception of smell	GO:0007608	2.86E-07
	sensory perception of chemical stimulus	GO:0007606	3.83E-07
	positive regulation of biological process	GO:0048518	3.69E-06
	regulation of protein phosphorylation	GO:0001932	9.61E-06
	regulation of phosphate metabolic process	GO:0019220	1.23E-05
Unique to Cry2 knockdown			
Day 0	sensory perception of chemical stimulus	GO:0007606	2.48E-06
	sensory perception of smell	GO:0007608	1.61E-05
	neurological system process	GO:0050877	0.0197
	sensory perception	GO:0007600	0.0477
	anatomical structure development	GO:0048856	0.182
Day 3	sensory perception of chemical stimulus	GO:0007606	3.63E-09
	nitrogen compound metabolic process	GO:0006807	5.2E-08
	small molecule metabolic process	GO:0044281	5.05E-07
	cellular metabolic process	GO:0044237	5.81E-07
	cellular aromatic compound metabolic process	GO:0006725	1.67E-06
Day 5	negative regulation of biological process	GO:0048519	1.05E-09
	negative regulation of cellular process	GO:0048523	4.69E-09
	regulation of multicellular organismal process	GO:0051239	5.42E-08
	single-multicellular organism process	GO:0044707	2.9E-07
	regulation of metabolic process	GO:0019222	3.2E-07

C

Gene Name	Cry1 KD d0	Cry2 KD d0	Control
Pdgfb	3.64414	1.42597	1.28925
Srpk3	2.4738	0.0837234	0.389264
Mef2c	9.9333	5.45377	4.73853
Tmem8c	56.0597	19.4364	25.9513
Tnnc2	52.5134	15.4048	22.5019
Unc45b	3.21806	0.622719	1.0898
Myh3	28.8923	7.92271	10.9988
Asb2	1.62383	0.143142	0.739527
Fos	2.89222	2.11009	1.07484
Myom1	2.15062	0.584248	0.553912
Sox8	4.89027	3.39345	2.02162
Ankrd2	3.16961	0.497477	0.749631
Rnf165	1.03459	0.884614	0.229971
Chrng	45.0611	18.1336	13.4901
Tnni1	119.275	29.1413	40.3591
Myog	126.857	24.8798	41.552
Neb	5.92033	2.20951	1.87545
P2ry1	1.20066	0.0873125	0.157887
Casq2	21.2917	4.76115	8.21436
Col11a1	1.16825	0.268703	0.114478
Myoz2	2.64201	0.608452	0.577949
Lmod2	3.82104	1.09149	1.74595
Csrp3	4.21602	0	0.597977
Ryr1	4.36259	1.46478	1.67789
Mylpf	677.864	228.075	235.661
Tnni2	71.386	26.2358	30.7181
Acta1	264.395	52.8698	85.2098
Heyl	29.1419	17.574	9.30169
Sgcg	9.99904	3.48892	3.9587
Alpk3	1.14383	0.574932	0.349444
Smpx	6.42495	0.855687	0.773644
Mybph	46.0292	13.6283	18.2709
Trim72	6.44429	2.2691	1.67293
Lmod3	6.33085	2.6638	2.9436
Klhl31	1.20681	0.520883	0.577973
Eid2	2.50605	1.87726	1.24466
Ttn	3.41562	2.42825	1.41645
Actn2	1.4499	0.219203	0.545036
Smyd1	14.1181	5.32254	5.83401
Myh8	1.00322	0.1002	0.216463
Myh1	7.50119	0.866544	1.50278
Ryr3	1.32927	0.146207	0.327331
Mkx	1.49487	0	0.258782
Tnnt3	88.5619	24.2797	33.3768
Myl1	227.385	67.2128	74.6497
Tnnt1	70.8799	24.237	33.7336
Actc1	61.7528	10.8975	19.8112
Klhl40	4.51323	1.4545	1.55414
Klhl41	12.5266	4.67286	3.64608
Tnnc1	141.333	39.551	57.4244

D



E

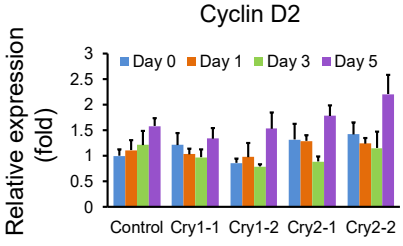


Figure S2. RNA-seq analysis of the KD cells, Related to Figure 4.

(A) Heat map comparing the transcriptome of KD cells.
(B) Enriched GO terms in each segment of the Venn diagram shown in Figure 4A. The GO terms mentioned in the text are highlighted in yellow.
(C) Genes belonging to the highlighted GO terms from the day 0 *Cry1* KD in (B). The FPKM (Fragments per Kilobase of Exon per Million Fragments Mapped) was compared between *Cry1* KD, *Cry2* KD, and control cells. The genes mentioned in the text are highlighted in yellow.
(D) Relative expression levels of cyclin D1 and cyclin D2 obtained from the RNA-seq data. The value with control cells on day 0 was defined as 1.0. The arrow indicates the data mentioned in the text.
(E) qRT-PCR results comparing the expression levels of *Ccnd2* during differentiation of KD cells. The value obtained with control on day 0 was defined as 1.0. Data are presented as mean + SD.

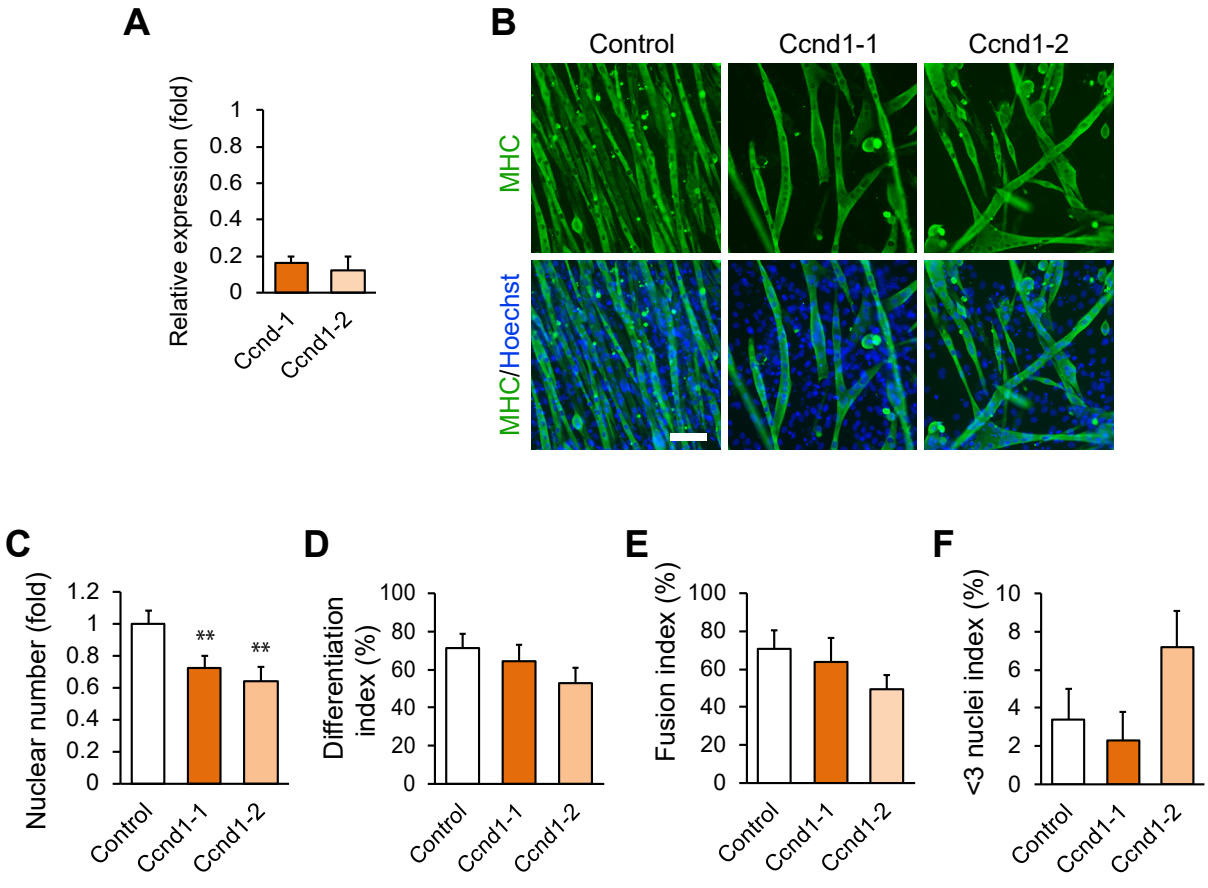

A
Relative expression (fold)
1
0.8
0.6
0.4
0.2
0
Ccnd-1
Ccnd1-2
B
Control
Ccnd1-1
Ccnd1-2
MHC
MHC/Hoechst
C
Nuclear number (fold)
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Control
Ccnd1-1
Ccnd1-2
D
Differentiation index (%)
100
80
60
40
20
0
Control
Ccnd1-1
Ccnd1-2
E
Fusion index (%)
100
80
60
40
20
0
Control
Ccnd1-1
Ccnd1-2
F
<3 nuclei index (%)
10
8
6
4
2
0
Control
Ccnd1-1
Ccnd1-2

Figure S3. Differentiation of C2C12 cells after KD of *Ccnd1*, Related to Figure 4.

(A) Relative expression level of *Ccnd1* in C2C12 cells after KD with two shRNA clones. The value obtained with the control shRNA was defined as 1.0.
(B) MHC staining of KD cells with two different shRNA clones on differentiation day 5. Bar, 100 μm
(C) Nuclear number of KD cells on differentiation day 5. The number with control shRNA was defined as 1.0.
(D-F) Differentiation index (D), fusion index (E), and <3 nuclei index (F) of *Ccnd1* KD cells on differentiation day 5.
** $p < 0.01$ with Student's t-test in comparison to control cells. Data are presented as mean + SD.

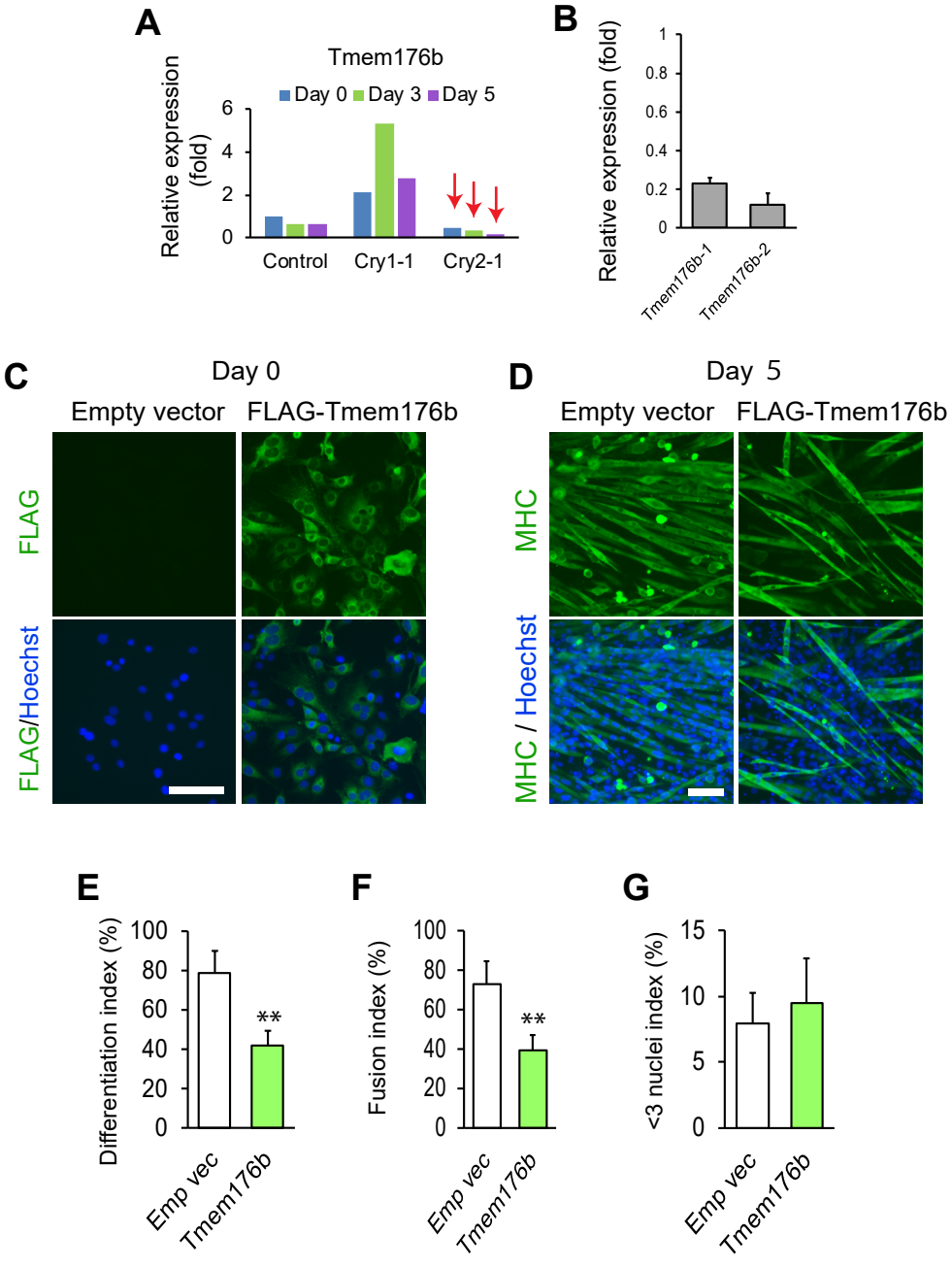

A
Tmem176b
Day 0
Day 3
Day 5
Relative expression (fold)
6
4
2
0
Control
Cry1-1
Cry2-1
B
Relative expression (fold)
1
0.8
0.6
0.4
0.2
0
Tmem176b-1
Tmem176b-2
C
Day 0
Empty vector
FLAG-Tmem176b
FLAG
FLAG/Hoechst
D
Day 5
Empty vector
FLAG-Tmem176b
MHC
MHC / Hoechst
E
Differentiation index (%)
100
80
60
40
20
0
**
Emp vec
Tmem176b
F
Fusion index (%)
100
80
60
40
20
0
**
Emp vec
Tmem176b
G
<3 nuclei index (%)
15
10
5
0
Emp vec
Tmem176b

Figure S4. Characterization of Tmem176b in C2C12 cells, Related to Figure 5.

(A) Relative expression levels of *Tmem176b* in *Cry1* and *Cry2* KD cells. The values were obtained from the RNA-seq data. The value with control cells on day 0 was defined as 1.0. The arrows indicate the data mentioned in the text.
(B) Relative expression level of *Tmem176b* in C2C12 cells after KD with two shRNA clones. The value obtained with the control shRNA was defined as 1.0.
(C) Immunofluorescence staining of the FLAG tag in undifferentiated C2C12 cells transduced with FLAG-Tmem176b. Bar, 100 μm.
(D) Immunofluorescence staining of the FLAG tag in day 5 differentiated C2C12 cells transduced with FLAG-Tmem176b. Bar, 100 μm.
(E-G) Differentiation index (E), fusion index (F), and <3 nuclei index (G) of cells transduced with empty vector or *Tmem176b* cDNA on differentiation day 5.
** $p < 0.01$ with Student's t-test in comparison to the empty vector data. Data are presented as mean + SD in (B), (E), (F), and (G).

A

FLAG-Cry1-binding proteins

Accession	Coverage (%)	#Peptides	#Unique	Description
gi|126157504	10	22	22	serine/arginine repetitive matrix protein 2
gi|6752954	58	21	1	actin cytoplasmic 2
gi|22094119	9	18	18	unconventional myosin-XVIIIa
gi|6681031	18	13	7	cryptochrome-1 (Cry1)
gi|8394460	18	6	6	tropomodulin-3
gi|254587962	20	5	5	serine/threonine-protein phosphatase PGAM5 mitochondrial
gi|31981690	9	5	4	heat shock cognate 71 kDa protein
gi|329755241	8	7	7	gelsolin
gi|114326446	2	4	4	myosin-9
gi|19527344	8	3	3	serine/threonine-protein kinase 38
gi|33620739	22	3	3	myosin light polypeptide 6
gi|165905585	6	4	4	LIM domain and actin-binding
gi|33598964	2	3	3	myosin-10
gi|9845257	17	2	2	histone H1.2
gi|29789070	5	2	2	F-box/LRR-repeat protein 3 (Fbxl3)
gi|254540166	5	3	2	78 kDa glucose-regulated protein precursor
gi|226823279	1	2	2	period circadian protein homolog 1 (Per1)

B

FLAG-Cry2-binding proteins

Accession	Coverage (%)	#Peptides	#Unique	Description
gi|126157504	17	36	36	serine/arginine repetitive matrix protein 2
gi|157823889	34	17	3	actin gamma-enteric smooth muscle
gi|439253893	33	17	3	actin alpha skeletal muscle
gi|27312016	44	25	20	cryptochrome-2 (Cry2)
gi|22094119	15	31	31	unconventional myosin-XVIIIa
gi|226823279	9	10	10	period circadian protein homolog 1 (Per1)
gi|33598964	8	14	13	myosin-10
gi|114326446	4	7	6	myosin-9 isoform 1
gi|31981690	15	8	4	heat shock cognate 71 kDa protein
gi|8394460	20	7	7	tropomodulin-3
gi|19527344	22	9	9	serine/threonine-protein kinase 38
gi|165905585	10	6	6	LIM domain and actin-binding protein 1
gi|9845257	21	3	3	histone H1.2
gi|33620739	36	5	5	myosin light polypeptide 6
gi|165932375	7	2	2	plasminogen activator inhibitor 1 RNA-binding protein
gi|254587962	12	3	3	serine/threonine-protein phosphatase PGAM5 mitochondrial
gi|240849436	7	3	3	nexilin
gi|29789070	9	3	3	F-box/LRR-repeat protein 3 (Fbxl3)
gi|70906447	4	4	4	bcl-2-associated transcription factor 1 (Bclaf1)
gi|162461907	6	3	3	stress-70 protein mitochondrial
gi|68533246	5	3	3	thyroid hormone receptor-associated protein 3
gi|190194418	1	2	2	desmoplakin
gi|20544149	7	3	2	casein kinase I isoform delta (CK1δ)

C

Bclaf1 gi70906447

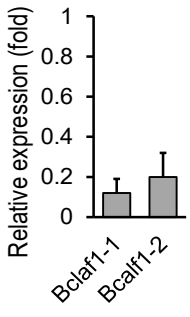
303 - TITPQNAPR

303 - TITPQNAPREESR

535 - MIASDSHRPEVK

620 - SPAVTLNER

D



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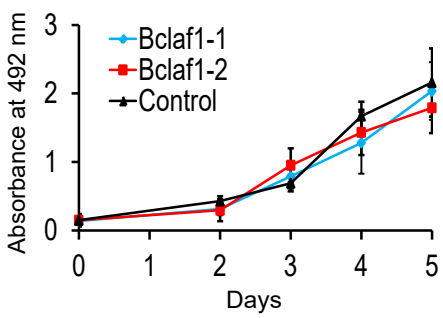

Figure S5. Immunoprecipitation of Cry1- and Cry2-interacting proteins from C2C12 cells, Related to Figure 6.

(A-B) Lists of proteins co-precipitated with FLAG-Cry1 (A) and FLAG-Cry2 (B). Proteins in which more than one peptide sequences were detected are listed. Known circadian regulators are highlighted in red. Bclaf1 is highlighted in yellow in (B). We adjusted peptide false discovery rate (FDR) to 0.5% and protein FDR to 1.0%.

(C) Detected peptide sequences of Bclaf1. The first peptide is a partial sequence of the second one. Amino acid number of the first residue in each peptide is written on the left.

(D) Relative expression levels of *Bclaf1* in KD cells.

(E) MTS assay of *Bclaf1* KD cells.

Data are presented as mean + or ± SD.

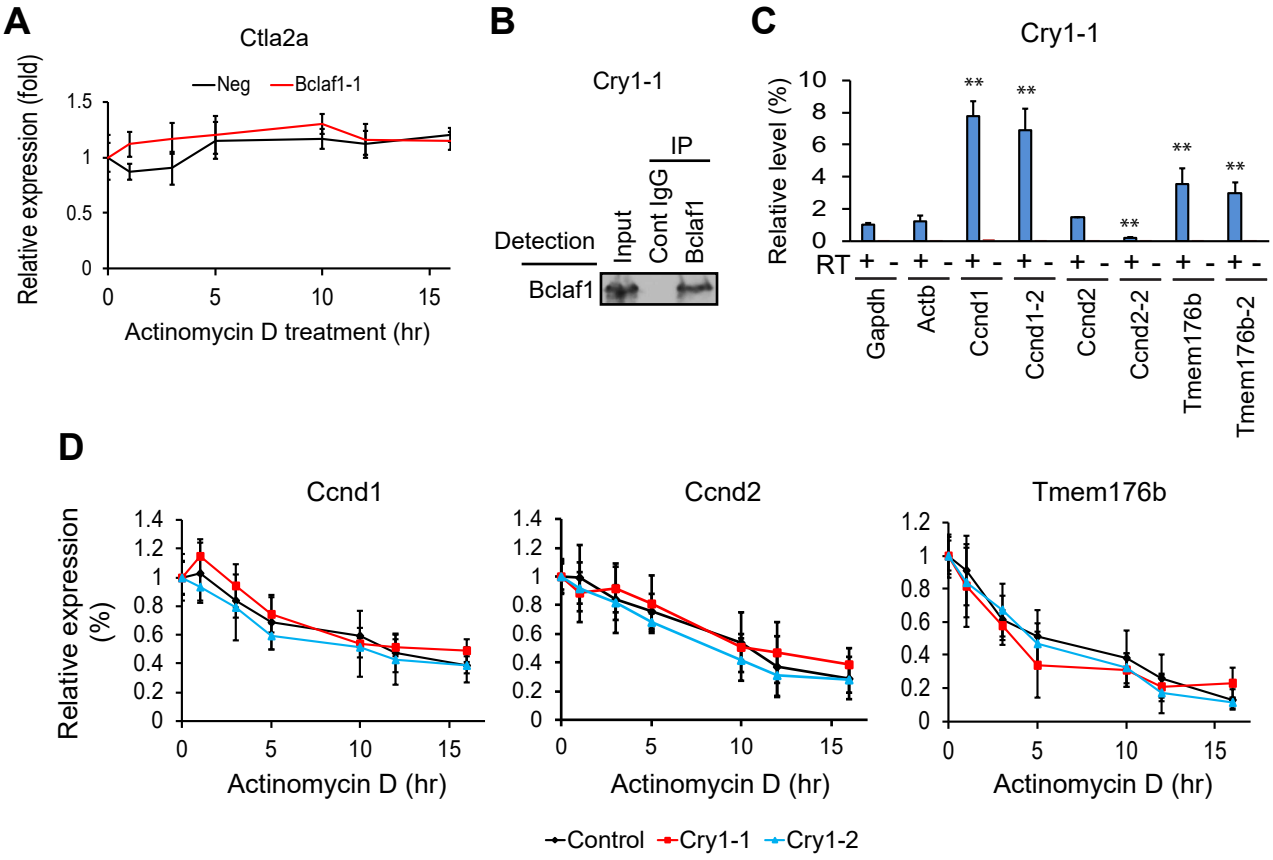
A
Ctla2a
Neg
Bclaf1-1
Relative expression (fold)
Actinomycin D treatment (hr)
B
Cry1-1
IP
Detection
Input
Cont IgG
Bclaf1
Bclaf1
C
Cry1-1
Relative level (%)
RT
Gapdh
Actb
Ccnd1
Ccnd1-2
Ccnd2
Ccnd2-2
Tmem176b
Tmem176b-2
D
Ccnd1
Ccnd2
Tmem176b
Relative expression (%)
Actinomycin D (hr)
Control
Cry1-1
Cry1-2

Figure S6. *Cry1* KD does not affect Bclaf1-mediated mRNA stabilization, Related to Figure 7.

(A) Relative expression levels of *Ctla2a* in *Bclaf1* KD cells treated with actinomycin D. Data are presented as mean ± SD. The value at 0 hr was defined as 1.0 for each cell type.
(B) Western blotting of immunoprecipitated Bclaf1 from differentiation day 3 of *Cry1* KD cells.
(C) Relative expression levels of mRNAs in the co-immunoprecipitated material with a Bclaf1 antibody. Results with and without reverse transcription (RT) are shown. Two PCR primer sets were used for *Ccnd1*, *Ccnd2*, and *Tmem176b*.
(D) Relative expression levels of three mRNAs in *Cry1* KD cells treated with actinomycin D. The expression levels were normalized against *Ctla2a* mRNA at each time point and subsequently against 0 hr for each gene.
** $p < 0.01$ with Student's t-test in comparison to control cells. Data are presented as mean + or ± SD.

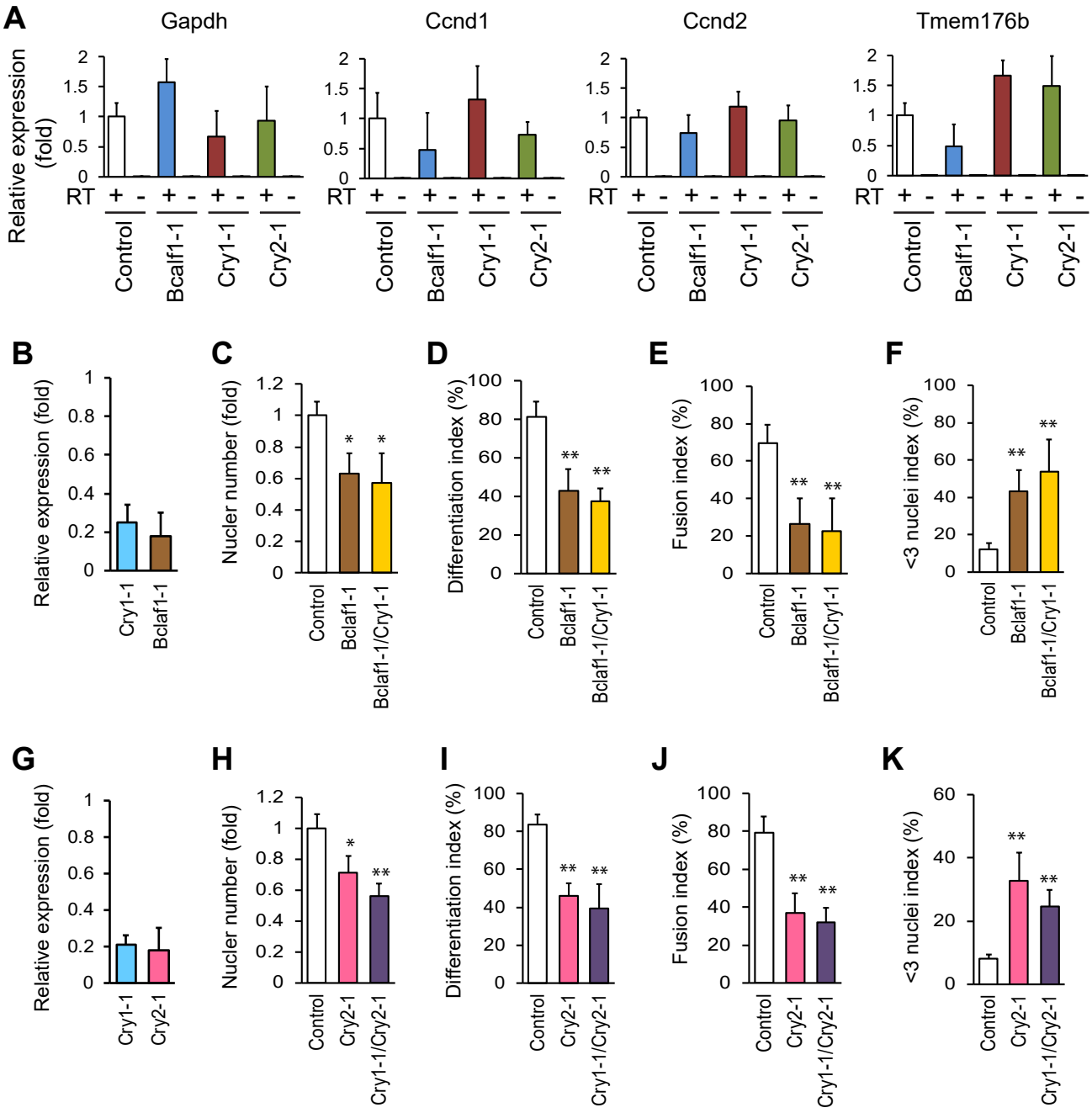
A
Gapdh
Ccnd1
Ccnd2
Tmem176b
Relative expression (fold)
RT
Control
Bcalf1-1
Cry1-1
Cry2-1
B
Relative expression (fold)
Cry1-1
Bclaf1-1
C
Nucler number (fold)
Control
Bclaf1-1
Bclaf1-1/Cry1-1
D
Differentiation index (%)
E
Fusion index (%)
F
<3 nuclei index (%)
G
Relative expression (fold)
Cry1-1
Cry2-1
H
Nucler number (fold)
Control
Cry2-1
Cry1-1/Cry2-1
I
Differentiation index (%)
J
Fusion index (%)
K
<3 nuclei index (%)

Figure S7. Effects of *Cry1*, *Cry2*, and *Bclaf1* KD on myoblast differentiation, Related to Figure 7.

(A) qRT-PCR of nascent mRNAs. Results with (+) and without (-) reverse transcription (RT) are shown.
(B) Relative expression levels of *Cry1* and *Bclaf1* in double KD cells.
(C) Nuclear numbers in *Bclaf1* KD and *Bclaf1/Cry1* double KD cells on differentiation day 5.
(D-F) Differentiation index (D), fusion index (E), and <3 nuclei index (F) in *Bclaf1* KD and *Bclaf1/Cry1* double KD cells on day 5.
(G) Relative expression levels of *Cry1* and *Cry2* in double KD cells.
(H) Nuclear numbers comparing *Cry2* KD and *Cry1*/*Cry2* double KD cells on day 5.
(I-K) Differentiation index (I), fusion index (J), and <3 nuclei index (K) comparing *Cry2* KD and double KD cells on day 5.
* $p < 0.05$ and ** $p < 0.01$ with Student's t-test in comparison to control cells. Data are presented as mean + SD.

Table S1. shRNA clones, Related to Experimental Procedures

Gene	Manufacturer	Catalog #
Control	Sigma-Aldrich	SHC016-1EA
Cry1-1	GE Life Sciences	TRCN0000176255
Cry1-2	GE Life Sciences	TRCN0000173610
Cry2-1	Sigma-Aldrich	TRCN0000240548
Cry2-2	Sigma-Aldrich	TRCN0000194121
Ccnd1-1	GE Life Sciences	TRCN0000026948
Ccnd1-2	GE Life Sciences	TRCN0000026881
Tmem176b-1	GE Life Sciences	TRCN0000105305
Tmem176b-2	GE Life Sciences	TRCN0000105306
Bclaf1-1	GE Life Sciences	TRCN0000084413
Bclaf1-2	GE Life Sciences	TRCN0000084416

Table S2. Sequences of qPCR primers, Related to Experimental Procedures

Gene	Forward	Reverse
MyoD	TGAGCAAAGTGAATGAGGCCTTCG	TGCAGACCTTCGATGTAGCGGAT
Myogenin (*Myog*)	CCCTATTTCTACCAGGAGCCCCAC	GCGCAGGATCTCCACTTTAGGCAG
Myomaker (*Mymk*)	ATCGCTACCAAGAGGCGTT	CACAGCACAGACAAACCAGG
MHC (*Myh3*)	CACCTGGAGAGGATGAAGAAGAA	AAGACTTGACTTTCACTTGGAGTTTATC
Ckm	CTCAGCAAGCACAACAATCAC	GATGACATCGTCCAGAGTGAAG
Gapdh	TGCACCACCAACTGCTTAG	GATGCAGGGATGATGTTC
Ccnd1	CAGAGGCGGATGAGAACAAG	GAGGGTGGGTTGGAAATGAA
Ccnd1-2	GCCGAGAAGTTGTGCATCTA	AGGTTCCACTTGAGCTTGTT
Ccnd2	CTCCCGCAGTGTTCCTATTT	TCACAGACCTCTAGCATCCA
Ccnd2-2	CAGGAGCTGCTGGAGTGGGAACTG	AACTTGAAGTCGGTAGCGC
Tmem176b	CCTGGAGATTGTTGTGTCTGT	AGCTTCCTCTCTGACTCTTCT
Tmem176b-2	GCAAACCAGTGTTCCCTACT	ATCATCACTGTATCGCACTGTC
Bmal1	CAACCCATACACAGAAGCAAAC	CATCTGCTGCCCTGAGAATTA
Per1	CAGGATGTGGGTGTCTTCTATG	GTGAAGTCCTTGAGACCTGAAC
Cry1	CTCAGTCCTTATCTCCGCTTTG	CCACAGGAGTTGCCCATAAA
Cry2	GATGCCGATTTCAGTGTGAATG	GGCAGTAGCAGTGGAAGAAT
Bclaf1	ACACAGAGGAGACAGAGGATTA	CTCAGTATTCCGGTGAGATGAAG
Ctla2a	GGCAAGACCAGCTTCTACAT	CAGGAGCCATTTCTCCTCTATTC
Actb	GAGGTATCCTGACCCTGAAGTA	CACACGCAGCTCATTGTAGA

Supplemental Experimental Procedures

Knockout Mice

Cry1$^{+/-}$ mice (B6.129P2-Cry1^{tm1Asn}/J, stock # 016186) and *Cry2*$^{+/-}$ mice (B6.129P2-Cry2^{tm1Asn}/J, stock# 016185) were purchased from Jackson Laboratory. Genotyping to detect the mutated *Cry1* and *Cry2* alleles was performed by PCR using the primers described on the web site of Jackson Laboratory. Age-matched littermate wild type (WT) mice were used as controls. Mice were housed in a specific pathogen free (SPF) environment and were monitored by the Research Animal Resources (RAR) staff of the University of Minnesota. Mice were provided access to drinking water and standard chow ad libitum under a 14 hr-light and 10 hr-dark cycles except for the 12 hr-light and 12 hr-dark experiments to isolate TA muscles described below. Mice were euthanized by CO_2 inhalation or KCl injection after anesthesia with 2-4% isoflurane (Phoenix). These methods are consistent with the recommendations of the Panel of Euthanasia of the American Veterinary Medical Association.

Culture of C2C12 cells

Mouse myoblast C2C12 cells were purchased from American Type Culture Collection (ATCC, CRL-1772) and maintained with 10% FBS in DMEM in an incubator at 37°C with 5% CO_2. Differentiation was induced as follows. On day -2, cells were seeded at $1x10^5$ cells/well in a 12-well plate. On day 0 the wells became confluent. The cells were rinsed twice with phosphate buffered saline (PBS) and cultured with 1% insulin-transferrin-selenium (ITS) in DMEM. Medium was changed with fresh DMEM with ITS every two days.

Synchronization of Circadian Rhythms of Undifferentiated and Proliferating C2C12 Cells

C2C12 cells were seeded at $8x10^4$ cells/well in a 12-well plate in 10% FBS in DMEM on day -1. On day 1, 10 μM forskolin was added between -1 and 0 hrs. Cells were washed with PBS twice and fresh 10% FBS in DMEM was added at 0 hr. Cells were harvested for qRT-PCR or fixed with 4% formaldehyde for immunofluorescence staining every 4 hrs for 44 hrs. In addition, cells were pulse-labelled with 0.5 μM EdU (5-ethynyl-2'-deoxyuruidine) with a Click-iT EdU Alexa Fluor 448 imaging kit (Invitrogen, C10337) for 30 min before each 4 hr time point.

Synchronization of Circadian Rhythms of Differentiating C2C12 Cells

C2C12 cells were seeded at $1x10^5$ cells/well in a 12-well plate in 10% FBS in DMEM on day -2. On day 0, forskolin was added between -1 and 0 hrs. Cells were washed with PBS twice and then differentiation was started with 5% horse serum (HS) in DMEM at 0 hr. Synchronization was maintained better with HS than with ITS. Cells were harvested for qRT-PCR, fixed for immunofluorescence staining, or treated with EdU as described above every 4 hrs for 120 hrs. Only a half of medium was replaced at 60 hr to preserve circadian rhythms.

MTS Cell Proliferation Assay

C2C12 cells were seeded at $1x10^3$ cells/well in a 96-well plate. On the day of measurement, 20 μl of MTS solution (Promega, CellTiter 96 Aqueous One Solution Cell Proliferation Assay, G3581) was added to 100 μl of culture in each well. After incubation for 2 hr at 37°C, absorbance at 492 nm was measured with an LD400 spectrophotometer (Beckman Coulter). The value obtained from blank wells without cells were subtracted from the absorbance values. Average $\pm$ SD was obtained from three independent experiments.

Circadian Gene Expression in TA Muscles

WT, *Cry1*$^{-/-}$, and *Cry2*$^{-/-}$ male mice aged 8-12 weeks old were entrained at 12hr-light and 12hr-dark cycles (6:00-18:00 light and 18:00-6:00 dark) for two weeks before experiments. This means that Zeitgeber Time 0 (ZT0) corresponds to 6:00 and ZT12, 18:00. TA muscles were isolated every 4 hr starting at ZT2. Total RNA was isolated with a Direct-zol RNA MiniPrep with TRI-Reagent (Zymo Research, R2015). qRT-PCR was performed as described above with two TA muscles each.

Muscle Injury

Male mice of 6-8 weeks old were anesthetized using 2-4% isoflurane. We injected 50 μl 1.2% $BaCl_2$ in 0.9% NaCl into the left TA muscle of four WT, *Cry1*$^{-/-}$, and *Cry2*$^{-/-}$ mice each between ZT4 and ZT8 when *Cry1* and *Cry2* mRNA levels were low. This was because peak timing of these mRNA levels was different among WT, *Cry1*$^{-/-}$, and *Cry2*$^{-/-}$ mice (Fig. 4C), leaving the lower timing more consistent. Mice were euthanized 3, 4, 5, and 7 days post injection and the TA muscle was extracted between ZT4 and ZT8. Cryosections with a thickness of 10 μm were prepared for immunofluorescence, Hematoxylin Eosin (HE), and Sirius red staining.

Preparation of Primary Myoblasts
Satellite cell-derived primary myoblasts were obtained from adult hind limb muscle of 2-month-old WT, *Cry1*$^{-/-}$, and *Cry2*$^{-/-}$ mice as described previously (Motohashi et al., 2014). Briefly, muscles were minced and digested with collagenase type 2 (Worthington, CLS-2) to obtain dissociated muscle cells. Satellite cells were then purified with MS columns (Miltenyi Biotec, 130-042-201) and LD columns (Miltenyi Biotec, 130-042-901) by negative selection with antibodies against CD31-PE (eBioscience, 12-0311), CD45-PE (eBioscience, 12-0451), and Sca1-PE (eBioscience, 12-5981), followed by anti-PE MicroBeads (Miltenyi Biotec, 130-048-801). This was followed by positive selection with an antibody against biotin-conjugated integrin α7-biotin (Miltenyi Biotec, 130-102-125) and anti-biotin MicroBeads (Miltenyi Biotec, 130-090-485). Isolated satellite cells were cultured on dishes coated with rat tail collagen (BD Biosciences, 354236) in myoblast growth medium consisting of HAM's F-10 medium with 20% fetal bovine serum (FBS), 10 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, PHG0263), penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C with 5% CO_2. Low- passage satellite cell-derived primary myoblasts (typically less than eight passages) were used for immunostaining. Differentiation medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% HS, penicillin, and streptomycin) was used for myogenic differentiation.

Gene Knockdown
293FT cells (Invitrogen, R70007) were seeded at $3x10^5$ cells/well in a 12-well plate with 10% FBS in DMEM on day 1. Cells were transfected with a 0.5 μg pLKO.1 vector encoding an shRNA sequence (GE Life Science, Table S1) along with 0.2 μg each of pCMV-VSV-G (Addgene, 8454), pRSV-Rev (Addgene, 12253), and pMDLg/pRRE (Addgene, 12251) with 2.75 μl Lipofectamine 2000 (Invitrogen, 11668019) on day 2. Culture medium was replaced with fresh DMEM with 10% FBS 5 hr later. On day 5 the medium containing lentivirus was harvested and filtered through a 0.45 μm syringe filter. C2C12 cells that had been seeded in 12-well plates on day 4 were transduced with 400 μl of the virus suspension with 0.8 μg/ml polybrene (Sigma, H9268) and 400 μl DMEM with 10% FBS. Culture medium was replaced with fresh DMEM with 10% FBS on day 6. Virus-integrated cells were selected with 1 μg/ml puromycin dihydrochloride (MB Bio, 100552) between days 7 and 14. The proliferated cells were frozen in liquid nitrogen or used in differentiation studies.

Gene Subcloning
The FUW-tetO-hOCT4 (FUW) vector used was obtained from Addgene (20726). The zeocin resistance gene was replaced with a puromycin resistance gene. The FUW vector was further modified with the removal of the *hOCT4* sequence. Additionally, an adaptor with the restriction sites PacI, EcoRI, and BamHI was subcloned into the EcoRI site of FUW. The Flag x3-Linker sequence shown below were obtained from GeneArt (Invitrogen) and amplified from their pcDNA3 vector and subcloned into FUW.
Flag x3-Linker
GAATTCATGGACTACAAGGACGACGACGACAAGGATTACAAGGATGATGATGATAAGGACTATAAGG
ACGATGATGACAAAGGCGGCGGAGGCAGCGGATCCCTCGAG

The *Tmem176b* gene were amplified from cDNA prepared from C2C12 cells and inserted into the BamHI site at the 3' side of the linker in the FUW-Flag x3-Linker vector using the Gibson method.

Gene Overexpression
The FUW-tetO-FLAG-Tmem176b vector was transfected into 293FT cells to prepare lentivirus as described above. The FUW vector and FUW-M2rtTA (Addgene 20342) were used at 0.25 μg each in place of the pLKO.1 vector. Tetracyclin-free FBS was used to prevent premature induction of the transgenes. C2C12 cells were transduced as described above to establish cell lines. The transgenes were induced with 2 μg/ml doxycycline for four days before use for immunofluorescence staining and immunoprecipitation.

Actinomycin D Treatment
Cells were treated with 5 μg/ml actinomycin D (Sigma-Aldrich, A1410) for 17 hr to arrest transcription to study the stability of mRNA. Ctla2a was used as control mRNA due to its long half-life (687 min) in C2C12 cells (Lee et al., 2010).

Immunofluorescence Staining of C2C12 Cells
Transduced C2C12 cells were fixed with 4% paraformaldehyde for 15 min on day 3 and 5 during differentiation and

permeabilized with 0.5% Triton X-100 in PBS for 5 min. Cells were then incubated with the antibody against myosin heavy chain (MHC, Developmental Studies Hybridoma Bank, MF20, 1:200 dilution) and the secondary antibody Alexa Fluor 488 goat anti-mouse IgG (H+L) (Thermo Fisher Scientific, A11029, 1:200 dilution) for 1 hr each at 25°C. DNA was counterstained with 5 μg/ml Hoechst 33342 (Sigma, B2261). Fluorescence signal was captured with a LUCPlanFLN 20x objective lens (Olympus) with 0.45 Ph1 aperture and a C11440-42U digital camera (Hamamatsu) attached to an IX73P2F microscope (Olympus). Adobe Photoshop CS6 was used for image processing. Differentiation index was defined as a percentage of nuclei (Hoechst-stained structure) existing within MHC(+) cells among 1,000 nuclei in total. Fusion index is a percentage of nuclei located in MHC(+) cells that contained two or more nuclei in each cell among the same 1,000 nuclei. <3 nuclei index is a percentage of nuclei that were located in MHC(+) cells containing one or two nuclei among all MHC(+) cell nuclei.

Immunofluorescence Staining of Primary Myoblasts

Cells were fixed with 2% formaldehyde and were blocked with 1% bovine serum albumin in PBS. Cells were then stained with anti-MyoD (Santa Cruz Biotechnology, sc-760, 1:200 dilution) and anti-MHC antibodies. After staining, they were incubated with the secondary antibodies Alexa Fluor 488 donkey anti-mouse IgG (Thermo Fisher Scientific, A21202, 1:200 dilution) and Alexa Fluor 594 donkey anti-rabbit IgG (Thermo Fisher Scientific, A21207, 1:200 dilution). DNA were counterstained with 4',6'-diamidine-2'-phenylindole dihydrochloride (DAPI, 10236276001).

Single Muscle Fiber Culture and Immunostaining

Single muscle fibers were isolated by 0.2% collagenase type I digestion (Sigma-Aldrich, C0130) of extensor digitorum longus muscles for 90 min at 37 ºC. Isolated single muscle fibers were transferred and cultured on 5% HS-coated tissue culture dishes with DMEM supplemented with 10% HS and 1% chicken embryo extract (MP Biomedicals, 092850145) for 72 hr. Single muscle fibers were fixed with 2% paraformaldehyde in PBS for 20 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 10% bovine serum albumin in PBS for 30 min. The fibers were stained with primary antibodies (anti-Pax7, Developmental Studies Hybridoma Bank, 1:5 and anti-MyoD, Santa Cruz Biotechnology, sc-304, 1:500) and secondary antibodies (Alexa Fluor 488 donkey anti-rabbit IgG (H+L), Thermo Fisher Scientific, A21206, 1:500 and Alexa Fluor 568 donkey anti-mouse IgG (H+L), Thermo Fisher Scientific, A10037, 1:500). Nuclei were counterstained with DAPI.

Staining of EdU Uptake

EdU uptake was assessed by pulse labeling C2C12 cells with 0.5 μM EdU for 30 min, followed by fixation with 4% formaldehyde and detection with a Click-iT EdU Alexa Fluor 448 imaging kit following the instructions provided by the manufacturer. Frequency of EdU uptake was calculated by observing 1,000 nuclei.

Western Blotting

Protein transferred to an Immobilon P membrane (Millipore Sigma, IPVH00010) was detected with primary antibodies against FLAG (Sigma, F1804, 1:200 dilution), Cry1 (Alpha Diagnostic International, CRY11-A, 1:200 dilution), Cry2 (Alpha Diagnostic International, CRY12-A, 1:200 dilution), and Bclaf1 (Bethyl Laboratory, A300-608A, 1:200 dilution) with mouse anti-rabbit IgG-HRP light chain specific (Jackson ImmunoResearch 211-032-171, 1:1000 dilution), goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology sc-2004, 1:1000 dilution), and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005, 1:1000 dilution). The chemiluminescence signal was detected with a SuperSignal West Dura kit (Thermo Fisher Scientific, 34075) and X-ray films.

Quantitative RT-PCR (qRT-PCR)

RNA was isolated from cells with a Quick-RNA MiniPrep kit (Zymo Research, R1055). cDNA was synthesized using 1.0 μg of total RNA with ProtoScript II First Strand cDNA Synthesis Kit (NEB, E6560L) and random hexamers. Real-time quantitative PCR was performed using a GoTaq qPCR Master Mix (Promega, A6002) in a 96-well PCR plate in a Mastercycler realplex^2 thermocycler (Eppendorf). Primer sequences are listed in Table S2. mRNA expression levels were analyzed by normalizing expression values to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) expression. Mean ± SD of three independent experiments were calculated.

Immunofluorescence Staining of TA Sections

Sections were first fixed with 2% paraformaldehyde for 10 min, followed by permeabilization with 0.2% Triton X-100 in PBS for 5 min. The sections were treated with two blocking reagents: 3% Mouse-on-Mouse Blocking Reagent (Vector MKB-2213) in PBS for 1 hr and 5% FBS in PBS for 30 min. Mouse anti-embryonic myosin heavy chain (eMHC, Developmental Studies Hybridoma Bank, F1.652-f, 1:5 dilution) and rabbit anti-laminin (Sigma, L9393,

1:2000 dilution) antibodies were used as primary antibodies diluted in 5% FBS in PBS. After 1 hr incubation, sections were washed twice with 0.01% Triton-X100 in PBS. The secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (H+L) (Thermo Fisher Scientific, A11029, 1:200 dilution) and Alexa Fluor 555 goat anti-rabbit IgG (H+L) (Thermo Fisher Scientific, A21429, 1:200 dilution) diluted in 5% FBS in PBS were used along with 5 μg/ml Hoechst 33342 (Sigma, B2261) for 1 hr. Sections were mounted using Fluorescent Mounting Medium (DAKO, S302380-2). Fluorescence images were captured using Metamorph Basic software (Molecular Devices) and an ORCA-flash4.0LT camera (Hamamatsu) attached to an IX73 microscope (Olympus) with a 20X LUCPlan FL N lens. Images were processed with Photoshop and Illustrator CS6 (Adobe).

Hematoxylin Eosin (HE) Staining

Sections were first fixed using 2% formaldehyde for 5 min. Sections were then treated as follows: deionized water for 1 min, Harris Modified Hematoxylin (Thermo Fisher Scientific, SH26-500D) for 2 min, tap water for 1 min, deionized water for 1 min, Eosin-Y (Thermo Fisher Scientific, 22-220-104) for 5 min, 95% ethanol for 30 sec, 100% ethanol for 2 min twice, and xylene for 10 min twice. Sections were mounted using Permount (Thermo Fisher Scientific, SP15-100). Images were captured with cellSens Entry 1.11 software (Olympus) and a DP26 camera (Olympus) attached to the microscope described above.

Sirius Red Staining

The Sirius red solution was made as 1% Direct Red 80 in 1.3% picric acid. TA sections were fixed with acetone, pre-chilled at -20°C, for 10 min. The sections were then washed in deionized water for 1 min, stained with Sirius red for 15 min, and rinsed with 0.5% acetic acid for 1 min. The sections were subsequently washed with 100% ethanol for 2 min and twice with xylene for 10 min. Finally, the sections were mounted with Permount for taking images as described for HE staining. Quantifications were done using the entire section area with ImageJ (Shimizu-Motohashi et al., 2015).

Immunoprecipitation

Whole cell extracts were prepared by resuspending $4x10^6$ cells with 300 μl lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 2 mM $MgCl_2$, 1% NP40, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 1.5mM pepstatin A, and 150 u/ml benzonase). The extracts were incubated for 30 min at 25°C with 5 μl Dynabeads Protein G (Thermo Fisher Scientific, 10004D) that had been washed with 0.1% Tween 20 in PBS and then loaded with 3 μg of anti-FLAG M2 monoclonal antibody (Sigma F1804) or normal mouse IgG (Santa Cruz Biotechnology, sc-2025). After incubation the beads were washed three times with 500 μl of 0.1% Tween 20 in PBS. Bound proteins were eluted by incubation with 30 μl of 100 μg/ml 3X FLAG peptides (Sigma, F4799) in PBS with 0.1% Tween 20 for 10 min on ice. Elution was repeated three times in total and they were combined for mass spectrometry or western blotting.

Endogenous Cry1, Cry2, and Bclaf1 were immunoprecipitated with the antibodies described above. Co-precipitated proteins were eluted by incubating the beads in 30 μl of 1X Laemmli buffer (50 mM Tris-HCl, pH6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue, and 25 mM dithiothreitol) at 95°C for 5 min. Eluted proteins were applied to SDS-PAGE for western blotting.

Mass Spectrometry

Proteins eluted from Dynabeads Protein G were run into a Criterion 8-16% Tris-HCl gradient gel (Bio-Rad Laboratories, 3450038) for 25 min at 25 mA constant current. The gel was fixed with 40% ethanol and 10% acetic acid for 30 min and then stained with Imperial Protein Stain (Thermo Fisher Scientific, 24615). The stained protein regions were excised and in-gel trypsin digestion was performed (Shevchenko et al., 1996). The dried peptide mixtures were solubilized in 2% acetonitrile and 0.1% trifluoroacetic acid in water and cleaned with the Stage Tip protocol (Rappsilber et al., 2003) and dried in vacuo.

Approximately 1.5 μg of sample was injected for a mass spectrometry analysis. Spectra were acquired on an Orbitrap Fusion (Thermo Fisher Scientific) coupled to an Easy-nLC 1000 (Thermo Fisher Scientific) ultrahigh pressure liquid chromatography pump. Peptides were separated on an in-house packed 100 μm internal diameter, 20 cm column containing ReproSil-Pur C18 resin (3μm, 120 Å, Dr. Maisch GmbH, Germany). Liquid chromatography solvents employed were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with a gradient consisting of 2 min of 2-8% B, 40 min of 8-30% B, 1 min of 30-90% B, 5 min of 90% B, 1 min of 90-2% B, and 5 min of 2% B with a flow rate of 330 nl/min.

Mass spectrometric data was acquired in top 8 data dependent mode. The MS1 spectra data was collected at a resolution of 60,000, with an automated gain control (AGC) target of 400,000 and a max injection time of 50 ms.

Precursor ions were filtered according to charge state (2-7 *z*) and an intensity of >130,000 were selected for MS/MS, in which a dynamic exclusion window of 10 seconds and ± 10 ppm mass accuracy was employed for previously examined precursor ions. Isolation of MS2 precursor ions was performed using a quadruple mass filter set to 1.6 *m/z* window and fragmentation of precursor ions was performed using an HCD-normalized collision energy of 35 with first mass set to 100 *m/z*. The MS/MS scan was acquired with Orbitrap resolution of 15,000, an AGC target of 50,000, and a maximal injection time of 100 milliseconds.

RNA Immunoprecipitation

RNA immunoprecipitation was performed with an EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Millipore Sigma, 17-701) following the instruction. Non-crosslinked cell extract was prepared from $1x10^7$ non-transduced C2C12 cells on differentiation day 3 with the kit. Bclaf1was immunoprecipitated with 5 µg of anti-Bclaf1 antibody (Bethyl Laboratory, A300-608A) for 4 hr at 4°C. Normal rabbit IgG (Santa Cruz Biotechnology, sc-2027) was used as a control. Purified RNA was applied to qRT-PCR as described above.

qPCR analysis of nascent mRNAs

Nascent RNA was pulse-labeled with 5-ethynyl uridine for 1 hr, biotinylated, and purified with streptavidin magnetic beads with a Click-iT Nascent RNA kit (Thermo Fisher Scientific, C10365). RT-qPCR was performed as described above.

RNA-Seq

1) Sample Quality Assessment

Total RNA was prepared from KD C2C12 cells before differentiation (day 0) and day 3 and 5 during differentiation. shRNA clone #1 was used for Cry1 and Cry2 KD. Total RNA isolates were quantified with a Quant-iT RiboGreen RNA Assay Kit (Thermo Fisher Scientific, R11490). RNA integrity was assessed by generating an RNA Integrity Number (RIN) with capillary electrophoresis using a BioAnalyzer 2100 (Agilent). All samples passed the initial quality control, having over 1 µg of RNA and RIN over 8.

2) Library creation: Samples were converted to Illumina sequencing libraries using a Truseq RNA Sample Preparation Kit (Illumina, RS-122-2001). Briefly, polyA (+) RNA was purified from 1 µg total RNA with oligo-dT-coated magnetic beads, fragmented, and reverse-transcribed into cDNA. The cDNA was further fragmented, blunt-ended, and ligated to indexed (barcoded) adaptors and amplified with 15 cycles of PCR. Final library size distribution was validated with capillary electrophoresis and quantified with a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, P11496) and quantitative PCR. Indexed libraries were then normalized, pooled, and size-selected to 320 bp ± 5% with a LabChip XT (PerkinElmer).

3) Cluster generation and sequencing: Libraries were hybridized to a single read flow cell and individual fragments were clonally amplified by bridge amplification on a cBot (Illumina). Once clustering was completed, the flow cell was loaded onto a HiSeq 2500 (Illumina) and sequenced using Illumina's SBS chemistry. Upon completion of read 1, an 8 bp forward and 8 bp reverse (i7 and i5) index read was performed.

4) Primary analysis and de-multiplexing: Base call (.bcl) files for each cycle of sequencing were generated by Real Time Analysis (RTA) software (Illumina, https://support.illumina.com/downloads/hcs-2-2-68.html). The base call files and run folders were then exported to servers maintained at the Minnesota Supercomputing Institute (MSI). Primary analysis and de-multiplexing were performed using bcl2fatstq software version 2.17.1.14 (Illumina, https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html). The de-multiplexed FASTQ files were used for subsequent analyses.

5) Sequence analysis: Over 5 million reads were generated per library and the average quality score passing quality filter were above Q30. Raw sequences were analyzed using a customized pipeline (gopher-pipelines; https://bitbucket.org/jgarbe/gopher-pipelines/overview) developed and maintained by the MSI. Briefly, quality controls were performed on each FASTQ files using FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) before and after adapter trimming with Trimmomatic version 0.33 (http://www.usadellab.org/cms/index.php?page=trimmomatic) (Bolger et al., 2014). Post-trimming sequences were aligned to GRCm38/mm10 reference genome using HISAT2 version 2.0.2 (https://ccb.jhu.edu/software/hisat2/index.shtml) (Kim et al., 2015). Transcript abundance was then estimated using subread version 1.4.6 (http://subread.sourceforge.net/) (Liao et al., 2014).

Differential gene expression was determined using Cufflinks version 2.2.1 (http://cole-trapnell-lab.github.io/cufflinks/) (Trapnell et al., 2012). Pathway analysis of differentially expressed genes were performed by functionally annotate the genes and perform overrepresentation enrichment test using PANTHER (http://pantherdb.org/) (Mi et al., 2013).

6) Heat maps: Heat maps were generated using *DESeq2* (Version 1.10.1, http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html) and *pheatmap* version 1.0.8 (https://cran.r-project.org/web/packages/pheatmap/index.html) packages in R version 3.2.3 (https://www.r-project.org/) (Love et al., 2014). Briefly, read counts generated by subread are filtered to include genes with at least 5 reads in at least 1 sample. The remaining reads were then log transformed. Heat maps were generated using the log transformed values with *pheatmap* package. Hierarchical clustering was performed using euclidian distances and average linkage clustering method.

QUANTIFICATION

Image analysis

For TA muscle sections stained for eMHC, randomly selected 2,000 myofibers from two mice totaling 4,000 myofibers from each strain were used in the histograms in Figure 1C and in the statistical analysis. The pixels depicting myofibers expressing eMHC were classified as foreground and everything else as background. These images were then made binary and the area of each myofiber was found with ImageJ (NIH). These areas were then sorted based on size to make a histogram.

For the HE-stained sections, randomly selected 500 myofibers with centrally located nuclei from four mice totaling 2,000 myofibers for each mouse strain are shown in the histograms in Figures 1E and 1G and were used in statistical analysis. Cross section images of myofibers were separated using the Pixel Classification program of the ilastik 1.2 software (ilastik). The pixels depicting myofibers with centrally located nuclei were classified as foreground and everything else as background. ImageJ was then used to make these images binary and to find the cross-sectional area of the myofibers. Myofibers were then sorted based on size to make histograms. The raw number of myofibers within each size range was divided by the total number of myofibers to give the frequency, which is shown in Figures 1C, 1E, and 1G.

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