

Creb coactivators direct anabolic responses and enhance performance of skeletal muscle

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Abstract

During the stress response to intense exercise, the sympathetic nervous system (SNS) induces rapid catabolism of energy reserves through the release of catecholamines and subsequent activation of protein kinase A (PKA). Paradoxically, chronic administration of sympathomimetic drugs (β -agonists) leads to anabolic adaptations in skeletal muscle, suggesting that sympathetic outflow also regulates myofiber remodeling. Here, we show that β -agonists or catecholamines released during intense exercise induce Creb-mediated transcriptional programs through activation of its obligate coactivators Crtc2 and Crtc3. In contrast to the catabolic activity normally associated with SNS function, activation of the Crtc/Creb transcriptional complex by conditional overexpression of Crtc2 in the skeletal muscle of transgenic mice fostered an anabolic state of energy and protein balance. Crtc2-overexpressing mice have increased myofiber cross-sectional area, greater intramuscular triglycerides and glycogen content. Moreover, maximal exercise capacity was enhanced after induction of Crtc2 expression in transgenic mice. Collectively these findings demonstrate that the SNS-adrenergic signaling cascade coordinates a transient catabolic stress response during high-intensity exercise, which is followed by transcriptional reprogramming that directs anabolic changes for recovery and that augments subsequent exercise performance.

Keywords cAMP-response element-binding protein; Creb-regulated transcriptional coregulators; hypertrophy; skeletal muscle; sympathetic nervous system

Subject Categories Metabolism; Physiology

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Introduction

The sympathetic nervous system (SNS) controls the “fight or flight” response by releasing energy stores and enhancing cardiovascular dynamics during high-intensity exercise (HIE). Catecholamines

released by the SNS bind and activate β -adrenergic receptors (BARs), initiating a series of molecular events that increase intracellular cAMP, which in turn liberates the catalytic subunit of protein kinase A (PKA) to induce rapid signaling pathways (Altarejos & Montminy, 2011). In skeletal muscle, PKA initiates catabolism of glycogen and intramuscular triglyceride (IMTG) energy stores in the sarcoplasm by phosphorylating of the regulatory enzymes phosphorylase kinase and hormone-sensitive lipase (Chesley *et al.*, 1996; Jocken & Blaak, 2008). These SNS-mediated catabolic effects are rapid and transient mechanisms for countering energetic stress.

β -Adrenergic receptor agonists (β -agonists) are used clinically as sympathomimetic drugs and in research as tool compounds to determine how skeletal muscle responds to sympathetic activation. When used acutely, β -agonists parallel the ability of catecholamines to induce catabolism of energy reserves in skeletal muscle; however, prolonged administration of the same compounds induces an anabolic state and cause muscle hypertrophy (Choo *et al.*, 1992; Mersmann, 1998; Ryall *et al.*, 2007). It remains unclear whether the anabolic properties of β -agonists are only a pharmacological effect or whether these observations suggest that sympathetic outflow also directs skeletal muscle anabolism.

Recently studies have suggested that the transcriptional arm of β -adrenergic signaling has roles in regulating muscle hypertrophy. First, exercise or β -agonists induce expression of multiple *Pgc-1 α* (*Ppargc1a*) splice variants in both skeletal muscle and cultured myocytes via activation of an alternative promoter located ~13 kb upstream of the proximal promoter (Baar *et al.*, 2002; Miura *et al.*, 2008; Norrbom *et al.*, 2011; Ruas *et al.*, 2012). Second, muscle-specific overexpression of one of the isoforms, *Pgc-1 α 4*, causes muscle hypertrophy in mice and primary myotubes, where silencing *Pgc-1 α 4* attenuates clenbuterol-mediated hypertrophy of primary myotubes (Ruas *et al.*, 2012). Third, mice having loss of BAR signaling via muscle-specific $G\alpha_{(s)}$ deficiency have decreased total muscle mass and attenuated expression of total *Pgc-1 α* (Chen *et al.*, 2009). Finally, genetic ablation of all BARs in mice also attenuates *Pgc-1 α* expression (Bachman *et al.*, 2002). Thus, transcriptional responses to β -adrenergic signaling and exercise promote muscle hypertrophy, but the mechanism of transcriptional control is unknown.

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β -Agonists such as clenbuterol or isoproterenol direct gene expression via the activation of the cAMP-response-element-binding protein (Creb) transcription factor and associated co-regulators, Creb-regulated transcriptional coregulators (Crtc) and Creb-binding protein (CBP) or its paralog p300 [reviewed in (Altarejos & Montminy, 2011)]. Disrupting assembly of the Creb transcriptional complex in skeletal muscle, by forced overexpression of a dominant-negative Creb molecule (A-Creb) that prevents Creb DNA binding, causes a severe dystrophic phenotype (Ahn *et al*, 1998; Berdeaux *et al*, 2007). However, neither Crtc coactivators nor Creb have been shown mediate hypertrophic responses by β -agonists or exercise.

Herein, we report that Crtc/Creb-directed transcription is initiated by β -agonists and in response to catecholamines released during exercise. Further calcium and β -adrenergic signaling is revealed to converge on the Crtc/Creb complex to increase their transcriptional output, providing a novel mechanism that integrates the level of cardiodynamic stress with the intensity of muscle contraction in working muscle. Moreover, overexpression of Crtc2 or Crtc3 coactivators alone is shown to be sufficient to activate Creb-dependent transcription without β -adrenergic signaling. Remarkably, conditional expression of Crtc2 coactivators in the striated muscle of transgenic mice or in primary myocytes is sufficient to induce anabolic changes that drive myofiber hypertrophy, including increases in the levels of intramuscular glycogen and triglycerides. In contrast, acute PKA activation by β -agonists or HIE increases catabolism and depletes intramuscular glycogen stores in Crtc-expressing myotubes and mice. Collectively, these findings establish that the SNS governs anabolism in skeletal muscle as a mechanism of recovery and adaptation via the Crtc/Creb transcriptional complex.

Results

Enforced expression of Crtc is sufficient to activate Creb signaling

There are three mammalian Creb-regulated transcriptional coregulators – Crtc1, Crtc2 and Crtc3 – that have unique and sometimes overlapping expression patterns. The β -agonists isoproterenol (Iso) triggered the dephosphorylation and rapid translocation of Crtc2 and Crtc3 to the nucleus in cultured primary myotubes and C₂C₁₂ myotubes, while Crtc1 was not detected by immunoblotting (Fig 1A–B). Absolute quantitation of transcripts of each coregulator in gastrocnemius, quadriceps and C₂C₁₂ myotubes confirmed that only *Crtc2* and *Crtc3* are expressed in skeletal myocytes (Supplementary Fig S1). Isoproterenol stimulation also increased phospho-Creb and phospho-Atf1 in a temporal manner, hallmarks of transcriptional activation (Fig 1A; Gonzalez & Montminy, 1989).

Previous studies in other cell types have shown that overexpression of the Crtc coactivators activates Creb-dependent transcription independent of PKA signaling (Conkright *et al*, 2003a; Sreaton *et al*, 2004). This occurs by overwhelming the regulatory mechanisms that retain the coactivators in the cytoplasm, where nuclear localization is sufficient for transcriptional activation (Supplementary Fig S2) (Sreaton *et al*, 2004). We used a luciferase

reporter gene regulated by cAMP response elements (CRE) and having a minimal TATA core promoter element to assess the effects of clenbuterol or isoproterenol on Creb transcriptional activity in primary myocytes, with or without Crtc2 and Crtc3 overexpression. Overexpression of either Crtc2 or Crtc3 increased luciferase activity to levels seen with either β -agonist (Fig 1C). Further, propranolol, a β -adrenergic antagonist, abolished the action of the β -agonists yet did not block downstream Crtc2- or Crtc3-mediated transcription (Fig 1C). Crtc2- or Crtc3-mediated transcription was Creb dependent, as the effects of Crtcs were attenuated by co-expression of the dominant-negative A-Creb molecule (Fig 1C). Finally, primary myocytes stimulated with isoproterenol or transduced with adenovirus expressing Crtc2 or Crtc3 increased the relative number of transcriptions for the canonical Crtc/Creb-responsive genes *Nr4a1*, *Nr4a3* and *Sik1* (Conkright *et al*, 2003a; Sreaton *et al*, 2004; Kanzleiter *et al*, 2010; Pearen *et al*, 2012), similar to those found following stimulation with isoproterenol (Fig 1D). Thus, β -agonists activate the Crtc/Creb transcriptional complex and overexpression of Crtc2 or Crtc3 mimics adrenergic-dependent transcriptional activation without receptor activation.

The sympathetic nervous system activates the Crtc/Creb transcriptional complex

As β -agonists activated the Crtc/Creb transcriptional complex, we next examined whether a physiological stimulus would parallel pharmacological activation. Catecholamines released by the SNS in response to HIE bind and activate BARs. As expected, mice exercised to exhaustion had significantly higher levels of epinephrine and norepinephrine in the quadriceps muscles and plasma spillover relative to rested controls, along with a commensurate increase in intracellular levels of cAMP (Fig 2A–B). HIE provoked increases in the levels of phospho-Creb and phospho-Atf1, while causing the dephosphorylation of Crtc2 and Crtc3, 30 min post-exercise (Fig 2C), and led to increases in the mRNAs of the canonical Crtc/Creb-responsive genes *Nr4a1*, *Nr4a3*, *Ppargc1a* (*Pgc1- α*) and *Sik1* (Herzig *et al*, 2001; Conkright *et al*, 2003a; Sreaton *et al*, 2004; Kanzleiter *et al*, 2010; Pearen *et al*, 2012) in the quadriceps muscle (Fig 2D–E). An extended kinetic analysis showed that maximal induction of these transcripts occurred between 1 and 2 h post-exercise and that transcript levels returned to resting levels after 4–8 h (Supplementary Fig S3A). Dephosphorylation and redistribution of Crtc2 followed a similar temporal pattern (Supplementary Fig S3B and C). Thus, in response to a single bout of intense exercise, the Crtc/Creb transcriptional complex activates skeletal muscle target genes in a prototypical fashion, where a burst phase is followed by an attenuation of transcription (Michael *et al*, 2000; Mayr & Montminy, 2001).

If the Crtc/Creb transcriptional complex is indeed activated during HIE by the SNS/adrenergic signaling pathway, then administration of β -blockers should block the transcriptional activation of Crtc/Creb-dependent genes. Indeed, expression of *Pgc1- α* and *Sik1* was also markedly induced in the tibialis anterior muscle with exercise, and this response was suppressed by the administration of the β -blocker propranolol (Fig 2E). Notably, electroporation of a Crtc3 expression construct directly into the tibialis anterior muscle was sufficient to override these effects of propranolol

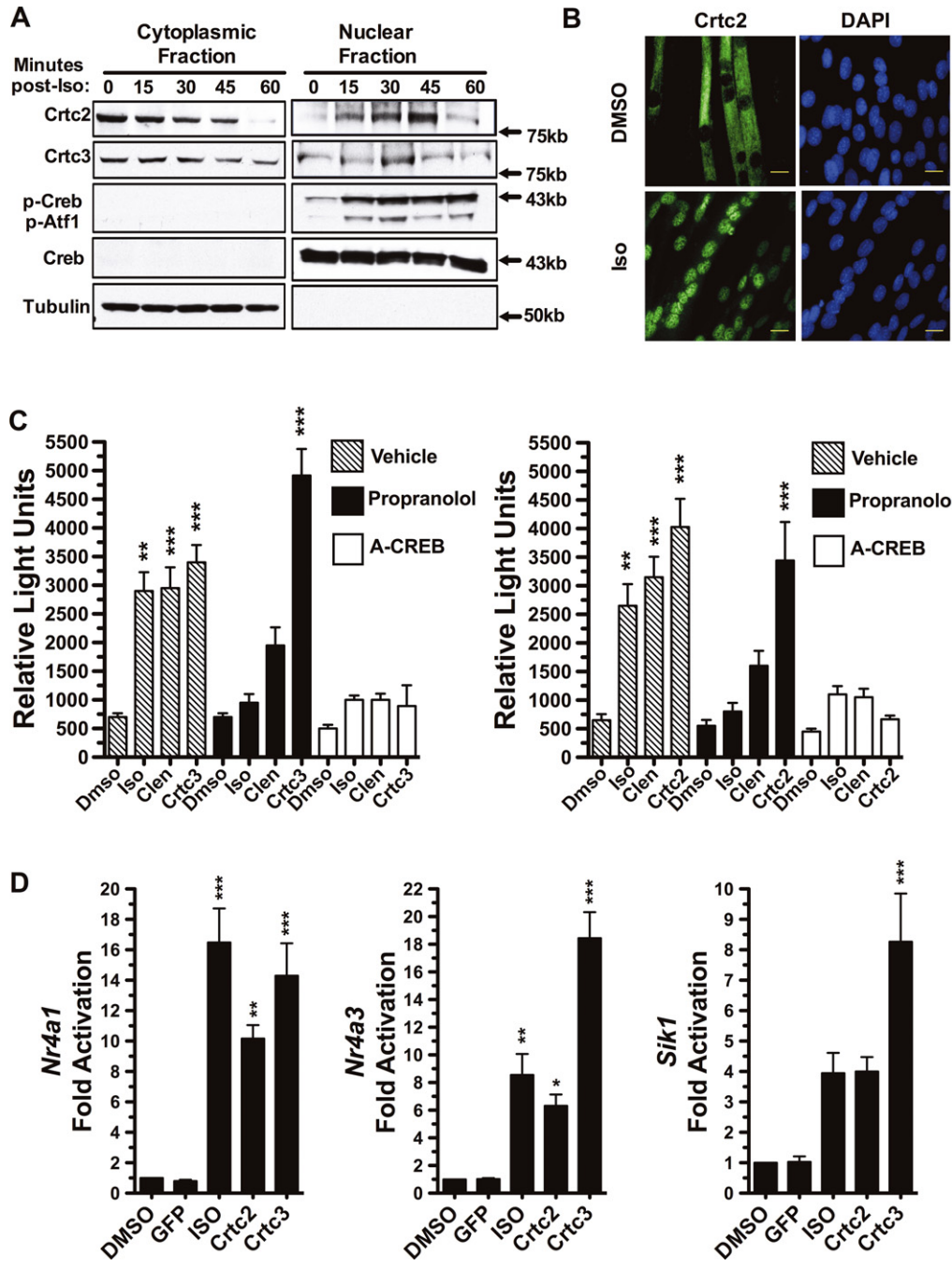


Figure 1. Crtcs are activated by β -agonists and mimic adrenergic-dependent transcription in skeletal myocytes when overexpressed.

A Effect of β -agonist on Crtc2 and Crtc3 subcellular localization. Immunoblots of Crtc2, Crtc3, phospho-Creb/Atf1, total Creb or tubulin from cytoplasmic and nuclear extracts of cultured primary myotubes treated with 100 nM isoproterenol (Iso) for 0, 15, 30, 45 or 60 min.

B Immunofluorescence of endogenous Crtc2 (green) and nuclear staining with DAPI (DNA, blue) in murine C2C12 myotubes treated with vehicle (DMSO) or 100 nM Iso for 15 min. Scale bar: 20 μ m.

C Transient transfection assays in primary mouse myocytes with a CRE-luciferase reporter gene and an expression vector for Crtc2, Crtc3 alone or with A-Creb. Myocytes were also stimulated with isoproterenol, clenbuterol (clen) or DMSO for 4 h prior to luciferase assay as indicated. Propranolol was incubated 30 min prior to stimulation, each bar is the mean \pm s.e.m. of 4 wells.

D Relative fold induction of Nr4a1, Nr4a3, Irs2 and Sirk1 RNA transcripts by rtPCR from primary myocytes treated with DMSO, 100 nM isoproterenol (Iso), transfected with control adenovirus or virus expressing either Crtc2 or Crtc3. Values were normalized to Rpl-23. Each bar is the mean \pm s.e.m. of 4 wells.

Data information: One-way ANOVA was conducted and *P*-values were calculated with Tukey's *post hoc* test and are represented by **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 versus DMSO.

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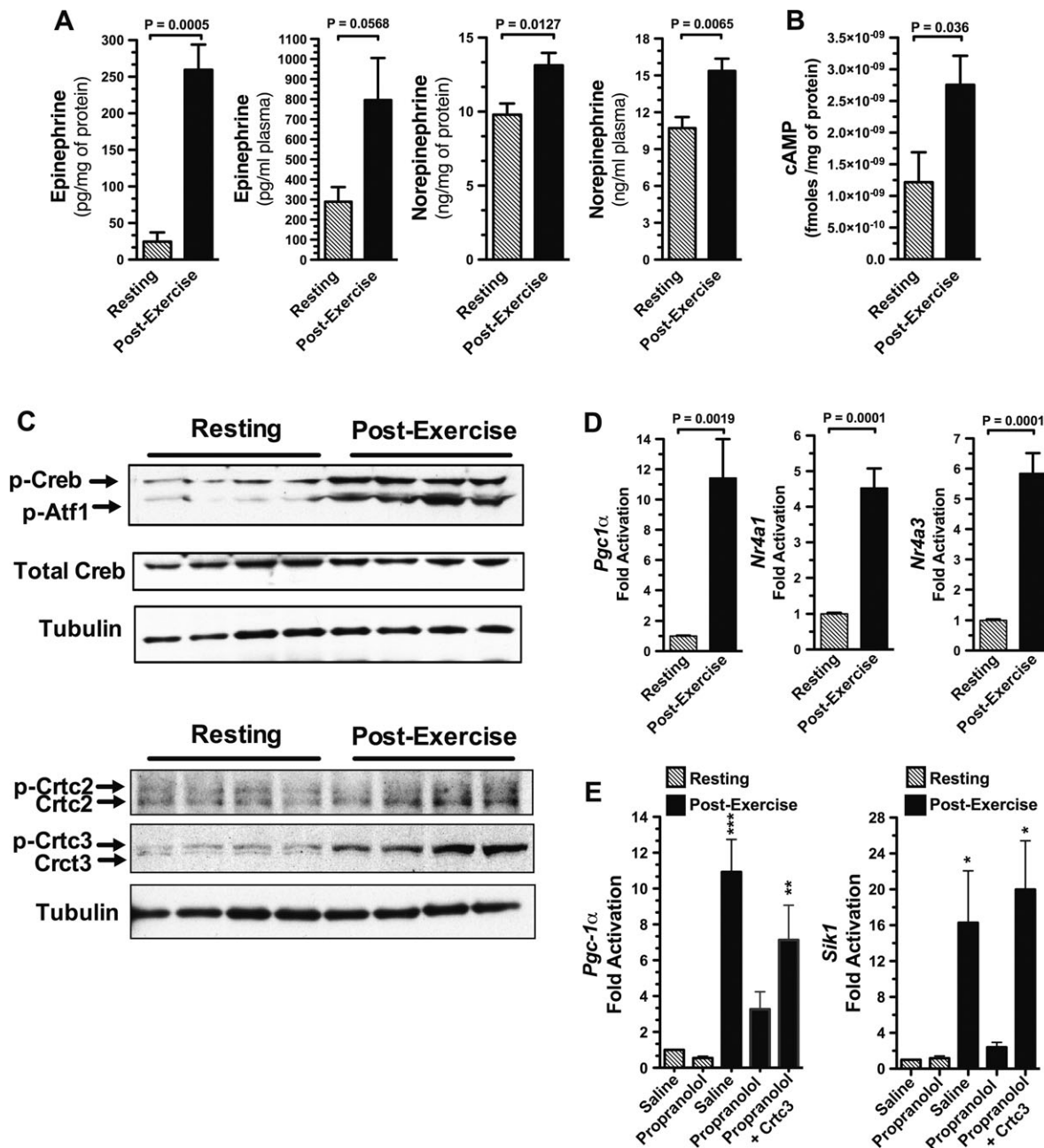


Figure 2. High intensity exercise activates Creb and the Crtc coactivators.

A Levels of norepinephrine and epinephrine from plasma or in quadriceps muscle normalized to total protein from mice either at rest or 30 min post-exercise. Each bar is mean ± s.e.m., *n* = 4 male mice at 8 weeks of age. *P*-value calculated by a two-tailed unpaired *t*-test.

B Levels of cAMP in quadriceps muscle. Values were normalized to total protein in the indicated animals rested or 30 min post-exercise. Each bar is mean ± s.e.m., *n* = 4 male mice at 8 weeks of age. *P*-value calculated by a two-tailed unpaired *t*-test.

C Immunoblots showing levels of phospho-Cre/Atf1, Creb, Crtc2, Crtc3 and tubulin from animal rested or 30 min post-exercise.

D Fold induction of *Pgc-1α*, *Nr4a1* and *Nr4a3* transcripts in quadriceps muscle from the indicated animals after 30 min of intense exercise. Transcript levels were measured by qRT-PCR, and values were normalized to *Rpl-23* mRNA and expressed relative to those present in resting control animals; each bar is mean ± s.e.m. *n* = 4 male mice at 8 weeks of age. *P*-values calculated by a two-tailed unpaired *t*-test.

E Crtc3 overrides the suppression of gene expression by the adrenergic antagonist propranolol in exercised animals. Fold induction of *Pgc-1α* and *SIK1* mRNAs was determined by qRT-PCR from the tibialis anterior (TA) muscle. Crtc3 expression was induced by electroporation of the Crtc3 plasmid into the tibialis anterior muscle. Propranolol or saline were injected i.p. 30 min before exercise. Values were normalized to levels of *Rpl-23* transcripts and are expressed relative to those in control (saline/resting) TA muscle; each bar is the mean ± s.e.m. of 4 mice. One-way ANOVA was conducted and *P*-values were calculated with Tukey's *post hoc* test and are represented by **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 versus saline.

Source data are available online for this figure.

(Fig 2E). Thus, enforced Crtc expression circumvents upstream BAR signaling *in vivo*, and SNS release of catecholamines in response to the stress of exercise initiates Crtc/Creb-mediated gene expression.

The Crtc coactivators integrate SNS/adrenergic and cholinergic signaling

The systemic action of catecholamines that is manifest during exercise suggests that the SNS lacks the specificity needed to direct adaptive changes only to the worked muscle (Lambert *et al*, 2010; Roatta & Farina, 2010). However, Crtc2 functions as a coincidence regulator of cAMP and Ca²⁺ signaling pathways in many cell types (Screaton *et al*, 2004; Baxter *et al*, 2011; Ch'ng *et al*, 2012), and during HIE, cholinergic signaling is specific to the worked muscle. Here, acetylcholine depolarizes the membrane of myocytes and liberates calcium (Ca²⁺) stores from the endoplasmic reticulum, which can be mimicked by treatment with KCl (Macías *et al*, 2001). Thus, we tested whether calcium and cAMP signaling pathways converged on Crtc-dependent gene transcription by co-treating primary myocytes with isoproterenol and KCl. Isoproterenol activated Crtc/Creb-dependent expression of Nr4a1 and Nr4a3, and this response was markedly augmented by co-treatment with KCl (Fig 3A). Moreover, Crtc2 or Crtc3 overexpression was sufficient to induce Nr4a1 and Nr4a3 to levels induced by co-stimulation of primary myocytes with isoproterenol and KCl (Fig 3B). The dependency of Creb and the Crtc coactivators was illustrated by attenuating the transcriptional response to co-stimulation with Iso and KCl by the co-expression of A-Creb (Supplementary Fig S4). Further, increasing intracellular Ca²⁺ augmented the duration of beta-agonist-dependent phosphorylation of Creb and dephosphorylation of Crtc2 (Fig 3C), which lengthens the duration of target gene activation, prolonging the burst phase of Creb-mediated transcription (Michael *et al*, 2000; Mayr & Montminy, 2001). Thus, the two pathways that are simultaneously activated by HIE, the cholinergic and adrenergic-dependent signaling pathways, are integrated by Crtc coactivators in working muscle, suggesting that Crtc coactivators may function as physiological drivers of the adaptive response to exercise.

The Crtc coactivators induce skeletal myofiber hypertrophy

We reasoned that conditional expression of the CRTC coactivators in transgenic mice could be used as a genetic tool to separate the rapid non-genomic effects of SNS/adrenergic signaling from the later transcriptional axis in a tissue-specific manner. To this end, we generated double transgenic mice (DTg) that inducibly express a constitutively active form of Crtc2, Crtc2tm, in muscle when administered doxycycline (Dox). The TRE-Crtc2tm transgenic mice contain an ORF with three point mutations (S171A, S275A and K625A) that increase the activity and stability of Crtc2 (Screaton *et al*, 2004; Dentin *et al*, 2007; Jansson *et al*, 2008), under the control of tetracycline-responsive elements (TRE). Skeletal muscle specificity was conferred when this transgenic line was crossed with second mouse that harbors a transgene for the reverse tetracycline transactivator (rtTA) regulated by the 1256[3Emut] MCK promoter (Grill *et al*, 2003).

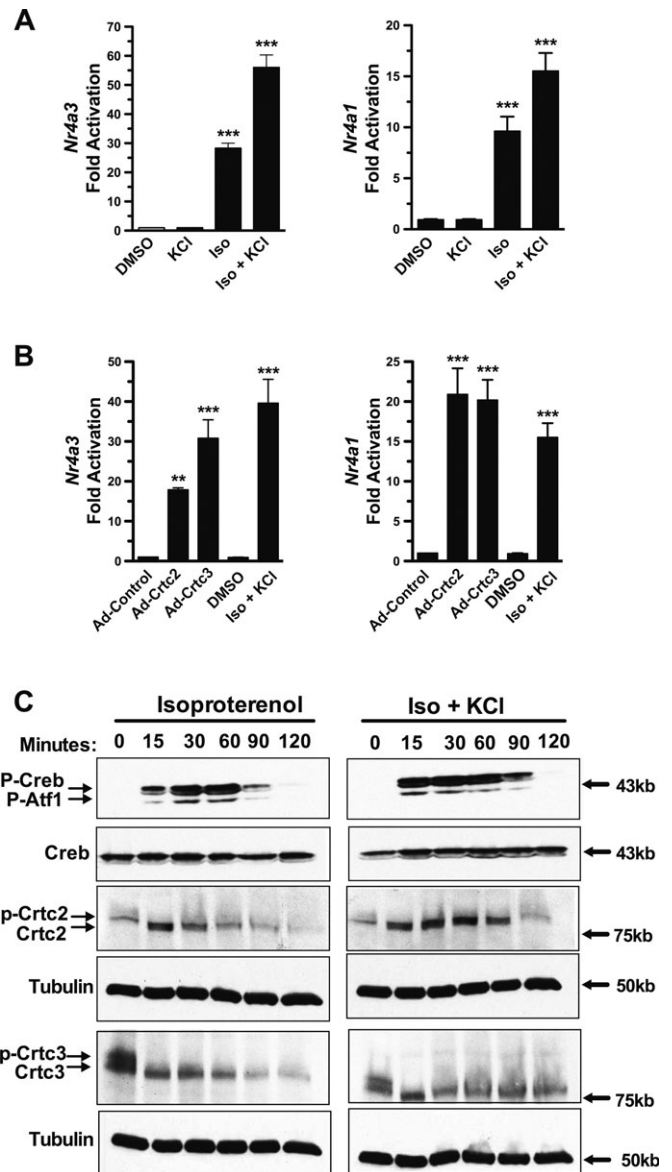


Figure 3. Adrenergic and cholinergic signaling pathways converge on the Crtc coactivators to potentiate target gene transcription.

A Fold induction of Nr4a1 and Nr4a3 RNA transcripts, quantified by qRT-PCR, in primary myotubes treated with 40 mM KCl, isoproterenol (Iso), Iso plus KCl or vehicle (DMSO) for 45 min. Values were normalized to those of Rpl-23 mRNA and were expressed relative to DMSO control; each bar is the mean \pm s.e.m. of 4 wells. One-way ANOVA was conducted and *P*-values were calculated with Tukey's *post hoc* test and are represented by ****P* < 0.001 versus vehicle (DMSO).

B Fold induction of Nr4a1 and Nr4a3 transcripts, quantified by qRT-PCR, in primary myotubes treated with 40 mM KCl plus Iso for 45 min, and in myotubes transduced with adenovirus expressing GFP, Crtc2 or Crtc3 for 24 h. One-way ANOVA was conducted, and *P*-values were calculated with Tukey's *post hoc* test and are represented by ***P* < 0.01 and ****P* < 0.001 versus Ad-Control.

C Immunoblot analyses of levels of phospho-Creb, total Creb, Crtc2, Crtc3 and tubulin in total lysates of primary myotubes stimulated for 0, 15, 30, 60, 90 or 120 min with either 100 nM Isoproterenol or isoproterenol and 40 mM KCl.

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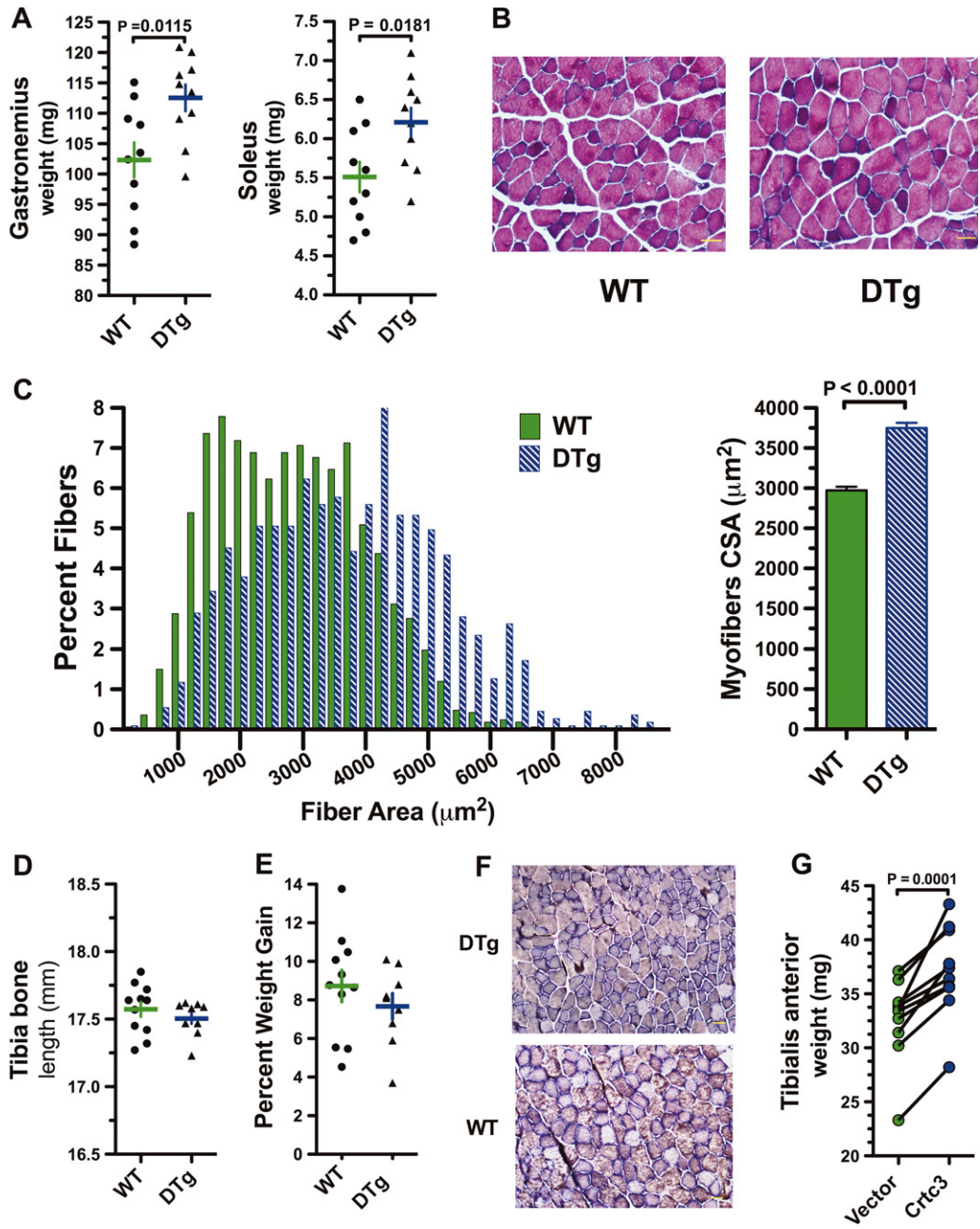


Figure 4. Activation of the Crtc coactivators induces skeletal myofiber hypertrophy.

A Gastrocnemius and soleus muscle weight in wild-type (WT) and double transgenic (DTg) mice 36 days post-Dox administration. Data are represented as a scatter plot with the mean \pm s.e.m. indicated, $n = 10$ mice. P -values calculated by a two-tailed unpaired t -test.

B Histological analysis of myofibers from gastrocnemius muscle sections with hematoxylin and eosin (H & E) staining. Scale bar: 100 μ m.

C Distribution of cross-sectional areas (CSAs) of WT and DTg mice after Dox (left panel) and mean CSAs of muscle fibers (right panel). Data are represented as mean \pm s.e.m. P -values calculated by a two-tailed unpaired t -test.

D Tibia bone length of WT and DTg mice after dox. Data are represented as a scatter plot with the mean \pm s.e.m. indicated, $n = 10$ per group.

E Percent weight gain of WT and DTg mice after Dox. Data are represented as a scatter plot with the mean \pm s.e.m. indicated, $n = 10$ per group.

F Histological analysis of myofibers from gastrocnemius muscle sections by myosin ATPase staining. Scale bar: 500 μ m.

G Wet weight of TA muscles after 10 days of Crtc3 expression. The muscle was electroporated with either empty vector or with a Crtc3 expression plasmid. Each line represent the difference in mass relative to the contralateral muscle of each animal, $n = 10$ per group. P -values calculated by a two-tailed paired t -test.

Doxycycline (Dox) administration to DTg mice induced *Crtc2* transgene expression two- to fivefold relative to endogenous levels of *Crtc2* mRNA expressed in striated, but not cardiac muscle

(Supplementary Fig S5A). Increased accumulation of FLAG-Crtc2 was also evident after 3–7 days of Dox administration of transgenic mice (Supplementary Fig S5B and C). In striated muscle, Crtc2

overexpression led to significant increases in the mass of the gastrocnemius and soleus muscle relative to age- and weight-matched Dox-treated wild-type (WT) littermates (Fig 4A). Quantitative analysis of fiber size in histological sections of gastrocnemius muscle confirmed increased average cross-sectional area (CSA) of the fibers (Fig 4B–C). The skeletal structure was similar between experimental groups as indicated by the similar tibia bone length and body weights (Fig 4D–E).

Fiber type transitions occur primarily during development and only to a limited extent in response to chronic training (Simoneau et al, 1985; d’Albis et al, 1989; Lynch et al, 1995; Fry, 2004); however, several studies have reported that extended administration of β -agonists fosters a slow-to-fast muscle fiber transition (Maltin et al, 1986; Agbenyega & Wareham, 1990; Hayes & Williams, 1994). However, an alteration in the ratio of fiber types was not evident by myosin ATPase staining of gastrocnemius muscle of DTg mice treated with Dox (Fig 4F). We therefore tested whether forced overexpression of Crtc3 would also produce fiber hypertrophy using a second approach, electric-pulse-mediated gene transfer. Notably, electric transfer of a Crtc3 expression vector provoked marked increases in wet muscle weight or the tibialis anterior muscle compared with muscle pulsed with a control vector (Fig 4G). Thus, modest overexpression of either Crtc2 or Crtc3 drives hypertrophic phenotypes similar to those seen with administration of β -agonists, but without receptor activation (Shi et al, 2007; Koopman et al, 2010).

Crtc/Creb coordinates signaling circuits that drive muscle cell anabolism and hypertrophy

To investigate the mechanisms by which activation of the Crtc/Creb complex induces fiber hypertrophy, mouse primary myotubes were

transduced with adenovirus expressing either Crtc2 or Crtc3. As predicted, forced overexpression of Crtc2 or Crtc3 also provoked hypertrophy in cultured myotubes (Fig 5A and Supplementary Fig S6A), including elevated levels of MHC protein (Fig 5B). Myotubes expressing Crtc3 typically had a greater hypertrophic response, yet Crtc2 adenovirus co-expresses GFP, which only allows examination of transduced myotube and the with use of fixatives that could affect fiber size. Finally, overexpression of either Crtc2 or Crtc3 caused a marked increases in the ratio of total protein to DNA in the myotube, establishing that hypertrophy rather than hyperplasia leads to increased fiber size (Fig 5C).

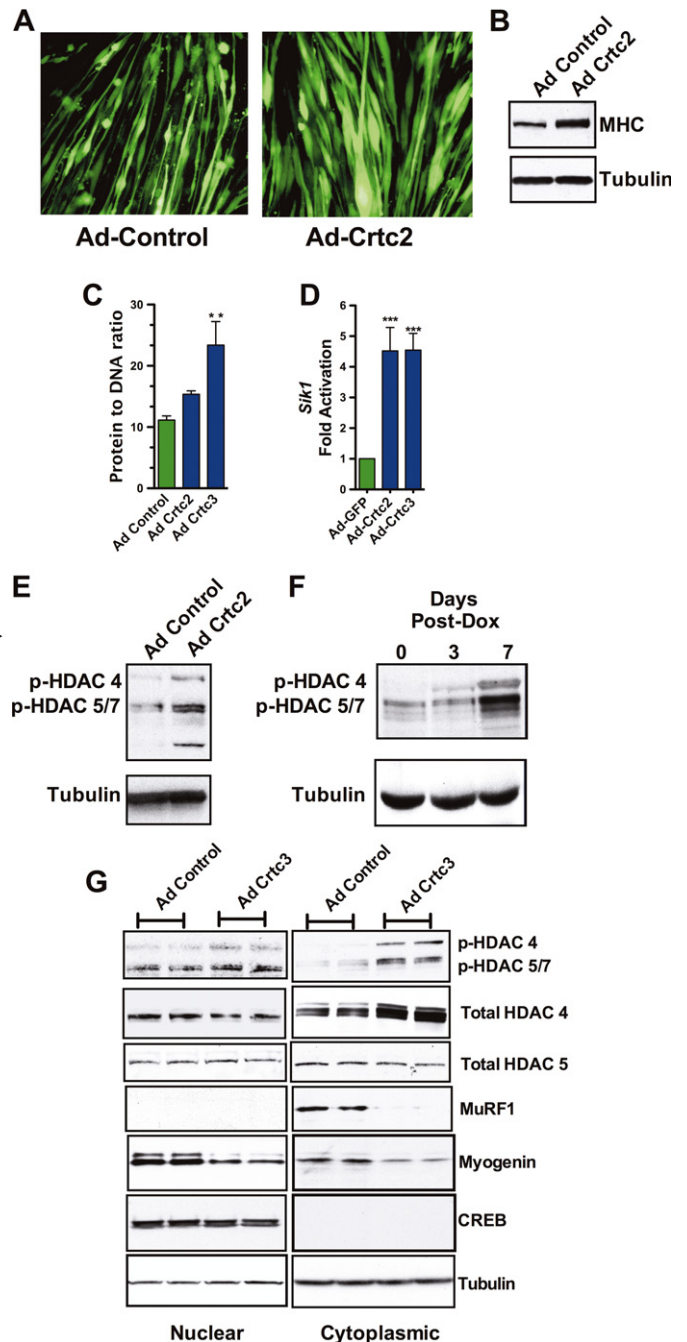


Figure 5. Crtc coactivators induce signaling pathways that regulate protein degradation.

- A Fluorescence microscopy of primary myotubes transduced with adenovirus expressing GFP alone (Ad-Control) or Crtc2 and GFP (Ad-Crtc2).
- B Immunoblots of MHC and tubulin from primary myotubes transduced with adenovirus expressing GFP alone (Ad-Control) or Crtc2 and GFP (Ad-Crtc2).
- C Total protein and DNA were isolated and quantified from control, Crtc2- or Crtc3-expressing myotubes and expressed as total protein divided by DNA. Data are represented as mean \pm s.e.m. ($n = 4$ wells per group). One-way ANOVA was conducted and P -values were calculated with Tukey’s *post hoc* test and are represented by $**P < 0.01$ versus Ad Control.
- D Relative fold induction of Sik1 RNA transcripts by rtPCR from primary myocytes transduced with control adenovirus or adenovirus expressing Crtc2 or Crtc3. Values were normalized to *Rpl-23* mRNA. Each bar is the mean \pm s.e.m. of 4 wells. One-way ANOVA was conducted and P -values were calculated with Tukey’s *post hoc* test and are represented by $***P < 0.001$ versus Ad Control.
- E Immunoblots of phospho-HDAC4, phospho-HDAC 5/7 or tubulin from extracts of primary myotubes transduced with control adenovirus or adenovirus expressing *Crtc2*.
- F Immunoblots of phospho-HDAC4, phospho-HDAC 5/7 or tubulin from extracts of gastrocnemius muscle isolated from double transgenic mice after 0, 3 or 7 days of Dox administration.
- G Immunoblots of phospho-HDAC4, phospho-HDAC 5/7, total HDAC 4, total HDAC5, Murf1, myogenin, Creb or tubulin from nuclear (left) and cytoplasmic (right) extracts of primary myotubes transduced with control adenovirus or adenovirus expressing *Crtc3*. Sample replicates for Ad control and Ad *Crtc3* are shown.

Source data are available online for this figure.

Myotube hypertrophy triggered by forced expression of Crtc2 or Crtc3 was associated with the inhibition of the proteasomal degradation pathway. Specifically, overexpression of either Crtc coactivator in primary myocytes increased expression of *Sik1* (Fig 5D), which phosphorylates and inactivates the class II histone deacetylases HDAC4, HDAC5 and HDAC7 by directing their export into the cytoplasm (Takemori *et al*, 2009). Indeed, expression of Crtc2 and Crtc3 triggered the phosphorylation and cytoplasmic localization of HDAC4, HDAC5 and HDAC7 (Fig 5E and F and Supplementary Fig S6B and C). In skeletal muscle, inactivation of HDACs attenuates protein degradative pathways by indirectly preventing the expression of myogenin and the E3 ubiquitin ligase MuRF1, which are required for the ubiquitylation step in the degradation of muscle protein (McKinsey *et al*, 2000). Suppression of myogenin and MuRF1 was evident by immunoblotting, demonstrating their control by Crtc/Creb signaling network (Fig 5G). The currently accepted model by which β -agonists induce muscle hypertrophy is attributed to non-canonical mechanisms of GPCR signaling that are not dependent of cAMP signaling or Creb-dependent transcription (Lynch & Ryall, 2008). Specifically, β -agonists signal through the $G_{\beta\gamma}$ subunits of the G-protein tetramer and have been proposed to increase PI3 kinase activity and potentiate phosphorylation and activation of Akt1 kinase (Chesley *et al*, 2000; Erbay & Chen, 2001; Schmidt *et al*, 2001; Park & Chen, 2005; Kline *et al*, 2007), which then increases protein synthesis in myofibers by regulating components of the mTOR signaling network (Cuenda & Cohen, 1999; Erbay & Chen, 2001; Sneddon *et al*, 2001; Park & Chen, 2005; Koopman *et al*, 2010). Our data rather indicate that the Crtc/Creb transcriptional complex stimulates muscle growth via Pgc-1 α 4/Igf-1/Akt1 signaling pathway. First, of the four known Pgc-1 α splice variants, muscle-specific overexpression of the Pgc-1 α 4 isoform induced Igf1 expression and hypertrophy in primary myocytes (Ruas *et al*, 2012). Here, enforced expression of Crtc2 or Crtc3 mimics exercise-mediated induction of the Pgc-1 α 4 splice variant (Fig 6A). While full-length Pgc-1 α (Pgc-1 α 1) is regulated by p38 MAP kinase phosphorylation of ATF2 in skeletal muscle (Cao *et al*, 2004; Akimoto *et al*, 2005, 2008), the Pgc-1 α 4 isoform is induced via activation of an alternative promoter located ~13 kb upstream of the proximal promoter. This distal promoter contains putative cAMP-responsive elements (CREs) (Miura *et al*, 2008; Yoshioka *et al*, 2009; Ruas *et al*, 2012) and is regulated by exercise and by β -agonists. We cloned 500-bp fragments of the distal and proximal Pgc-1 α promoters to determine whether Creb and the Crtc coactivators selectively activated either promoter. Only the distal promoter was responsive to isoproterenol stimulation (Fig 6B). Further, chromatin immunoprecipitation (ChIP) analyses of the distal and proximal Pgc-1 α promoters, along with the *Nr4a1* and *Gapdh* promoters as positive and negative controls, respectively, confirmed that endogenous Creb, Crtc2 and Crtc3 are enriched at the distal Pgc-1 α promoter relative to the proximal promoter following treatment of myocytes with isoproterenol (Fig 6C and Supplementary Fig S7A). Overexpression of Crtc2 preferentially increased RNA transcripts originating from the distal promoter (Supplementary Fig S7B and C), consistent with previous reports stimulating myocytes with either the adenylate cyclase agonist, forskolin or clenbuterol (Miura *et al*, 2008; Ruas *et al*, 2012).

Second, as the Pgc-1 α 4 isoform induces *Igf1* expression, we examined the expression of *Igf1*, *Pdpk1* (*Pdk1*) and *Akt1* in primary myocytes and transgenic mice expressing Crtc2. Notably, there were marked increases in *Igf1*, *Pdpk1* and *Akt1* transcripts in primary myocytes overexpressing *Crtc2* or *Crtc3* (Fig 6D) and in the gastrocnemius muscle of Dox-treated double transgenic mice (Fig 6E). Increased protein levels of the Akt1 activating kinase Pdpk1 were evident, yet despite a clear and reproducible induction in Akt1 transcripts, only a very marginal change in Akt1 protein was detected in *Crtc2* or *Crtc3* overexpression (Fig 6F). However, *Crtc3* overexpression triggered an increase in Akt phosphorylation, which is required for activation of Akt kinases (Fig 6G) (Alessi *et al*, 1997). Thus, the Crtc/Creb complex orchestrates muscle hypertrophy by coordinately regulating both the protein degradation and muscle growth pathways.

The Crtc coactivators augment intramuscular energy storage

Repetitive exercise increases energy reserves by promoting the intramuscular storage of both neutral lipids and glycogen. Glycogen content in skeletal muscle is an excellent predictor of exercise capacity, and depletion of glycogen is a primary cause of muscle fatigue (Bergström *et al*, 1967; Green *et al*, 1995). In efforts to spare glycogen catabolism, both endurance exercise and high-intensity exercise lead to increased storage of intramuscular triglycerides (IMTGs) (Stepto *et al*, 2002; Hawley *et al*, 2011). Crtc overexpression was sufficient to provoke this anabolic response in skeletal muscle of mice, and in cultured myotubes. The gastrocnemius muscle of DTg mice contained greater IMTGs relative to wild-type mice following Dox treatment (Fig 7A–B). Diacylglycerol O-acyltransferase 1 (*Dgat1*) catalyzes the committed step in triacylglycerol synthesis, and muscle-specific transgenic mice overexpressing *Dgat1* display increased IMTGs (Liu *et al*, 2007; 2009; Zhang *et al*, 2010). *Dgat1* expression elevated in the gastrocnemius muscle of Dox-treated DTg mice relative to wild-type mice (Fig 7C). Similar to the transgenic mice, myotubes overexpressing *Crtc3* esterified more fatty acids to triglycerides (Fig 7D–E), and there were robust increases in the levels of *Dgat1* mRNA and protein in Crtc2- and Crtc3-expressing myofibers (Fig 7F–G). Accordingly, myotubes ChIP analyses showed recruitment of the Crtc/Creb complex to the *Dgat1* promoter in isoproterenol-stimulated myocytes, comparable to that of canonical Creb/Crtc target such as *Nr4a1* (Fig 7H and Supplementary Fig S8). Thus, Crtc-mediated increases in *Dgat1* are associated with the accumulation of intramuscular triglycerides in Crtc-expressing myofibers.

Exercise performance is dependent on glycogen stores as an energy source, and in the short-term, PKA activation initiates glycogenesis (Galbo *et al*, 1975), as evidenced by the low glycogen content in the muscle of mice that were run to exhaustion (Fig 8A). Glycogen content is not only replenished during rest but can increase to supraphysiological levels prior to training as a mechanism of dampening the energetic stress of future bouts of exercise (Green *et al*, 1995). Notably, glycogen content was dramatically increased in the muscle of DTg mice expressing Crtc2, and in the tibialis anterior (TA) muscle of mice transiently expressing Crtc3 (Fig 8B and Supplementary Fig S9).

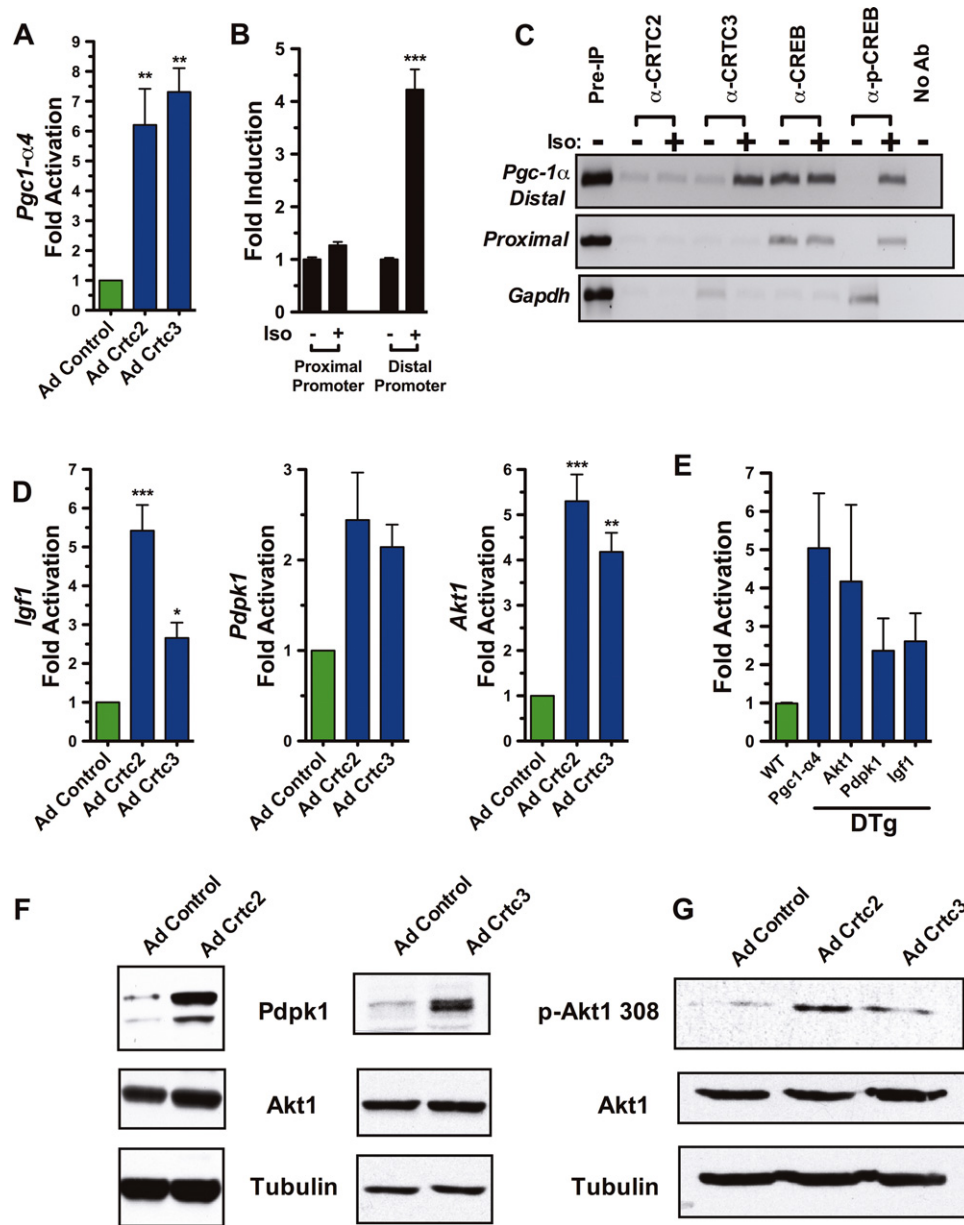


Figure 6. Crtc coactivators induce signaling pathways that regulate protein synthesis.

- A** Relative fold induction of *Pgc1-α4* transcripts by qRT-PCR from primary myocytes transduced with control adenovirus or adenovirus expressing Crtc2 or Crtc3. Values were normalized to *Rpl-23* mRNA. Each bar is the mean \pm s.e.m. of 4 wells. One-way ANOVA was conducted and *P*-values were calculated with Tukey's *post hoc* test and are represented ***P* < 0.01 versus Ad Control.
- B** Transient transfection assays in myocytes with a -51 to +500 *Pgc1α* proximal or distal promoter-luciferase reporter genes. Myocytes were also stimulated with isoproterenol or DMSO vehicle control for 4 h prior to luciferase assay as indicated. Each bar is the mean fold induction \pm s.e.m. of three experiments. One-way ANOVA was conducted and *P*-values were calculated with Tukey's *post hoc* test and are represented by **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 versus Iso.
- C** Chromatin immunoprecipitation (ChIP) of the *Pgc-1α* distal, *Pgc-1α* proximal and *Gapdh* promoters with anti-Creb, anti-Phospho-Creb, anti-Crtc2 or anti-Crtc3 in isoproterenol stimulated mouse myotubes. Myotubes were stimulated with isoproterenol prior to ChIP as indicated. Pre-immunoprecipitation (Pre-IP) control DNA (left) and no antibody control (right).
- D** Relative fold induction of *Pgc1-α4*, *Pdpk1*, *Akt1* and *Igf1* transcripts by qRT-PCR from gastrocnemius muscle isolated from double transgenic mice or wild-type mice after 7 days of Dox administration. Values were normalized to *Rpl-23* mRNA. Each bar is the mean \pm s.e.m. of 4 wells, of two animals. One-way ANOVA was conducted and *P*-values were calculated with Tukey's *post hoc* test and are represented by **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 versus Ad Control.
- E** Relative fold induction of *Pdpk1*, *Akt1* and *Igf1* RNA transcripts by qRT-PCR from primary myocytes transduced with adenovirus control or adenovirus expressing Crtc2 or Crtc3. Values were normalized to *Rpl-23* mRNA. Each bar is the mean \pm s.e.m. of 4 wells.
- F** Immunoblots of Pdpk1, Akt1 and tubulin from primary myotubes expressing Crtc2 or Crtc3.
- G** Immunoblots of phospho-Ser 308 of AKT1/2 and tubulin from myocytes expressing Crtc2 or Crtc3.

Source data are available online for this figure.

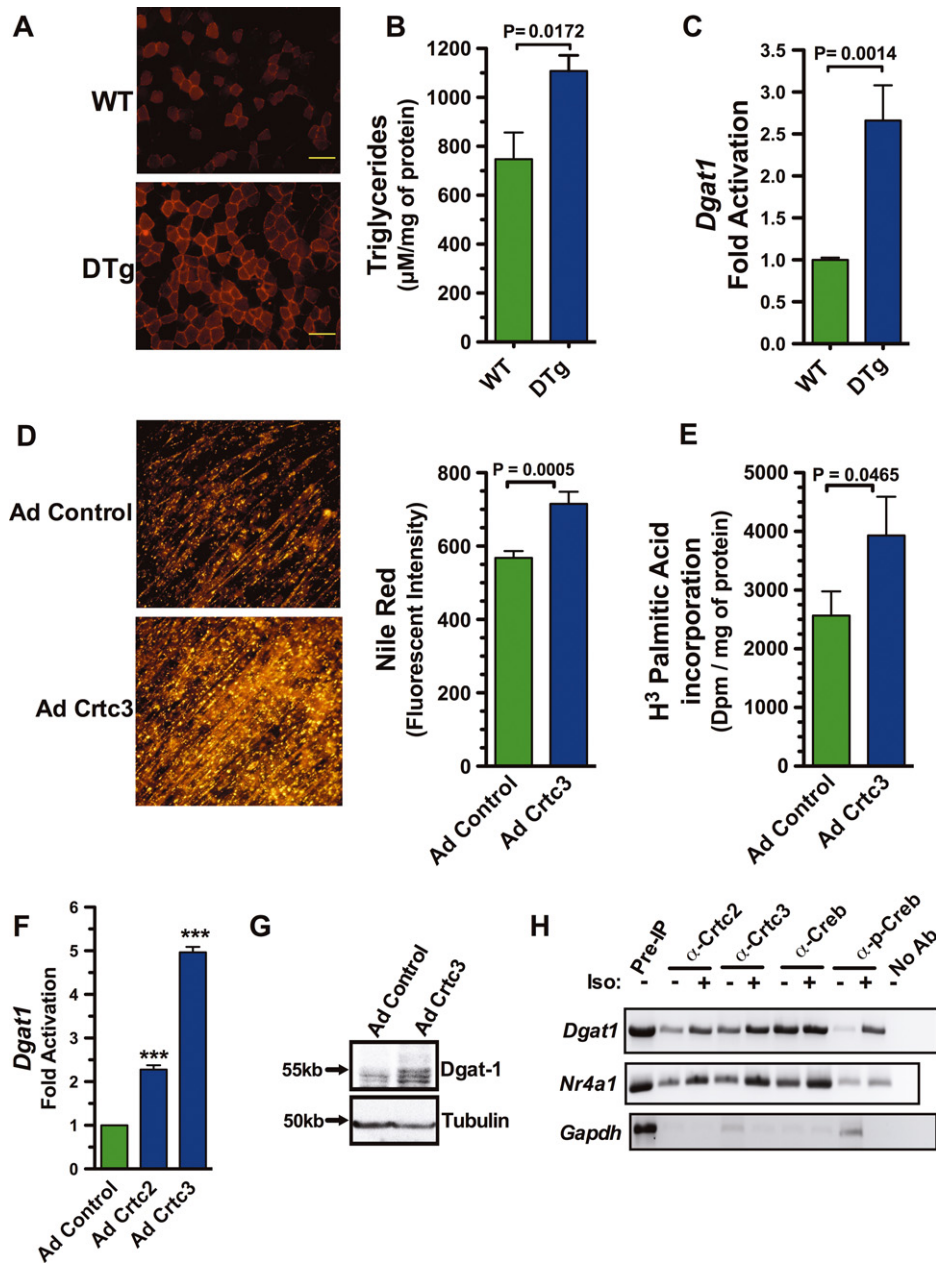


Figure 7. Crtc expression increases intramuscular triglycerides.

A Histological analysis of neutral lipids with Nile Red in muscle sections of WT and DTg mice after Dox treatment. Scale bar: 100 μ m.

B Triglyceride levels in gastrocnemius muscle of WT and DTg mice after dox. Data are represented as a scatter plot with the mean \pm s.e.m. indicated, $n = 6$ per group. P -value calculated by a two-tailed unpaired t -test.

C Relative fold induction of *Dgat1* mRNA by qRT-PCR from gastrocnemius muscle isolated from double-transgenic or WT mice after 7 days of Dox treatment. Values were normalized to *Rpl-23*. Each bar is the mean \pm s.e.m. of 4 wells, of two animals. P -value calculated by a two-tailed unpaired t -test.

D Neutral lipids visualized in primary myotubes expressing Crtc3 by Nile Red (left) and quantified by fluorescent intensity (right). Each bar is the mean \pm s.e.m. of 4 wells. P -value calculated by a two-tailed unpaired t -test.

E Incorporation of 3 H-palmitic acid in primary myotubes expressing Crtc3. Each bar is the mean \pm s.e.m. of 4 wells. P -value calculated by a two-tailed unpaired t -test.

F Relative fold induction of *Dgat1* mRNA, quantified by qRT-PCR analyses of primary skeletal myocytes transduced with adenovirus expressing Crtc2 or Crtc3 versus myocytes transduced with control adenovirus. Values were normalized to levels of *Rpl-23* transcripts. One-way ANOVA was conducted and P -values were calculated with Tukey's *post hoc* test and are represented by $***P < 0.001$ versus Ad Control.

G Immunoblots of Dgat1 and tubulin proteins in myotubes expressing Crtc3.

H Chromatin immunoprecipitation (ChIP) of the *Dgat1*, *Nr4a1* and *Gapdh* promoters using anti-Creb, anti-Phospho-Creb, anti-Crtc2 or anti-Crtc3. Myotubes were stimulated with isoproterenol (+) or DMSO (-) for 45 min prior to ChIP. Pre-immunoprecipitation (Pre-IP) control DNA (left) and no antibody control (right).

Source data are available online for this figure.

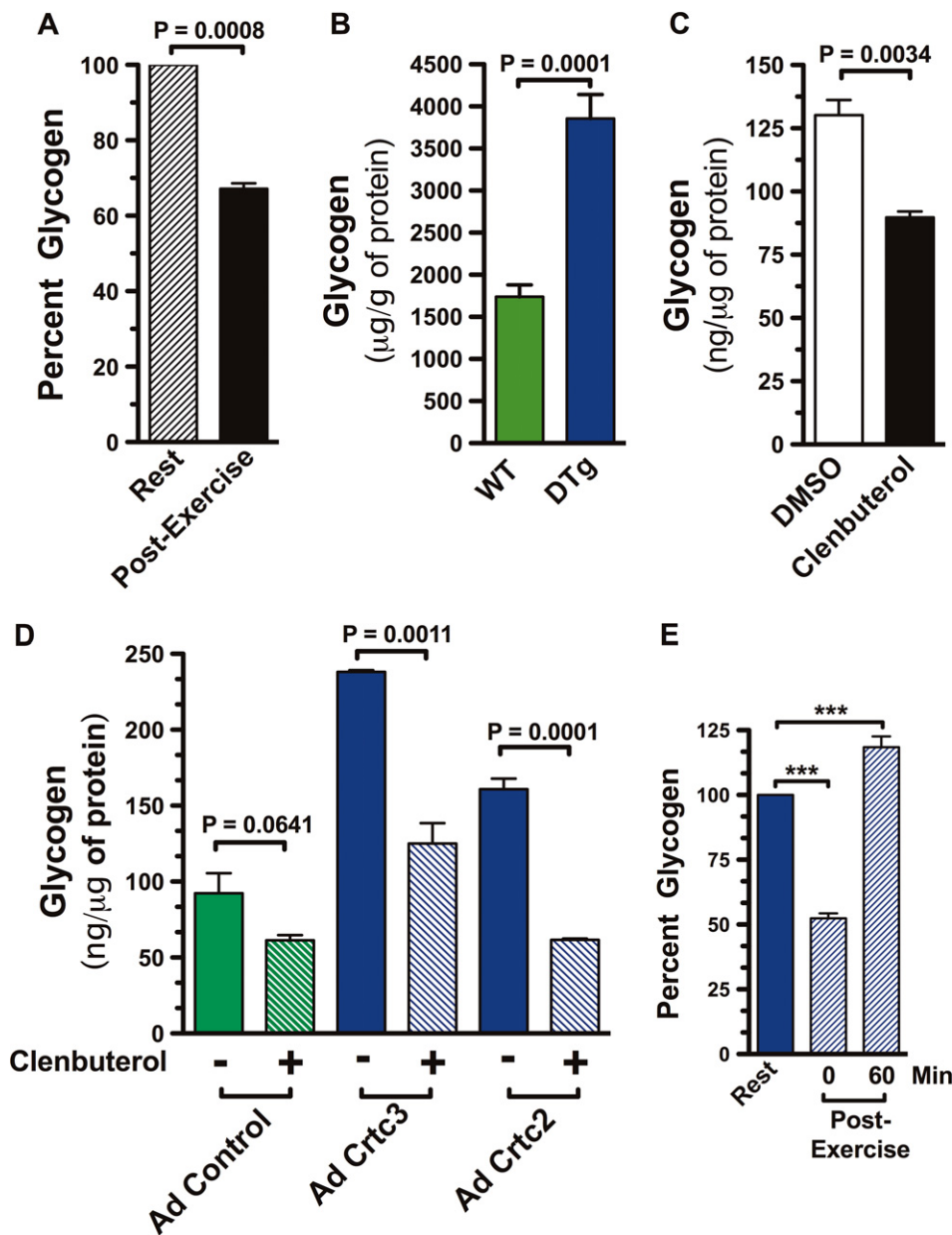


Figure 8. Crtc expression increases available intramuscular glycogen.

- A Percentage of glycogen depleted in quadriceps muscle of mice post-exercise. Glycogen levels were normalized to total protein for each mouse at rest or 30 min after exercise. Each bar is the mean \pm s.e.m. of $n = 4$ male mice at 8 weeks of age. P -value calculated by a two-tailed unpaired t -test.
- B Glycogen levels in the gastrocnemius muscle of WT and DTg mice after Dox treatment. Each bar is the mean \pm s.e.m. of glycogen normalized to total protein of 8 animals. P -value calculated by a two-tailed unpaired t -test.
- C Glycogen levels in primary myocytes after treatment with clenbuterol or vehicle (DMSO) for 30 min. Each bar is the mean glycogen \pm s.e.m. of 4 wells normalized to total protein. P -value calculated by a two-tailed unpaired t -test.
- D Glycogen levels in primary myocytes transduced with adenovirus expressing Crtc2, Crtc3 or GFP control for 48 h and then stimulated with clenbuterol for 30 min. Each bar is the mean glycogen \pm s.e.m. of 4 wells normalized to total protein. P -value calculated by a two-tailed unpaired t -test.
- E Percentage of glycogen depleted in quadriceps muscle of Crtc2-expressing mice post-exercise. Glycogen levels were quantitated and normalized to total protein during rest or immediately after exercise or 60 min post-exercise. Each bar is the mean \pm s.e.m. of $n = 3$ male mice at 10–12 weeks of age. One-way ANOVA was conducted and P -values were calculated with Tukey's *post hoc* test and are represented by *** $P < 0.001$ versus Rest control.

These opposing effects of the SNS/adrenergic signaling cascade suggested a model whereby rapid cAMP/PKA signaling mediates glycogen catabolism followed by the subsequent activation of Crtc/Creb transcriptional program during recovery, which mediates the

adaptive anabolic response. In accord with this newly identified duality in SNS/adrenergic function, the anabolic action of Crtc2 or Crtc3 on glycogen accumulation was reversed after 30 min of clenbuterol stimulation as effectively as control adenovirus infected

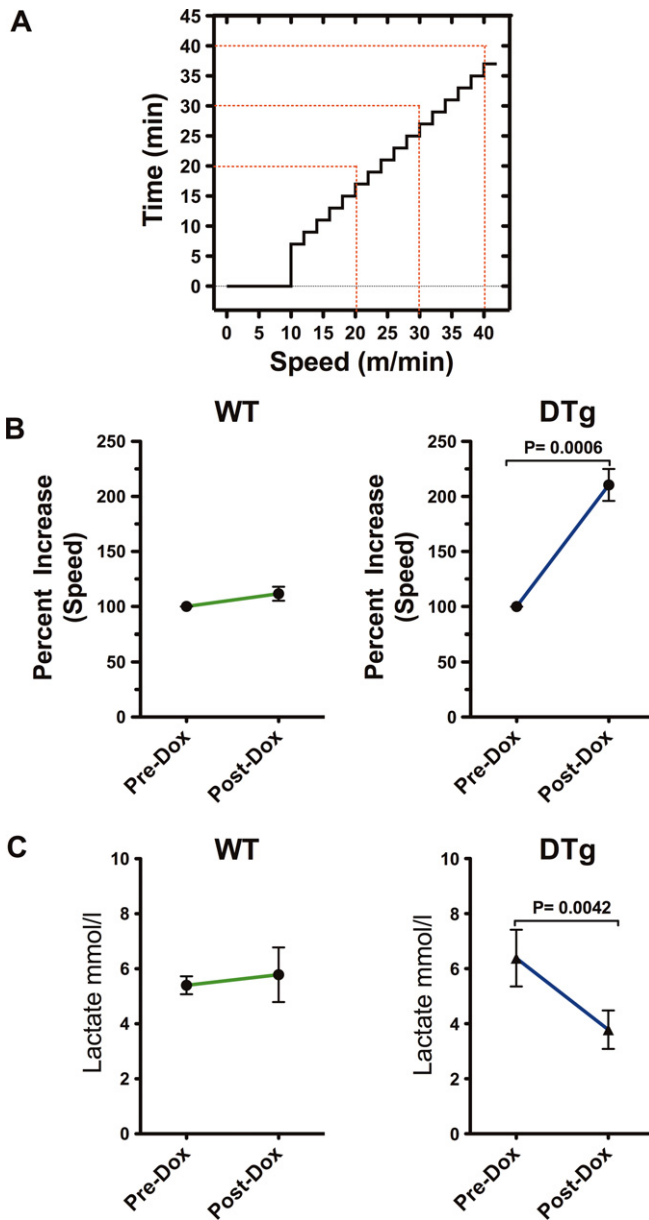


Figure 9. Crtc2 expression augments exercise performance.

- A** Exercise protocol for evaluating within-animal performance. Exercise capacity was assessed by determining the maximal speed achieved when the speed increased by 2 m/min every 2 min until exhaustion.
- B** Maximal exercise capacity before and after Dox administration. Maximal exercise capacity was determined by averaging the maximum speed achieved in three independent trails of an exercise stress test before and after Dox treatment for 2 weeks. Each symbol is the mean percent increase in the speed for each mouse, $n = 5$ mice per experimental group. P -values calculated by a two-tailed paired t -test.
- C** Blood lactate concentration in response to exercise before and after Dox administration. Each symbol is the mean blood lactate concentration of each experimental group, $n = 5$ mice per group. P -values calculated by a two-tailed paired t -test.

myotubes *ex vivo* (Fig 8C–D). Importantly, a bout of high-intensity exercise after Crtc2 overexpression reversed the elevated glycogen levels in the skeletal muscle (Fig 8E).

Crtc2 reduces metabolic stress and improves exercise capacity in skeletal muscle

Since Crtc2 overexpression fosters many of the same phenotypic changes that occur in response to the onset of a new exercise regimen, we reasoned that DTg mice would experience less stress during forced exercise. Metabolic stress and exercise capacity were tested using continuous running with an escalating velocity protocol (Fig 9A). Mice were tested for exhaustion during three trials prior to induction of the Crtc2 transgene followed by an additional three trials 2 weeks after administration of Dox. Both groups displayed signs of a training or learning effect, but the DTg mice exhibited a significant increase in performance that did not occur with the wild-type cohort (Fig 9B). Moreover, lower plasma lactate levels occurred after 20 min of exercise when the Crtc2 transgene was expressed, suggesting that the same amount of work induced less metabolic stress in these mice (Fig 9C).

Discussion

β -Adrenergic receptor (β -AR) agonists induce skeletal muscle hypertrophy (Choo *et al*, 1992; Mersmann, 1998) and attenuate muscle atrophy due to sarcopenia (Ryall *et al*, 2007), denervation (Maltin *et al*, 1989; Hinkle *et al*, 2002) or neuromuscular-induced dystrophy (Harcourt *et al*, 2007; Zeman *et al*, 2000). These studies and others have spawned enthusiasm in the therapeutic application of β -agonists for muscle wasting (Ryall & Lynch, 2008). The precise mechanism by which β -agonists activate the Akt signaling pathway to promote muscle hypertrophy is however unclear, despite the fact that some have suggested that the $G_{\beta\gamma}$ subunits of the G-protein tetramer activate PI3K to then activate Akt1 (Chesley *et al*, 2000; Erbay & Chen, 2001; Schmidt *et al*, 2001; Park & Chen, 2005; Kline *et al*, 2007). Rather, here we show that β -agonists or exercise activate the Crtc/Creb transcription and, using genetic approaches, establish that initiating Crtc/Creb signaling without activation of the adrenergic receptor is sufficient to induce adaptive changes, including hypertrophy and increased exercise capacity. Several upstream activators of Akt1, including Igf1 and Pdk1 (Musaró *et al*, 2001; Rommel *et al*, 2001), are upregulated by Crtc/Creb signaling, but whether the hypertrophy observed is dependent on their function is unclear.

Activation of the Crtc/Creb transcriptional complex also inhibited the proteasomal degradation pathway, and this likely also contributes to the phenotypes manifest in Crtc2 transgenic mice. In skeletal muscle, inactivation of class II HDACs impairs protein degradative pathways via effects on the expression of myogenin and the E3 ubiquitin ligase MuRF1, which are required for the ubiquitylation of muscle protein (McKinsey *et al*, 2000). Crtc2 or Crtc3 overexpression appears to inactivate the histone deacetylases HDAC4, HDAC5 and HDAC7 via their induction of SIK1, which phosphorylates these HDACs and directs their export into the cytoplasm (Berdeaux *et al*, 2007; Takemori *et al*, 2009). Accordingly, myogenin and MuRF1 expression was suppressed in primary myocytes and transgenic mice expressing Crtc2. Thus, the Crtc/Creb transcriptional complex sustains muscle hypertrophy by coordinating the signaling circuits that decrease protein degradation and those that promote protein synthesis.

Despite the clear pharmacological effects of β -AR agonists, the SNS has not been previously thought to direct the anabolic adaptations fostered by exercise, largely due to the systemic effects of catecholamines. We show here that the Crtc/Creb transcriptional complex is activated in response to SNS cues, including high-intensity exercise, and that the Crtc coactivators integrate signals from both calcium and catecholamine pathways in muscle cells *ex vivo* and *in vivo*. During exercise, acetylcholine depolarizes the membrane of the worked muscle, liberating Ca^{2+} stores from the ER into the sarcolemmal space. Calcium not only directs contraction, but also functions as a second messenger that regulates the protein phosphatase calcineurin, which dephosphorylates NFAT transcription factors (Bassel-Duby & Olson, 2006). When catecholamines increase cAMP signaling in contracting myofibers, the two signaling pathways converge to activate the Crtc coactivators. This dual signaling integration is highly conserved, occurring in diverse tissues such as hippocampus (Ch'ng *et al*, 2012), cortex (Baxter *et al*, 2011) and islet cells (Screaton *et al*, 2004). The convergence of these two exercise-induced signaling pathways provides a mechanism to direct the appropriate adaptation primarily to the working muscle.

Skeletal muscle exhibits remarkable plasticity to exercise, which consists of any infinite combination of intensity, duration and resistance; however, most exercise research focuses on effects of these extremes of training regimes. This is best exemplified by comparing the differences in adaptations that result from chronic aerobic training versus resistance training (Egan & Zierath, 2013). Our data unexpectedly show adaptations that occur in both of these modalities, which are generally thought to be mutually exclusive. For example, Crtc2 expression increases myofiber cross sectional area with elevated triglyceride and glycogen stores but also stimulated the expression of genes known to induce mitochondrial biogenesis, including *Pgc-1 α* and *Nr4A3*. Moreover, it is unlikely that the SNS has a major role in these classically studied modalities since catecholamine levels progressively decrease in response to the same stimulus when the absolute workload is held constant (Winder *et al*, 1978). If SNS/Crtc/Creb signaling coordinates an adaptive response to exercise, the question remains, in what context and time frame does this occur? The distinctive pattern of responses seen with Crtc/Creb activation reflects the unique role of catecholamines in coordinating the stress response to novel stimuli or high-intensity training. In support of this concept, several studies have shown that the onset of aerobic exercise increases myofiber hypertrophy along with mitochondrial biogenesis. For example, mice provided free wheel running access for 2 weeks have a significant increase in mitochondrial enzyme expression and generally show hypertrophy of hindlimb muscle (Allen *et al*, 2001, 2002; Harrison *et al*, 2002; Konhilas *et al*, 2005). Moreover, 12 weeks of cycle ergometer training induces myofiber hypertrophy in untrained young men, older men and older women (Harber *et al*, 2009, 2012). Thus, Crtc/Creb coordinates a unique cohort of adaptive responses tailored to the novel and intense stimuli that activate the SNS, and as training continues, the role of the SNS in directing adaptations quickly diminishes as other signaling pathways predominate.

The data presented herein suggest new functions for the SNS in coordinating both the immediate catabolic response to mobilize resources and in the anabolic adaptive changes in exercised muscle. In our model, the rapid action and transcriptional components of

the SNS-adrenergic signaling cascade are regulated in a sequential, biphasic manner (Supplementary Fig S10), suggesting a revision of the current concept of the SNS as driving solely catabolism during exercise (Egan & Zierath, 2013). Specifically, although transient elevations in cAMP and PKA regulate catabolism of energy stores during exercise, following exercise activation of the Crtc/Creb transcriptional complex induces genes that direct structural and metabolic remodeling. In support of this model, elevated levels of glycogen induced by forced expression of Crtc2 or Crtc3 are reversible by acutely activating PKA with clenbuterol, or by a bout of HIE. These adaptive Crtc/Creb-driven events during rest would thus prepare the muscle for the next repetitive bout of exertion and suggest that the Crtc/Creb transcriptional complex plays a specialized role in coordinating the adaptive response to the stress or intensity of exercise to restore homeostasis. Transgenic mice showed a clear improvement in maximal exercise capacity during treadmill-based stress test after induction of the *Crtc2* transgene. This is consistent with the well-known fact that athletic performance is greatly enhanced by reintroduction of high-intensity interval training and periodic changes in exercise routines (Fairbairn *et al*, 1991; Smith *et al*, 2003; Babraj *et al*, 2009; Richards *et al*, 2010). Thus, the SNS is exquisitely tailored to direct skeletal muscle adaptations that are proportional to the stress of the exercise, and our work challenges the current paradigm of SNS as primarily being mediator of rapid catabolic changes in skeletal muscle, as it clearly also controls adaptive anabolic responses via the Crtc/Creb complex.

Materials and Methods

Transgenic mice

TRE-Crtc2tm mice were generated by cloning FLAG-Crtc2 (S171A, S275A, K628R) into pTRE-Tight. Oocyte injections were conducted into pseudopregnant C57Bl6 mice at the University of Cincinnati. Skeletal muscle specificity was conferred when this transgenic line was crossed with second mouse transgenic strain that harbors a transgene for the reverse tetracycline transactivator (rtTA) that is regulated by the 1256[3Emut] MCK promoter (Grill *et al*, 2003). For skeletal muscle-specific expression, *Crtc2tm* DTg mice were treated with DOX (1.0 g/kg chow, Bio serv). Wild-type littermates were used as controls and were treated with DOX in the same manner.

Electric-pulse-mediated gene transfer

Electric-pulse-mediated gene transfer was conducted with an ECM 830 square wave using non-invasive platinum-coated tweezertrodes according to the manufacturer's guidelines. Fifty micrograms of purified plasmid DNA was injected with a 29-G needle in the tibialis anterior muscle. Immediately after the plasmid injection, four pulses of 40 mV lasting for 100 ms with an interval of 200 ms between pulses were applied. The polarity was then reversed and four additional pulses were performed.

Mouse exercise protocols

Eight-week-old male C57BL/6J mice were acclimated and pre-trained on a treadmill for 2 weeks prior to high-intensity exercise.

Mice were run at 10 m/min for 15 min followed by an incremental increase of 1 m/min over the next 5 min until a speed of 20 m/min was reached. Mice were considered exhausted if they had an irregular gate or stayed on the shock grid for two continuous seconds. Mice were rested 30 min post-exercise before euthanization and tissue biopsy.

Exercise capacity was determined utilizing a treadmill-based exercise stress test that consisted of an escalating belt speed that increased by 2 m/min every 2 min at a 15-degree incline after an initial warm-up at 20 m/min for 5 min. The maximum speed achieved despite extra stimulus was recorded. Each mouse was evaluated in three independent tests separated by 1-week intervals prior to the administration of Dox, followed by three additional tests after 2 weeks of Dox administration. All animal protocols were reviewed and approved by the Institutional Animal Care and Use (IACUC) of The Scripps Research Institute, Florida.

Primary myocyte isolation and cell culture

Primary myoblasts were isolated from P1 to P3 day old C57BL/6J pups as described (Springer *et al*, 1997) and cultured on collagen-coated plates in expansion media consisting in 1:1 F-10/DMEM (Invitrogen) with 20% fetal calf serum and 25 µg/ml of bFGF. Sub-confluent myoblasts were differentiated into multinucleate myotubes using a 1:1 F-10/5 mM glucose DMEM supplemented with 4% heat-inactivated horse serum (Hyclone) for up to 5 days. C₂C₁₂ mouse myoblasts from American Type Collection were expanded in DMEM supplemented with 10% fetal calf serum.

Pharmacological agents

Isoproterenol 100 nM, clenbuterol 45 nM and propranolol 10 µM.

Transfections and luciferase assays

Mouse primary myoblasts were transfected with the using Lipofectamin LTX and plus reagent (Invitrogen) following the manufacturer's guidelines. Luciferase assays were carried out as described (Conkright *et al*, 2003b) using BrightGlo (Promega) reagent and an EnVision Reader (PerkinElmer).

Adenoviruses

The control GFP-expressing, Crtc2-expressing and Crtc3-expressing adenoviruses have been described (Koo *et al*, 2005; Wu *et al*, 2006). For all experiments, myotubes were infected after 72 h of differentiation with 4×10^8 viral particles per ml per well for 24–48 h.

Immunoblotting and subcellular fractionation

Immunoblot analysis was performed as described (Conkright *et al*, 2003a). Cytoplasmic and nuclear biochemical fractionation of the myotubes was performed using the Active Motif nuclear extract kit (Active Motif) following the manufacturer's protocol. Whole-cell extracts were prepared in HEPES whole-cell lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 1× phosphoSTOP complete inhibition cocktail tablets (Roche), and 1X Complete-EDTA Free protease inhibitor cocktail (Roche). Frozen muscle tissue was homogenized

in lysis buffer T-PER tissue protein extraction reagent (Thermo Scientific) by a polytron homogenizer and sonication. The protein concentration was determined using the protein assay reagent (Bio-Rad). 60–80 µg of protein was separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. The antibodies used in this study were as follows: CRT3 (C35G4)Rb, CREB (48H2)Rb, pAKT(Thr308) (244f1)Rb and pAKT(Ser473) (971s) were from Cell Signaling Technology; PGC1α ab54481 Rb and PDPK1 ab31406 Rb were from Abcam; Nur77 #2518 Rb was from Novus Biological and Epitomics; and CRT2 #A300-638A Rb was from Bethyl Laboratories.

Tissue and cellular glycogen content

Glycogen concentration of primary myotubes was measured using an assay kit from Biovision.

Measurement of signaling molecules

Catecholamines were quantitated using the 2-CAT (Epinephrine/Norepinephrine) Research ELISA™ Kit from Rocky Mountain Diagnostic. cAMP was quantitated with a LANCE assay from PerkinElmer following the manufacturer's protocol.

RNA and qRT-PCR

Total RNA from tissue or cells was harvested using Rneasy RNA Kit (Qiagen) and cDNA prepared using Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Relative abundance of cDNAs was determined a Roche LightCycler 480 and normalized the resulting data to *Rpl-23* ribosomal RNA as described (Conkright *et al*, 2003b). Primers used were *Nr4a1* (5'-ttctgctcaggctgact', 5'-gattctgcagctcttcacc-3'); *Nr4a3* (5'-tcagccttttggagctgt', 5'-tgaagtcgatgcaggacaag-3'); *Sik1* (5'-cccttattatccccctgga-3', 5'-cttacttcagagaagggg-3'); *Dgat1* (5'-tggttaacctggccacaatc-3', 5'-ttggagtatgatgccagagca-3'); *Pdpk1* (5'-tcacagatttggaaacagcaa-3', 5'-tgagcagctctggagaaca-3'); and *Ppargc1a* isoform 1 (5'-atggcttgggacatg-3', 5'-gttcaggaagatctgg-3'). *Ppargc1a* isoform 4 (5'-TCACACCAA ACCCACAGAAA-3', 5'-CTGGAAGATATGGCACAT-3'), *Igf1* (5'-TT TTGCCTCATTATCCCTG-3', 5'-TCTCCTTTGAGCTTCGTTT-3').

Histology

Dissected muscle tissue was mounted in OCT medium (TissueTek) and frozen in liquid-nitrogen-cooled isopentane. Tissue sections were stained with hematoxylin and eosin for overall morphology, or ATPase activity for fast and slow fiber by standard methods. The cross-sectional area of muscle fibers was determined using ImageJ software.

Chromatin immunoprecipitation

C₂C₁₂ cells were stimulated with isoproterenol for 30 min before cross-linking in 1% formaldehyde as described (Amelio *et al*, 2007). We performed 28 cycles of PCR with the primers flanking the CRE sites of each promoter: *Dgat1* (5'-GAACCGCAAGACGTCAGC-3', 5'-GAGGCTGCGATGCTGCGG-3'); *Nr4a1* (5'-AAGAAATAGCAGGCTGG TTGGG-3', 5'-CAGGTAAGAGAAAGGAAGGGGTATG-3'); *Gapdh*

(5'-GAAAAGGAGATTGCTACG-3', 5'-GCAAGAGGCTAGGGGC-3'); and *Pparg1* distal promoter (5'-TCATTGAGCAGTACTCCCAGG-3', 5'-CA AACCCTACATACCAGCAGC-3').

Statistical analysis

Two-tailed Student's *t*-test was used to determine *P*-values between datasets assuming Gaussian distribution. Data are reported as the mean \pm s.e.m., along with calculated *P*-values. One-way ANOVA was applied to compare means of three or more groups followed by Tukey's *post hoc* test for comparing pairs of means. The calculated *P*-values are represented by **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

Supplementary information for this article is available online: <http://emboj.embopress.org>

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Author contributions

NB and MDC initiated the research and planned all experiments. NB performed most of the experiments and supervised the assistance of KAK, RH and MBL with cell culture and immunoblotting. NB and KAK developed and characterized the transgenic mice. ALA conducted the ChIP experiments and cloned the *Pgc-1 α* promoter constructs. NB, NK and MDC interpreted the data. NB, KAK, KN and MDC contributed to the writing of the manuscript.

Conflict of interest

N.E. Bruno and M.D. Conkright declare a patent application related to this work. The remaining authors declare that they have no conflict of interest.

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