Supplementary Information for

Activation of the Crtc2/Creb1 transcriptional network in skeletal muscle enhances weight loss during intermittent fasting

Nelson E. Bruno[1]*, Jerome C. Nwachukwu[1], Sathish Srinivasan[1], Richard Hawkins, David Sturgill[3], Gordon L. Hager[3], Stephen Hurst[4], Shey-Shing Sheu[4], Michael D. Conkright[5], Kendall W. Nettles[1]*

Correspondence: Nelson E Bruno, nbruno@scripps.edu; Kendall Nettles, knettles@scripps.edu

SUPPLEMENTARY FIGURES

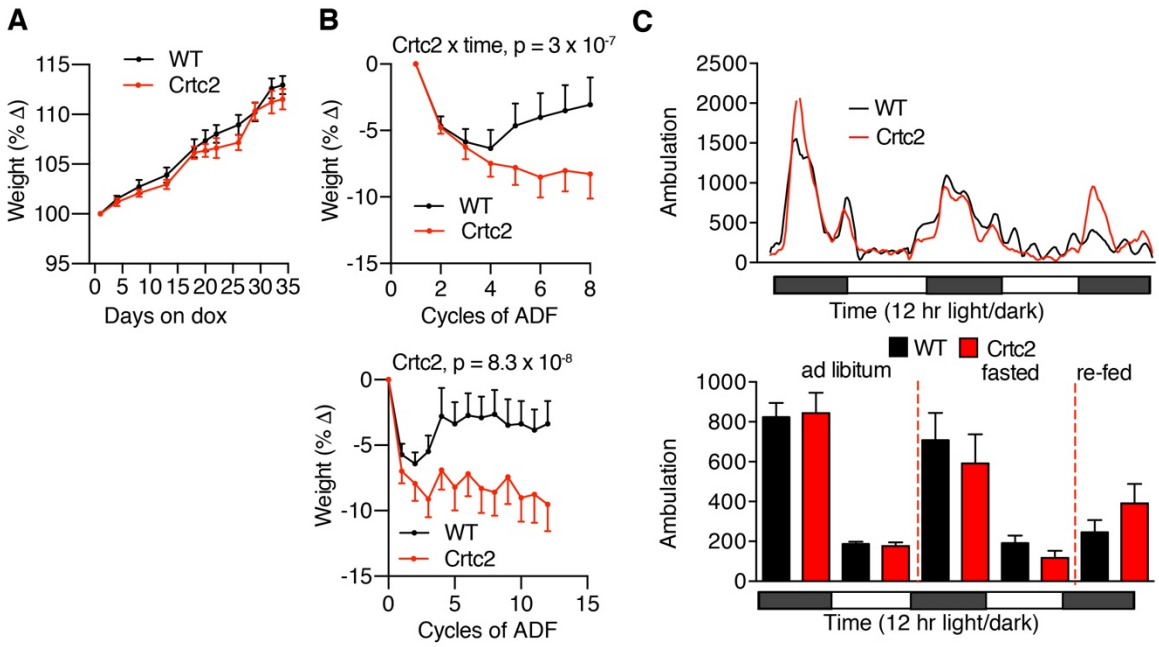
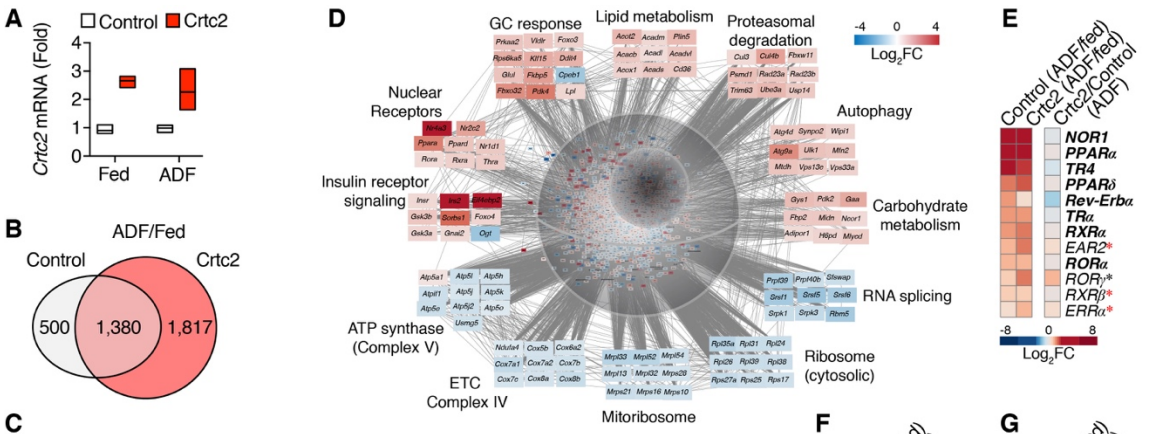


Fig. S1. Effects of skeletal muscle overexpression of Crtc2 on the response to ADF.
(**A**) Weight gain after treatment of 18-week old WT and Crtc2 transgenic mice (n=8) with doxycycline.
(**B**) Changes in body weight during ADF.
(**C**) Average ambulatory activity during ad libitum feeding, fasting and re-feeding, n = 8 mice per group. 12-hr averages of the data. There were no significant effects of Crtc2.
A–C) Data shown as mean ± SEM.



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Biological process/function — No. of DEGs — *Crtc2 regulated >20% of DEGs

Source	GO term	Total	Regulated by ADF: Control	Regulated by ADF: Crtc2	Regulated by ADF: Both	Crtc2/ Control (fasted)
M.E. Pipkin	Transcription factors	324	155	284	124	42
EpiFactors	Coregulators	154	84	135	68	14
GO:0042255	Ribosome assembly	20	16	15	11	2
NURSA	Nuclear hormone receptors (NHRs)	13	8	11	6	1
GO:0006629	Lipid metabolic process	258	128	223	103	43
GO:0006635	Fatty acid beta-oxidation	26	16	22	13	4
GO:0008286	Insulin receptor signaling pathway	37	25	32	21	6
Kuo et al.	Glucocorticoid response	1,145	563	980	436	192
GO:0043161	Proteasomal protein degradation	104	57	91	46	9
GO:0000502	Proteasome complex	30	21	26	17	0
GO:0006914	Autophagy	105	59	98	52	13
GO:0042752	Regulation of circadian rhythm	22	14	19	11	4
GO:0004672	Protein kinase activity	133	65	127	62	22
GO:0008380	RNA splicing	71	50	61	42	3
GO:0003009	Skeletal muscle contraction	20	14	18	12	1
GO:0042594	Response to starvation	37	23	36	23	2
GO:0005746	Mitochondrial respiratory chain	51	42	14	8	23*
MitoCarta2.0	Mitochondrial proteins	368	220	246	116	78*
GO:0030433	Ub-dependent ERAD pathway	26	11	23	9	5
GO:0006954	Inflammatory response	105	30	86	17	41*
GO:0006468	Protein phosphorylation	385	174	339	140	91*
GO:0007249	I-kappaB kinase/NF-kappaB cascade	53	18	46	13	12*

Fig. S2. Transcriptional control of fasting and weight loss and its regulation by Crtc2.

(**A**) Mice TA muscles were transduced with GFP control or Crtc2 expression vector. Crtc2 mRNA levels in the TA of mice fed ad libitum (Fed), or subject to ADF (Fast) were compared by qPCR. $N = 3$ mice per group.

(**B**) Venn diagram showing the numbers of DEGs identified by mRNA-seq comparing the effects of ADF in control versus Crtc2-transduced TA muscles.

(**C**) Biological processes and functions regulated by ADF and Crtc2. Gene ontology (GO) analysis suggests that ADF regulates several processes/ functions in a Crtc2-sensitive manner. The numbers of DEGs involved in each process are shown. See SI Appendix, **Dataset S1** for a complete list of represented GO annotations.

(**D**) Examples of ADF-regulated transcriptional programs and mRNAs in control TA muscles.

(**E–G**) Gene expression profiles in control and Crtc2-transduced TA muscles of mice subjected to ADF relative to the ad libitum fed mice (columns 1–2), and effect of Crtc2 transduction relative to control during ADF (column 3). Expression profiles of genes that encode **E**) nuclear receptors, **F**) other transcription factors, and **G**) transcriptional coregulators. ADF-regulated genes in control muscle appear in **bold**. *Crtc2-regulated genes in mice subjected to ADF. *ADF-regulated genes in Crtc2-transduced muscle but not control. FC, fold change. Also see SI Appendix, **Dataset S1**

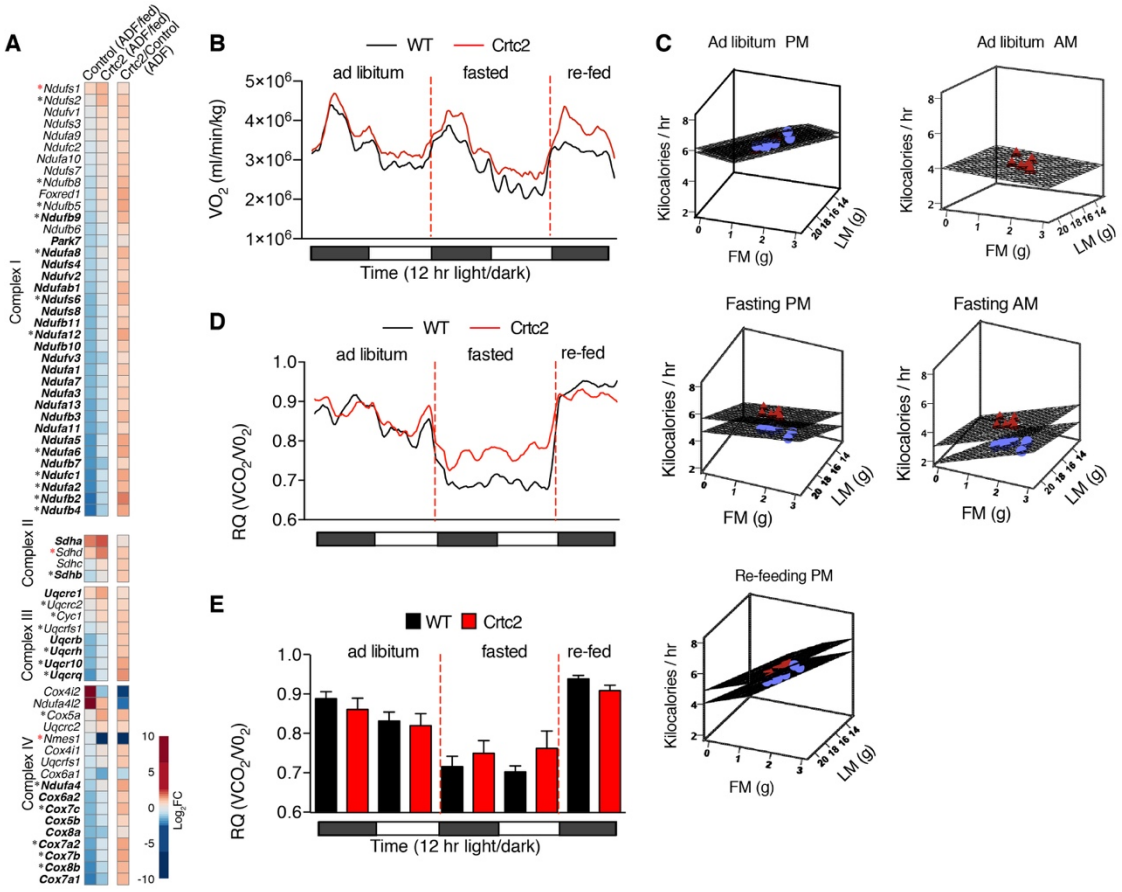


Fig. S3. Electron transport chain gene expression and whole-body energetics.

(**A**) Expression profiles of genes that encode the electron transport chain. The mRNA levels in control and Crtc2-transduced TA muscles of mice subjected to ADF relative to the ad libitum fed mice (columns 1–2), and effect of Crtc2 transduction relative to control during ADF (column 3) are shown. ADF-regulated genes in control muscle appear in **bold**. *Crtc2-regulated genes in mice subjected to ADF. *ADF-regulated genes in Crtc2-transduced muscle but not control. FC, fold change

(**B**) VO_2 (and VCO_2) were measured continuously for 72 hrs. in a CLAMS animal monitoring system. N = 8 per group

(**C**) Total energy expenditure (EE) for WT and Crtc2 mice was calculated from VO_2 and VCO_2. The data was adjusted by repeated measures ANCOVA in with lean mass (LM) and fat mass (FM) as covariants at the following values: LM = 17.01 g; and FM = 1.62 g. Dot plot graphs shows adjusted means derived from repeated measure general linear model ANCOVA, using 5 consecutive 12-hr time intervals: PM-ad libitum; AM-ad libitum; PM-Fast; AM-Fast; and Re-feed EE as dependent variables, and LM and FM as covariates. The lack of intersection of the planes graphically demonstrates lack of covariation.

(**D**) The respiratory quotient was determined during the CLAMS experiment described in panel B.

(**E**) 12-hr light/dark averages of the data from panel D. Data are mean +SEM.

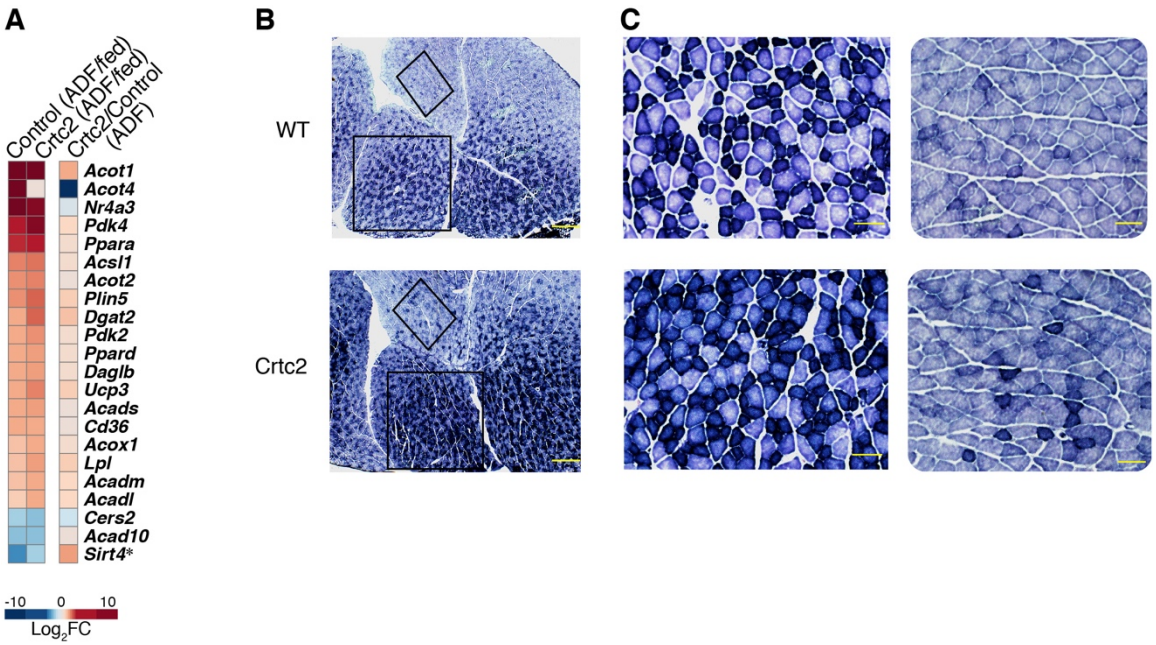
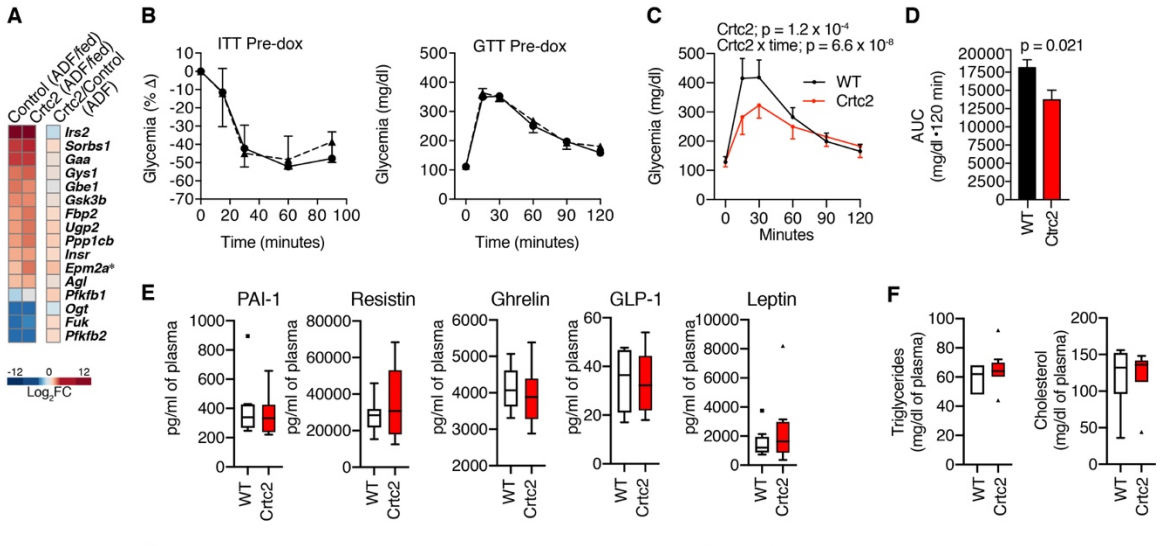


Fig. S4. Analysis of succinate dehydrogenase in skeletal muscle fibers.

(**A**) ADF upregulated a panoply of genes involved in the transport, oxidation, and esterification of fatty acids. Key among these are: *Ppard* and *Ppara,* the chief regulators of fat metabolism in muscle; *Cd36* that regulates plasma membrane fatty acid transport and utilization to spare glucose; *Ucp3,* which is implicated in b-oxidation as well as protection from triglyceride accumulation in muscle (1); *Pdk2* and *Pdk4,* which promote fatty acid oxidation over glycolysis (2); and multiple enzymes involved in Acyl-CoA metabolism. *Acox1* is a peroxisomal Acyl-CoA oxidase, while *Acad10*, *Acadl*, *Acadm* are members of the acyl-coenzyme A dehydrogenase family that are critical for effective beta-oxidation in the mitochondria. *Acsl1*, *Acot1*, *Acot2*, and *Acot4* regulate the generation and hydrolysis of Acyl-CoA. Differential expression of *Dgat2*, *Daglb*, *Plin5* and other genes that regulate lipid storage and accumulation highlights their importance in lipid homeostasis during fasting. Notably, the lipoprotein lipase (*Lpl*) gene, which controls a rate limiting step in lipoprotein metabolism, was upregulated by ADF more robustly in Crtc2-transduced legs than in control legs, suggesting an improved post prandial metabolism of triglyceride-rich lipid particles. *Sirt4*, a mitochondrial sirtuin that regulates the rate of fatty acid oxidation in muscle, was also substantially suppressed during fasting but significantly less so in the Crtc2-overexpressing legs which may favor the higher lipid accumulation that we previously described in muscle overexpressing Crtc2 (3).

(**B–C**) Histological analysis of succinate dehydrogenase in myofibers from gastrocnemius muscle sections of WT and Crtc2 mice after Dox treatment. Boxes highlight areas of lighter and darker staining **B**) Scale bar = 2 mm. **C**) Scale bar = 100 μm. Close up from boxed areas in **B**)



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Pathway name	# Entities found	# Entities total	FDR
Extracellular matrix organization	45	329	3.98E-14
Assembly of collagen fibrils and other multimeric structures	17	67	2.12E-14
MET promotes cell motility	10	45	5.98E-07
Elastic fibre formation	12	46	6.13E-11
Regulation of Insulin-like Growth Factor (IGF) transport and uptake	13	127	2.47E-05
Neutrophil degranulation	26	480	2.35E-05
Immune System	79	2823	7.75E-06
Signaling by Receptor Tyrosine Kinases	23	622	7.13E-04
Signaling by Interleukins	25	639	0.049

Fig. S5. Glucose tolerance test and plasma feeding hormones in Crtc2 mice fed ad libitum.

(**A**) Expression profiles of genes that regulate carbohydrate metabolism in mice described in **Figure 2**. These included genes involved in glycogen metabolic processes, including synthesis (*Gys1*, *Fbp2*, *Ugp2*, *Epm2a*, and *Ppp1cb*), branching (*Gbe1*), and breakdown (*Gaa*, *Agl*, *Gsk3b*) of glycogen, as well as insulin signaling genes, *Irs2*, *Sorbs1,* and *Insr.* Genes necessary for glycolysis and fructose metabolism were suppressed by ADF, including *Pfkfb1, Pfkfb2*, and *Fuk*. *Ogt*, an important inducer of insulin resistance in skeletal muscle via the inhibition of AKT phosphorylation was also downregulated.

(**B**) Insulin and glucose tolerance tests (ITT and GTT) on WT (solid line) and Crtc2 (dashed line) mice pre-doxycycline treatment

(**C–D**) GTT on WT and Crtc2 transgenic animals treated with dox for 1 week. Mice were fasted for 16 hrs. before i.p. injection of 20% glucose. n =10 mice per group.

(D) Area under the curve (AUC) above baseline glucose for each experimental group. Data are shown as mean + SEM.

(**E–F**) Analysis of plasma proteins regulating feeding and triglycerides and cholesterol in Crtc2 expressing and WT mice.

(**G**) Reactome.org analysis of enriched pathways from a list of 171 putative secreted proteins that were differentially expressed in Crtc2 or WT mice after ADF as described in **Figure 2**.

References SI

1. C. Aguer *et al.*, Muscle uncoupling protein 3 overexpression mimics endurance training and reduces circulating biomarkers of incomplete beta-oxidation. *FASEB J* **27**, 4213-4225 (2013).
2. S. Zhang, M. W. Hulver, R. P. McMillan, M. A. Cline, E. R. Gilbert, The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility. *Nutr Metab (Lond)* **11**, 10 (2014).
3. N. E. Bruno *et al.*, Creb coactivators direct anabolic responses and enhance performance of skeletal muscle. *The EMBO journal* **33**, 1027-1043 (2014).