Supplementary Figures

The massed-spaced learning effect in non-neural human cells

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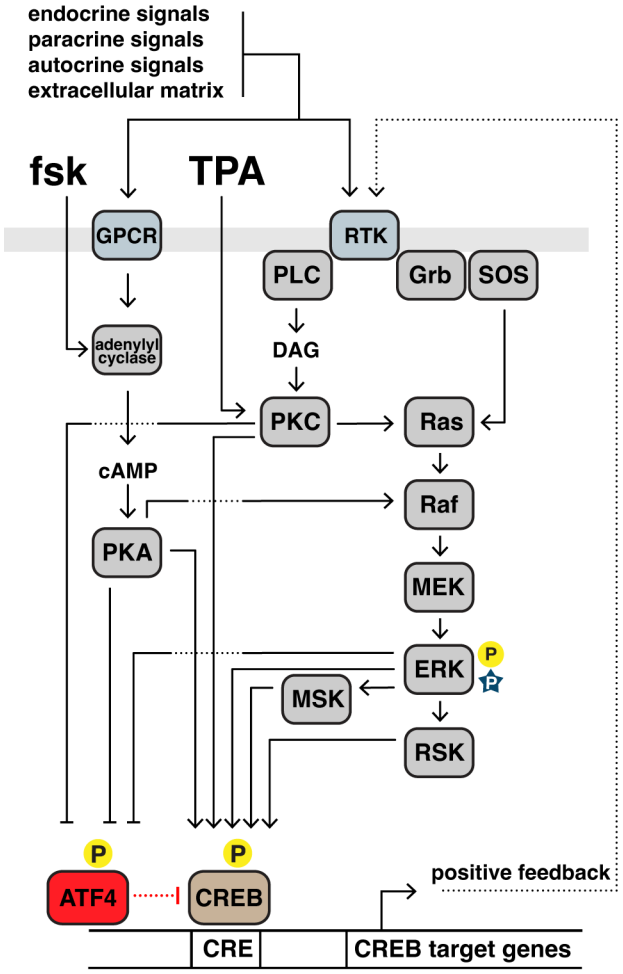
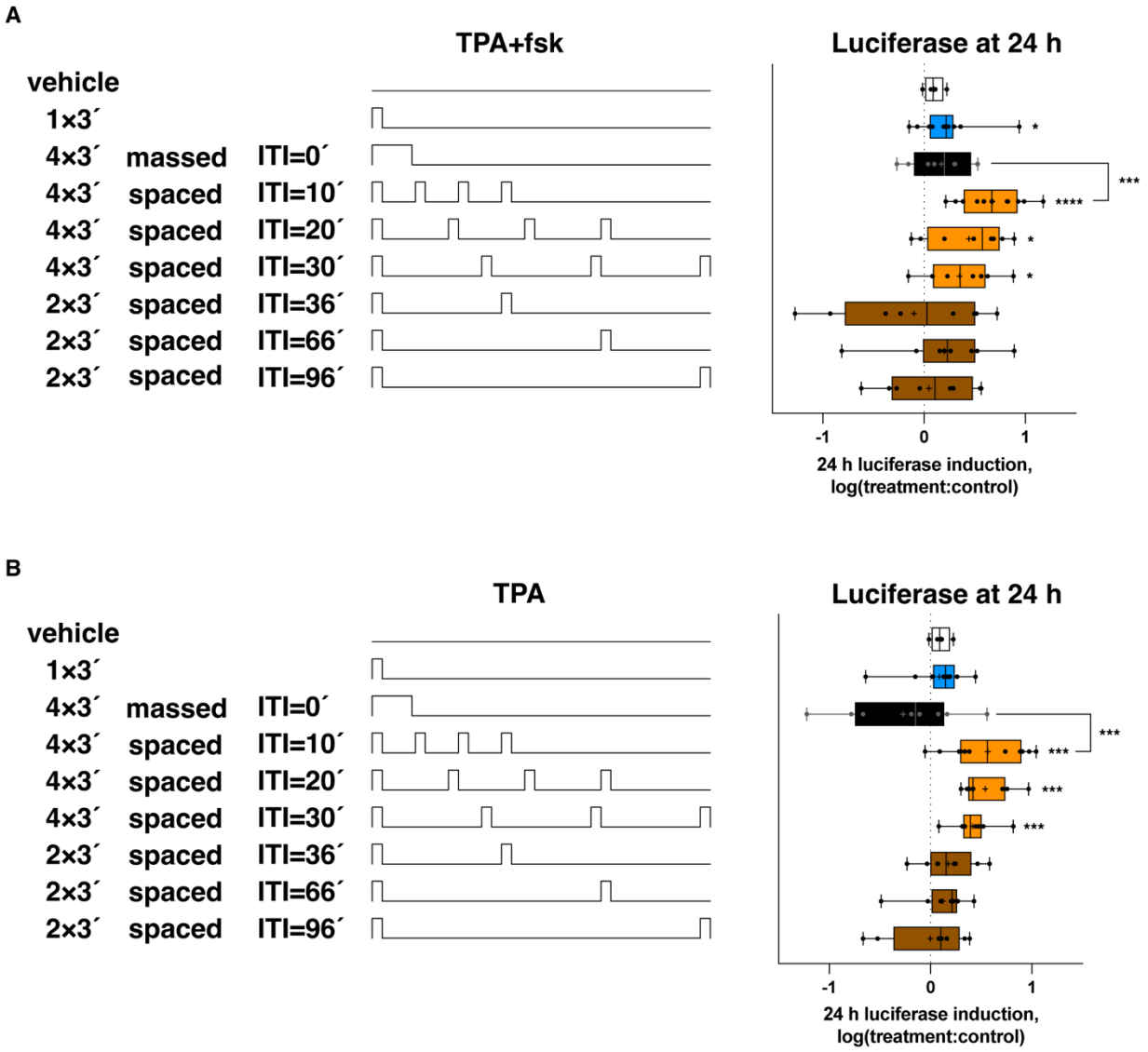
Supplementary Figure S1 (related to Fig. 1 and Fig. 2)



Select signaling pathways relevant to this study. Forskolin (fsk) acts by activating adenylate cyclase, which produces the second messenger cyclic AMP and activates PKA. Typically, adenylyl cyclase is activated downstream of G-protein coupled receptors (GPCRs) responding to a variety of environmental factors. PKA is known to both directly phosphorylate CREB[1], and also indirectly induce CREB-dependent expression by phosphorylating ATF4, leading to its degradation and derepression of CREB[2,3]. PKA can also influence the ERK signaling cascade at various points, most notably via the inhibition of Raf[4]. The phorbol ester TPA directly activates PKC in place of the second messenger diacyl glycerol, which is typically generated by phospholipase C (PLC) downstream of receptor tyrosine kinases (RTK) that respond to first messengers such as hormones and growth factors. PKC also activates the ERK

cascade by phosphorylating Raf. The ERK cascade itself, besides being influenced by PKA and PKC, is most typically activated via the RTK/Grb/SOS complex, an alternative output of receptor tyrosine kinases besides phospholipases. When activated by double phosphorylation, ERK can influence CREB-dependent transcription in multiple ways. It can activate downstream effectors, ribosomal S6 kinases (RSK)[5] and mitogen and stress-activated protein kinases (MSK)[6], which in turn phosphorylate CREB; it can also directly phosphorylate CREB[5]. A positive feedback loop exists between CREB-dependent transcription and ERK activation, possibly acting through a secreted autocrine messenger that persistently acts upon RTKs[7].

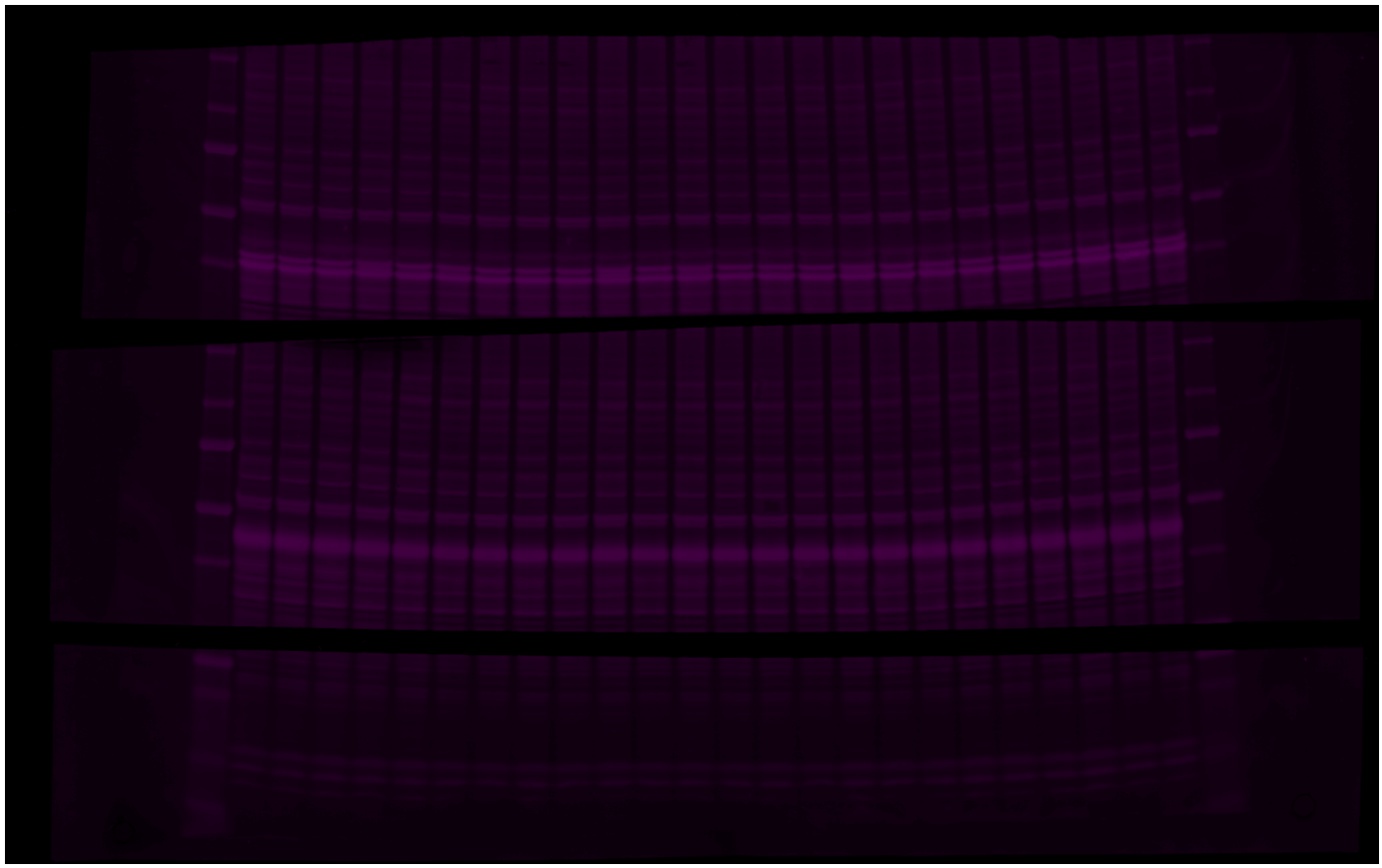
Supplementary Figure S2 (Extended Fig. 3A–C)

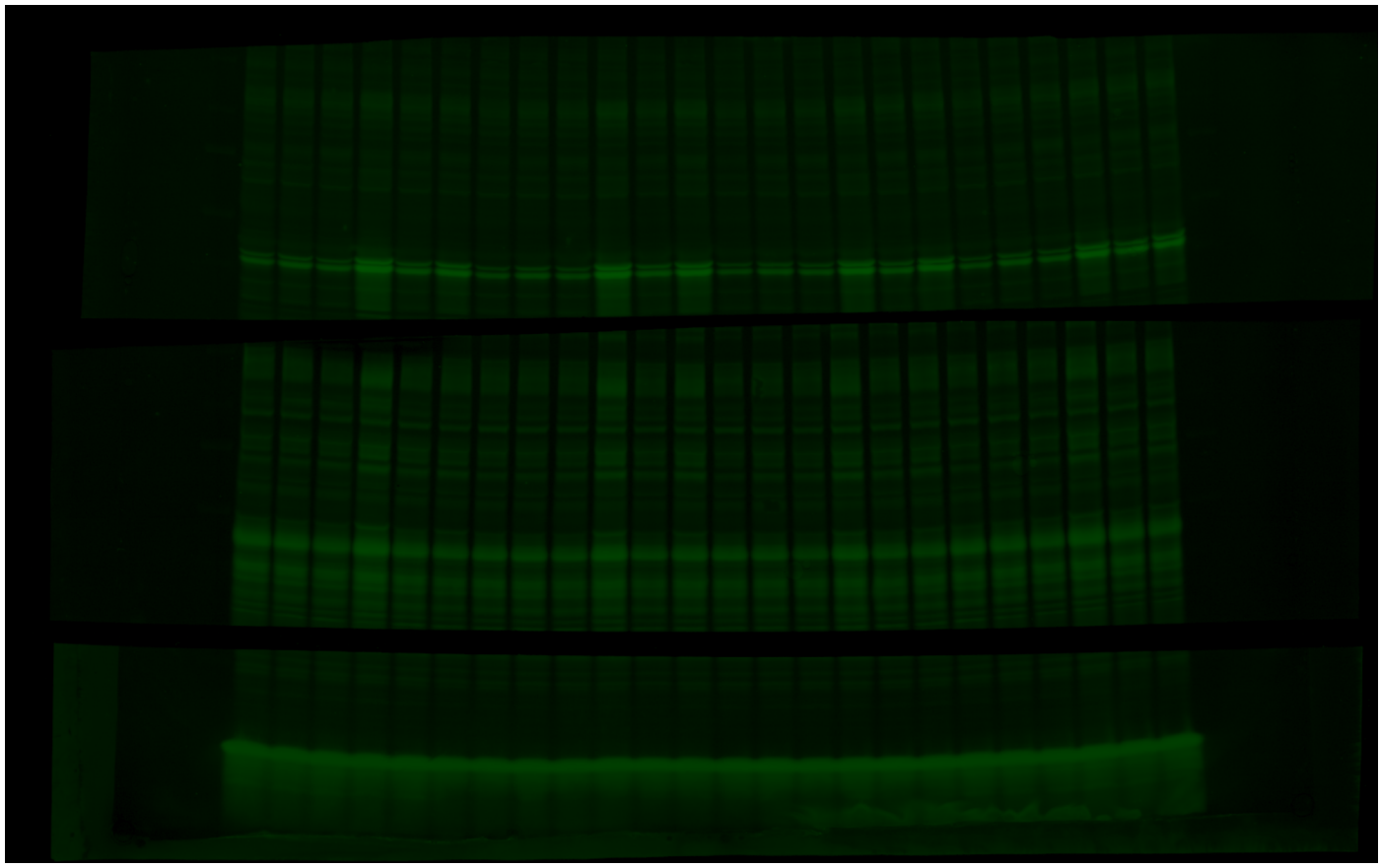


Massed-spaced effect in CRE-luc cells. A combination of Fsk and TPA (A), or TPA alone (B), were administered in various temporal patterns outlined on the left. Agonists were delivered either as a single pulse (1x3'), as a "massed" pulse (4x3' without ITI), as four spaced pulses (4x3', ITI=10, 20, or 30 min) or as two spaced pulses (2x3', ITI matching the intervals between the first and last pulse in the four-pulse protocols). CRE-luc induction was measured 24 h from the onset of treatment. N=4 for Vehicle, N=11 for 4×TPA+fsk (ITI 10 min) and 1×TPA+fsk, N=12 for 4×TPA (ITI 10 min) and 1×TPA, and N=8 for the remaining data points. Asterisks by data bars indicate p-values in two-tailed single-sample Student's *t* tests (Theoretical mean=0; 1xTPA+fsk, p=0.0299; 4xTPA+fsk

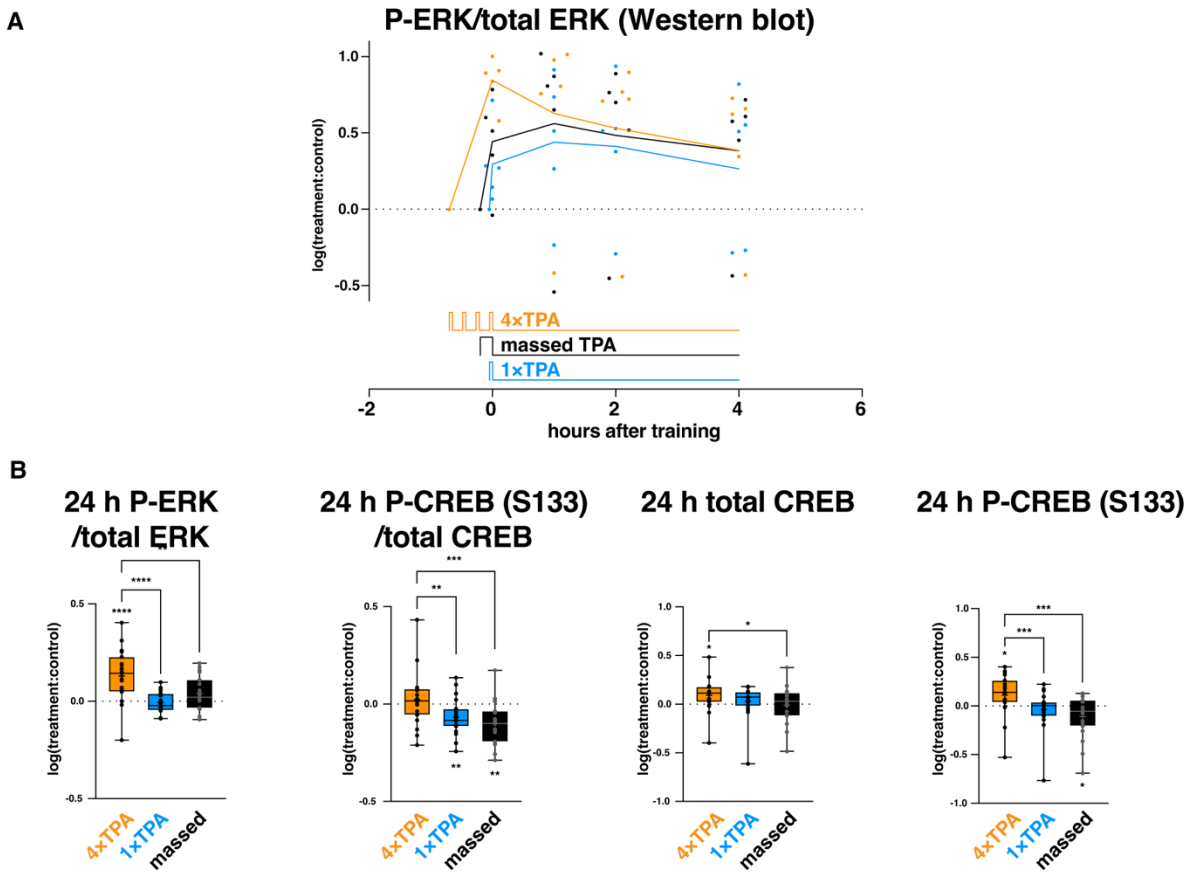
(ITI 10 min), p<0.0001; 4xTPA+fsk (ITI 20 min), p=0.0137; 4xTPA+fsk (ITI 30 min), p=0.0244; 4xTPA (ITI 10 min), p=0.0003; 4xTPA (ITI 20 min), p=0.0004; 4xTPA (ITI 30 min), p=0.0009). Asterisks between bars indicate p-values in two-tailed paired Student's *t* tests (4xTPA+fsk (ITI 10 min) vs M TPA+fsk, p=0.0009; 4xTPA (ITI 10 min) vs M TPA, p=0.0016). Summary p-values: *, p<0.05; ***, p<0.001; ****, p<0.0001. Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Source data are provided as a Source Data file.

Supplementary Figure S3 (uncropped gel images for Fig. 3D)



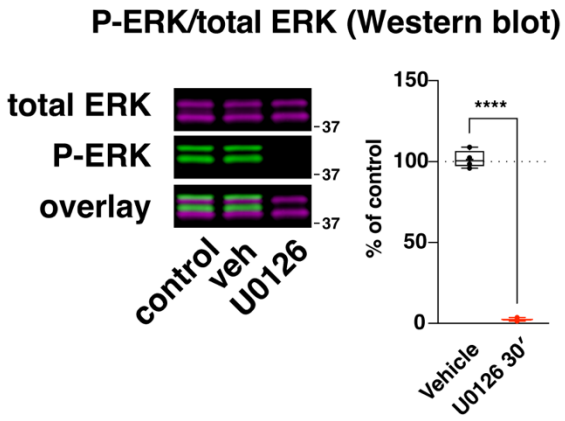


Supplementary Figure S4 (related to Fig. 3D)



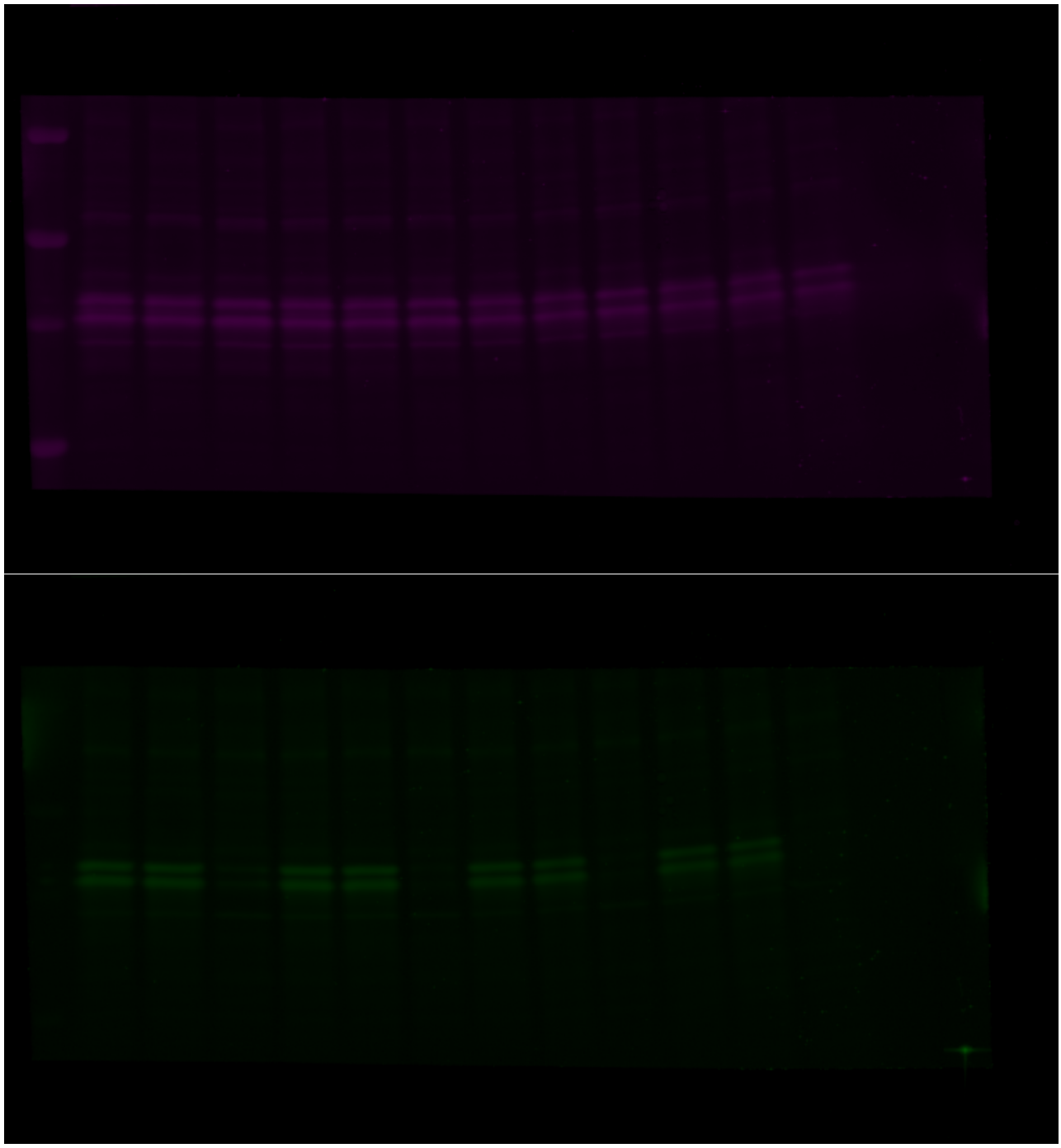
A. ERK phosphorylation (measured by Western blot as a ratio of P-ERK to total ERK) at 0, 1, 2 and 4 h after the treatments with 4×, 1× or massed TPA. N=5 independent experiments. B, P-ERK/total ERK, P-CREB (S133)/total CREB, total CREB/H3 and P-CREB (S133)/H3 ratios measured by Western blot in luc-CRE cells 24 h after treatment with 4×, 1× or massed TPA. (Histone H3 is used as a loading control.) *, $p<0.05$ in a two-tailed ratio paired Student's *t* test (same data as in Fig. 3D). Asterisks above or below data points indicate p-values in two-tailed single-sample Student's *t* tests. Asterisks between data points indicate p-values in two-tailed ratio paired Student's *t* tests. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Source data and exact p-values are provided in the Source Data file.

Supplementary Figure S5 (related to Fig. 3G)

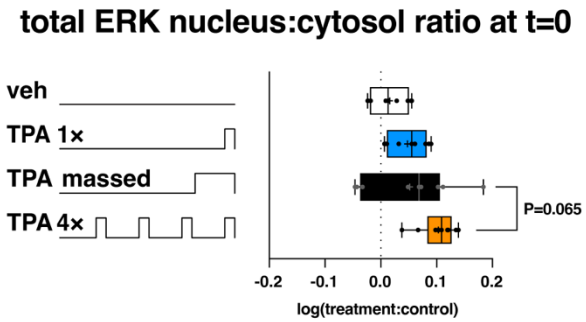


Western blot of total CRE-luc cell lysates treated with either vehicle or U0126 for 30 min, confirming that this treatment leads to an almost complete removal of P-ERK from the cell, thus establishing the baseline in D. N=4 independent experiments analyzed on the same Western blot. Asterisks represent p-value in a two-tailed unpaired Student's t test (Vehicle vs U0126 30', $p<0.0001$). Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Uncropped images shown in Fig. S6. Source data are provided as a Source Data file.

Supplementary Figure S6 (uncropped gel images for Fig. S5)

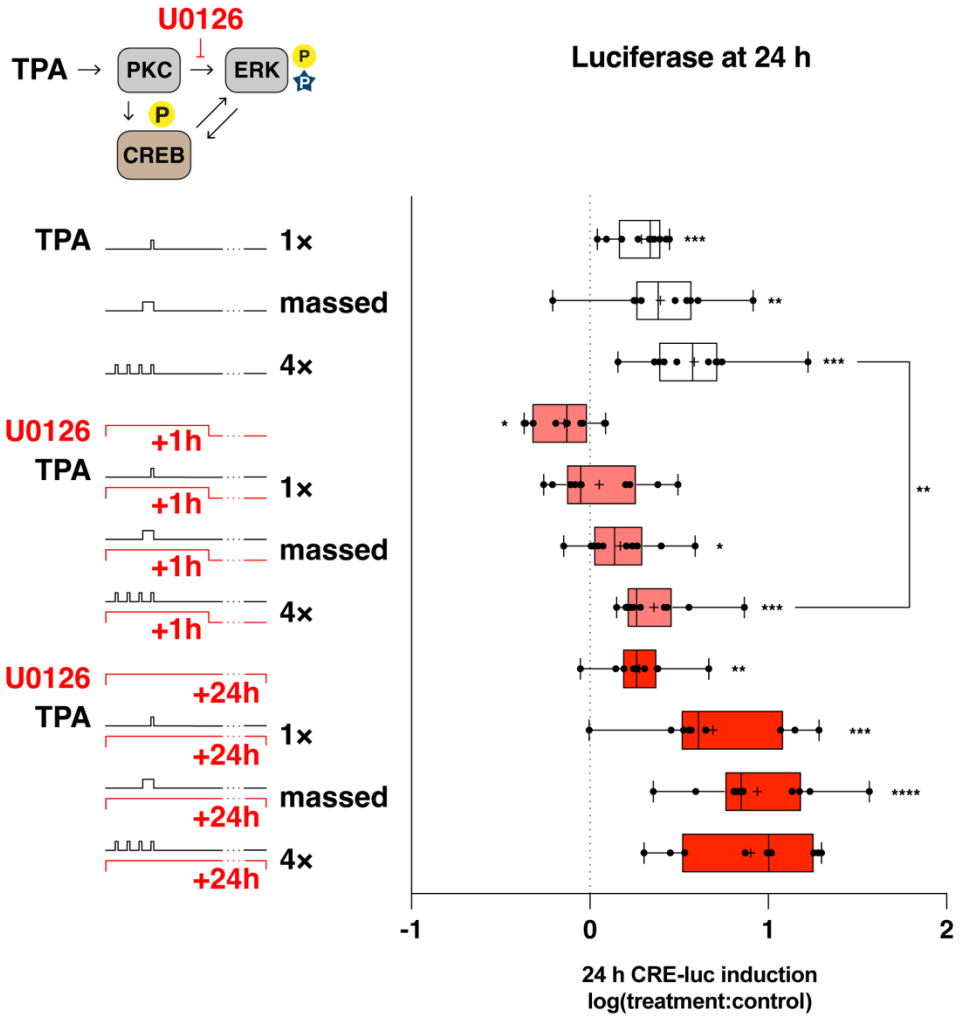


Supplementary Figure S7 (related to Fig. 3F–H)



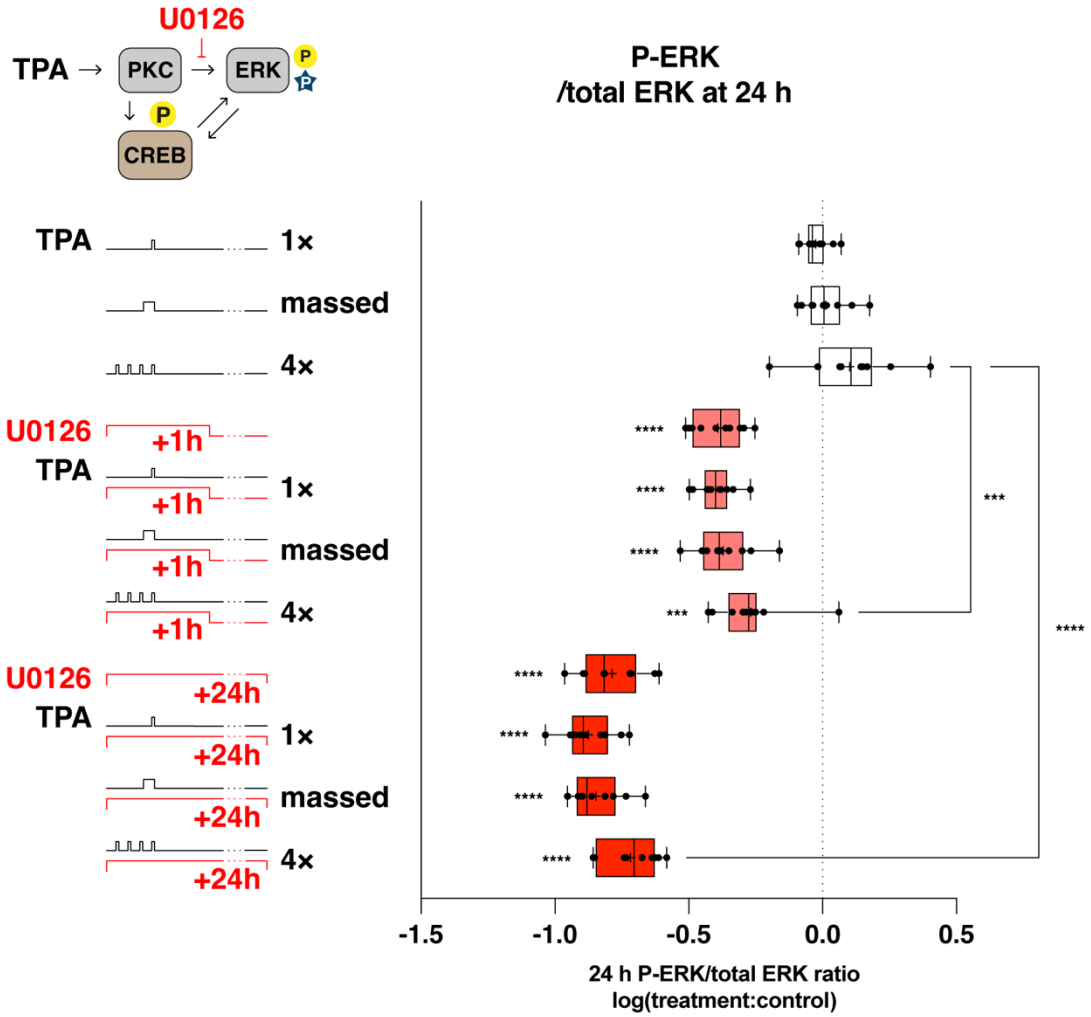
Quantification of nuclear-to-cytosolic ratio of total ERK as shown in 3F. N=9 biological replicates from 3 independent experiments. P-value in a two-tailed paired Student's t test shown: 4xTPA vs M TPA, p=0.065. Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Source data are provided as a Source Data file.

Supplementary Figure S8 (extended Fig. 4A)



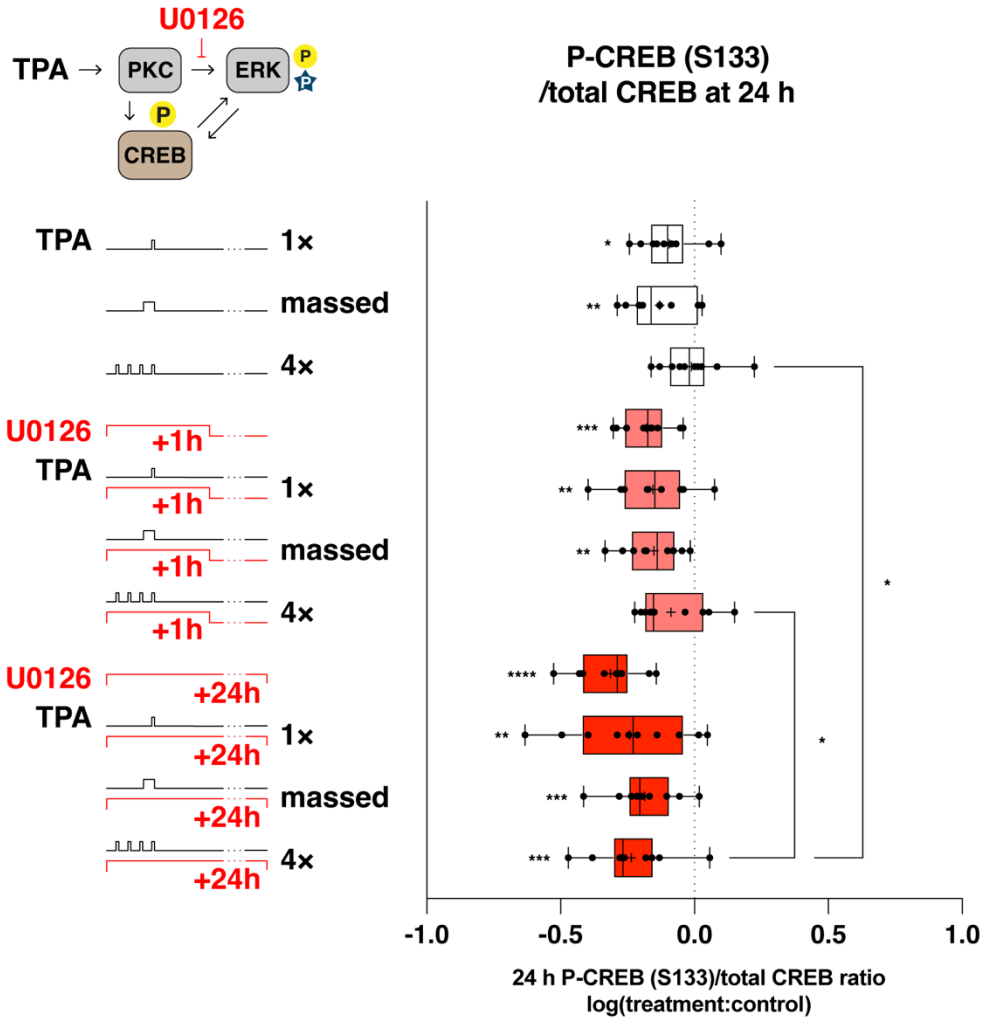
A, luc-CRE cells were treated with 1×, 4×, or massed TPA, and incubated in the presence of U0126 for 1 h or 24 h as indicated on the left. Luciferase expression was measured 24 h after TPA treatment. B, ratio of luciferase induction, as in A, elicited by 4× or massed TPA treatments, with or without U0126. N=10 independent experiments. Asterisks by data bars indicate p-values in single-sample Student's *t* tests. Asterisks between bars indicate p-value in a two-tailed ratio paired Student's *t* test. *, p<0.05; **, p<0.01; ***, p<0.001. Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Source data and exact p-values are provided in the Source Data file.

Supplementary Figure S9 (related to Fig. 4A)



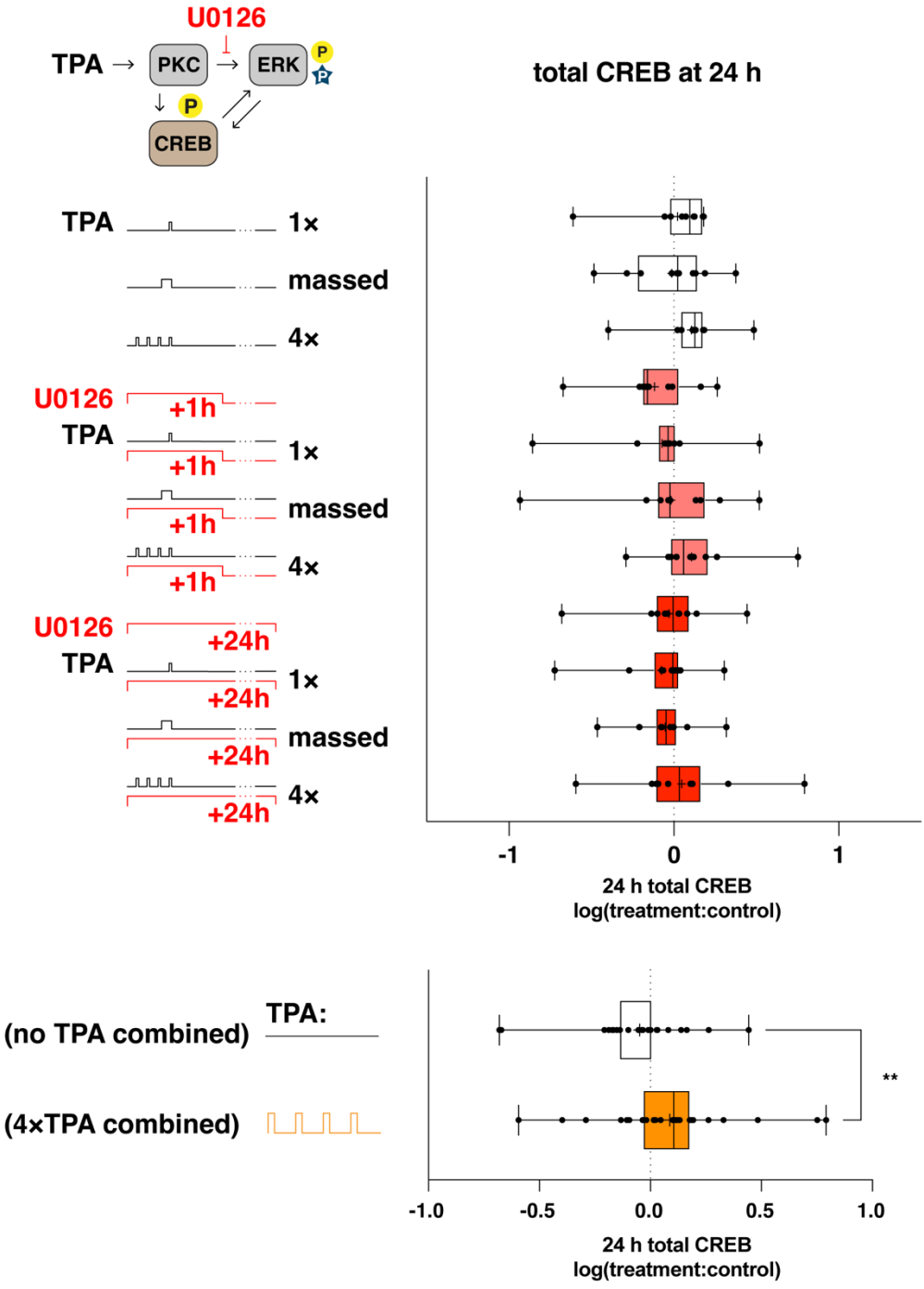
Cells were treated as in Fig. S8 and as indicated on the left, lysed 24 h after TPA treatment, and P-ERK/total ERK ratios were measured by Western blot. Asterisks by data bars indicate p-values in single-sample Student's *t* tests. N=10 independent experiments. Asterisks between bars indicate p-values in Šidák's post-hoc tests following repeated measures ANOVA. **, p<0.01; ***, p<0.001, ****, p<0.0001. Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Source data and exact p-values are provided in the Source Data file.

Supplementary Figure S10 (related to Fig. 4A)



Cells were treated as in Fig. S8 and as indicated on the left, lysed 24 h after TPA treatment, and P-CREB/total CREB ratios were measured by Western blot. N=10 independent experiments. Asterisks by data bars indicate p-values in single-sample Student's *t* tests. Asterisks between bars indicate p-values in Šidák's post-hoc tests following repeated measures ANOVA. *, p<0.05; **, p<0.01; ***, p<0.001. Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Source data and exact p-values are provided in the Source Data file.

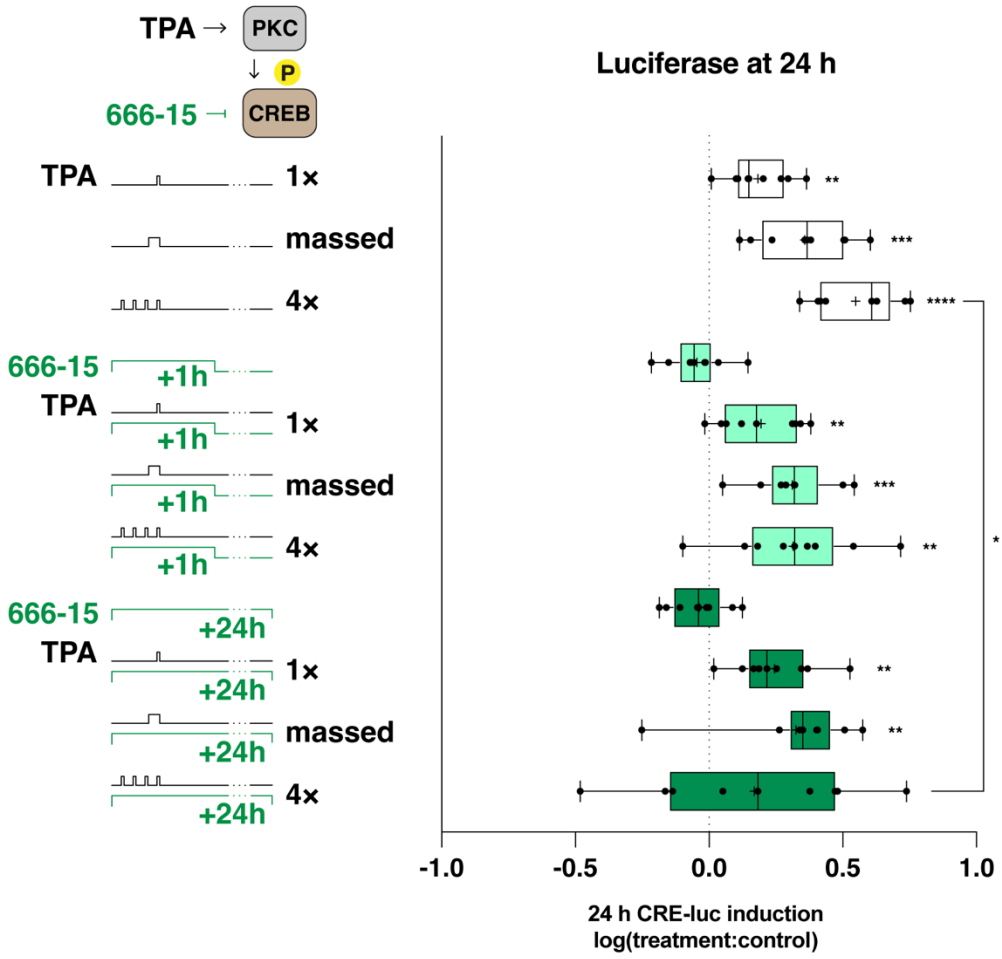
Supplementary Figure S11 (related to Fig. 4A):



A, cells were treated as in Fig. S8 and as indicated on the left, lysed 24 h after TPA treatment, and total CREB levels measured by Western blot (histone H3 was used as

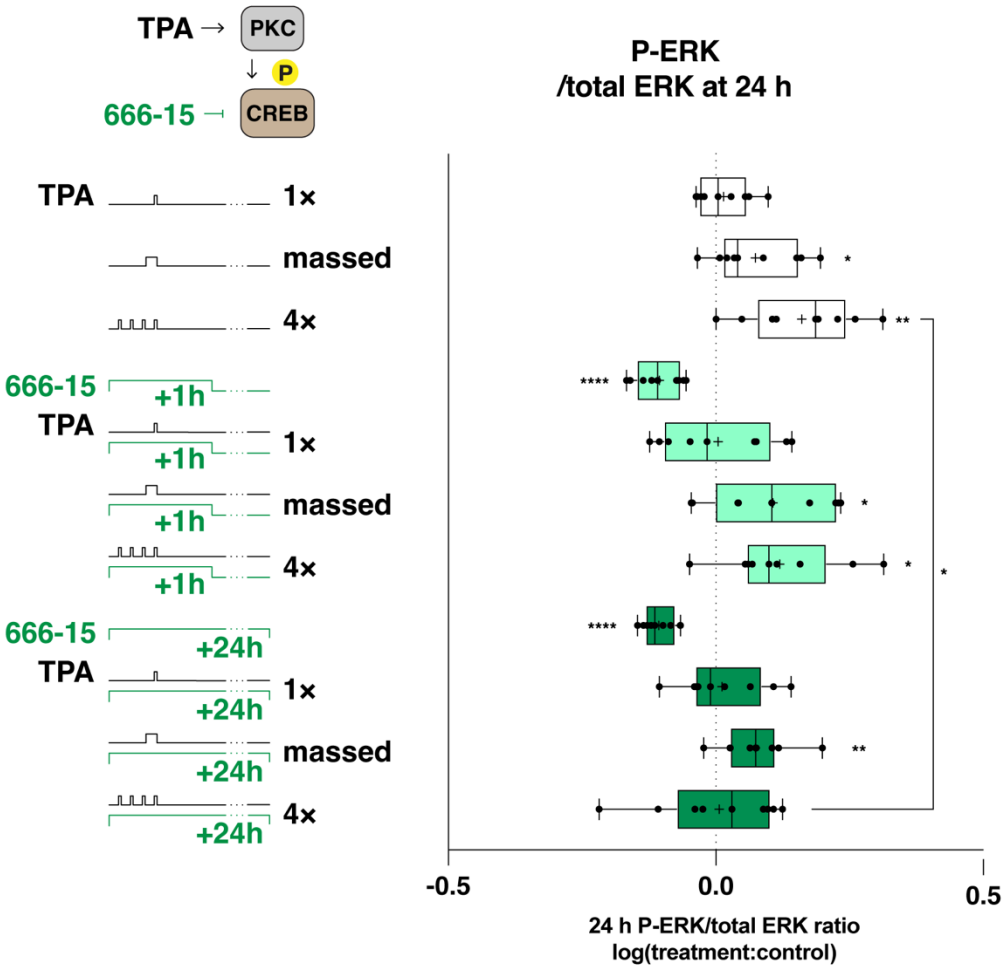
loading control). B, data from A combined based on the TPA treatment paradigm. N=10 independent experiments. Asterisks indicate p-value in a two-tailed ratio paired Student's *t* test. **, $p<0.01$. Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Source data and exact p-values are provided in the Source Data file.

Supplementary Figure S12 (extended Fig. 4A):



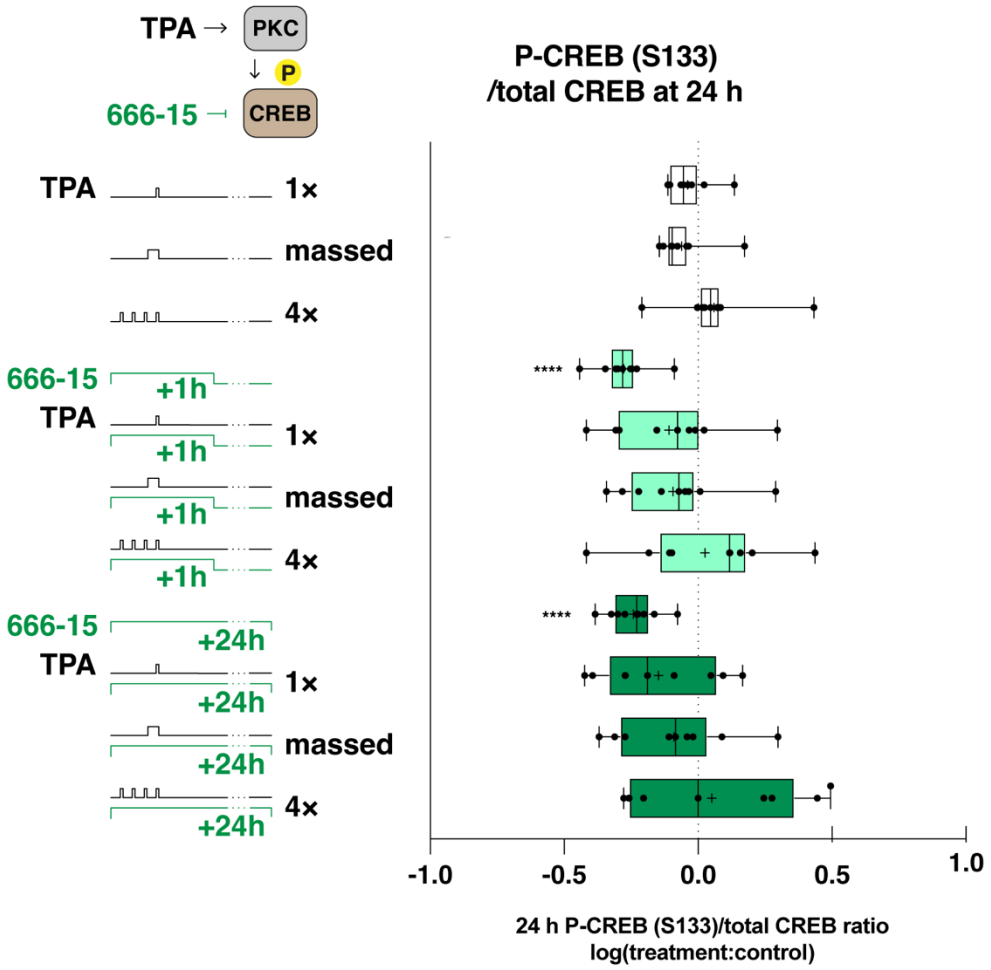
A, luc-CRE cells were treated with 1×, 4×, or massed TPA, and incubated in the presence of 666-15 for 1 h or 24 h as indicated on the left. Luciferase expression was measured 24 h after TPA treatment. B, ratio of luciferase induction, as in A, elicited by 4× or massed TPA treatments, with or without 666-15. Asterisks by data bars indicate p-values in single-sample Student's *t* tests. Asterisks between bars indicate p-values in Šidák's post-hoc tests following repeated measures ANOVA. N=9 independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001. Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Source data and exact p-values are provided in the Source Data file.

Supplementary Figure S13 (related to Fig. 4A):



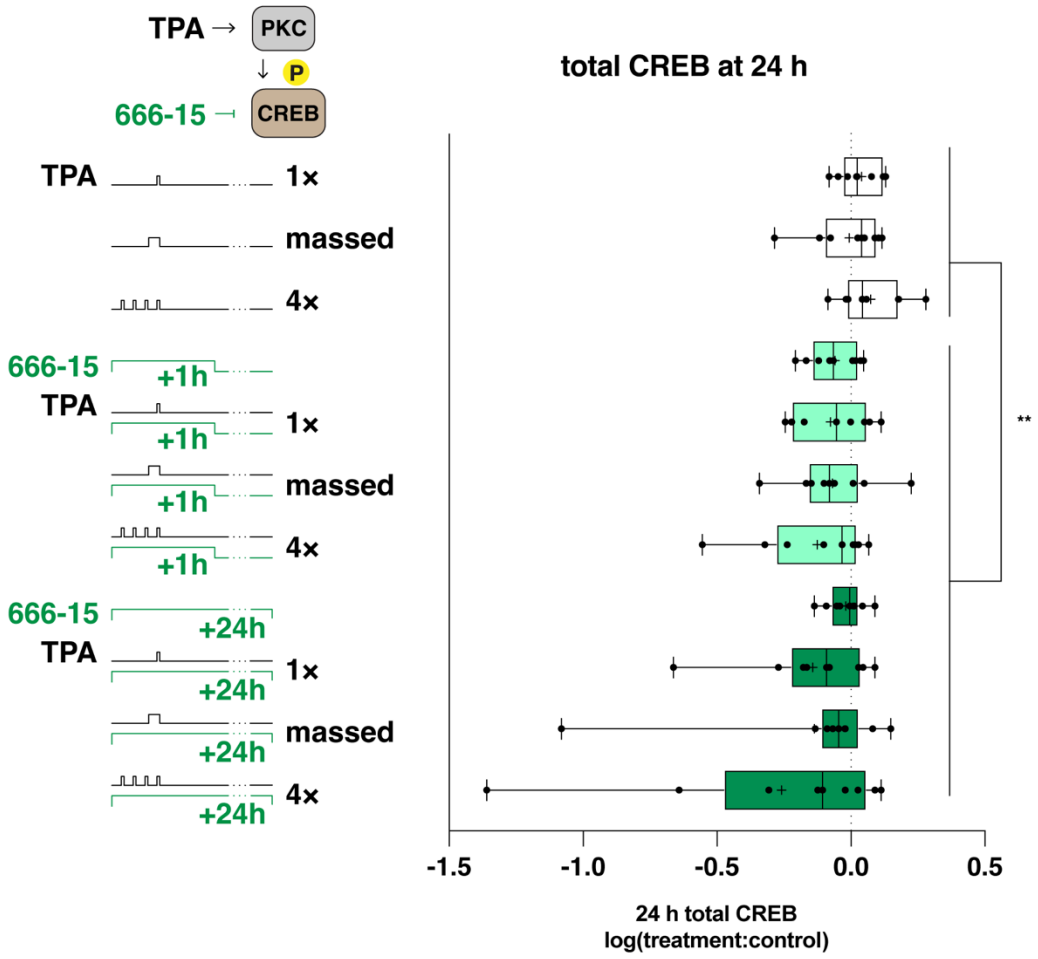
Cells were treated as in Fig. S12 and as indicated on the left, lysed 24 h after TPA treatment, and P-ERK/total ERK ratios were measured by Western blot. Asterisks by data bars indicate p-values in single-sample Student's *t* tests. Asterisk between bars indicates p-value in a two-tailed ratio paired Student's *t* test. N=9 independent experiments. *, p<0.05; ****, p<0.0001. Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Source data and exact p-values are provided in the Source Data file.

Supplementary Figure S14 (related to Fig. 4A):



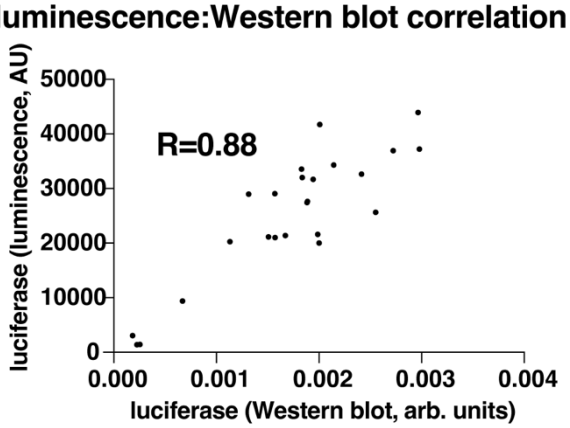
Cells were treated as in Fig. S12 and as indicated on the left, lysed 24 h after TPA treatment, and P-CREB (S133)/total CREB ratios were measured by Western blot. Asterisks by data bars indicate p-values in single-sample Student's *t* tests. N=9 independent experiments. ****, $p<0.0001$. Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Source data and exact p-values are provided in the Source Data file.

Supplementary Figure S15 (related to Fig. 4A):



Cells were treated as in Fig. S12 and as indicated on the left, lysed 24 h after TPA treatment, and total CREB levels measured by Western blot (histone H3 was used as loading control). Asterisks indicate p-value in a two-tailed unpaired Student's *t* test when all data are averaged across 666-15-treated and 666-15-untreated samples. N=9 independent experiments. **, $p<0.01$. Box-and-whisker plots show all values (points) with range (whiskers), interquartile range (box), median (center line) and mean ("+" sign within box). Source data and exact p-values are provided in the Source Data file.

Supplementary Figure 16 (related to Methods)



Correlation between levels of luciferase assayed in the same samples by luminescence and Western blot. R value indicates Pearson's correlation coefficient. Source data are provided in the Source Data file.

References

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