

SUPPLEMENTARY INFORMATION

SI TABLES

Table S1. List of annotated phosphorylation sites

The list of 7936 identified phosphorylation sites was annotated using ProteoConnections. The following data fields are reported: accession number, gene symbol, description, species, residue, position, site localization confidence, peptide sequence, peptide modifications, Mascot peptide score, known phosphorylation site, kinase motifs and predictions, phosphorylation binding motif, NLS, NES, protein domains, disordered regions prediction, secondary structure prediction, solvent accessibility prediction, PDB structure, glycosylation predictions, and site conservation in other species.

Table S2. Kinetic profiles of phosphorylated peptides

Kinetic profiles of identified phosphorylated peptides for cytosolic (3,015 profiles) and nuclear (5,222 profiles) fractions are reported with the following values: peptide m/z, retention time, charge, sequence, modifications, Mascot peptide score, protein accession, gene symbol, name, peptide start and stop position, modification positions on protein, ProteoConnections protein and kinetic graph links, average peptide abundance for each time point, number of replicates where the peptide is found, fold-change(treated/control) and p-value (two-tailed Student t-test).

Table S3. Kinetic profiles of candidate ERK1/2 substrates

Kinetic profiles of candidate ERK1/2 substrates are reported for cytosolic (73 profiles) and nuclear (171 profiles) fractions. Values are the same as in Table S2. In addition, annotations for each phosphorylation site are included in the file (as in Table S1) with two additional columns to show the presence of potential DEF and D domain motif in the protein. Substrates validated by *in vitro* kinase assays are highlighted in green.

Table S4. Gene Ontology analysis of candidate ERK1/2 substrates

Gene Ontology enrichment analyses were performed using the tools included in ProteoConnections. The following fields are reported: GO term identifier, ontology, ontology name, definition, depth, p-value (Fisher's exact test), number in candidate ERK1/2 substrates list, number in the rat proteome, ratio, enrichment, and IPI protein identifiers.

Table S5. ERK1/2 substrates validated by *in vitro* kinase assays

List of six ERK1/2 substrates validated by *in vitro* kinase assays. The following data fields are reported: gene symbol, description, molecular function, residue position, cellular fraction, $\Sigma \log_{10}(\text{Stimulated } t_i / \text{Control } t_{0 \text{ min}})$ and $\Sigma \log_{10}(\text{PD184352 } t_i / \text{DMSO } t_i)$ (t_i : 0, 5, 15, 60 min time points).

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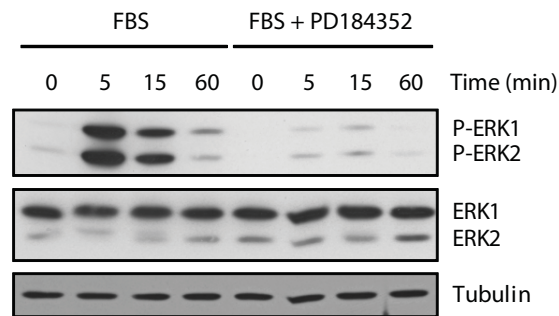


Figure S1. PD184352 inhibits the activating phosphorylation of ERK1/2

IEC-6 intestinal epithelial cells were starved of serum for 24 hours, treated or not with 2 μ M PD184352 for 1 h, and then stimulated with 10% fetal bovine serum for 5, 15 or 60 min. The expression and activating phosphorylation of ERK1/2 were analyzed by immunoblotting. Tubulin was used as loading control.

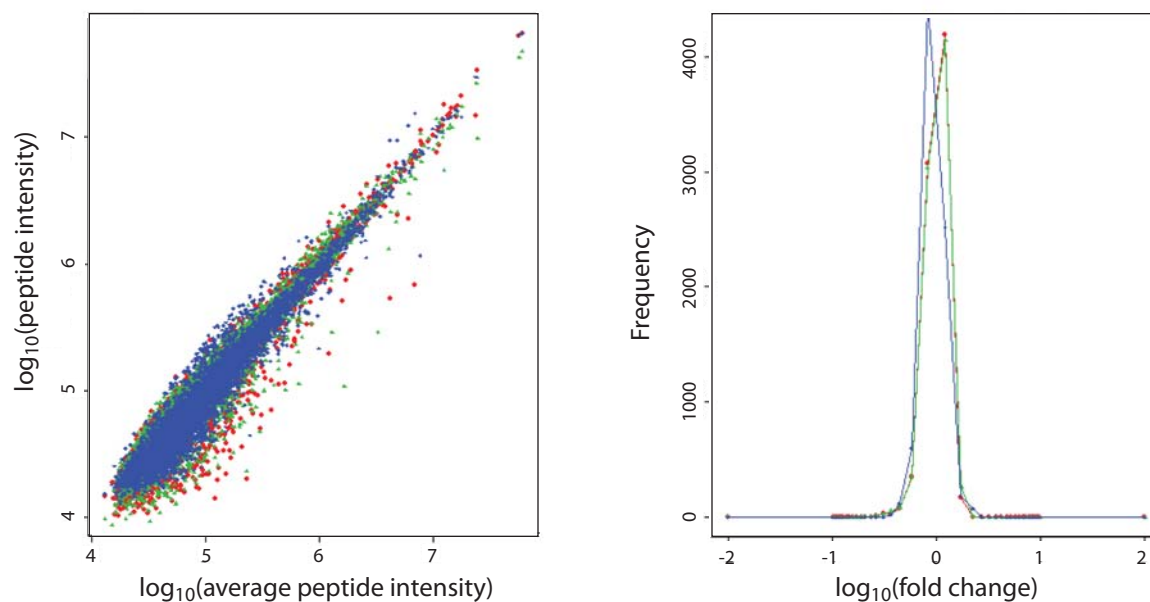


Figure S2. Reproducibility of label-free phosphopeptide quantification

TiO₂ enriched phosphopeptides were analyzed in triplicates by nanoLC-MS/MS on LTQ-Orbitrap and quantified by a label-free method. Phosphopeptides were detected from raw MS spectrum and aligned between replicates. Shown are biological replicates of the PD184352-treated cytosolic fraction stimulated for 5 min condition to demonstrate the reproducibility of the experiment. 95% of phosphopeptides show less than two-fold change and the coefficient of variation (CV) of the measured peak intensities for the replicates was on average 37%.

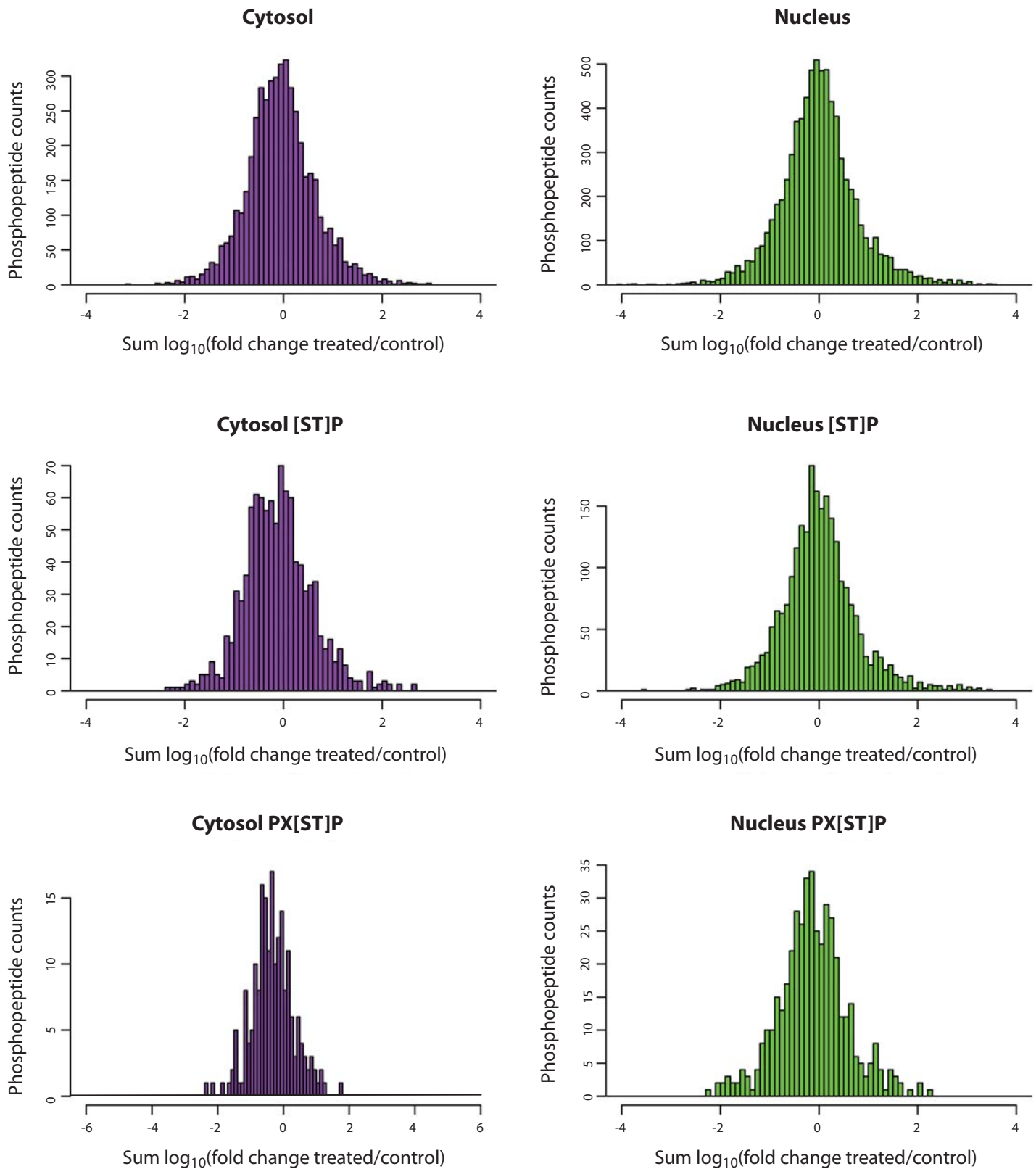


Figure S3. Distributions of changes in phosphorylation site abundance after MEK1/2 inhibition
 The distributions show the measured $\log_{10}(\text{PD184352}/\text{control})$ of phosphopeptides for the four experimental time points.

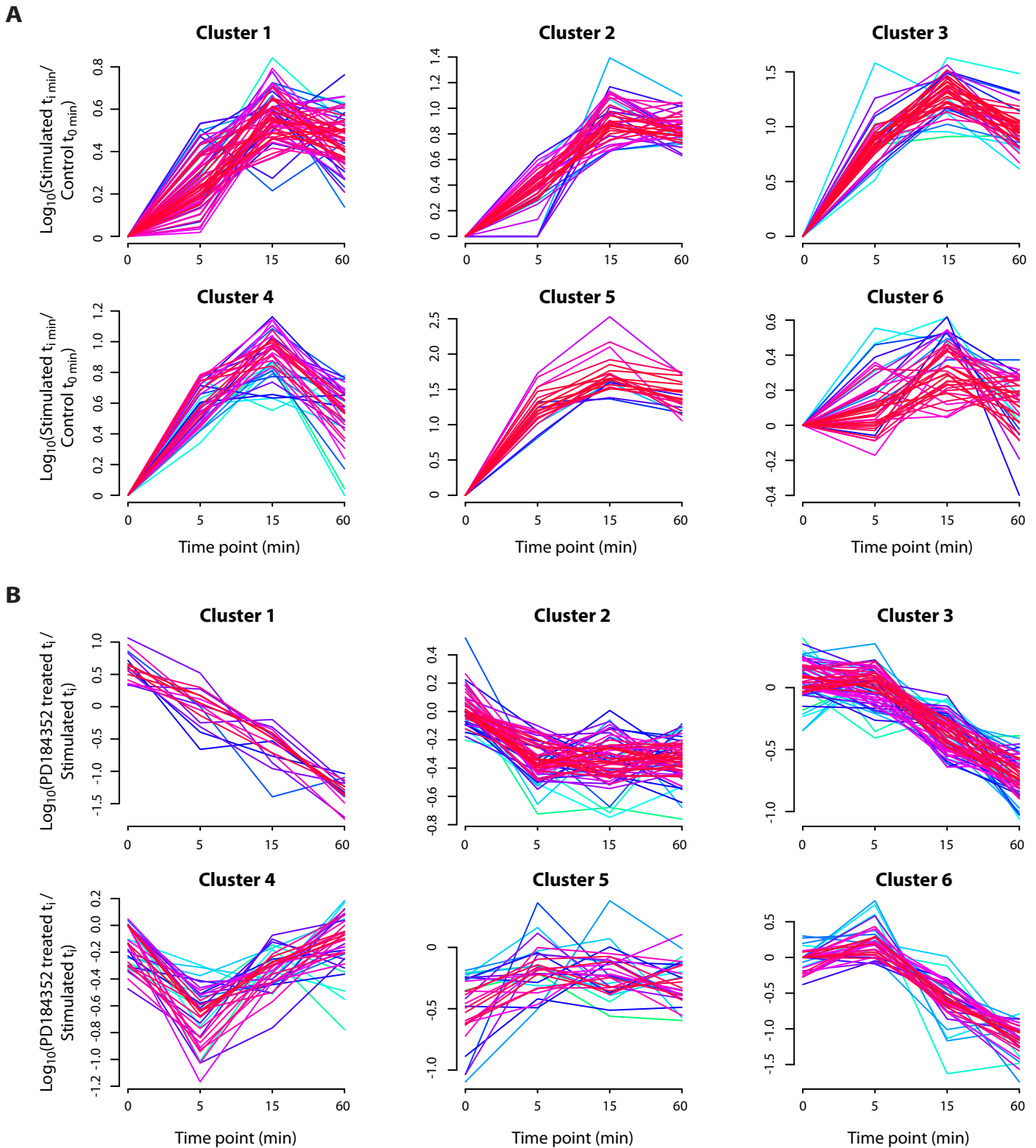
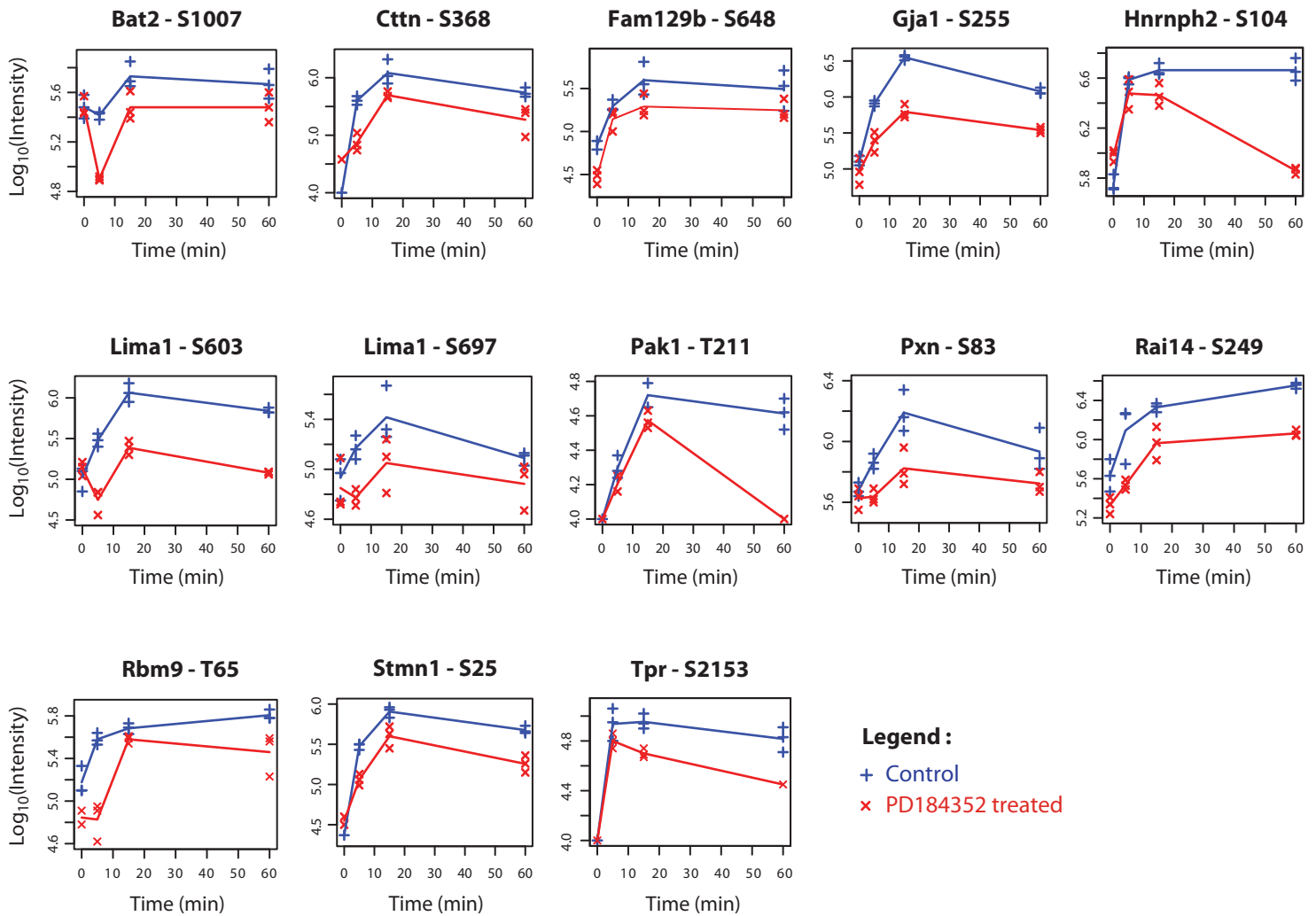


Figure S4. Kinetic profiles of the phosphorylation of candidate ERK1/2 substrates

Phosphorylation profiles of selected candidate ERK1/2 substrates after serum stimulation (A) and PD184352 inhibition (B). Soft-clustering of kinetic profiles (fuzzy c-means clustering MFuzz R package, $c=6$, $m=1.5$) was done to show 6 patterns (arbitrary chosen number) of phosphorylation change trends for both experimental groups. Fuzzy c-means clustering is a soft-clustering algorithm that distinguishes itself from hard-clustering algorithm by providing membership probability value to each member of the clusters (high and low memberships are shown with a gradient from red and green).

A**B**

Gene symbol	Name	Position	References
Bat2	Large proline-rich protein BAT2	S1007 (mouse)	Carlson et al. Sci Sign 2011
Ctnn	Cortactin isoform B	S405 (human)	Campbell et al. Cancer res 1999; Martinez-Quiles et al. Mol Cell Biol 2004
Fam129b	Niban-like protein 1	S646 (human)	Old et al. Mol Cell 2009; Kosako et al. Nat Struct Mol Biol 2009
Gja1	Gap junction alpha-1 protein	S255 (rat)	Warn-Cramer et al. J Biol Chem 1996
Hnrnp2	Heterogeneous nuclear ribonucleoprotein H2	S104 (mouse)	Carlson et al. Sci Signal 2011
Lima1	Epithelial protein lost in neoplasm	S602, S697 (mouse)	Han et al. Mol Cell Biol 2007
Pak1	Serine/Threonine-protein kinase PAK1	T211 (rat)	Sundberg-Smith et al. J Biol Chem 2005
Pxn	Paxillin	S83 (mouse)	Ku and Meier. J Biol Chem 2000; Ishibe et al. Moll Cell 2004
Rai14	Isoform 2 of Ankyrcorbin	T249 (mouse)	Carlson et al. Sci Sign 2011
Rbm9	RNA-binding protein 9	T65 (mouse)	Carlson et al. Sci Sign 2011
Stmn1	Stathmin	S25 (human)	Marklund et al. J Biol Chem 1993
Tpr	Translocated promoter region	S2153 (mouse)	Carlson et al. Sci Sign 2011; Eblen et al. J Biol Chem 2003 Allen et al. Nat Methods 2007

Figure S5. Identification of previously known ERK1/2 substrates

(A) Kinetic analysis of the site-specific changes in phosphorylation for the twelve known ERK1/2 substrates in response to serum stimulation and MEK1/2 inhibition. (B) The name of the twelve substrates and the sites phosphorylated by ERK1/2 are indicated with the original references.

Gene symbol	Name	Position (this study)	Phosphorylation site (other studies)	AS-ERK2 (Carlson et al.)	Biochemical validation (reference)
Marcks	Myristoylated alanine-rich C-kinase substrate	S138	S101 (Old et al. Mol Cell 2009)	-	Kinase assay (Schönwasser et al. FEBS Lett 1996)
Smarca5	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	S65	-	-	Kinase assay (Sif et al. Genes Dev 1998)
Dync1li1	Cytoplasmic dynein 1 light intermediate chain 1	T513	NI ^a (Kosako et al. Nat Struct Mol Biol 2009)	S516	Kinase assay (Kosako et al. Nat Struct Mol Biol 2009)
Nup153	Nuclear pore complex protein Nup153	S1087	S334 (Pan et al. Mol Cell Proteomics 2009)	T610	Kinase assay and phosphospecific antibodies (Kosako et al. Nat Struct Mol Biol 2009)
Nup214	Nuclear pore complex protein Nup214	S932	S430, T437 (Pan et al. Mol Cell Proteomics 2009) NI ^a (Kosako et al. Nat Struct Mol Biol 2009)	-	Kinase assay (Kosako et al. Nat Struct Mol Biol 2009)
Ahctf1	Protein ELYS	S1080 S1305 S1335	-	T1954	-
Ahnak	Neuroblast differentiation-associated protein AHNAK	S94 S178 S1107 S2134 S2181 S4751 S4767 S5397	-	T510	-
Map1b	Microtubule-associated protein 1B	S1494	-	T1784	-
Aim1	Absent in melanoma 1 protein	S452	-	T655	-
Cdc42ep1	Cdc42 effector protein 1	S194	-	S19 S113 T197	Kinase assay (Carlson et al. Sci Signal 2011)
Cdc42ep2	Cdc42 effector protein 2	S137 S141	-	S110	-
Phf2	Lysine-specific demethylase PHF2	S357 S505	-	S651	-
Rai1	Retinoic acid-induced protein 1	S1114	-	T1051	-
Tnks1bp1	182 kDa tankyrase-1-binding protein	S757	-	T131 T1025	-
Wiz	Widely-interspaced zinc finger-containing protein	S323 S328	-	S288	-

^a NI, site not identified

Figure S6. Previously reported ERK1/2 substrates with unmapped or distinct phosphorylation sites

Each protein is described with the phosphorylation site(s) identified in this study (this study), the phosphorylated residue(s) identified in other studies, the type of biochemical validation and the original references.

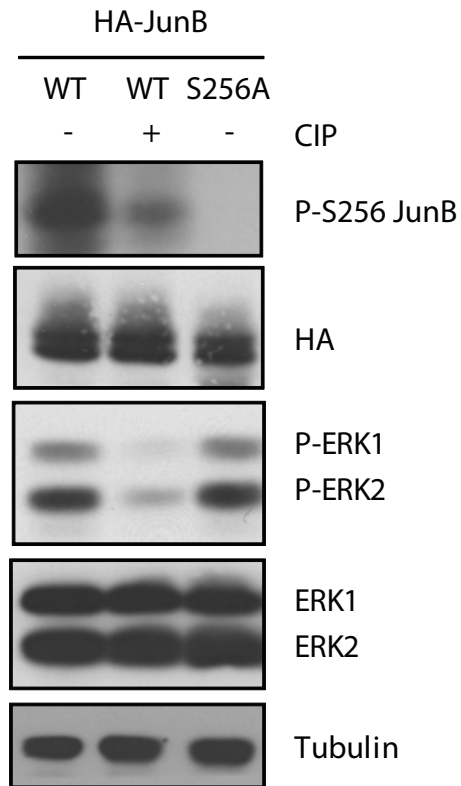


Figure S7. Validation of the phospho-JunB(Ser256) specific antibody

HEK 293 cells were transfected with HA-JunB wild type (WT) or S256A mutant. Cellular extracts were treated or not with calf intestinal phosphatase (CIP) (20 U for 50 μ g of extract) for 1 h at 37°C. Phosphorylation of JunB was monitored by immunoblotting with anti-JunB (Ser256) antibody (Sigma). Phosphorylation of ERK1/2 was used as a positive control of dephosphorylation.

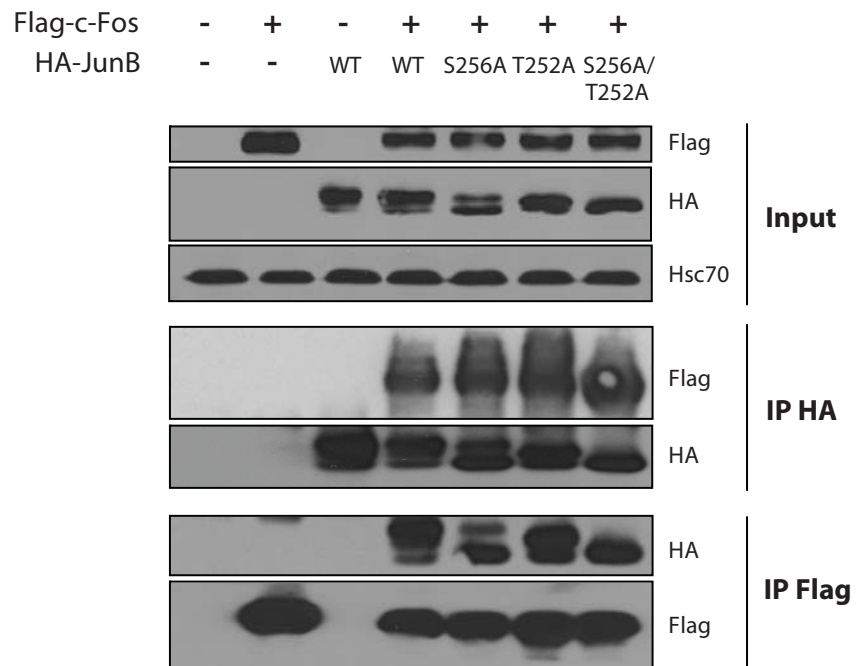


Figure S8. Impact of Ser256 and Thr252 phosphorylation of the interaction of JunB with c-Fos
 HEK 293 cells were transfected with the indicated constructs. After 36 h, the cells were lysed and JunB or c-Fos were immunoprecipitated using anti-HA or anti-Flag antibodies, respectively. Total lysates and immunoprecipitates were analyzed by immunoblotting.

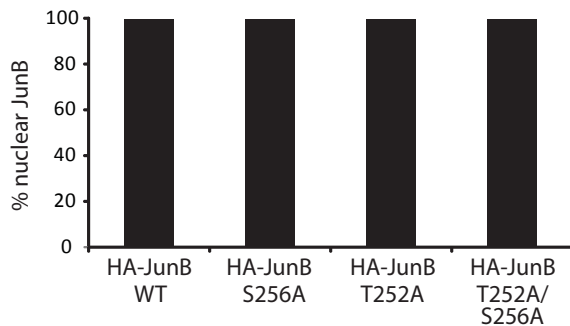
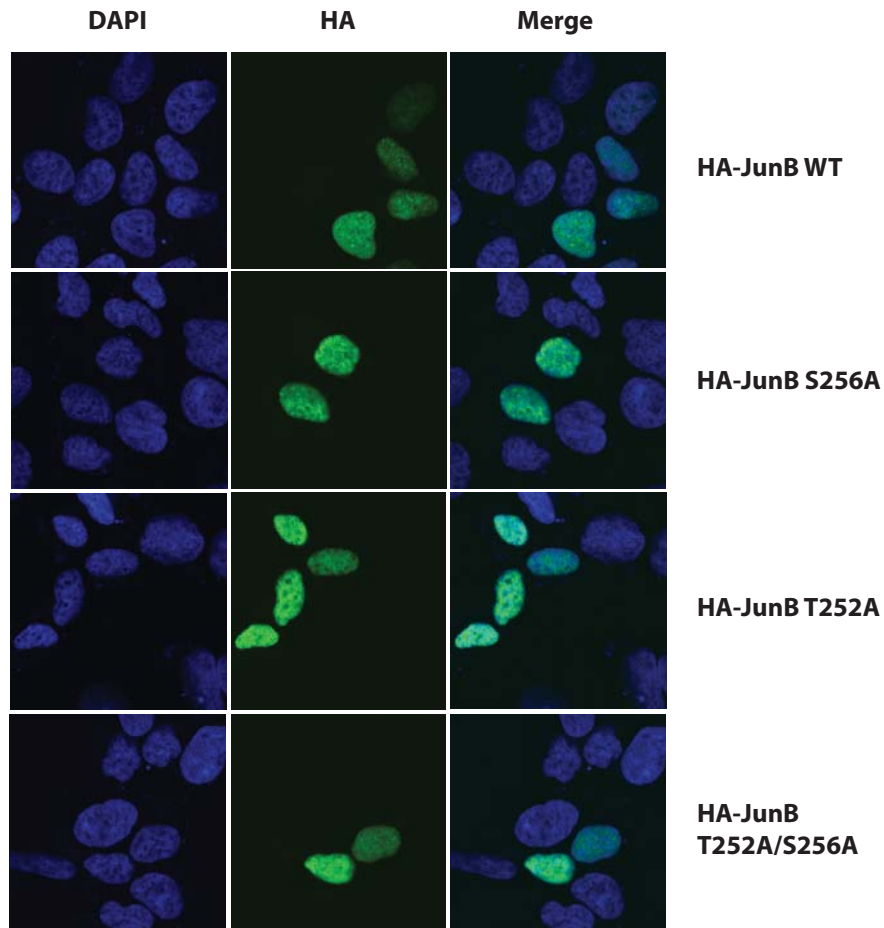


Figure S9 . Phosphorylation of Ser256 and Thr252 does not regulate the nuclear localization of JunB

HEK 293 cells were transfected with the indicated HA-tagged JunB constructs and analyzed by immunofluorescence with anti-HA antibody. Nuclei were stained with DAPI. JunB WT and mutants were localized in the nucleus of 100% of cells.

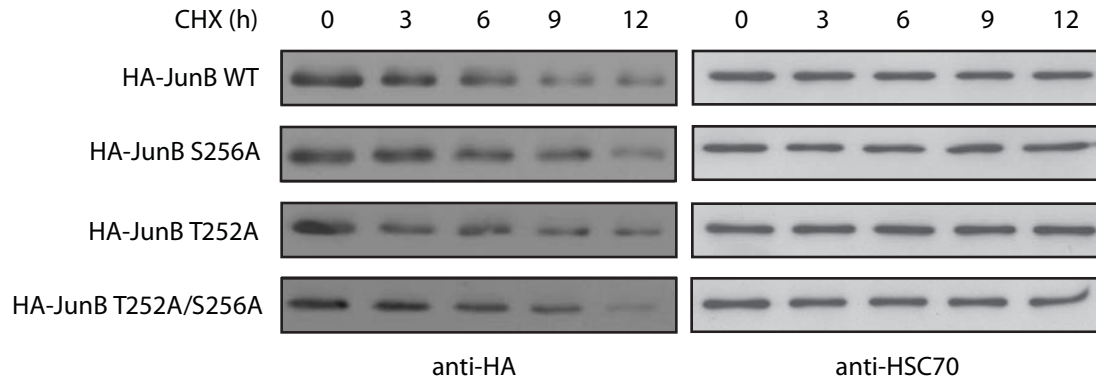
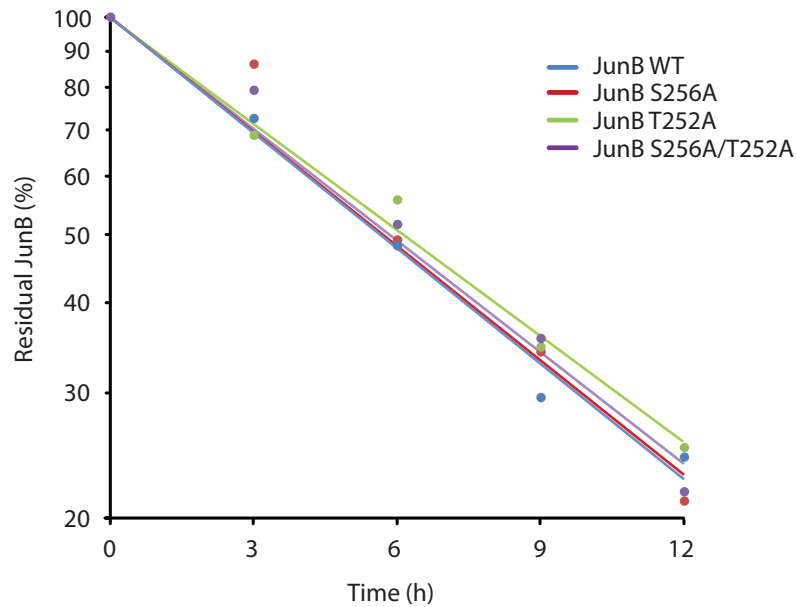
A**B**

Figure S10. Phosphorylation of Ser256 and Thr252 does not regulate the stability of JunB in proliferating NIH 3T3 cells

(A) NIH 3T3 cells were transfected with the indicated HA-tagged JunB constructs and treated with cycloheximide (50 $\mu\text{g}/\text{ml}$) for the indicated times. The expression of ectopic JunB was monitored by immunoblotting with anti-HA antibody. (B) Quantification of immunoblotting data shown in A. JunB levels were measured by densitometric analysis of immunoblots.

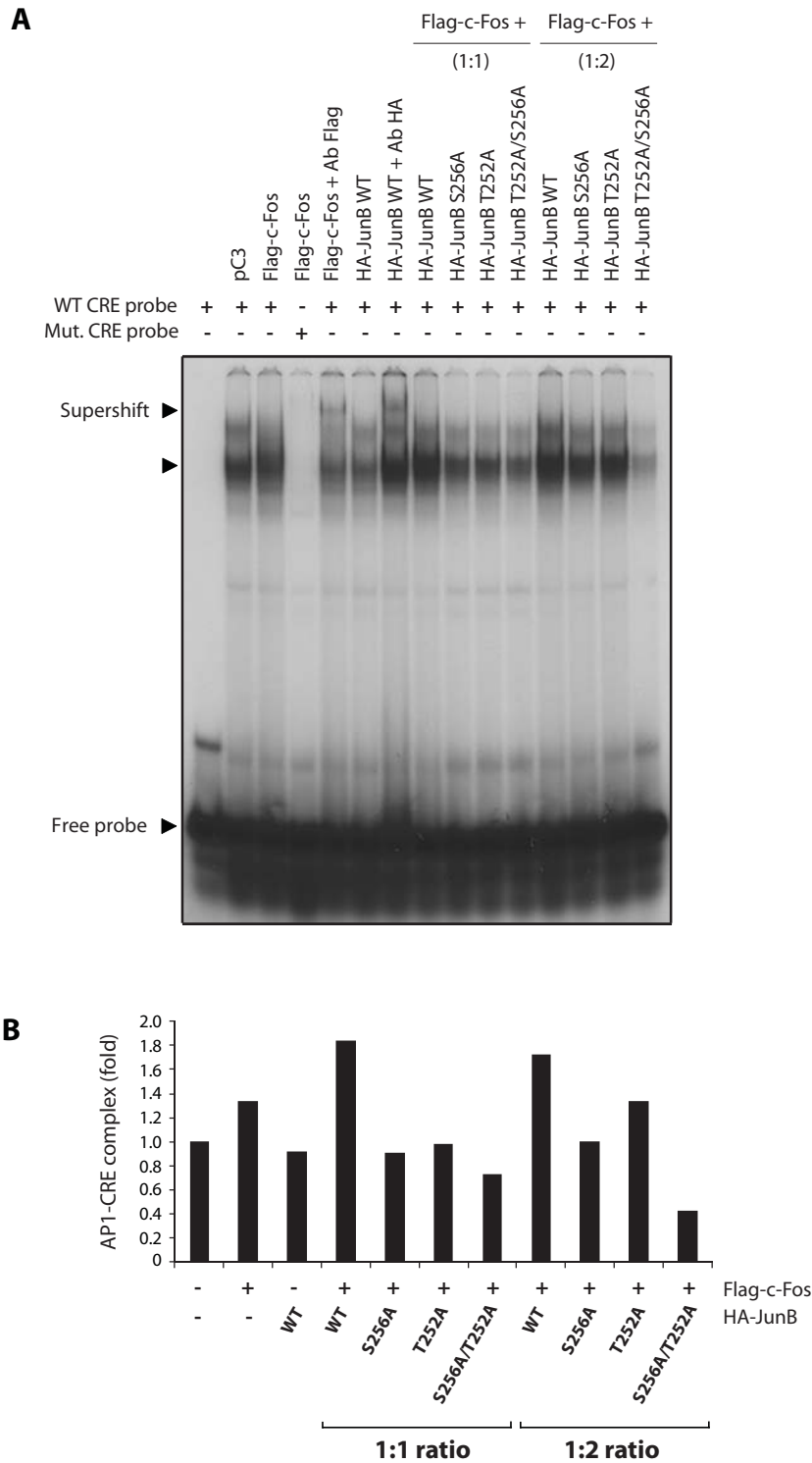


Figure S11. ERK1/2 phosphorylation of JunB on Ser256 promotes cooperative DNA binding of JunB/c-Fos heterodimers.

(A) HEK 293 cells were transfected with the indicated constructs and nuclear extracts were analyzed by EMSA using a radiolabeled CRE probe. Supershift analyses were performed by incubating nuclear extracts with Flag or HA antibody. Results are representative of 3 experiments. (B) Quantification of EMSA results in A by densitometric analysis. The results are normalized to control cells transfected with empty pcDNA3.