

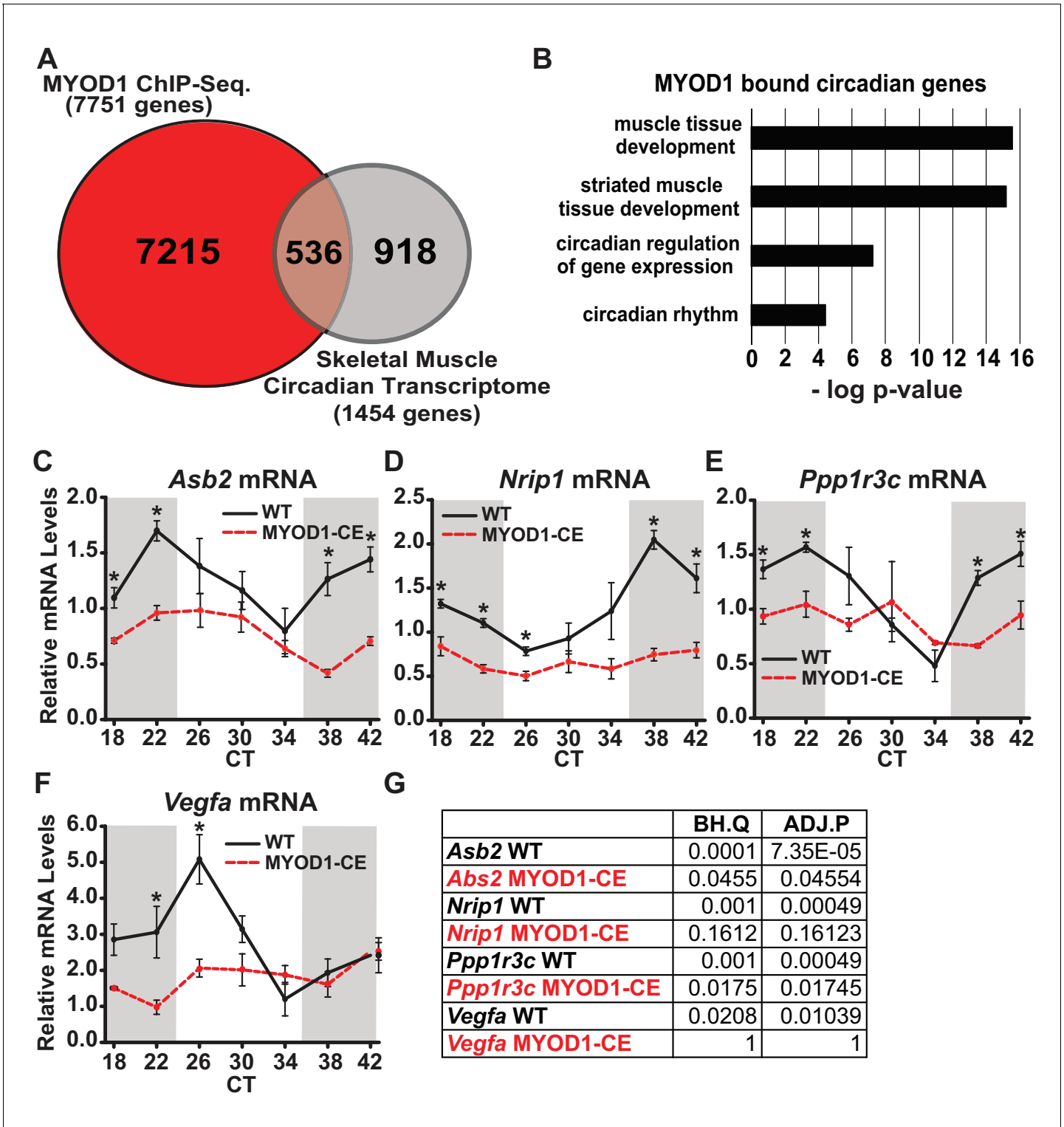


---

## Figures and figure supplements

MYOD1 functions as a clock amplifier as well as a critical co-factor for downstream circadian gene expression in muscle

**Brian A Hodge et al**

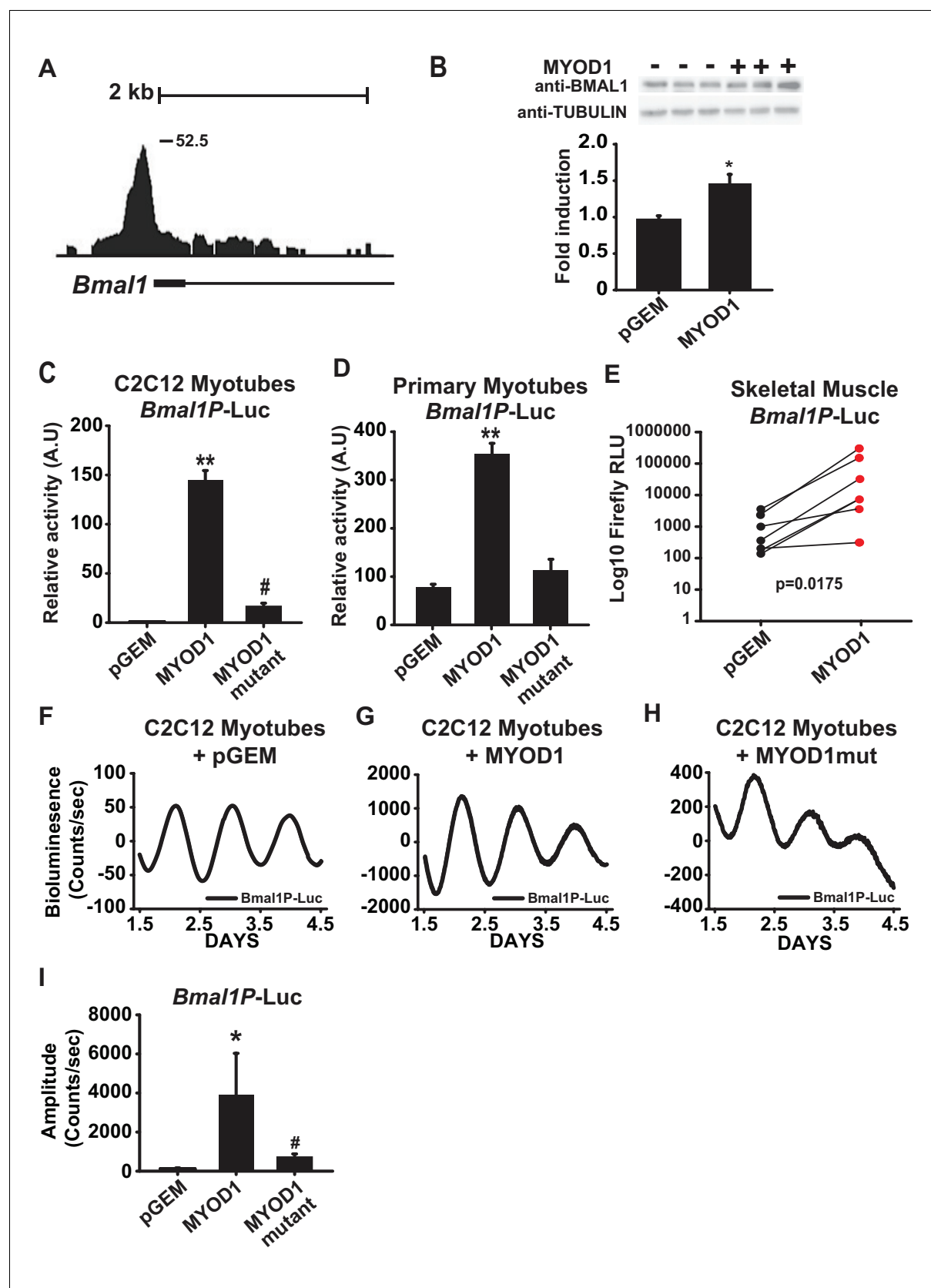


**Figure 1.** MYOD1 binding coverage on skeletal muscle circadian genes. (A) Overlap of genes bound by MYOD1 (red) and circadian genes (grey) in adult skeletal muscle (JTK\_CYCLE p-value < 0.03). (B) Gene-ontology enrichment terms for MYOD1-bound, circadian genes in adult skeletal muscle. (C–F) Temporal mRNA expression profiles of MYOD1-bound, circadian genes in adult skeletal muscle from either MYOD1-CE (dotted red) or C57BL/6J (solid black, wildtype) mice. Dark shading indicates the relative dark/active phase as these mice were reared in DD at the time of collection. At each time-point RT-PCR expression values are displayed as average fold-change relative to the *Rpl26* house-keeping gene ± SEM (n = 3). Relative gene expression was calculated by the standard curve method. Results were analyzed with one-way ANOVA comparing WT vs. MYOD1-CE, \* indicates a p-value < 0.05. Figure 1 continued on next page

Figure 1 continued

value less than 0.05. (G) JTK\_CYCLE statistics for the RT-PCR results corresponding to the temporal expression values in C-F. 'BH.Q' column reports false discover rates and 'ADJ.P' reports adjusted p-values.

DOI: <https://doi.org/10.7554/eLife.43017.002>



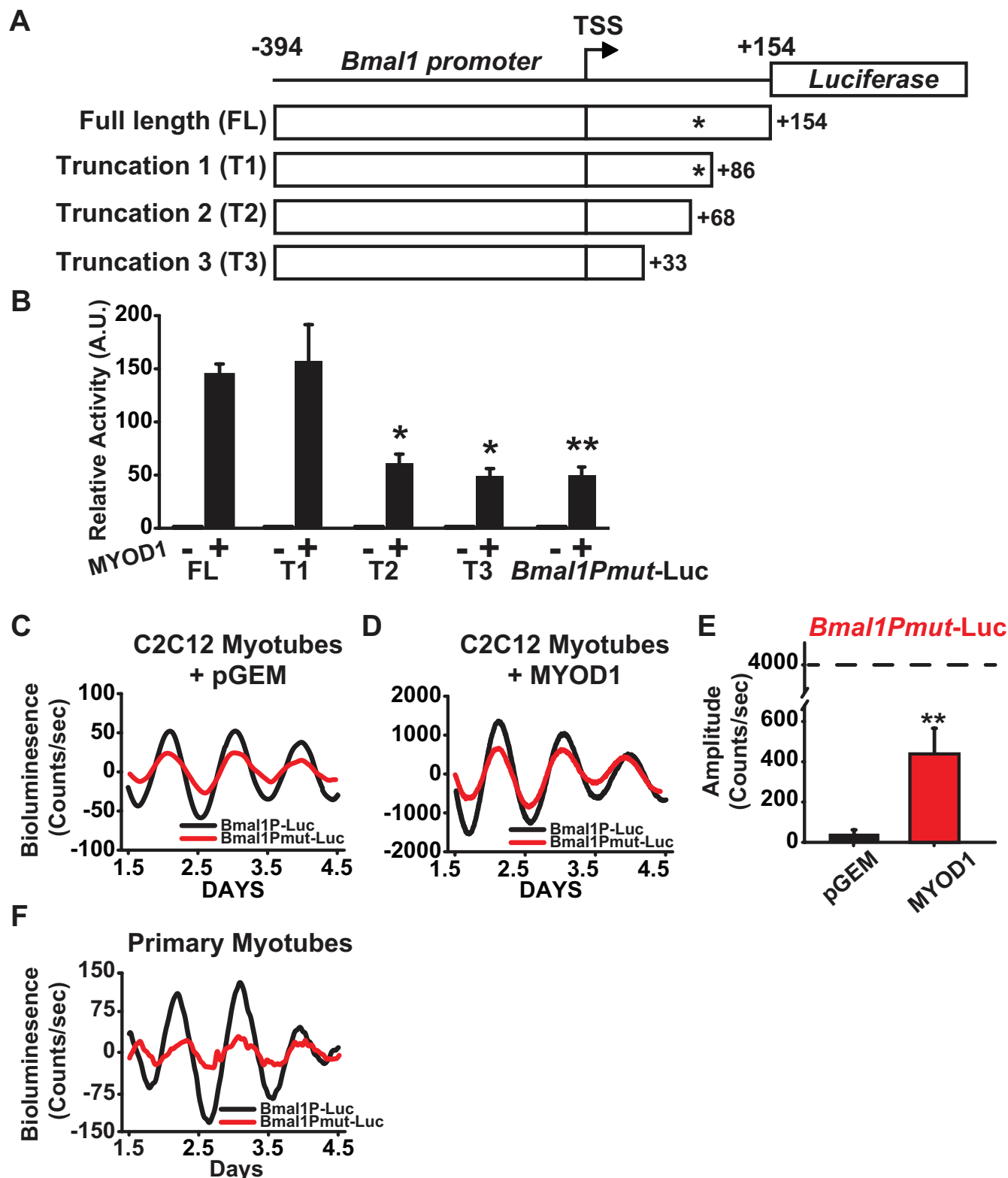
**Figure 2.** MYOD1 transactivates the *Bmal1* promoter and enhances its circadian amplitude in C2C12 myotubes. (A) UCSC genome browser visualization (mm10 genome) of MYOD1 binding tags within the *Bmal1* locus. (B) Representative BMAL1 western blots from C2C12 myoblasts

Figure 2 continued on next page

## Figure 2 continued

transiently transfected with 150 ng pGEM empty vector (-) or MYOD1 expression vector (+). Densitometric values are expressed as average fold-change of BMAL1 over the Tubulin loading control which was unchanged with MYOD1 transfection  $\pm$  SEM (n = 3). Results were analyzed with one-way ANOVA, \* indicates a p-value less than 0.05 (p = 0.025). *Bmal1P*-Luc luminescence in C2C12 myotubes (2C, n = 4 biological replicates) and skeletal muscle primary myotubes (D, n = 3 biological replicates) with transient transfection of pGEM control, MYOD1, or MYOD1mut expression vectors. Luciferase activity for each co-transfection is plotted as average fold-change in relation to the pGEM empty vector control  $\pm$  SEM. Results were analyzed using one-way ANOVA, \*\* indicates a p-value less than 0.001. # indicates a p-value less than 0.01 comparing MYOD1 vs MYOD1mut. (E) *Bmal1P*-Luc luminescence in electroporated skeletal muscle. *Bmal1P*-Luc activity was normalized to *Renilla* luciferase as an electroporation control with the right-leg receiving MYOD1 expression vector (red circles) and the left leg receiving the pGEM empty vector control (black circles). The p-value statistic was calculated by performing a Mann-Whitney non-parametric t-test. Note, one outlier was removed from each group based upon the Robust regression and outlier removal (ROUT) test, with a false discovery rate of <0.01. *Bmal1P*-Luc Dual-Luciferase activities in skeletal muscle primary myotubes (F-H) Representative *Bmal1P*-Luc driven bioluminescence recordings in synchronized C2C12 myotubes co-transfected with pGEM control (F, n = 7), MYOD1 (G, n = 6), or MYOD1mut (H, n = 4) expression vectors. Luminescence recordings are expressed as average counts/sec (base-line subtracted) (I) Average *Bmal1P*-Luc amplitudes  $\pm$  SEM calculated by JTK\_CYCLE from 1.5 to 4.5 days post-synchronization. Results were analyzed with one-way ANOVA, \*\* indicates p-value less than 0.001, # indicates p-value less than 0.002 (n = 4 biological replicates per group).

DOI: <https://doi.org/10.7554/eLife.43017.003>



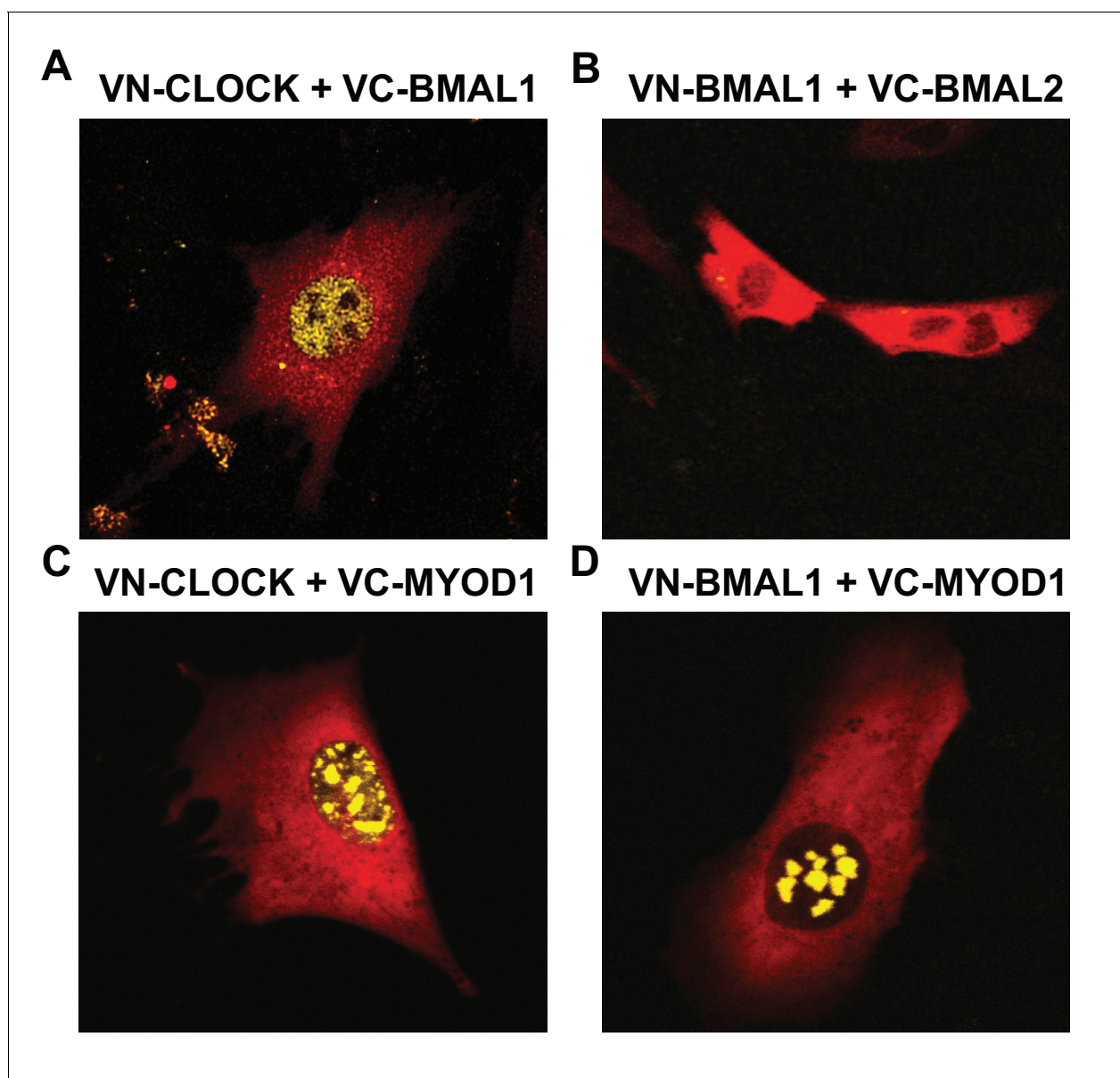
**Figure 3.** A non-canonical E-box within the *Bmal1* promoter is required for amplification by MYOD1. (A) Representative diagram of the *Bmal1P*-Luc promoter truncation constructs. TSS indicates transcriptional start site. \* indicates the relative location of the non-canonical E-box element. FL indicates

Figure 3 continued on next page

## Figure 3 continued

the full-length *Bmal1P*-Luc reporter. T1, T2 and T3 indicate the truncated reporters. **(B)** Dual-luciferase activities from the full-length *Bmal1P*-Luc, T1-T3, and *Bmal1Pmut*-Luc reporters (n = 4/group). Luciferase activity for each co-transfection is plotted as average fold-change in relation to the pGEM empty vector control  $\pm$ SEM (n = 4). Results were analyzed using one-way ANOVA, \*\* indicates a p-value less than 0.001 in relation to the FL *Bmal1P*-Luc + MYOD1 result (p value < 0.001), \* indicates a p-value less than 0.029 in relation to the FL *Bmal1P*-Luc +MYOD1 result. **(C-D)** Representative *Bmal1P*-Luc (black) and *Bmal1Pmut*-Luc (red) driven bioluminescence recordings in synchronized C2C12 myotubes co-transfected with the pGEM control vector **(C)**, or MYOD1 expression vector **(D)**. Luminescence recordings are expressed as average counts/sec (base-line subtracted). **(E)** Average *Bmal1Pmut*-Luc amplitudes calculated by JTK\_CYCLE from 1.5 to 4.5 days post-synchronization (n = 3/group). Data are displayed as average amplitude  $\pm$  SEM. Results were analyzed with one-way ANOVA, \*\* indicates p-value less than 0.001. **(F)** Representative *Bmal1P*-Luc (black) and *Bmal1Pmut*-Luc (red) driven bioluminescence recordings in synchronized skeletal muscle primary myotubes. Luminescence recordings are expressed as average counts/sec (base-line subtracted) for n = 3 biological replicates per group.

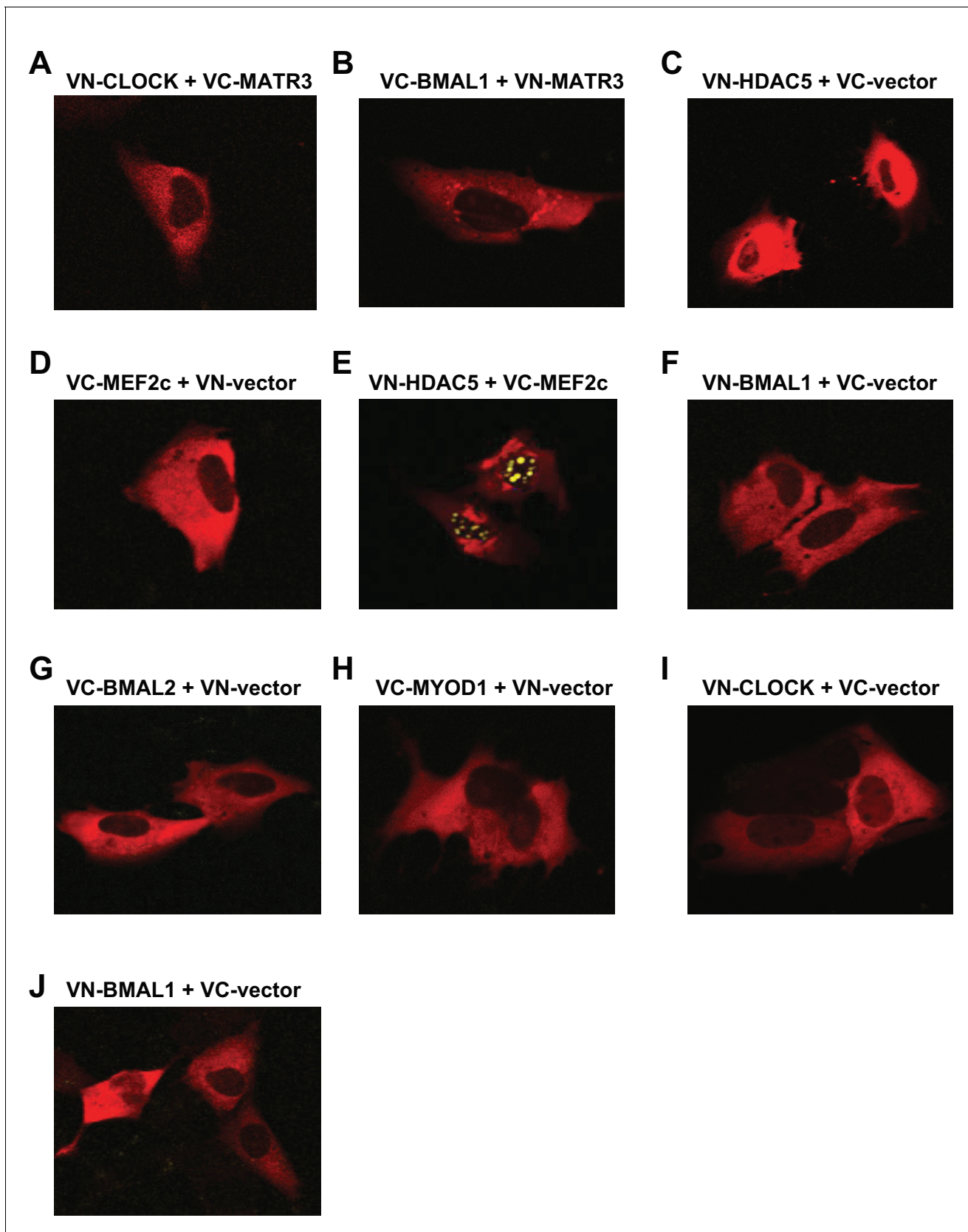
DOI: <https://doi.org/10.7554/eLife.43017.005>



**Figure 4.** MYOD1 colocalizes with BMAL1 and CLOCK in C2C12 myonuclei. Representative images from the BiFC assay performed in C2C12 myoblasts co-transfected with (A) VN-CLOCK and VC-BMAL1, (B) VN-BMAL1 and VC-BMAL2, (C) VN-CLOCK and VC-MYOD1, and (D) VN-BMAL1 and VC-MYOD1. mCherry expression plasmids (red fluorescence signal) were co-transfected in each experiment to visualize the myoblasts and ensure successful transfection. Yellow fluorescence signals indicate positive co-localization via the formation of the Venus Luciferase.

DOI: <https://doi.org/10.7554/eLife.43017.006>



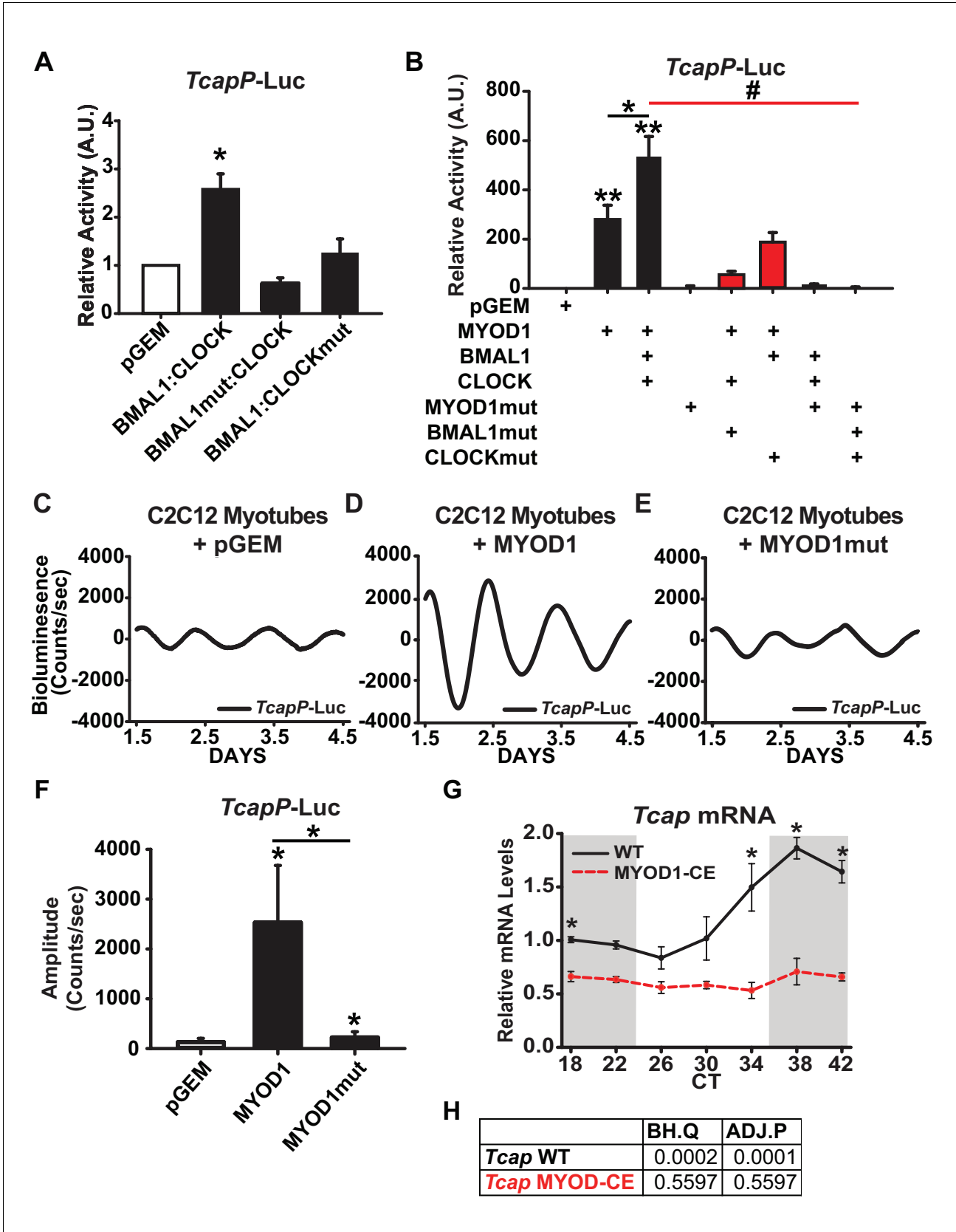


**Figure 4—figure supplement 1.** Representative control images for BiFC experiments in C2C12 myotubes. Interactions were visualized in C2C12 cells by bimolecular fluorescence complementation assay (BiFC). (A) VN-HDAC5 and VC-MATR3; (B) VC-BMAL1 and VN-MATR3; (C) VN-HDAC5 and VC-vector. Figure 4—figure supplement 1 continued on next page

Figure 4—figure supplement 1 continued

vector; (D) VC-MEF2C and VN-vector; (E) VN-HDAC5 and VC-MEF2C; (F) VN-BMAL1 and VC-vector; (G) VC-BMAL2 and VN-vector; (H) VC-MYOD1 and VN-vector; (I) VN-CLOCK and VC-vector; (J) VN-BMAL1 and VC-vector.

DOI: <https://doi.org/10.7554/eLife.43017.007>

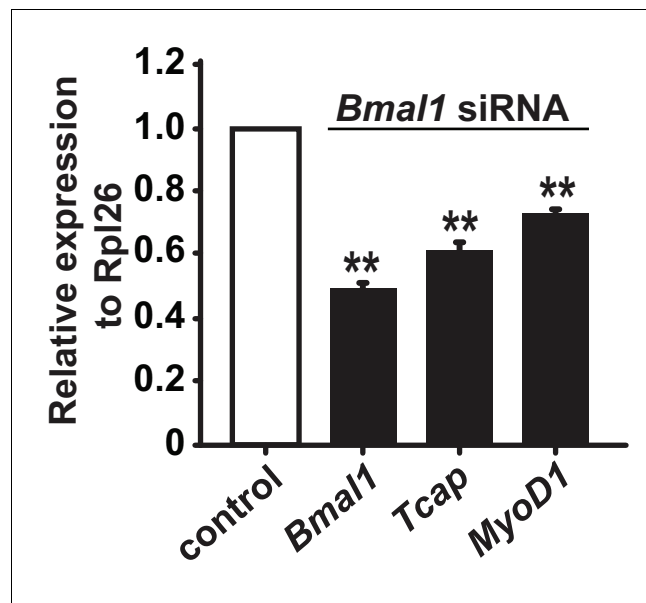


**Figure 5.** *TcapP-Luc* is synergistically activated by MYOD1 and BMAL1:CLOCK. (A) *TcapP-Luc* Dual-Luciferase activity responses from C2C12 myotubes co-transfected with BMAL1 +CLOCK, BMAL1mut +CLOCK, and BMAL1 +CLOCKmut vectors (n = 4 biological replicates/group). Luciferase activity for Figure 5 continued on next page

## Figure 5 continued

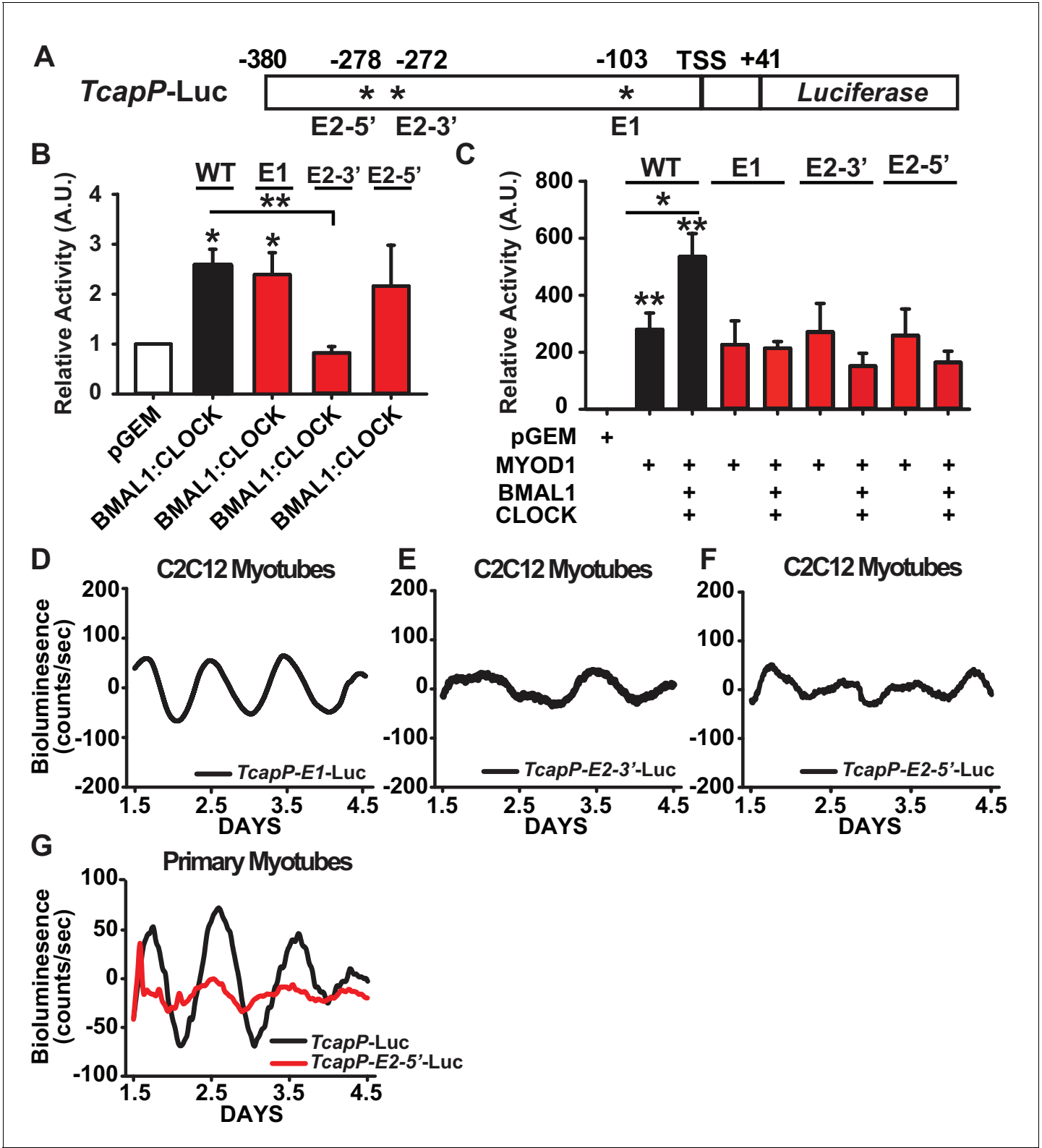
each co-transfection is plotted as average fold-change in relation to the pGEM empty vector control  $\pm$  SEM. Results were analyzed using one-way ANOVA, \* indicates  $p = 0.029$ . (B) *TcapP*-Luc Dual-Luciferase activity responses from C2C12 myotubes co-transfected with MYOD1 alone, MYOD1 +BMAL1+CLOCK, or variations of BMAL1mut, CLOCKmut, and MYOD1mut vectors. Luciferase activity for each co-transfection is plotted as average fold-change in relation to the pGEM empty vector control  $\pm$  SEM ( $n = 4$ ). Results from MYOD1 and MYOD1 +BMAL1:CLOCK co-transfections were analyzed using one-way ANOVA, \*\* indicates a p-value less than 0.001 in relation to the pGEM control. Results from the mutant co-transfection experiments (red) were analyzed using one-way ANOVA. # indicates a p-value less than 0.01 relative to the MYOD1 +BMAL1:CLOCK result. (C–E) Representative *TcapP*-Luc driven bioluminescence recordings in synchronized C2C12 myotubes co-transfected with the pGEM control (C), MYOD1 (D), or MYOD1mut (E) expression vectors. Luminescence recordings are expressed as average counts/sec (base-line subtracted). (F) Average *TcapPmut*-Luc amplitudes calculated by JTK\_CYCLE from 1.5 to 4.5 days post-synchronization. Data are displayed as average amplitude  $\pm$  SEM ( $n = 3$  biological replicates/group). Results were analyzed with one-way ANOVA, \* indicates p-value less than 0.05. (G) *Tcap* temporal mRNA expression profiles from muscles of MYOD1-CE (dotted red) or C57BL/6J (solid black) mice. Dark shading indicates relative dark/active phase as these mice were reared in DD at the time of collection. At each time-point RT-PCR expression values are displayed as average fold-change relative to the *Rpl26* house-keeping gene  $\pm$  SEM ( $n = 3$ ). Results were analyzed with one-way ANOVA comparing WT vs. MYOD1-CE at each time-point, \* indicates a p-value less than 0.05. (H) JTK\_CYCLE statistics for the RT-PCR results corresponding to *Tcap*'s temporal expression values. 'BH.Q' column reports false discover rates and 'ADJ.P' reports adjusted p-values.

DOI: <https://doi.org/10.7554/eLife.43017.008>



**Figure 5—figure supplement 1.** Effect of *Bmal1* siRNA mediated knockdown on *MyoD1* and *Tcap* expression. Relative mRNA expression levels for *Bmal1*, *Tcap*, and *MYOD1* in C2C12 myotubes transfected with either *Bmal1* siRNA (black bars) or a control non-targeting siRNA-A vector (white bar). Results are displayed as average fold-change relative to the control siRNA-A  $\pm$  SEM ( $n = 4$  biological replicates). Results were analyzed with one-way ANOVA, \*\* indicates a p-value less than 0.001.

DOI: <https://doi.org/10.7554/eLife.43017.009>



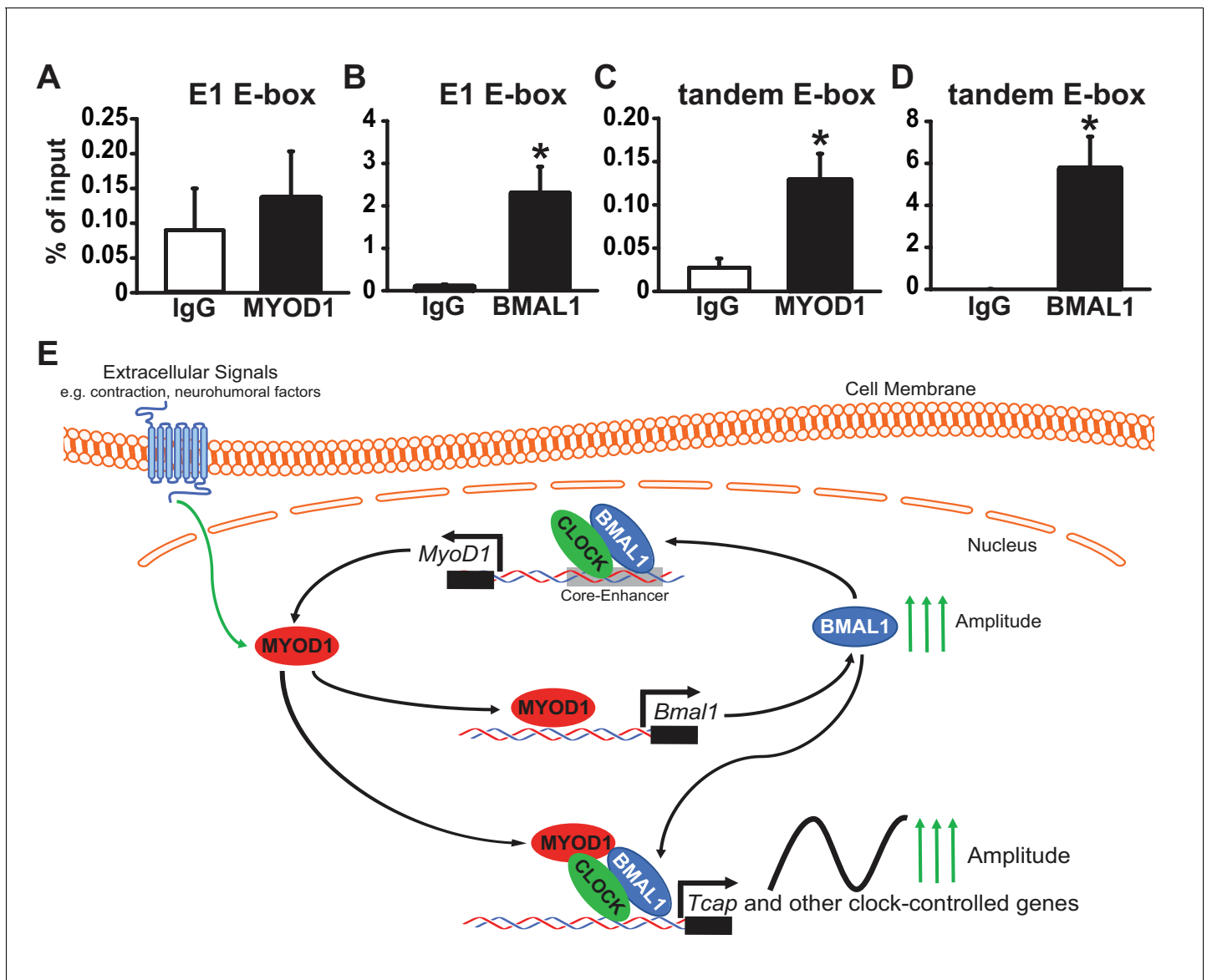
**Figure 6.** Synergistic activation of the *Tcap* promoter by MYOD1 + BMAL1:CLOCK requires the tandem E-box element. (A) Graphical representation of the *TcapP-Luc* promoter:reporter construct. TSS indicates transcript start site. E1 E-box is located at –103 and E2 tandem E box is located –272 (E2 3') and –278 (E2 5') from the TSS. (B) Dual-Luciferase activity responses from the wildtype *TcapP-Luc* and the *Tcap* E-box mutants in C2C12 myotubes co-transfected with BMAL1:CLOCK. Luciferase activity for each co-transfection is plotted as average fold-change in relation to the pGEM empty vector

Figure 6 continued on next page

## Figure 6 continued

control  $\pm$  SEM (E2-3' and E2-5' n = 4, E1 n = 3). Results were analyzed using one-way ANOVA, \*\* indicates p-value less than 0.01 in relation to the WT *TcapP*-Luc response, \* indicates a p-value less than 0.05 in relation to the pGEM control vector. (C) Dual-Luciferase activity responses from the *Tcap* E-box mutants with co-transfection of MYOD1 alone or MYOD1 +BMAL1:CLOCK. Luciferase activity for each co-transfection is plotted as average fold-change in relation to the pGEM empty vector control  $\pm$  SEM (n = 3). Results were analyzed using one-way ANOVA. In comparison to the pGEM control all co-transfections were significantly elevated (p-value < 0.05), \*\* indicates a p-value of less than 0.01 in relation to the pGEM control, and \* indicates a p-value of less than 0.05 comparing *TcapP*-Luc with over-expression of MYOD1 vs MYOD1+BMAL1:CLOCK. In comparison to the pGEM control all co-transfections were significantly elevated (p-value<0.05). . No statistical differences were observed for each of the *Tcap* mutant reporters comparing MYOD1 alone to MYOD1 +BMAL1:CLOCK. (D-F) Representative bioluminescence recordings from the *TcapP*-E1-Luc (D), *TcapP*-E2-3'-Luc (E), and *TcapP*-E2-5'-mut-Luc (F) in synchronized C2C12 myotubes. (G) Representative *TcapP*-Luc (black, n = 3 biological replicates) and *TcapPmut*-E2-5'-Luc (red, n = 3 biological replicates) driven bioluminescence recordings in synchronized skeletal muscle primary myotubes. Luminescence recordings are expressed as average counts/sec (base-line subtracted).

DOI: <https://doi.org/10.7554/eLife.43017.010>



**Figure 7.** MYOD1 and BMAL1 bind to the *Tcap* tandem E-box element in adult skeletal muscle. (A–B) Chromatin Immunoprecipitation-PCR with anti-MYOD1 and -BMAL1 antibody pulldowns (and IgG controls) to detect binding of MYOD1 and BMAL1 within *Tcap*'s E1 E-box element (A, B) or the tandem E-box (C, D, primers contain 3' and 5' E-boxes). Pull-downs were performed with extracts from adult mouse quadriceps muscles collected at ZT 2 (2 hours after lights on). Data are displayed as an average % of input  $\pm$  SEM ( $n = 3$  samples/group). Results were analyzed with a one-way ANOVA and \* denotes a p-value ranging from 0.02 to 0.037. (E) Graphical model of depicting the role of MYOD1 in modulating core clock gene expression and working as a co-factor with BMAL1:CLOCK to amplify downstream circadian genes in skeletal muscle. MYOD1 activity is modulated by extracellular signals and it amplifies *Bmal1* expression via direct transcriptional activation. BMAL1:CLOCK in turn form a positive feedback loop to regulated the circadian expression of *MyoD1* by targeting the core-enhancer element. MYOD1 and BMAL1:CLOCK work in a synergistic fashion to amplify the expression of circadian genes.

DOI: <https://doi.org/10.7554/eLife.43017.011>