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2019-07-15

Calcineurin broadly regulates the initiation of skeletal muscle-specific gene expression by binding target promoters and facilitating the interaction of the SWI/SNF chromatin remodeling enzyme

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Witwicka H, Nogami J, Syed SA, Maehara K, Padilla-Benavides T, Ohkawa Y, Imbalzano AN. (2019). Calcineurin broadly regulates the initiation of skeletal muscle-specific gene expression by binding target promoters and facilitating the interaction of the SWI/SNF chromatin remodeling enzyme. Open Access Articles. <https://doi.org/10.1128/MCB.00063-19>. Retrieved from <https://escholarship.umassmed.edu/oapubs/3925>

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1 **Calcineurin broadly regulates the initiation of skeletal muscle-specific gene expression by**
2 **binding target promoters and facilitating the interaction of the SWI/SNF chromatin**
3 **remodeling enzyme**

4

5 Running title: Calcineurin globally promotes myogenic gene expression

6

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17 **ABSTRACT**

18 Calcineurin (Cn) is a calcium-activated serine/threonine protein phosphatase that is broadly
19 implicated in diverse cellular processes, including the regulation of gene expression. During
20 skeletal muscle differentiation, Cn activates the NFAT transcription factor but also promotes
21 differentiation by counteracting the negative influences of protein kinase C beta (PKC β) via
22 dephosphorylation and activation of BRG1, an enzymatic subunit of the mammalian SWI/SNF
23 ATP-dependent chromatin remodeling enzyme. Here we identified four major temporal patterns
24 of Cn-dependent gene expression in differentiating myoblasts and determined that Cn is broadly
25 required for the activation of the myogenic gene expression program. Mechanistically, Cn
26 promotes gene expression through direct binding to myogenic promoter sequences and
27 facilitating the binding of BRG1, other SWI/SNF subunit proteins, and MyoD, a critical lineage
28 determinant for skeletal muscle differentiation. We conclude that the Cn phosphatase directly
29 impacts the expression of myogenic genes by promoting ATP-dependent chromatin remodeling
30 and formation of transcription-competent promoters.

31 INTRODUCTION

32 Myoblast differentiation is an essential process during skeletal muscle development
33 where mononuclear myoblasts withdraw from the cell cycle and undergo fusion and other
34 morphological changes to form multi-nucleated myotubes. This process is coordinated by the
35 family of myogenic regulatory factors (MRFs) that include MyoD, myogenin, Myf5, and Mrf4 in
36 cooperation with the MEF family of transcription factors and other auxiliary transcriptional
37 regulators. MRFs regulate the commitment, determination, and differentiation of skeletal muscle
38 progenitor cells. The ability of MRFs to drive the myogenic gene expression needed for
39 differentiate requires remodeling of chromatin at target genes that depends on the recruitment of
40 histone modifying and chromatin remodeling complexes that alter nucleosome structure and the
41 local chromatin environment (1-3).

42 The SWI/SNF (SWItch/Sucrose Non-Fermentable) complexes are large, multiprotein,
43 ATP-dependent chromatin remodeling enzymes (4-6) that alter nucleosome structure to promote
44 transcription, replication, recombination and repair (7-10). The chromatin remodeling activity of
45 the SWI/SNF enzyme is required for the initiation of many developmental and differentiation
46 programs (11-14) including activation of myogenic genes upon differentiation signaling (15, 16).
47 Mammalian SWI/SNF complexes contain one of two related ATPase subunits, either Brahma
48 related gene 1 (Brg1) or the ATPase Brahma (Brm), and a collection of at least 9 to 12 associated
49 protein known as Brg1/Brm-associated factors (Bafs) (4, 17, 18). Mammalian SWI/SNF enzyme
50 function can be influenced by the assembly of different combination of Baf subunits around the
51 different ATPases (12, 19). Furthermore, signal transduction pathways promote specific
52 posttranslational modifications of SWI/SNF subunit proteins that influence enzyme activity (15,
53 20-23). In skeletal muscle differentiation, the p38 mitogen-activated protein kinase (MAPK)

54 phosphorylates the Baf60c subunit, which then allows the recruitment of the rest of SWI/SNF
55 remodeling complex to myogenic promoters (24). Our group previously showed that casein
56 kinase 2 (CK2) phosphorylates Brg1 to regulate *Pax7* expression and to promote myoblast
57 survival and proliferation (21), while protein kinase C β 1 (PKC β 1) phosphorylates Brg1, which
58 represses chromatin remodeling function and, consequently, myogenesis (20).

59 Calcineurin (Cn) is a serine/threonine phosphatase that is regulated by changes in the
60 intracellular concentration of Ca^{2+} (25). Cn is a heterodimer formed by association of catalytic
61 subunit A (CnA) and regulatory subunit B (CnB) (26, 27). Its mechanism of action has been
62 characterized extensively in lymphocytes, where activated Cn dephosphorylates Nuclear Factor
63 of Activated T-cell (NFAT) transcription factors. Dephosphorylated NFAT translocates to the
64 nucleus and binds to promoter regions of target genes to regulate gene expression (28-31). In
65 skeletal muscle, Cn-dependent binding of NFAT to target promoters controls skeletal muscle
66 fiber type and primary muscle fiber number during development (32, 33) and growth of
67 multinucleated muscle cells (34-38). Cn is also required for the initiation of skeletal muscle
68 differentiation (39), by mechanisms that are independent of NFAT (40, 41). More recently, we
69 reported novel functions for Cn in chromatin remodeling. We showed that Cn is bound to
70 Brg1 at the myogenin promoter and that it dephosphorylates Brg1 shortly after cells start the
71 differentiation process to positively promote differentiation (20).

72 The aim of the current study was to explore the global effect of Cn on gene expression
73 in myoblasts. We demonstrate that inhibition of Cn in myoblasts globally down-regulates
74 expression of genes important for muscle structure and function. We identified four major
75 temporal patterns of Cn-dependent gene expression. Mechanistically, we show that Cn acts as

- 76 a chromatin binding regulatory protein, interacting with Brg1 to facilitate SWI/SNF enzyme
- 77 and MyoD binding to myogenic gene regulatory sequences.

78 **RESULTS**79 **RNA-seq identification of genes differentially expressed in myoblasts treated with calcineurin**
80 **inhibitor FK506**

81 To better understand the involvement of calcineurin in the gene regulation program
82 underlying myogenesis, we compared the transcriptomes of C2C12 myoblasts at three time
83 points of differentiation (0, 24, and 72 h post-induction of differentiation) in cells treated with
84 the Cn inhibitor, FK506, with those of control cells. FK506 inhibits the phosphatase activity of
85 Cn by binding to the immunophilin FKB12; the drug-bound FKB12 binds to and blocks Cn
86 function (28). C2C12 myoblasts treated with FK506 showed significant inhibition of myotube
87 formation at 48 and 72h time points compared to the DMSO control, confirming prior results
88 demonstrating that inhibition of Cn activity blocked myoblast differentiation (Fig. 1A; (39-41)).
89 Myogenic gene expression is temporally regulated, with different genes expressed with different
90 kinetics during differentiation (42-44). We analyzed gene expression 24 and 72 h after the
91 induction of differentiation to distinguish the impact of Cn inhibition on genes expressed at early
92 or late times of myogenesis.

93 At 24h post-differentiation, 156 genes were differentially expressed in the presence of
94 FK506 [false discovery rate (FRD) < 0.05]. Fewer genes were up-regulated (53) than were
95 downregulated (103). At the later time point of differentiation (72h), 648 genes were
96 differentially expressed. More genes were up-regulated (373) than were downregulated (275)
97 (Fig. 1B, C). In confluent myoblasts at the onset of differentiation (0h), only 21 genes were
98 differentially expressed in the presence of FK506. The complete list of differentially expressed
99 genes determined by RNA-seq analysis is shown in **Supplementary Table S1**. Specific
100 myogenic genes were identified and labeled on the volcano plots in **Fig. 1B**. The results indicate

101 that myogenic genes are downregulated by calcineurin inhibition at the 24 and 72h time points.
102 Review of these genes determined that the genes largely encoded structural and functional
103 proteins expressed during differentiation. Specifically, the expression of genes encoding the
104 regulatory proteins MyoD, Myf5, Mef2A, and Mef2D were unaffected by Cn inhibition by
105 FK506, whereas the genes encoding myogenin, Mef2C, and Six1 were noted as being sensitive
106 to the Cn inhibitor. The MRF4 regulator was not expressed. In addition, we noted that none of
107 the genes encoding NFAT proteins or genes encoding mSWI/SNF subunits showed expression
108 changes due to Cn inhibition by FK506 (**Supplementary Table S1**).

109 We performed *de novo* motif analysis on the differentially expressed genes. The most
110 significantly enriched motifs at the 24h post-differentiation were for Mef2 transcription factors
111 and E-boxes bound by the MRFs Myf5, myogenin and MyoD, and we note the continued
112 presence of MRF and Mef2 protein binding sites at Cn-regulated genes at late times of
113 differentiation (**Fig. 1D**). These findings indicate that genes that require calcineurin for
114 expression are also regulated by myogenic regulatory factors, strongly reinforcing the connection
115 between Cn and expression of the myogenic gene program.

116 The top hits from a gene ontology (GO) analysis of genes up- and down-regulated by
117 calcineurin inhibition at the 24 and 72h time points is shown in **Fig. 2A**. GO terms identified by
118 side by side comparison of up- and down-regulated genes at each time point are presented in **Fig.**
119 **2B**. Genes related to muscle differentiation and function were down-regulated in cells treated
120 with FK506 (**Figs. 2A, B**). In contrast, we observed up-regulation of genes associated with
121 ossification, response to interferons and viruses, and cardiovascular and blood vessel
122 development. These results are consistent with documented roles for Cn in the regulation of
123 NFAT function in these processes (45-47). **These results are also consistent with our finding that**

124 the most significantly enriched DNA binding motif at 72h post-differentiation was the interferon
125 regulatory factor (IRF) binding sequence (Fig. 1D) and with findings that IRFs regulate
126 expression of vascular cell adhesion molecule 1 (VCAM-1) receptor that mediates cell-cell
127 adhesion and is important for myoblast fusion (48, 49).

128

129 **Global expression analysis reveals four major temporal gene expression patterns that are**
130 **dependent on Cn**

131 We continued our analysis by identifying the groups of genes that showed treatment-
132 specific changes in gene expression over time. In total, 308 genes were identified. We analyzed the
133 expression patterns of these differentially expressed genes using clusterProfiler (50). Genes
134 differentially expressed over time were clustered into 4 major groups by k-means clustering, and
135 graphically presented by heat map (Fig. 3A) and expression kinetics (Fig. 3B). We performed GO
136 analysis for the differentially expressed genes in these 4 clusters to gain additional insight into the
137 biological processes regulated by calcineurin during myoblast differentiation (Fig. 3C). Cluster 1
138 included 95 genes that were down-regulated during differentiation but were unchanged in the
139 presence of the Cn inhibitor. GO terms significantly enriched for this group of differentially
140 expressed genes related to cell migration, motility and adhesion. In addition, Cluster 1 included
141 Inhibitor of differentiation (Id) proteins 1, 2 and 3 (Supplementary Table S2). Id proteins interact
142 with MyoD and related MRFs prior to differentiation to repress transcription activation activity. Id1
143 and Id3 have previously been identified as genes repressed by Cn during the activation of skeletal
144 muscle cell differentiation (41). Cluster 2 contained 80 genes that were up-regulated during
145 myoblast differentiation but were inhibited or delayed in the presence of FK506. GO terms
146 significantly enriched for Cluster 2 genes were related to muscle structure and function. Cluster 3

147 was composed of 108 genes that were expressed at relatively constant levels across the
148 differentiation time course but that were significantly up-regulated in the presence of FK506
149 expression. GO analysis of Cluster 3 genes identified genes implicated in immune response of cells.
150 Cluster 4 had 25 genes with an unusual profile. These genes were up-regulated in the presence of
151 FK506 in proliferating cells and in cells at the onset of differentiation, but were down-regulated as
152 differentiation advanced. No specific GO terms were identified for Cluster 4. A complete list of
153 genes in each of the clusters with their log fold change and FDR values are shown in
154 **Supplementary Table S2**. These data suggests that calcineurin regulates multiple and diverse
155 groups of genes during myoblast differentiation. We measured mRNA expression levels of two
156 randomly chosen genes from each of clusters 1, 3 and 4 by RT-qPCR analysis (**Fig. 4A**) to confirm
157 the gene expression kinetics revealed by the cluster analysis.

158 For further analysis, we focused on genes that were down-regulated in the presence of
159 FK506 and are important for muscle structure and/or function (**Fig. 1B**). We first measured
160 mRNA expression level of several such genes to validate RNA-seq and cluster analysis results:
161 *Myog*, *Ckm*, *Myh4*, *Mymk*, *Tnnt3*, *Acta1* and *Cav3* were analyzed by RT-qPCR. As expected,
162 each of these genes were increased over the course of the differentiation time course and each
163 was significantly down-regulated by exposure to the Cn inhibitor, FK506 (**Fig. 4B**). These
164 results confirmed that inhibition of Cn during myoblast differentiation impairs expression of
165 multiple myogenic genes that are normally activated during differentiation.

166

167 **Inhibition of calcineurin function prevents its binding to myogenic promoters**

168 We previously showed that Cn associates with Brg1 and binds to the myogenin promoter
169 early after the start of the differentiation process (20). We hypothesized that the same model

170 might be true for other myogenic genes. Alternatively, Cn may directly regulate the myogenin
171 promoter but indirectly regulate other downstream genes and via the dependency of other genes
172 on myogenin for completing the differentiation process (51, 52).

173 We first determined whether the Cn inhibitor could alter the nuclear localization or
174 chromatin association of Cn or Brg1. Immunoblot analysis of subcellular fractions is shown in
175 **Figs. 5A, B.** Cn is present in both cytosolic and nuclear fractions as well as in chromatin and its
176 localization is not changed when cells are treated with FK506. Brg1 is associated only with the
177 nuclear and the chromatin fractions and, similar to Cn, its localization is not affected by FK506.
178 Immunoblots of control proteins show the purity of the fractions. TATA Binding Protein (TBP)
179 is associated with the chromatin and the nuclear fractions while GAPDH is located only in the
180 cytosolic fraction. These data demonstrate that the effects due to inhibition of Cn activity are not
181 due to gross mislocalization of Cn or Brg1 within the cell.

182 We performed chromatin immunoprecipitation assays (ChIP) on the same panel of
183 myogenic genes examined in Fig. **4B.** PCR primers for ChIP were designed to amplify the E-box
184 containing regions that bind to MyoD and MyoD-related lineage determining factors (53) within
185 1.5 kb upstream of the TSS of each gene (**Fig. 6A**) and regulate gene expression (53-61). The
186 promoter of the *Pdx1* gene, which encodes the pancreatic and duodenal homeobox 1
187 transcriptional activator, served as a negative control sequence for all ChIP experiments, as was
188 done in prior work (62). The *Pdx1* E box is bound by members of the upstream (USF) family of
189 transcription factors (63), and is not bound by MyoD or related factors in proliferating or
190 differentiating myoblasts (62). *Pdx1* is expressed in pancreas, duodenum, gall bladder, stomach,
191 and small intestine (64). Our RNA-seq data confirmed that *Pdx1* was not expressed in
192 proliferating or differentiating myoblasts.

193 Cn did not interact with myogenic genes regulatory sequences in proliferating myoblasts
194 (GM), was weakly or not bound in undifferentiated cells at the start of differentiation (0h DM),
195 and was bound at all myogenic gene promoters in differentiated (48h DM) cells (**Fig. 6B**).
196 **FK506 treatment of cells greatly reduced Cn binding.** We conclude that Cn binding to myogenic
197 promoters is a general occurrence and FK506-mediated inhibition of Cn function impairs its
198 ability to interact with target gene promoters. The results suggest that Cn is a direct regulator of
199 many myogenic genes.

200

201 **Cn inhibition blocks the interaction of the ATPase Brg1 and other subunits of the**
202 **mammalian SWI/SNF complex with myogenic promoters**

203 We also performed ChIP assays to determine whether Brg1 recruitment to myogenic
204 regulatory sequences was dependent on calcineurin. We observed recruitment of Brg1 to
205 regulatory sequences in differentiated (48h DM) cells at all the tested myogenic genes. In cells
206 treated with FK506, Brg1 binding was significantly diminished at all promoters (**Fig. 7A**). A
207 subset of the myogenic promoters was tested for the binding of Baf170, and Baf250A/Arid1A,
208 which are other subunits of the mammalian SWI/SNF enzyme complex. The results show that
209 binding of these other subunits paralleled Brg1 binding in that Cn inhibition blocked interaction
210 of these proteins to the promoters (**Fig. 7B**). These findings indicate that Cn activity is necessary
211 for the interaction of mammalian SWI/SNF chromatin remodeling complexes with regulatory
212 sequences of myogenic genes.

213

214 **Mutation of Brg1 sites of Cn activity prevents its interaction with myogenic gene promoters**

215 In previous work, we showed that PKC β -mediated phosphorylation of Brg1 prior to the
216 onset of differentiation was counteracted by Cn-mediated dephosphorylation and activation of
217 Brg1 immediately after the onset of differentiation (20). Serine amino acids targeted by
218 PKC β /Cn mapped to N- and C-terminal of the Brg1 bromodomain. Mutation of these sites to the
219 phosphomimetic amino acid glutamate (SE) prevented myogenesis, whereas mutation to the non-
220 phosphorylatable amino acid alanine (SA) had no effect on differentiation (20). These
221 experiments used primary myoblasts derived from Brg1-deficient mice that were reconstituted
222 with wildtype (WT)-, SA-, or SE-Brg1. We performed CHIP experiments in differentiating cells
223 and showed that Cn and Brg1 are bound to myogenic promoters in myoblasts expressing WT-
224 Brg1 (Fig. 8A, B). The SE-Brg1 mutant was incapable of binding; the repressive
225 phosphorylation of Brg1 caused by PKC β is mimicked by the glutamate substitutions, rendering
226 Cn incapable of activating Brg1. As expected, the SA-Brg1 mutant, which cannot be
227 phosphorylated at the relevant PKC β target amino acids, bound Brg1 and Cn to myogenic
228 regulatory sequences (Fig. 8A, B). These results are consistent with those obtained with the Cn
229 inhibitor, and they reinforce the conclusion that Cn function regulates Brg1 binding to chromatin
230 at myogenic gene regulatory sequences.

231

232 **Cn inhibition does not impact the ability of Cn and Brg1 to interact**

233 We previously showed that Cn and Brg1 can be co-immunoprecipitated from cell lysate
234 of differentiating cells (20). Inhibition of Cn function with FK506 had no impact on the ability of
235 these proteins to be isolated in complex with each other (Fig. 9A). As a complement to this
236 experiment, we looked at the interaction of Cn with Brg1 in proliferating and differentiating
237 myoblasts (Fig. 9B) and in differentiating myoblasts expressing WT-, SA-, or SE-Brg1 (Fig.

238 **9C). Cn interacted with Brg1 only in the differentiating myoblasts.** Cn could interact with WT-,
239 SA-, and SE-Brg1 mutants (**Fig. 9C**), despite the observation that SE-Brg1 was not competent
240 for interaction with chromatin. Brg1 can also form a complex with MyoD during differentiation
241 (15, 65). We examined if the Brg1 mutants could interact with MyoD and determined that the
242 Brg1-MyoD interaction was not disrupted by the Brg1 mutations (**Fig. 9C**). As an additional
243 negative control for our co-IP experiments, we used conditional Brg1 KO cells. When Brg1
244 expression was depleted by Cre recombinase, the Brg1-Cn interaction was not observed,
245 confirming the specificity of the pulldown (**Fig. 9D**). These results indicate that inhibition of Cn
246 function and mutation of the sites of Cn activity on Brg1 do not affect the interaction that exists
247 between these regulatory proteins. The continued existence of Brg1 protein in the presence of the
248 Cn inhibitor and when Cn-targeted residues are mutated to alanine or glutamine suggests that the
249 lack of appropriate phosphorylation or dephosphorylation does not have significant impact on the
250 steady-state levels of Brg1 and therefore is unlikely to be a major regulator of protein stability.

251

252 **Inhibition of calcineurin blocks MyoD binding to regulatory sequences of myogenic genes**

253 Recruitment of MyoD to myogenic promoters prior to the onset of differentiation can be
254 accomplished by different mechanisms, including gene-specific mechanisms (24, 65, 66). Our
255 RNA-seq data and our prior RT-qPCR analysis (20) showed that MyoD expression was not
256 changed by FK506 treatment. The continued presence of MyoD on myogenic promoters after the
257 onset of differentiation requires the Brg1 ATPase (15, 65). We would therefore predict that the
258 inhibition of Cn would affect the interaction of MyoD with myogenic gene regulatory sequences.
259 We assessed MyoD enrichment at the regulatory sequences of tested genes by ChIP. As shown in
260 **Fig. 10**, we observed enhanced enrichment of MyoD at all analyzed gene promoters in

261 differentiated cells compared to enrichment prior to or at the onset of differentiation.
262 Recruitment of MyoD at these regulatory sequences was attenuated by the Cn inhibitor. These
263 results support the conclusion that Cn is necessary for the stable binding of MyoD to the
264 myogenic gene regulatory sequences during differentiation.

265

266 **shRNA-mediated knockdown of Cn impaired myoblast differentiation, affected gene**
267 **expression and blocked binding of Cn, Brg1 and MyoD to myogenic regulatory sequences.**

268 The combination of an inhibitor Cn activity and mutation of residues on Brg1 that are Cn
269 substrates gave consistent results with regard to the mechanism of Cn function during myogenic
270 gene expression. Nevertheless, to provide additional confirmation, we reduced overall Cn levels
271 by shRNA-mediated targeting and repeated all of the experiments. Two distinct shRNAs specific
272 for the catalytic subunit of calcineurin A (*Ppp3ca*) were used. ShCn-1 targets the 3'UTR and the
273 shCn-2 targets the coding sequence. Cells infected with lentivirus expressing either of the
274 shRNAs targeting Cn or the control shRNA (shCtrl) were selected for puromycin and then
275 evaluated for Cn expression and cells differentiation (**Fig. 11A**). The shCtrl had no effect on Cn
276 expression and differentiation. Both shCn-1 and shCn-2 had significant reduction of Cn protein
277 and mRNA expression compared to the control. Myoblast differentiation was evaluated by
278 myosin heavy chain staining (**Fig. 11B**); we observed significant reduction in myotube formation
279 in 72h differentiated cells. Consistent with the reduction of Cn protein level and lack of
280 differentiation, mRNA expression of myogenic genes (cluster 2 genes from RNA-seq analysis)
281 *Myog*, *Ckm*, *Cav3*, *Myh4*, *Tnnt3*, *Acta1* and *Mymk* were significantly reduced in the Cn
282 knockdown cells (**Fig. 11C**). A representative gene from clusters 1, 3, and 4 (*Smahd1* (cluster1),
283 *Cxcl1* (cluster 3) and *Larp1b* (cluster 4)) were also affected by knocking down Cn (**Fig. 11D**).

284 Next, we analyzed recruitment of Cn, Brg1 and MyoD to four of the previously
285 interrogated myogenic regulatory sequences (**Fig. 12A-C**). As expected, all three proteins were
286 bound to the indicated regulatory sequences in the shCtrl differentiated cells. Cn binding to the
287 regulatory sequences in the Cn knockdown cells was impaired (**Fig. 12A**). Brg1 and MyoD
288 binding (**Fig. 12B, C**) were also impaired in the Cn knockdown cells, which demonstrates that
289 the reduction in Cn blocked the interaction of these protein with the myogenic regulatory
290 sequences. These results further support the conclusion that Cn promotes myoblast
291 differentiation by binding to myogenic regulatory sequences, facilitating binding of the Brg1
292 chromatin remodeling enzyme and the stable binding of MyoD and contributing to the activation
293 of the myogenic gene expression program.

294 **DISCUSSION**295 **Cn broadly contributes to the activation of the myogenic gene expression program during**
296 **differentiation**

297 The data presented here demonstrate that Cn plays a general role in regulating myogenic
298 gene expression during the myoblast differentiation. Its mechanism of action is via binding and
299 dephosphorylation of the Brg1 ATPase of the mammalian SWI/SNF chromatin remodeling
300 enzyme, which regulates the ability of Brg1 and other SWI/SNF enzyme subunits to stably
301 associate with myogenic promoters during differentiation. We and others have previously
302 demonstrated that Brg1-based SWI/SNF enzymes remodel chromatin at myogenic regulatory
303 sequences in differentiating cells (15, 16, 65). Failure of Cn to promote association of Brg1 and
304 associated mSWI/SNF enzyme subunits prevents the required enzymatic remodeling of promoter
305 chromatin structure and subsequent gene activation during differentiation.

306 The consistent observation of Cn binding to each of the myogenic promoters assayed
307 suggests that Cn is directly required for the activation of each target gene. The alternative
308 hypothesis was that Cn required indirectly via a requirement for activation of myogenin, which is
309 required for activation of myogenic gene products that promote terminal differentiation (67, 68).
310 In prior work examining other cofactors of myogenic gene expression, we determined that the
311 SWI/SNF chromatin remodeling enzyme was required for the expression of both myogenin and
312 subsequent gene expression (51). These new results spatially link Cn and SWI/SNF enzyme
313 binding to myogenic promoters, which is consistent with Cn function being required for
314 SWI/SNF enzyme function. In contrast, the Prmt5 arginine methyltransferase is required for
315 myogenin activation, but ectopic expression of myogenin promoted myogenic gene expression
316 and differentiation even in the absence of Prmt5 (69), indicating that the requirement for Prmt5

317 in later stages of the myogenic gene expression cascade was indirect. It is important to note,
318 however, that direct evidence that Cn-mediated dephosphorylation of Brg1 must occur while
319 bound to promoters is lacking. Future examination of Cn and PKC β 1 binding and function at
320 myogenic regulatory sequences will enable elucidation of mechanistic details about the timing,
321 dynamics, and spatial requirements for Brg1 phosphorylation and dephosphorylation.

322 The promoter binding capability of Cn is a novel function that is poorly understood.
323 There is no evidence that Cn contains a recognized DNA binding domain, raising the possibility
324 that it binds chromatin indirectly. The absence of Cn binding in the presence of the Cn inhibitor
325 suggests the possibility of auto-dephosphorylation as a necessary pre-requisite. However, prior
326 studies indicate that autodephosphorylation is slow and that phosphorylated Cn is more
327 efficiently dephosphorylated by protein phosphatase IIa (70). An alternative hypothesis is that
328 Cn inhibition alters the structure or function of a Cn binding partner, which directly or indirectly
329 results in loss of association with chromatin.

330

331 **Regulation of SWI/SNF enzyme by subunit composition and by phosphorylation state**

332 The diversity of mammalian SWI/SNF enzyme complex formation is due in part to the
333 existence of several subunits that exclusively or predominantly associate with subsets of enzyme
334 complexes (12, 19). The Baf250A subunit exists in a subset of SWI/SNF complexes known as a
335 SWI/SNF-A or BAF, which contains several unique subunits not found in the other major
336 subfamily of SWI/SNF complexes, referred to as SWI/SNF-B or PBAF complexes (71). The
337 presence of Baf250A at each of the promoters assays suggests that the A/BAF complex may be
338 the functionally relevant enzyme to promote skeletal muscle differentiation.

339 The literature on which specific enzyme complex(es) act at muscle-specific promoters is
340 limited. Brg1 has been identified at many myogenic promoters. Interference with Brg1 function
341 through expression of a dominant negative enzyme, injection of specific antibodies, or via
342 knockdown blocks myogenic gene expression and differentiation (15, 16, 24, 65, 72), but these
343 data do not distinguish between different types of SWI/SNF enzymes. The Brm ATPase binds to
344 the myogenin promoter in isolated mouse myofibers, but not in isolated satellite cells (73).
345 However, knockdown of Brm in cultured myoblasts had limited effect on differentiation-specific
346 gene expression while instead affecting cell cycle withdrawal (72). Those data indicate that
347 A/BAF complex function is a necessary pre-requisite for myoblast differentiation. In mouse
348 heart development, the B/PBAF specific subunits BAF200 and BAF180 are required (74-76), but
349 Baf250A knockout in mouse neural crest leads to embryonic death due to defective cardiac
350 development (77). Despite the intriguing ramifications of thousands of different potential
351 SWI/SNF enzyme compositions, comparison of complexes formed by A/BAF and B/PBAF-
352 specific subunits in the same cell type showed that genomic binding sites and transcriptionally
353 responsive genes largely overlapped, leading to the conclusion that the regulation of gene
354 expression by SWI/SNF enzymes is due to the combined effect of multiple SWI/SNF enzymes
355 (78). Muscle development and differentiation may similarly rely on multiple SWI/SNF enzyme
356 assemblies and may not be attributable to one specific enzyme complex.

357 Regulation of SWI/SNF chromatin remodeling enzyme activity, via control of the
358 phosphorylation state of different proteins within the enzyme complex, is an emerging
359 complexity that adds to the alarming complexity posed by the thousands of potential
360 combinatorial assemblies of the enzyme complex from its component subunit proteins (12, 19) .
361 Nevertheless, the evidence for this additional layer of regulation is clear. Amino acids **N-** and **C-**

362 **terminal** to the Brg1 bromodomain are phosphorylated by PKC β 1 in proliferating myoblasts and
363 dephosphorylated by Cn after the onset of differentiation signaling. Failure to remove the
364 phosphorylation prevents remodeling enzyme function and differentiation, while mutation of
365 these amino acids to prevent phosphorylation permits function even in the presence of a Cn
366 inhibitor (20). Here we demonstrate the generality of the requirement for Cn-mediated
367 facilitation of Brg1 function. The PKC β 1/Cn axis is joined by p38 kinase-mediated
368 phosphorylation of the Baf60c subunit that permits assembly of the SWI/SNF enzyme complex
369 on myogenic promoters (24). Most of the SWI/SNF subunits have been characterized as
370 phosphoproteins (79), suggesting that regulation of activity in response to differentiation
371 signaling may be influenced by other kinases and phosphatases as well.

372 The consequences of phosphorylation and dephosphorylation of the PKC β 1/Cn- targeted
373 amino acids on Brg1 structure remain to be determined. The fact that Brg1 protein remains
374 present when the relevant amino acids are mutated or when exposed to Cn inhibitor indicates that
375 the phosphorylation state of these amino acids does not **render the** Brg1 protein **unstable**. The
376 failure of other SWI/SNF subunits to remain associated with myogenic gene chromatin suggests
377 that the phosphorylation state may control Brg1 protein conformation, interactions with
378 chromatin, interactions with other SWI/SNF enzyme subunits, and/or interactions with other
379 cofactors that contribute to enzyme complex stability or chromatin binding. Additional
380 characterization will improve our understanding of Brg1 and SWI/SNF chromatin remodeling
381 enzyme function in differentiation and may also inform studies on the role of Brg1 in
382 oncogenesis, where Brg1 can be either mutated or overexpressed without mutation in different
383 types of cancer (80, 81).

384

385 **MATERIALS AND METHODS**

386 **Antibodies.** Rabbit antisera to Brg1 and MyoD were previously described (65, 82). Pan-
387 Calcineurin A (#2614), Baf170 (#12769), Baf250A (#12354), and TBP (#8515) antibodies were
388 from Cell Signaling Technologies (Danvers, MA). GAPDH antibody (#G9295) was from Sigma-
389 Aldrich (St. Louis, MO). MF20 antibody was from the Developmental Studies Hybridoma Bank
390 (Iowa City, IA). Brg1 antibody (G-7, #sc-17796) was from Santa Cruz Biotechnologies (Santa
391 Cruz, CA) and was used for western blotting and immunoprecipitation experiments.

392 **Cell culture.** C2C12 and HEK293T cells were purchased from ATCC (Manassas, VA) and were
393 maintained at subconfluent densities in Dulbecco's modified Eagle's medium (DMEM)
394 supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator at 37°C
395 in 5% CO₂. For differentiation, C2C12 cells at > 80% confluency were switched to DMEM
396 medium supplemented with 2% horse serum and 2 µg/ml of bovine insulin (Sigma-Aldrich).
397 FK506 (Cayman Chemical, Ann Arbor, MI) was added to the culture 24h before initiating
398 differentiation and maintained in the differentiation media at 2µM. Media containing FK506
399 was changed every day.

400 Mouse satellite cells were isolated from leg muscles of 3- to 6-week old Brg1 conditional
401 mice using Percoll sedimentation followed by differential plating as described previously (20).
402 Mice were housed in the animal care facility at the University of Massachusetts Medical
403 School and used in accordance with a protocol approved by the Institutional Animal Care and
404 Use Committee. Brg1 depleted primary myoblasts expressing wild type (WT) Brg1 or Brg1
405 mutated at sites of PKCβ1/Cn activity were generated as described (20). Primary myoblasts were
406 grown and differentiated as described (20) on plates coated overnight in 0.02% collagen
407 (Advanced BioMatrix, San Diego, CA).

408 **shRNA gene knockdown and lentivirus gene transfer.**

409 Calcineurin A, isoform A-targeting shRNA in pLKO.1 vector was obtained from Sigma Aldrich
410 (SHCLNG-NM_008913). shRNA sequences were as follows: shRNA 1:
411 CCGGGCCAGGAATTGGATTCAGTTTCTCGAGAACTGAATCCAATTCCTGGCTTTTT
412 G (TRCN0000081058); shRNA2:
413 CCGGCGCCAACCTTAACTCCATCAACTCGAGTTGATGGAGTTAAGGTTGGCGTTTTT
414 G (TRCN0000081060); The scrambled shRNA control sequence was:
415 CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG, generated by
416 Sarbassov et al., (83) and was obtained from Addgene, Cambridge, MA (plasmid # 1864). The
417 shRNA lentiviral constructs were co-transfected with pLP1, pLP2 and pVSVG packaging
418 vectors (Invitrogen, Carlsbad, CA) into HEK293T cells with Lipofectamine 2000 reagent
419 (Invitrogen). The viral supernatant was harvested after 48h incubation and filtered through a
420 0.45 μm syringe filter. To infect C2C12 cells, 2.7×10^5 cells were incubated with 5 ml of the
421 filtered viral supernatant supplemented with $8 \mu\text{g ml}^{-1}$ of polybrene (Sigma-Aldrich) for 24 h.
422 The transduced cells were subsequently selected in media containing $2 \mu\text{g/ml}$ puromycin
423 (Invitrogen) for 4 days.

424 **RNA-Seq and Data Analysis.** Total RNA from C2C12 cells treated with FK506 or with DMSO
425 was isolated from proliferating and differentiating cultures (time 0, 24h and 72h) with TRIzol,
426 and libraries were constructed as described (84). Five replicates were performed for each sample
427 except for the 72h sample treated with DMSO, which had only four replicates. The libraries were
428 sequenced using the Illumina HiSeq 1500 and the resulting reads were mapped onto the
429 reference mouse genome (GRCm38) by HISAT2 (ver. 2.2.6) (85). Read counting per gene was
430 performed with HTseq (ver. 0.6.1) (86) such that duplicates in unique molecular identifiers were
431 discarded. After converting UMI counts to transcript counts as described (87), differentially

432 expressed genes, (those with adjusted p-value <0.1) were extracted by the R library DESeq2
433 (version 1.10.1) (88). The differentially expressed genes and cluster analyses are listed in
434 **Supplementary Tables S1 and S2**. Gene ontology (GO) term identification was performed on
435 metascape: <http://metascape.org>. Cluster gene analysis was performed using clusterProfiler (ver.
436 3/10.0), a multi-purpose, open-source tool to identify and compare functional relationships
437 between genes (50). RNA-seq data were deposited at the Gene Expression Omnibus (GEO)
438 database under accession number GSE125914. The reviewer access token is krexcswllodxwn.
439 Motif analysis was performed using HOMER motif discovery software as described (89). The
440 promoters of genes that were differentially expressed genes upon FK506 treatment were
441 analyzed for the enrichment of known motifs. For each gene, we looked for motif enrichment up
442 to 1 kb upstream of the TSS.

443 **RT-qPCR gene expression analysis.** RNA was extracted using TRIzol Reagent (Invitrogen)
444 and the yield determined by measuring OD₂₆₀. 1µg of total RNA was subjected to reverse
445 transcription with a QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD). The
446 resulting cDNA was used for quantitative PCR using a Fast SYBR green master mix (Applied
447 Biosystems, Foster City, CA). Amplification reactions were performed in duplicate in 10 µl final
448 volume that included the following: 25 ng of template, 0.3 µM primers, 2× SYBR Green Master
449 Mix. Reactions were processed in QuantStudio3 (Applied Biosystems) using following primers:
450 *Acta1* forward 5'-cttcctttatcggtatggagtctgcgg-3', reverse 5'-ggggggcgatgatcttcag-3'; *Cav3*
451 forward 5'- tcaatgaggacattgtgaaggtaga-3', reverse 5'-cagtgtagacaacaggcgg-3'; *Ckm* forward 5'-
452 ctgtcctggaagctctcaacagc-3', reverse 5'-ttttgtgtcgttgccagatgcc-3'; *Cxcl1*: forward 5'-
453 accgagtcatagccacactc-3', reverse 5'-ctccgttacttggggacacc-3'; *Eef1A1* forward 5'-
454 ggcttcactgctcaggtgattac-3', reverse 5'-acacatggccttgccaggac-3'; *Id1*: forward 5'-

455 aggtgaggcggcatgtgtccag-3', reverse 5'-accctctaccactggaccg-3'; *Larp1b*: forward 5'-
456 cagtcaagcagccagaggaa-3; reverse 5'-ctctcccgtctccaactcg-3'; *Myh4* forward 5'-
457 ggctttgagatcttgactcaacacc-3', reverse 5'-gagaagatgccatcggttctcg-3'; *Mymk* forward 5'-
458 gagaagatgccatcggttctcg-3', reverse 5'-gtcggccagtgccatcaggga-3'; *Myog* forward 5'-
459 gtcccaaccagagatcattgtctc-3', reverse 5'-cccacttaaagccccctgctac-3'; *Pix3r1*: forward 5'-
460 tggatttctgggaagtacgg-3', reverse 5'-aagccgaagatccgtaaggc-3'; *Ppp3ca*: forward 5'-
461 ggtcgggtgtgcagtc-3', reverse 5'-atggaacggcttcaccacc-3'; *Rrp12*: forward: 5'-
462 aagccgaagatccgtaaggc-3'. reverse: 5'-agggcctttcaccacaaca-3'; *Samhd1*: forward 5'-
463 gaggcagctcattcgggtgta-3'; reverse 5'-tgtcaccatcctgtggct-3'; *Tnnt3* forward 5'-
464 tgacaagctgagggacaagg-3', reverse 5'-tgcttctgggcttgtaaat-3'. ΔC_t for each gene was calculated
465 and represents the difference between the C_t value for the gene of interest and that of the
466 reference gene, *Eef1A1*. Fold-changes were calculated using the $2^{-\Delta\Delta C_t}$ method (90).

467 **Chromatin immunoprecipitation (ChIP) assay.** Chromatin immunoprecipitation assays were
468 performed as previously described (20, 91) with some modifications. Briefly, cells (4×10^6)
469 were cross-linked with 1% formaldehyde (Ted Pella Inc., Redding, CA) for 10 minutes at room
470 temperature. After quenching the formaldehyde with 125 mM glycine for 5 minutes, fixed cells
471 were washed twice with PBS supplemented with protease inhibitor cocktail and lysed with 1 ml
472 buffer A (10 mM Tris HCl, pH 7.5, 10 mM NaCl, 0.5% NP40, 0.5 μ M DTT and protease
473 inhibitors) by incubation on ice for 10 minutes. The nuclei were pelleted, washed with 1 ml of
474 buffer B (20 mM Tris HCl, pH 8.1, 15 mM NaCl, 60 mM KCl, 1 mM CaCl₂, 0.5 μ M DTT) and
475 incubated for 30 minutes at 37°C in the presence of 1000 gel units of micrococcal nuclease
476 (#M0247S, NEB, Ipswich, MA) in 300 μ l volume of buffer B. The reaction was stopped by
477 adding 15 μ l of 0.5 M EDTA. Nuclei were pelleted and resuspended in 300 μ l of ChIP buffer

478 (100 mM Tris HCl, pH 8.1, 20 mM EDTA, 200 mM NaCl, 0.2 % sodium deoxycholate, 2%
479 Triton X 100 and protease inhibitors), sonicated for 10 minutes (medium intensity, 30 sec-on/ 30
480 sec-off) in a Bioruptor UCD-200 (Diagenode, Denville, NJ) and centrifuged at $21,000 \times g$ for 5
481 minutes. The fragmented chromatin was between 200–500 bp as analyzed on agarose gels.
482 Chromatin concentration was measured using Qubit 3 (Invitrogen). After preclearing with
483 protein A agarose, chromatin (2-4 μg) was subjected to immunoprecipitation with specific
484 antibodies listed above, or with anti-IgG as negative control at 4°C overnight, and
485 immunocomplexes were recovered by incubation with protein A agarose magnetic beads
486 (Invitrogen). Sequential washes of 5 minutes each were performed with buffers A-D (Buffer A:
487 50 mM Tris pH 8.1, 10 mM EDTA, 100 mM NaCl, 1% Triton X 100, 0.1% sodium
488 deoxycholate; Buffer B: 50 mM Tris pH 8.1, 2 mM EDTA, 500 NaCl, 1% Triton-X100, 0.1%
489 sodium deoxycholate; Buffer C: 10 mM Tris pH 8.1, 1 mM EDTA, 0.25 M LiCl_2 ; 1% NP-40,
490 1% sodium deoxycholate; Buffer D: 10 mM Tris pH 8.1, 1 mM EDTA), immune complexes
491 were eluted in 100 μl of elution buffer (0.1 M NaHCO_3 , 1% SDS) for 30 minutes at 65°C ,
492 incubated with 1 μl of RNase A (0.5 mg/ml) for 30 minutes at 37°C , and reverse cross-linked
493 by adding 6 μl of 5M NaCl and 1 μl of proteinase K (1 mg/ml) overnight at 65°C . DNA was
494 purified using the ChIP DNA clean & concentrator kit (Zymo Research, Irvine, CA). Bound
495 DNA fragments were analyzed by quantitative PCR using the SYBR Green Master Mix.
496 Quantification was performed using the fold enrichment method ($2^{-(\text{Ct sample} - \text{Ct IgG})}$) and shown as
497 relative to a control region, the promoter for the *Pdx1* gene. Primer sequences used were: *Acta1*
498 forward 5'-tggtgctgcccttccaagccatatt-3', reverse 5'-gcagacagctgggatactctccatat-3'; *Cav3*
499 forward 5'-cctaggtgtctcagtcagttta-3', reverse 5'-ctgccacgtagatcttgaaat-3'; *Ckm* forward 5'-
500 gacacccgagatgcctgtt-3', reverse 5'-gatccaccaggacagggtt-3'; *Myh4* forward 5'-

501 cacccaagccgggagaaacagcc-3', reverse 5'-gaggaaggacaggacagaggcacc-3'; *Mymk* forward 5'-
502 ctgacagcagggttagggct-3', reverse 5'-tgatgtacccttctcccc-3'; *Myog* forward 5'-
503 acaccaactgctgggtgcca-3', reverse 5'-gaatcacatgtaaccactgg-3'; *Tnnt3* forward 5'-
504 gcagctgacaccttctggaac-3', reverse 5'-attggccagcagatgggtgg-3'. The silent gene promoter *Pdx1*
505 was used as a negative control, as shown previously (62); forward 5'-
506 gaagtctccggacatctcccatacgaag-3', reverse 5'-ggattcatccacgggaaaggagctggac-3'. Primer
507 positions and localization of E-boxes for each regulatory sequence (53-61) are shown
508 schematically in Fig. 6A.

509 **Immunoprecipitation.** 100 mm dishes of 24h differentiated C2C12 cells treated with FK506 or
510 with DMSO or 100 mm dishes of primary myoblasts expressing wildtype or Brg1 mutants were
511 washed with ice-cold PBS twice and lysed in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.5,
512 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1mM CaCl₂ and supplemented
513 with complete protease inhibitors). Lysates were cleared by centrifugation and precleared with
514 PureProteome Protein A magnetic beads (Millipore) for 2 hours at 4°C. Next, cell extract was
515 incubated with 10 µl of Pan-Calceinurin A antibody (Cell Signaling Technologies) or 10 µl of
516 Brg1 antisera overnight at 4°C, followed by incubation with 100 µl with PureProteome Protein A
517 mix magnetic beads. After extensive washing of beads with washing buffer (24 mM Tris-HCl,
518 pH 7.5, 300 mM NaCl, 0.5% NP-40 and 1mM CaCl₂), precipitated proteins were eluted in
519 Laemmli buffer and detected by western blot analysis using chemiluminescent detection.

520 **Immunofluorescence.** Cells grown on coverslips were fixed with 3% paraformaldehyde,
521 permeabilized with 0.5% Triton X-100, and blocked with 1% BSA solution in PBS. Primary and
522 secondary antibodies were diluted in 1% blocking solution. The primary antibody against myosin
523 heavy chain, mAb MF20 was used at a dilution of 1:100. Goat anti-mouse secondary antibody

524 conjugated with Alexa 488 (Jackson Labs, West Grove, PA) were applied for 1 h at room
525 temperature. DAPI staining was used to visualize nuclei. The samples were mounted with
526 ProLong Gold antifade reagent (Life Technologies) and observed with inverted fluorescence
527 microscopy (Leica DMI6000). Leica LAS AF Lite software was used for recording and image
528 processing.

529 **Subcellular fractionation.** Cell fractionation was performed as described (92). Briefly, cell
530 pellets were resuspended in hypotonic buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM
531 KCl, 0.5 mM DTT supplemented with protease inhibitor cocktail). Cell suspension was
532 centrifuged, and the supernatant discarded. Intact pelleted cells were resuspended in buffer A and
533 pushed through a 26G needle to disrupt the cell membrane. Disrupted cell suspension was
534 centrifuged at 10,000 × g for 20 minutes and the supernatant was saved as the cytosolic fraction.
535 Pelleted nuclei were resuspended in buffer C (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 0.42 M
536 NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM DTT supplemented with complete protease
537 inhibitor cocktail) and disrupted by pushing 10 times through a 26G needle. The nuclear
538 suspension was centrifuged at 20,000 × g for 5 min and the supernatant was saved as the nuclear
539 extract. Chromatin isolation was performed as described previously (21). Cells were resuspended
540 in cytoskeleton buffer (CSK: 10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM
541 MgCl₂, 1 mM EGTA, 1 mM DTT, and 0.5% (v/v) Triton X-100 and complete protease inhibitor
542 cocktail). The cytosolic fraction was separated from soluble proteins by centrifugation and the
543 pellet was resuspended in CSK buffer. Chromatin was solubilized by DNase I digestion (New
544 England Biolabs) in CSK buffer for 15 min at 37 °C. 0.25 M (NH₄)₂SO₄ was added, and the
545 samples were incubated for 5 min at 4 °C, centrifuged at 5,000 × g for 3 min and supernatant was
546 saved as a soluble chromatin fraction.

547 **Statistical analysis.** All quantitative ChIP and RT-qPCR data are shown as mean +/- the
548 standard deviation (SD) of at least three (n=3) biological replicates for each experiment.
549 Statistical analysis was performed using GraphPad Prism Student's t- test (GraphPad Software
550 Inc.). For all analyses, a *P*-value of less than 0.05 was considered to be statistically significant.

551 **ACKNOWLEDGEMENTS**

552 This work was supported by NIH grant GM56244 to ANI, by a Faculty Diversity Scholars
553 Program award from the University of Massachusetts Medical School to TP-B, and by
554 MEXT/JSPS KAKENHI (JP25116010, JP17H03608, JP17K19356, JP18H04802 and
555 JP18H05527) and JST CREST JPMJCR16G1 to YO. We also thank the Advanced
556 Computational Scientific Program of the Research Institute for Information Technology, Kyushu
557 University, and the National Institute of Genetics for providing high-performance computing
558 resources.

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811

812 **FIGURE LEGENDS**

813 **Figure 1: Differential gene expression in myoblasts treated with Cn inhibitor.** (A) Myotube
814 formation in differentiating C2C12 cells is inhibited by FK506 treatment. The cells were fixed
815 and analyzed by immunofluorescence staining using the anti-myosin heavy chain monoclonal
816 Ab MF20 (green). The nuclei were visualized by DAPI staining (blue). Scale bar, 20 μ m. (B)
817 Volcano plots displaying differentially expressed genes between control (DMSO) treated and Cn
818 inhibitor (FK506) treated differentiated C2C12 cells. The y-axis corresponds to the mean
819 expression value of log₁₀ (p-value), and the x-axis displays the log₂ fold change value. The red
820 dots represent the up- and down- regulated transcripts between DMSO- and FK506-treated
821 samples (False Discovery Rate (FDR)<0.05). The black dots represent the expression of
822 transcripts that did not reach statistical significance (FDR>0.05). (C) A Venn diagram displaying
823 the number of genes up- and down- regulated by FK506 treatment at 24 and 72h post-
824 differentiation. (D) Transcription factor binding motifs identified within 1kb upstream of the TSS
825 of genes differentially expressed in cells differentiated for 24 and 72h in the presence of FK506.
826 Motifs displayed had *p* values ≤ 0.001 .

827

828 **Figure 2: Enriched GO term analysis of differentially expressed genes.** (A) Gene ontology
829 analyses on genes differentially expressed by FK506 treatment for 24 and 72h post-
830 differentiation. (B) GO analysis of the biological processes shows differences between
831 treatments (DMSO vs FK506) in muscle-related categories.

832

833 **Figure 3: Cluster analysis of differentially expressed genes at three differentiation time**
834 **points in myoblasts treated with Cn inhibitor.** (A) The heat map comparing differential

835 expression of 308 FK506 treatment-specific genes, categorized in four different clusters. Each
836 column represents an experimental sample (times 0, 24 and 72h in differentiation medium
837 (DM)) compared to the proliferating myoblast sample cultured in growth media (GM). Each
838 row represents a specific gene. The colors range from yellow (high expression) to magenta
839 (low expression) and represent the relative expression level value log₂ ratios. **(B)** Kinetic
840 expression patterns of the four clusters of genes. Lines represent the LOESS fitting for the
841 relative expression for all genes in the cluster. The grey shading represents 95% confidence
842 intervals. **(C)** Gene ontology analysis of differentially expressed genes within clusters 1-3 (C1,
843 C2, C3) identified the top enriched GO terms with the corresponding enrichment *p* values and
844 gene ratio.

845

846 **Figure 4: Cn regulates the expression of myogenic and other genes during myoblast**
847 **differentiation.** **(A)** Real time RT-PCR analysis validated expression changes of genes from
848 clusters 1, 3 and 4 in FK506 treated cells. **(B)** RT-qPCR showed that expression of cluster 2
849 genes *Myog*, *Ckm*, *Myh4*, *Mymk*, *Tnnt3*, *Acta 1*, and *Cav 3* is down-regulated in FK506-treated
850 C2C12 cells. GM, proliferating cells in growth medium. DM, differentiation medium for the
851 indicated time in hours (h). Data are the average of three or more independent samples
852 performed in duplicate and are presented as the mean +/- SD. Expression in DMSO-treated GM
853 samples were set to 1 and other values are relative to that sample. * $p \leq 0.05$, ** $p \leq 0.001$,
854 *** $p \leq 0.0001$ vs. GM or vehicle by Student's t-test.

855

856 **Figure 5: FK506 treatment did not affect intracellular (A) or chromatin localization (B) of**
857 **Cn and Brg1.** Representative western blots of CnA, Brg1 and marker proteins in cytosolic,

858 nuclear and chromatin fractions derived from proliferating and differentiating myoblasts. GM,
859 proliferating cells in growth medium. DM, differentiation medium for the indicated time in
860 hours.

861

862 **Figure 6: Cn binds to E-box containing regulatory sequences of myogenic genes during**
863 **myoblast differentiation. Cn binding is reduced by FK506 treatment. (A) A schematic**
864 **diagram of regulatory sequences of myogenic genes and localization of E-boxes and primers**
865 **used in ChIP assays. (B) ChIP assays were performed for Cn binding in C2C12 cells. GM,**
866 **proliferating cells in growth medium. DM, differentiation medium for the indicated time in hours**
867 **(h). Relative enrichment was defined as the ratio of amplification of the PCR product normalized**
868 **to control IgG and is shown relative to amplification of a non-specific control promoter region**
869 **(*Pdx1*). The data are average of at least 3 independent experiments performed in triplicate +/-**
870 **SD. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs. GM or vehicle by Student's t-test.**

871

872 **Figure 7: Cn inhibition reduces binding of the SWI/SNF subunits Brg1 (A), Baf170 and**
873 **Baf250A (B) to E-box containing regulatory sequences of myogenic genes during myoblast**
874 **differentiation. ChIP assays were performed for Brg1, Baf170 and Baf250A binding in C2C12**
875 **cells. GM, proliferating cells in growth medium. DM, differentiation medium for the indicated**
876 **time in hours (h). Relative enrichment was defined as the ratio of amplification of the PCR**
877 **product normalized to control IgG and is shown relative to amplification of a non-specific**
878 **control promoter region (*Pdx1*). The data are average of at least 3 independent experiments**
879 **performed in triplicate +/- SD. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs. GM or vehicle by**
880 **Student's t-test.**

881

882 **Figure 8: Phosphomimetic mutation of Brg1 amino acids that are dephosphorylated by Cn**
883 **reduces Cn and Brg1 binding to myogenic promoters in differentiating myoblasts.** ChIP
884 assays were performed for Cn or Brg1 binding in primary mouse myoblasts (C57Bl/6), in
885 primary mouse myoblasts deleted for the gene encoding Brg1 (Brg1c/c), or in primary mouse
886 myoblasts deleted for the gene encoding Brg1 that are expressing a wildtype (WT-Brg1), Brg1
887 containing a non-phosphorylatable amino acid at Cn-targeted sites (SA-Brg1), or Brg1
888 containing a phosphomimetic amino acid at Cn-targeted sites (SE-Brg1). Samples were collected
889 from proliferating cells in growth medium (GM) or at 24h post-differentiation (DM). Relative
890 enrichment was defined as the ratio of amplification of the PCR product normalized to control
891 IgG and is shown relative to amplification of a non-specific control promoter region (*Pdx1*). The
892 data are average of 3 independent experiments performed in triplicate +/- SD. * $p \leq 0.05$, ** p
893 ≤ 0.01 , *** $p \leq 0.001$ by Student's t-test.

894

895 **Figure 9: Interaction between Cn and Brg1 is differentiation-dependent and is not affected**
896 **by Cn inhibition or mutation of Brg1 amino acids that are targeted by Cn.** (A) Co-
897 immunoprecipitation of Cn and Brg1 from cell lysates from 48h differentiated C2C12 cells
898 treated with FK506. (B) Co-immunoprecipitation of Cn and Brg1 from cell lysates from
899 proliferating and 24h differentiated primary mouse myoblasts. (C) Co-immunoprecipitation of
900 Cn and Brg1 and MyoD and Brg1 from cell lysates from 24h differentiated primary C57Bl/6
901 myoblasts and from primary mouse myoblasts deleted for the gene encoding Brg1 that are
902 expressing a wildtype (WT-Brg1), Brg1 containing non-phosphorylatable amino acids at Cn-
903 targeted sites (SA-Brg1), or Brg1 containing phosphomimetic amino acids at Cn-targeted sites

904 (SE-Brg1). **(D)** Brg1 does not co-immunoprecipitate with Cn from Brg1 conditional myoblasts
905 depleted for Brg1 and subjected to differentiation. Cell lysate from each IP (2.5% of input)
906 served as a loading controls. The experiments were performed 3 times and representative gels are
907 shown.

908

909 **Figure 10: Inhibition of Cn reduced MyoD binding to regulatory sequences of myogenic**
910 **genes during myoblast differentiation.** ChIP assays were performed for MyoD binding in
911 C2C12 cells. GM, proliferating cells in growth medium. DM, differentiation medium for the
912 indicated time in hours (h). Relative enrichment was defined as the ratio of amplification of the
913 PCR product normalized to control IgG and is shown relative to amplification of a non-specific
914 control promoter region (*Pdx1*). The data are average of at least 3 independent experiments
915 performed in triplicate +/- SD. * $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$ vs. GM or vehicle by
916 Student's t-test.

917

918 **Figure 11: Knockdown of Cn recapitulates findings made with Cn inhibitors and mutation**
919 **of Brg1 amino acids that are targeted by Cn.** **(A)** Western blot and RT-qPCR analysis showed
920 shRNA-mediated knockdown of endogenous CnA in proliferating C2C12 cells. A scramble
921 shRNA construct (shCtrl) was used as a control. **(B)** Representative images of myosin heavy
922 chain staining in 72h differentiated cells expressing the control or Cn-targeting shRNAs. The
923 cells were fixed and analyzed by immunofluorescence using an anti-myosin heavy chain mAb
924 MF20 (*green*). The nuclei were visualized by DAPI staining (*blue*). Scale bar, 20 μm . **(C)** RT-
925 qPCR showed that expression of *Myog*, *Ckm*, *Myh4*, *Cav3*, *Mymk*, *Tnnt3* and *Acta1* is down-
926 regulated in Cn knockdown cells. GM, proliferating cells in growth medium. DM, differentiation

927 medium for the indicated time in hours (h). **(D)** RT-qPCR analysis validated expression of a
928 representative gene from RNA seq analysis clusters 1, 3 and 4 and confirmed that their
929 expression is changed in Cn knockdown cells compared to the scramble shRNA control. Data are
930 the average of three independent samples performed in duplicate and are presented as the mean
931 +/- SD. Expression in scramble shRNA expressing cells (panel A) or GM samples (panels C and
932 D) were set to 1 and other values are relative to those samples. * $p \leq 0.05$, ** $p \leq 0.001$,
933 *** $p \leq 0.0001$ by Student's t-test.

934

935 **Figure 12: Cn knockdown blocked binding of Cn (A), Brg1 (B) and MyoD (C) to E-box**
936 **containing regulatory sequences of myogenic genes during myoblast differentiation.** ChIP
937 assays were performed for Cn, Brg1 and MyoD binding in control and Cn knockdown C2C12
938 cells. Cn was knocked down by two independent shRNA constructs. GM, proliferating cells in
939 growth medium. DM, differentiation medium for the indicated time in hours (h). Relative
940 enrichment was defined as the ratio of amplification of the PCR product normalized to control
941 IgG and is shown relative to amplification of a non-specific control promoter region (*Pdx1*). The
942 data are average of at least 3 independent experiments performed in triplicate +/- SD. * $p \leq 0.05$,
943 ** $p \leq 0.01$, *** $p \leq 0.001$ by Student's t-test.

Figure 1

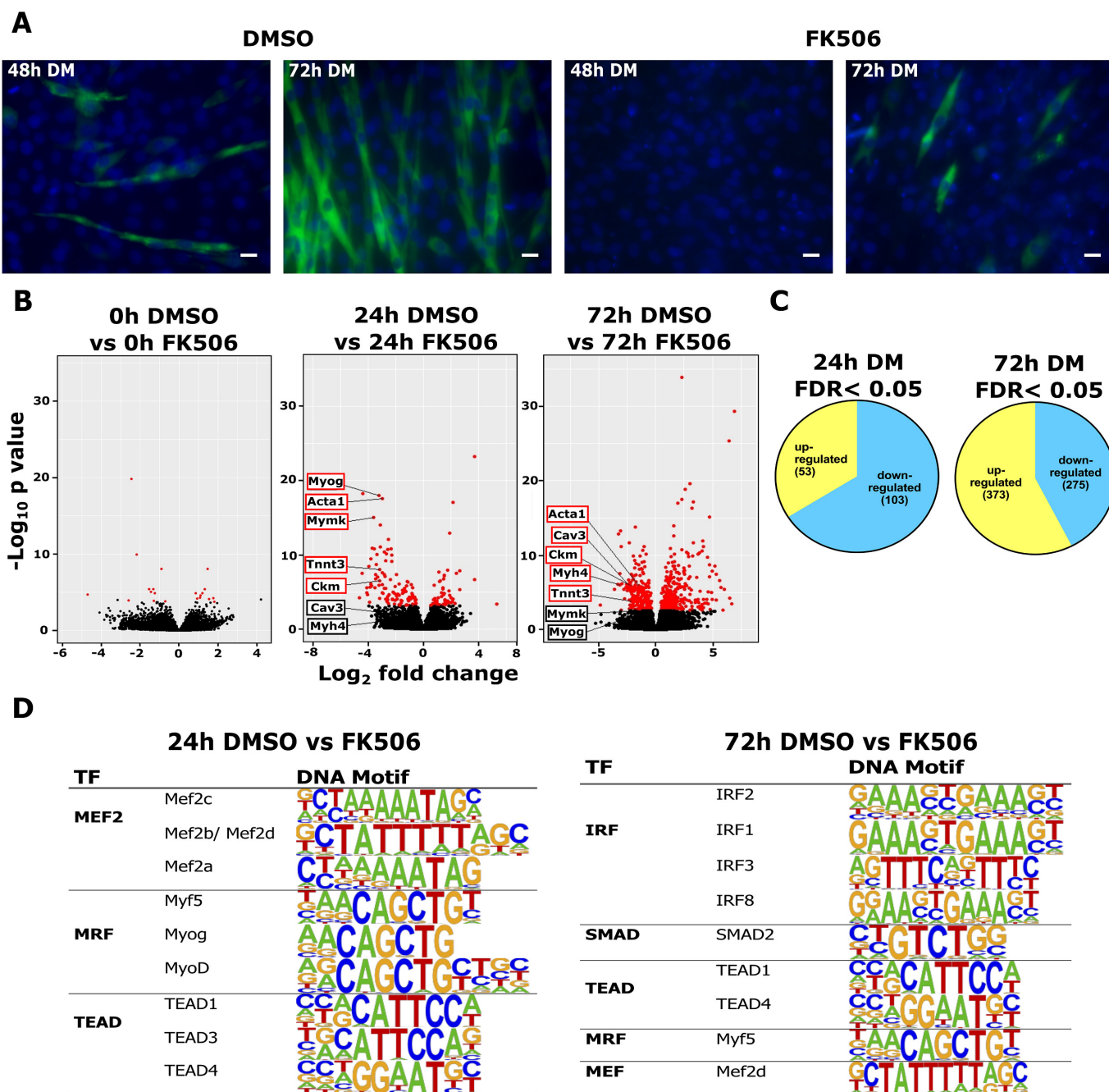


Figure 2

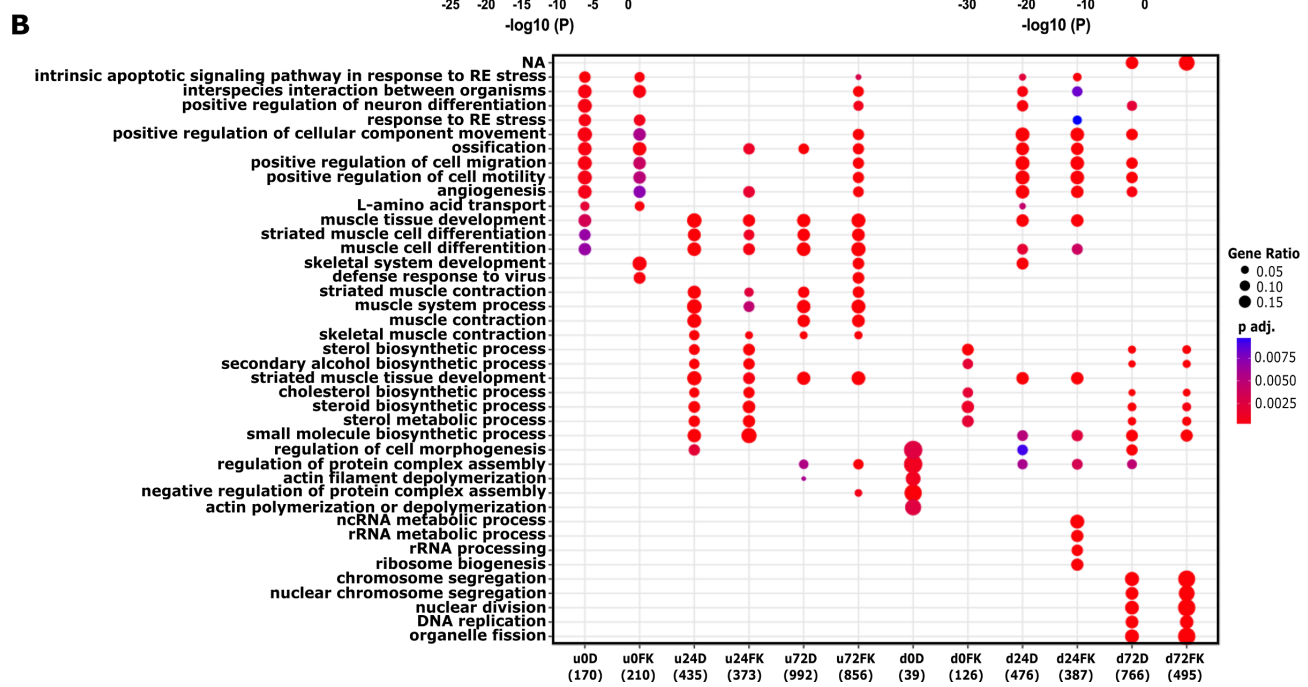
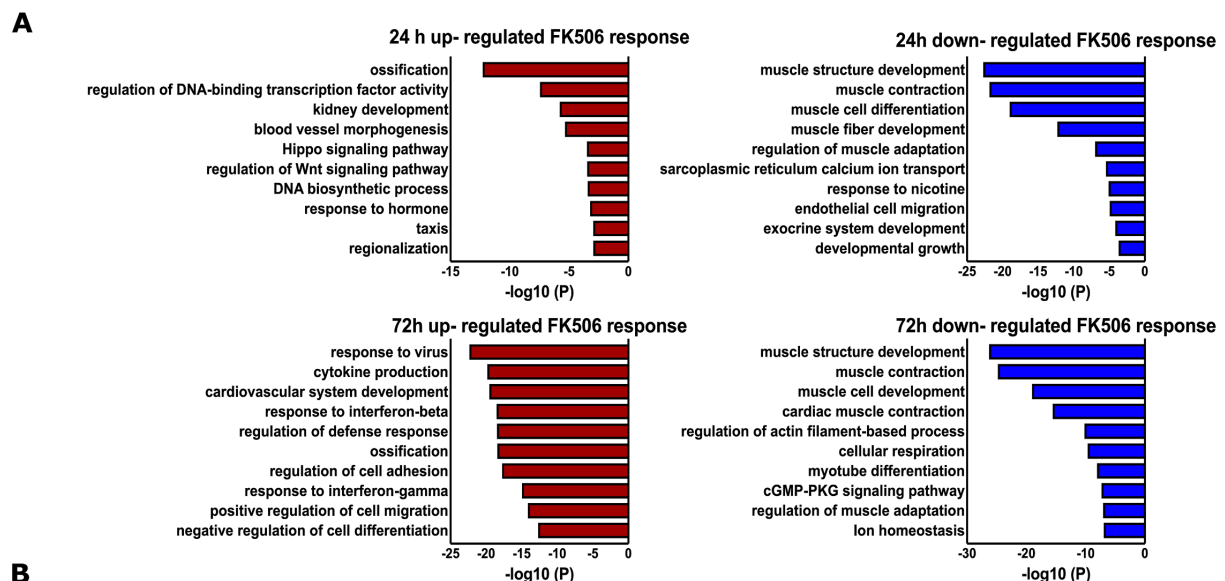


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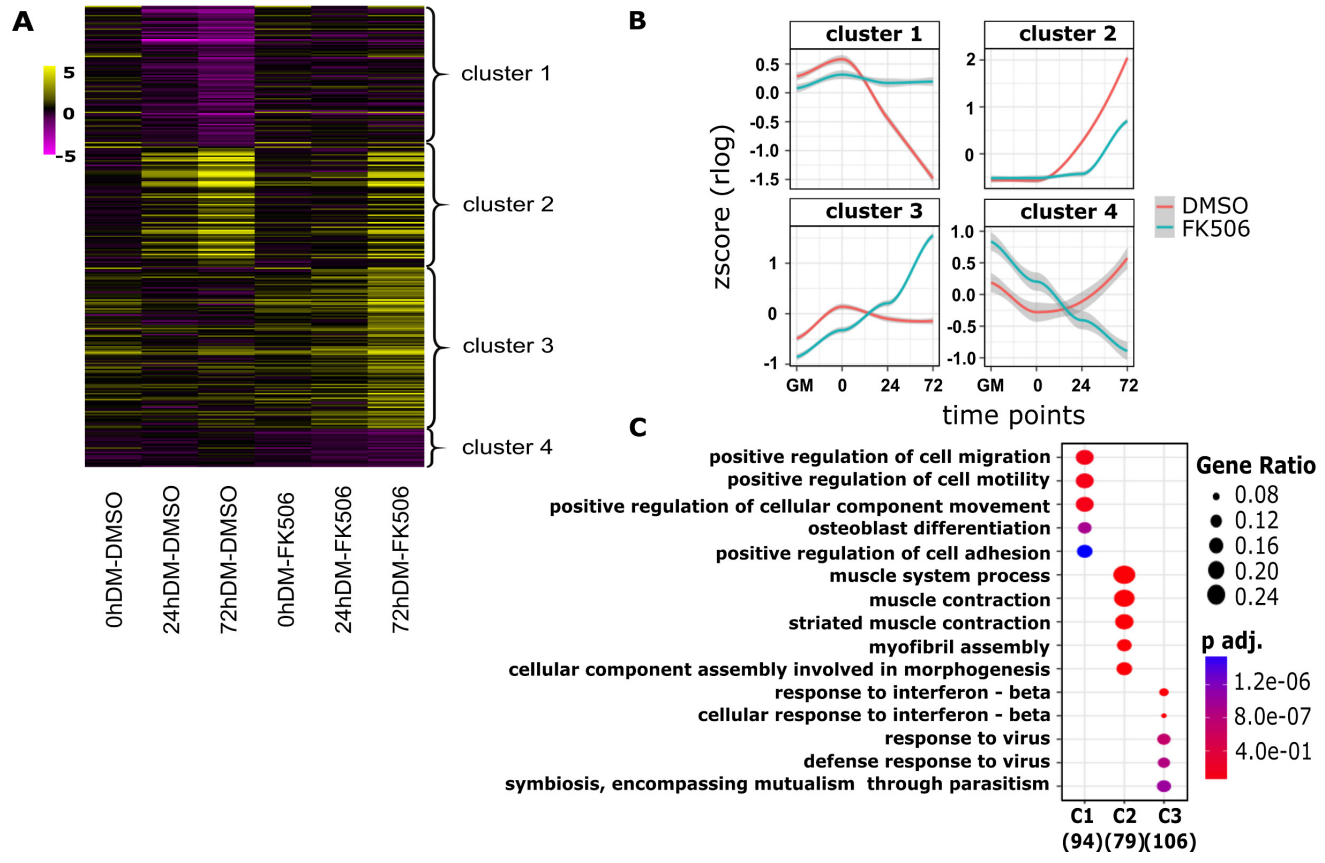


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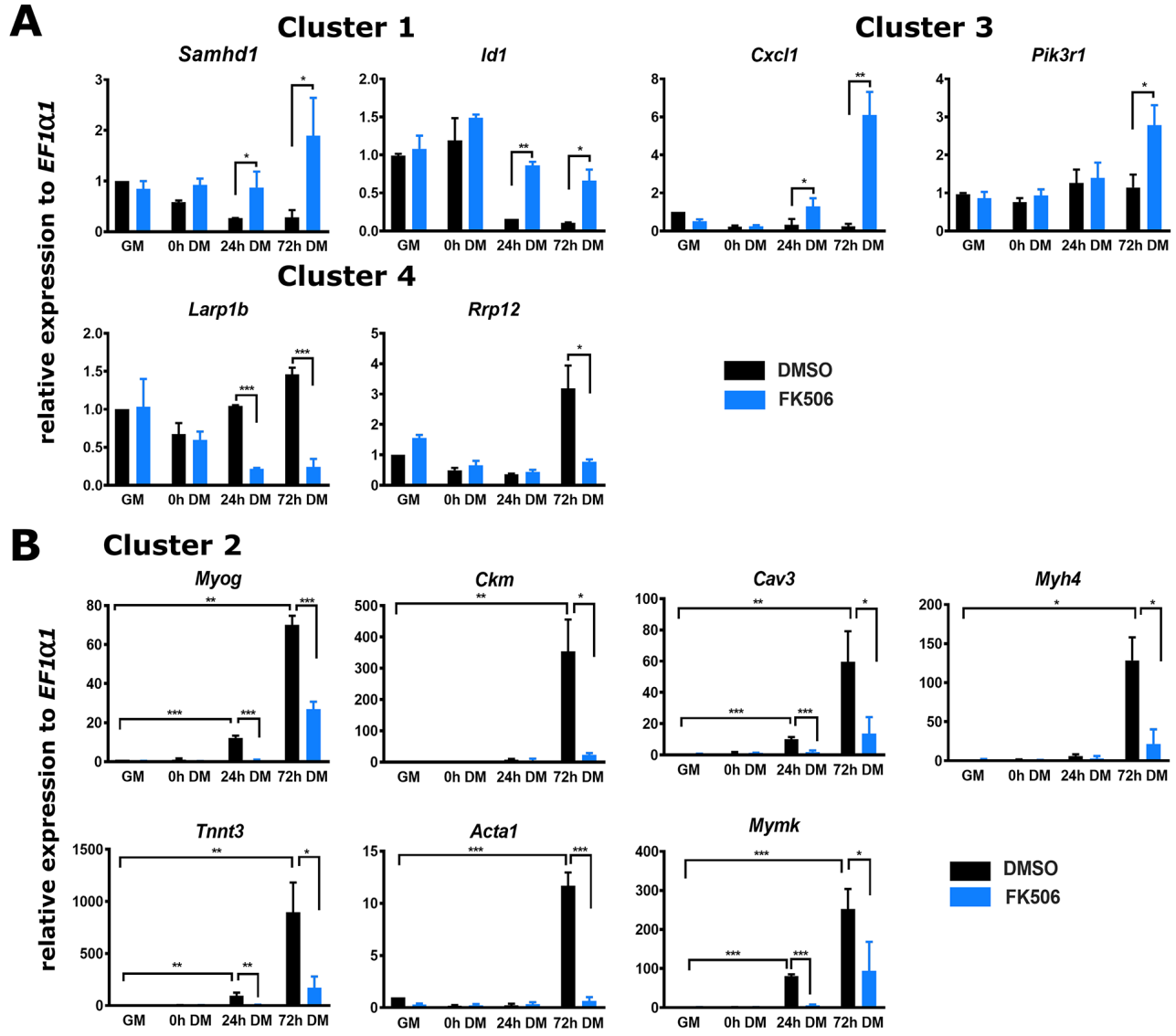


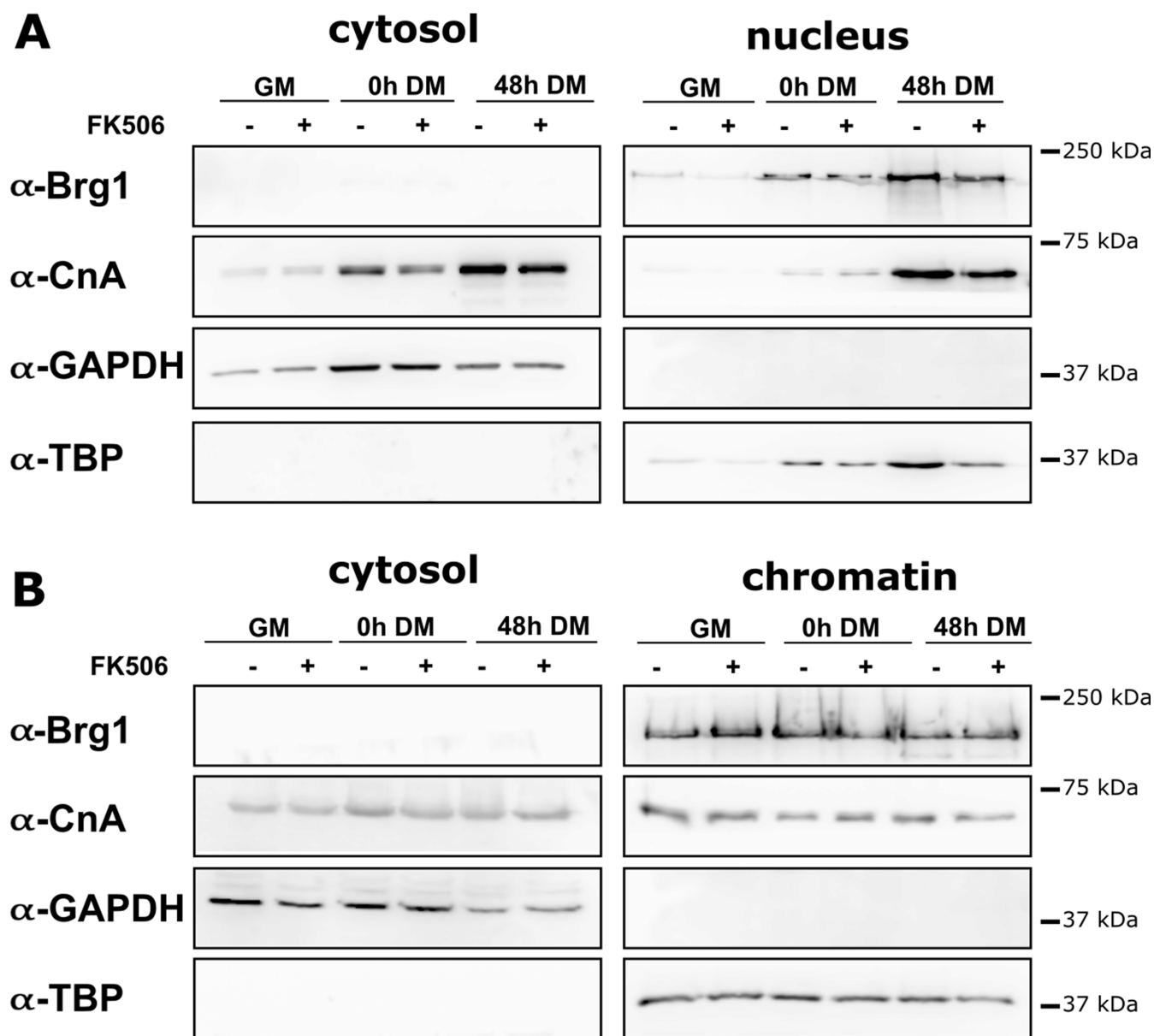
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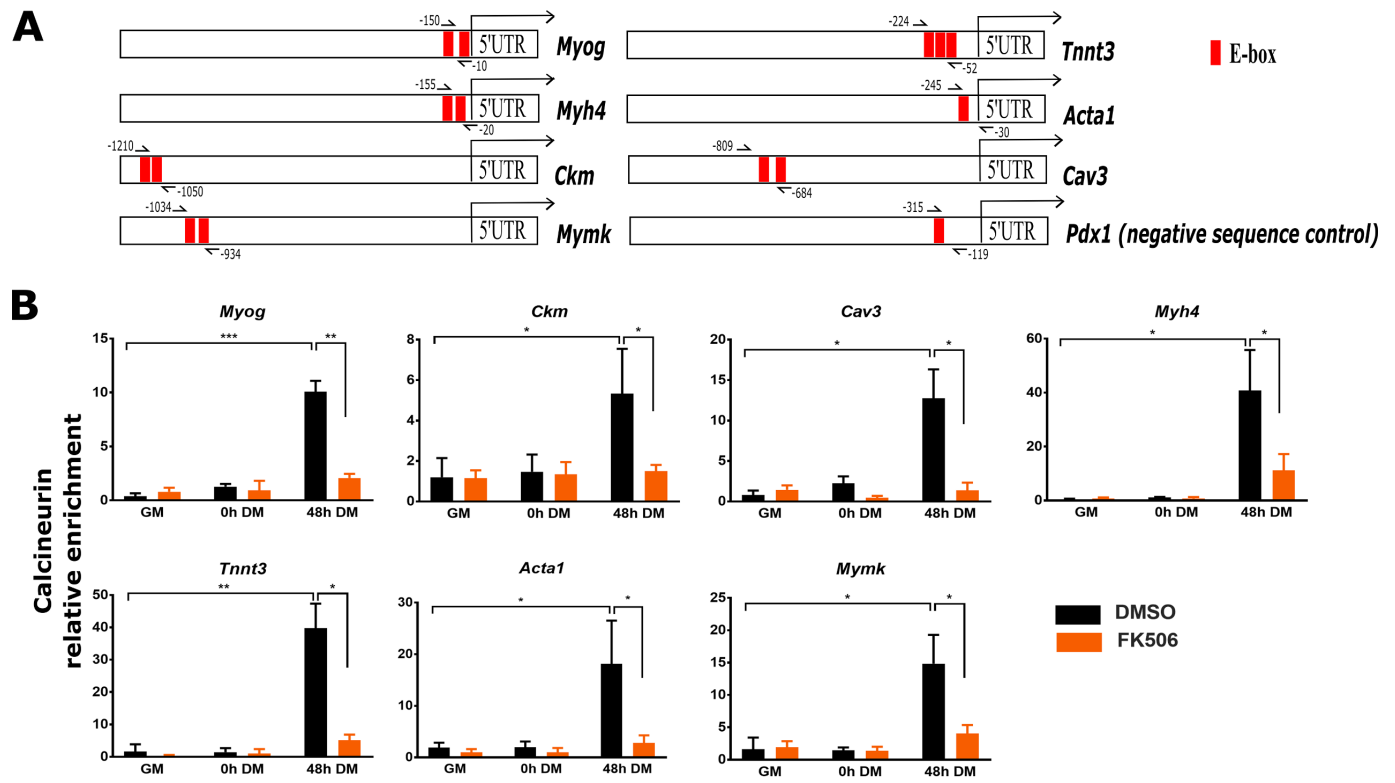
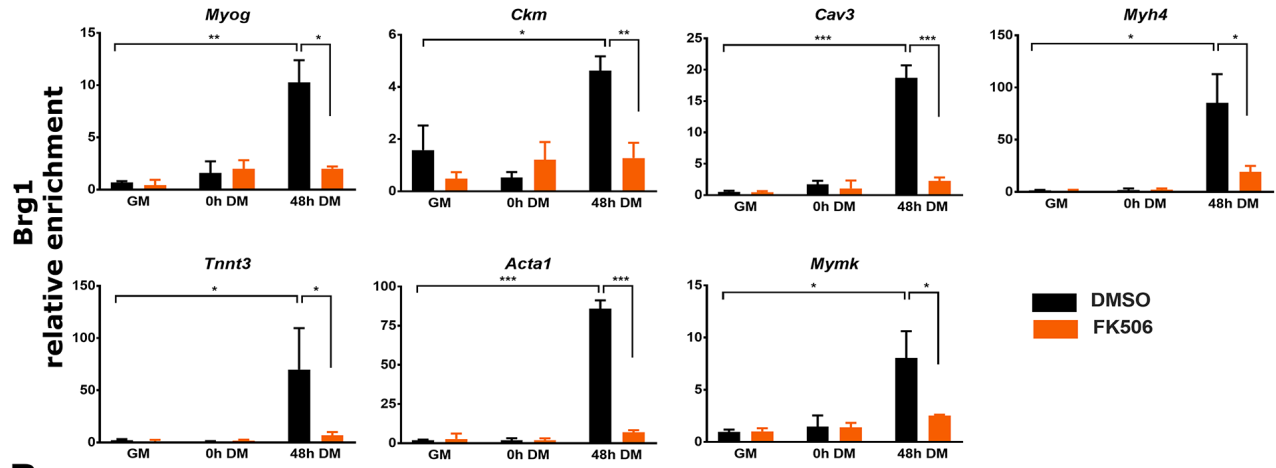
Figure 6

Figure 7

A



B

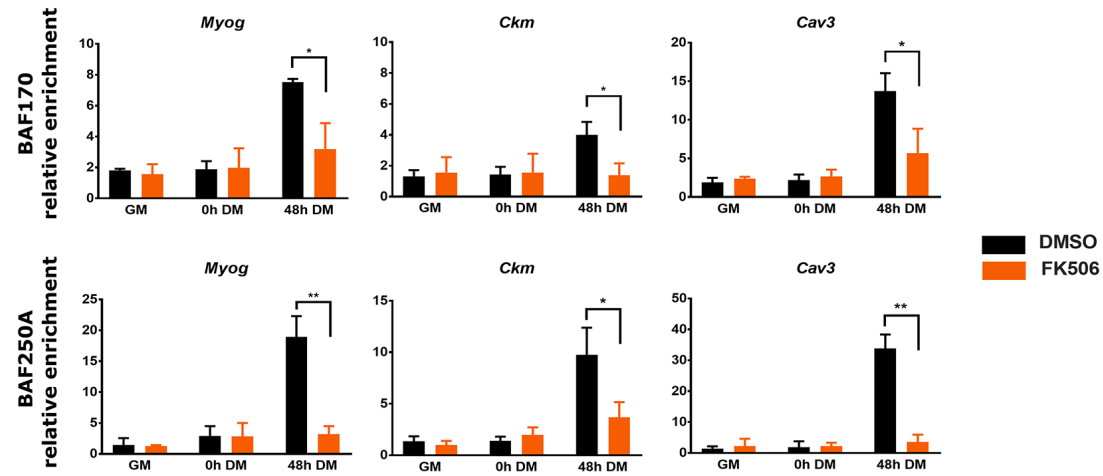


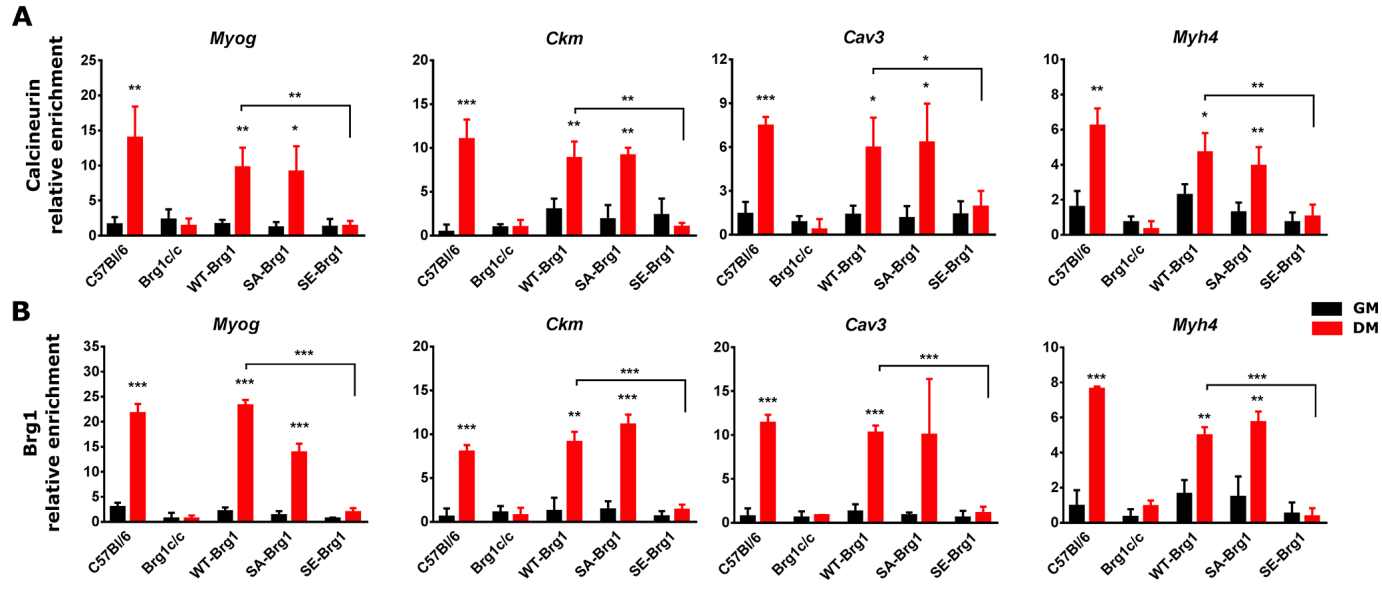
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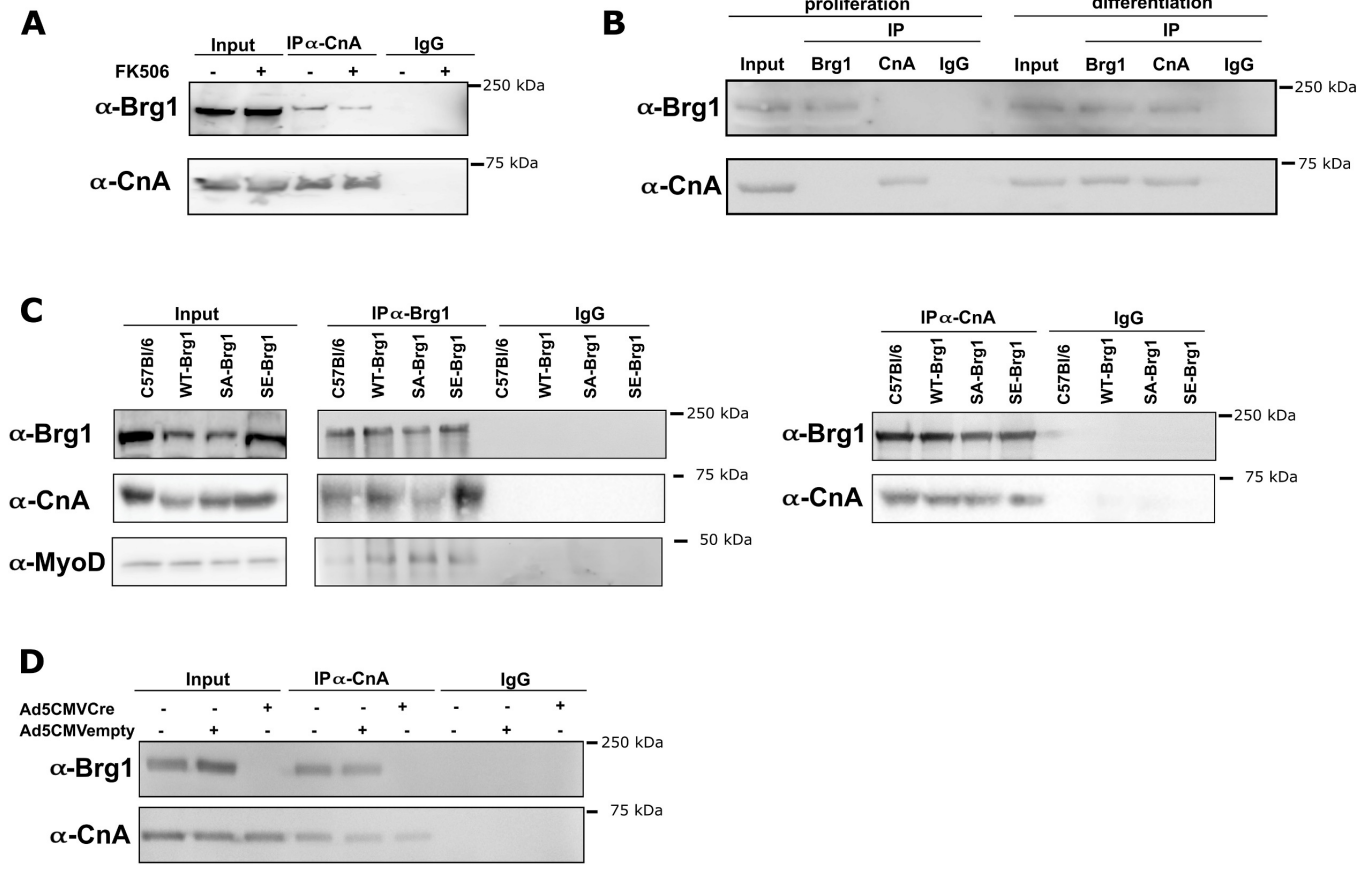
Figure 9

Figure 10

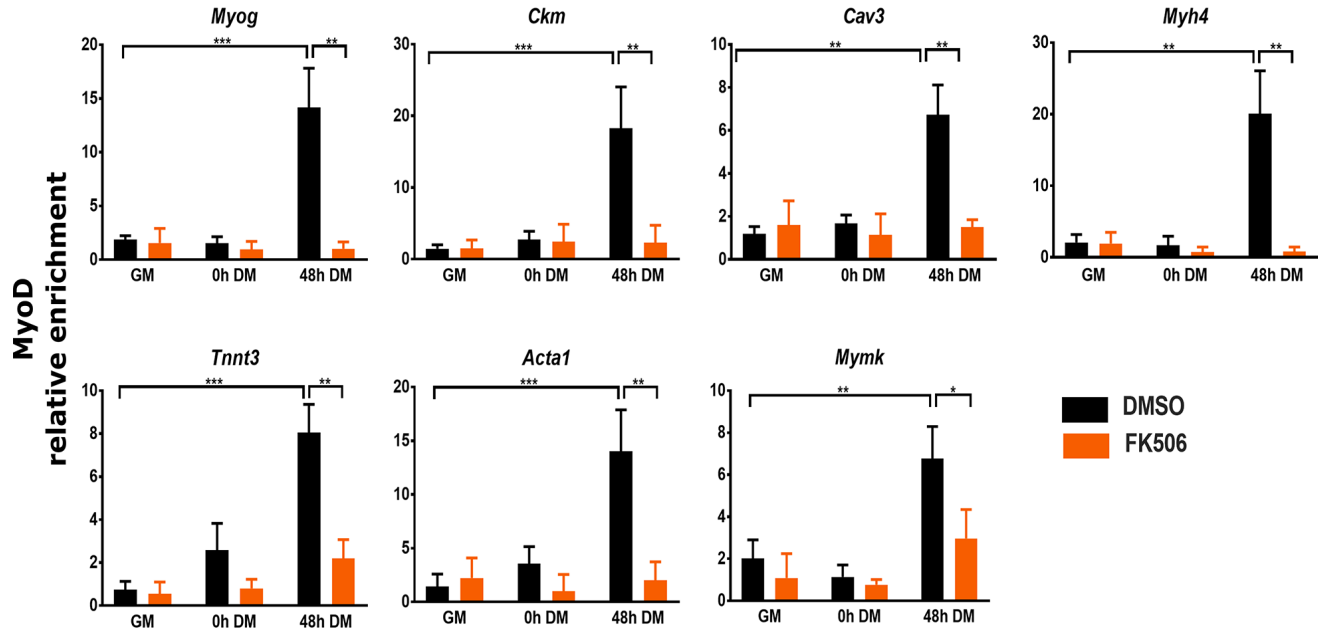


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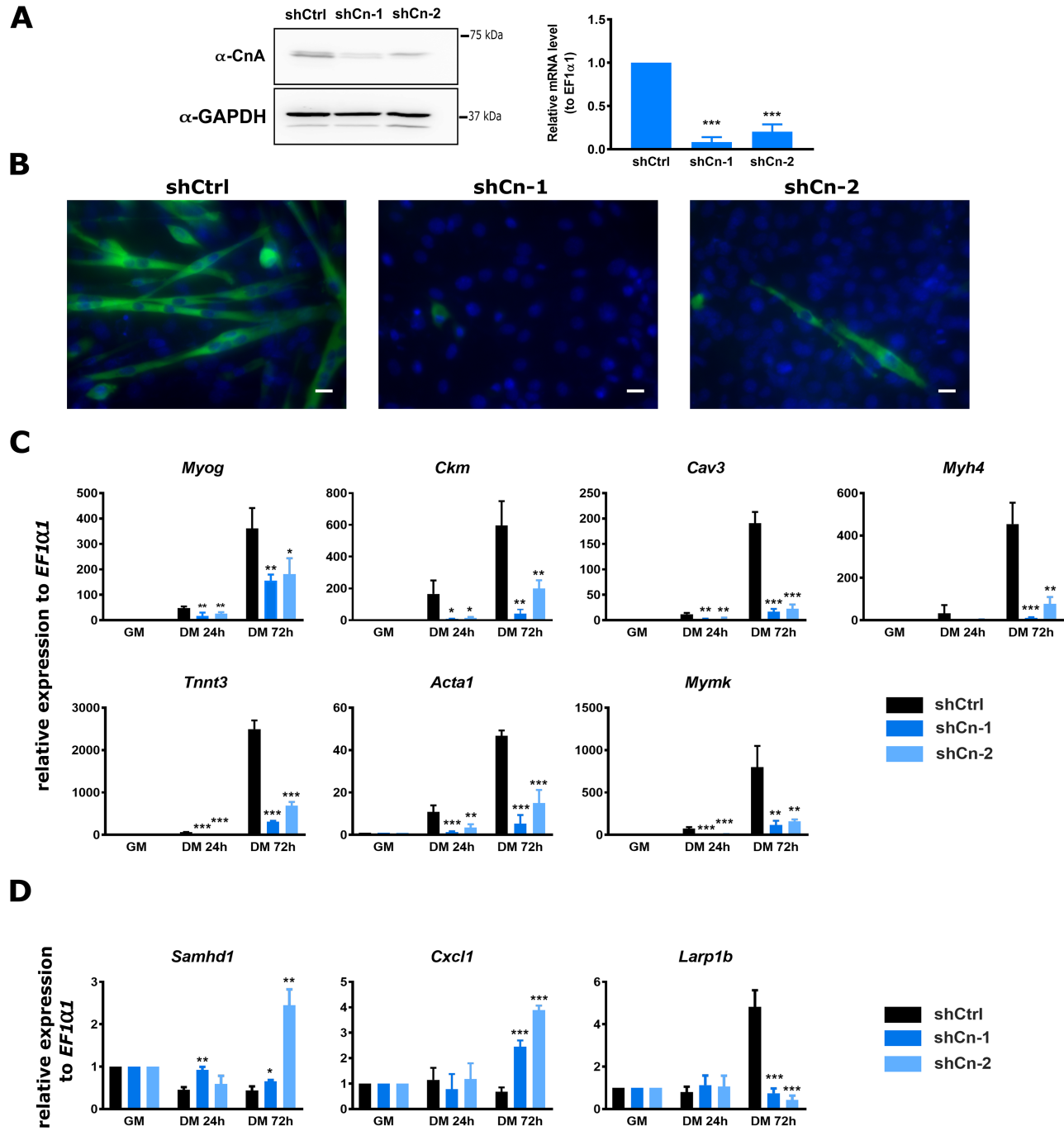


Figure 12

