Supporting Information

Supporting Information Corrected December 29, 2011

SI Materials and methods

Cell culture

Cos7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (10%), l-glutamine and penicillin/streptomycin. Primary cultures of neonatal rat cardiac ventricular myocytes (NRVMs) from 1- to 2-day-old Sprague-Dawley rats (Charles River Laboratories) were prepared as described previously (1). In brief, NRVMs were dispersed from the ventricles by digestion with collagenase type II (Worthington). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1,000 mg/L D-glucose, L-glutamine, and 110 mg/L sodium pyruvate (GIBCO), fetal bovine serum (10%), and penicillin/streptomycin at 37°C in a 5% CO_2 humidified atmosphere for 2–3 days. For inducer or inhibitor studies, cells were pretreated with various inducers or inhibitors for 30 min in serum-free medium. Primary cultures of adult rat cardiomyocytes were prepared from 200-250g male Sprague-Dawley rats as previously described (2). Briefly, rats were anesthetized, hearts excised and placed in Krebs buffer and perfused and digested with endotoxin-free collagenase II (Worthington). This protocol typically yielded approximately $4x10^6$ cells per heart, with ~85% surviving, rod-shaped Ca2+-tolerant cells. Myocytes were allowed to attach to 20 µg/ml laminin coated plates in Modified Eagle Medium (MEM) containing 2.5% FBS, 25mmol/L HEPES, 5 mmol/L taurine, 2mmol/L carnitine, 2mmol/L creatinine, 100 IU/ml penicillin, and 100 µg/ml streptomycin for 2 hrs before replacing with serum-free MEM containing 25mmol/L HEPES, 5 mmol/L taurine, 2mmol/L carnitine, 2mmol/L creatinine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Myocytes were pretreated with cAMP for 30 min and stimulated with PE for 3 hrs.

Materials

PMA (phorbol-12-myrisstate-13-acetate), Forskolin, Dibutyryl-cAMP, DibutyrylcGMP, Rolipram, Isoproterenol, Cilostamide, IBMX, EHNA and PKI (PKA Inhibitor 14-22 Amide) were from Calbiochem (San Diego, California). PE (R-(-)-Phenylephrine) was from Sigma. The antibodies for phospho-PKA substrate were purchased from Cell Signaling Technologies. The antibodies for 14-3-3 β (c-20) and HA-probe (Y-11) were from Santa Cruz, Inc. The antibodies for α-Actinin (sarcomeric), DAPI and Flag-probe were from Sigma. The antibody for phospho-HDAC5 (Ser259/498) was kindly provided by Dr. Timothy McKinsey and characterized as described previously (3).

Plasmids and transient transfection

GFP-HDAC5-WT, FLAG-HDAC5-WT, and YFP-HDAC7-WT have been described previously (4, 5). HDAC4, HDAC9, and constitutively active mutants of PKA, PKG, MEK1, Akt, MKK3, MKK4, IKK-β, and CAMK1 were purchased from Addgene Inc. GFP-HDAC4 and GFP-HDAC9 GFPHDAC5-S280A and GFP-HDAC5-S280D were

generated from GFP-HDAC5-WT with the Quick-change site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. For the S280A mutant, oligonucleotides 5'-GCTGAGCGGAGAAGCGCTCCCCTCCTGCGT-3' and 5'-ACGCAGGAGGGGAGCGCTTCTCCGCTCAGC-3' were used as primers for PCR. For S280D, the primers were 5'-GCTGAGCGGAGAAGCGATCCCCTCCTGCGT-3' and 5'-ACGCAGGAGGGGATCGCTTCTCCGCTCAGC-3'. By identical methods, YFP-HDAC7-K279S/N280S was generated from YFP-HDAC7-WT. For YFPHDAC7-K279S/N280S, the primers were 5'-CCTGGAGAGACGCAGCAGTCCCCTGCTCAGGA -3' and 5'-TCCTGAGCAGGGGACTGCTGCGTCTCTCCAGG -3'. Flag-HDAC5-S280A and Flag-HDAC5-S280D were generated from GFP-HDAC5-S280A and GFP-HDAC5-S280D, respectively. HA-PKA-CA-NLS was generated from HA-PKA-CA by PCR cloning. For HA-PKA-CA-NLS, primers were 5'-GCTAGTCCGGAATGTACCCATACG ATG TTCCAGATTACGCTATGGGCAACGCCGCCGCCGCCAA-3' and 5'-GCCCTCGAGCAAACTCAGTAAACTCCTTGCCA-3'. HcRed1-PKA-CANLS was generated from HA-PKA-CA-NLS. HDAC5 peptides containing amino acids 273–286 of WT and S280A mutant were cloned into pGEX5X-2 vector for *In vitro* phosphorylation assay. Transient transfection of plasmid DNA was performed using lipofectamine 2000 (Invitrogene) and 0.5 μg of the indicated plasmids.

Adenoviral construction and infection

Adenoviruses encoding Flag-tagged HDAC5 wild-type (WT), GFP-tagged HDAC5-WT, and YFP-tagged HDAC7-WT were generated as described previously (4, 5). Adenovirus expressing GFP-tagged HDAC5-S280A mutant, GFP-tagged HDAC5-S280D mutant and Flag-tagged HDAC5-S280A mutant were generated by using ViraPower Adenoviral Expression System (Invitrogen) according to the manufacturer's protocol. Adenovirus containing β-galactosidase (LacZ) or GFP was used as a control. NRVMs and Cos7 cells were infected with adenovirus expressing indicated proteins at the indicated multiplicity of infection (MOI) for 24 hours, and then treated with or without inhibitors or inducers for 30 min followed by the application of PMA or PE.

Small Interference RNA (siRNA) and Its Transfection

The siRNA duplex targeting rat PKACA and the scrambled siRNA control (a non-targeting siRNA pool) were purchased from Dharmacon, Inc (Illinois). For transfection of siRNA, NRVMs were transfected with PKACA siRNA using lipofectamine 2000 (Invitrogen, California) according to the manufacturer's protocol as described previously (6). PE stimulation was performed 48 hour after siRNA transfection.

Fluorescence images (subcellular localization)

For analysis of GFP-HDAC5 subcellular localization in NRVMs and Cos7 cells, cells were plated in the presence of adenovirus (multiplicity of infection, 50 to 100) on 35 mm dishes containing DMEM plus 10% FBS as previously described (3). After 24 hours culture, cells were maintained in serum-free DMEM medium for

additional 24 hours, and then exposed to various inducers or inhibitors (for 30 minutes) prior to PE or PMA stimulation for indicated times. The cells were washed with PBS and fixed with 3.7% formaldehyde in PBS. Images were captured at a magnification of x60, using a fluorescence microscope (Olympus BX51) equipped with a digital camera and Spot software system (RT Color Diagnostic Instruments). For analysis of the myocyte marker, sarcomeric α-actinin antibody and DAPI in neonatal cardiomyocyte, immunocytochemistry were performed according to a standard protocol. For subcellular localization of HDAC5, total around 200 cells were counted in each condition. For cardiomyocyte size, total myocyte surface areas were calculated using NIH Image J software, and expressed as the average of 100 randomly selected cells per condition.

***In vitro* phosphorylation assay**

For *in vitro* PKA-induced phosphorylation assays, GST-fusion peptide of HDAC5-WT or GST-tagged peptide of HDAC5-S280A were mixed in 500 µl of phosphate buffered saline (PBS) and incubated for 30 min at 4 °C. Slurry of glutathione-Sepharose 4B was subsequently added, followed by further incubation for 1 hour at 4 °C. GST-fusion peptides were incubated 20 min at 30°C in a 50µl final volume that contained 50 mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 200µM ATP, 0.1mM {γ-^{32}P}ATP (5µCi/tube) and 100ng of purified catalytic subunit of protein kinase A (PKA-CA, Calbiochem, NJ, USA). Reactions were terminated by addition of SDS sample buffer followed by boiling. The eluates were resolved by SDS–PAGE, transferred to PVDF membranes and visualized by autoradiography.

Western blot and immunoprecipitation

Cells were harvested in lysis buffer and clarified by centrifugation as described previously (4). The protein concentrations in the lysates were determined using the Bradford method (BioRad, Hercules, California). Immunoprecipitation were performed according to standard protocols as described previously (4). The immune complex samples or total cell lysates were resolved on SDS-PAGE according to standard protocols (4). For Western blots, the protein samples from total cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with appropriate primary antibodies. After incubating with fluorescence-conjugated secondary antibodies, immunoreactive proteins were visualized by Odyssey Infrared Imaging System (LI-COR Biotechnology, Nebraska). Densitometric analyses of immunoblots were performed with Odyssey software (LICOR Biotechnology). Results were normalized by arbitrarily setting the densitometry of control sample to 1.0.

Luciferase assay

To assess MEF2 and CREB transcriptional activation, we used adenovirus encoding 3xMEF2-dependent reporter gene in which three tandem repeats of MEF2 sites were located upstream of the thymidine kinase gene promoter and plasmid encoding CREB-dependent reporter gene. NRVMs or Cos7 cells cultured in 24-well were co-transfected with 3xMEF2 or CREB luciferase reporter gene and adenovirus encoding LacZ or GFP-HDAC5-WT, GFP-HDAC5-S280A mutant and

GFP-HDAC5-S280D mutant. At 48 hours post-transfection, cells were treated with forskolin or cAMP for 30min followed by PE for another 24 hours. The luciferase activities in cell lysates were determined using the Dual-Luciferase Reporter Assay kit (Promega) and Wallac 1420 multi-label counter (PerkinElmer) as described previously (4, 5).

RT-PCR

Total RNA was isolated from NRVM using using TRIzol (Invitrogen Corp.). Firststrand cDNA was synthesized with the SuperScript Preamplification System (Gibco-BRL). cDNA was amplified by PCR for 30 cycle (Applied Biosystem) as described previously (4, 5). Rat GAPDH served as an internal control. The following oligonucleotide primers were used in this study: rat ANF, sense 5'-ATGGGCTCCTTCTCCATCAC-3' and antisense 5'-ATCTTCGGTACCGGAAGCTG-3'; rat α-SMA, sense 5'-ACTGGGACGACATGGAAAAG-3' and antisense 5'-CATCTCCAGAGTCCAGCACA-3'; rat β-MHC (myosin heavy chain), sense 5'-CCTCGCAATATCAAGGGAAA-3' and antisense 5'-TACAGGTGCATCAGCTCCAG-3'; rat GAPDH, sense 5'-CGATGCTGGCGCTGAGTA -3' and antisense 5'-CGTTCAGCTCAGGGATGACC-3'. Experiments were repeated three times.

Statistics analysis

All values in the text and figures are expressed as means ± SEM at least three independent experiments from given n sizes. The significance of the results was assessed by a paired t-test between two groups. Differences among 3 or more groups were analyzed by contrast analysis, using the Super ANOVA. A p value <0.05 was considered significant.

References

1. Ago T, et al. (2008) A redox-dependent pathway for regulating class II HDACs and cardiac hypertrophy. (Translated from eng) Cell 133(6):978-993 (in eng).
2. Nadtochiy SM, Burwell LS, & Brookes PS (2007) Cardioprotection and mitochondrial S-nitrosation: effects of S-nitroso-2-mercaptopropionyl glycine (SNO-MPG) in cardiac ischemia-reperfusion injury. (Translated from eng) J Mol Cell Cardiol 42(4):812-825 (in eng).
3. Vega RB, et al. (2004) Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. Mol Cell Biol 24(19):8374-8385.
4. Ha CH, et al. (2008) Protein Kinase D-dependent Phosphorylation and Nuclear Export of Histone Deacetylase 5 Mediates Vascular Endothelial Growth Factor-induced Gene Expression and Angiogenesis. (Translated from eng) J Biol Chem 283(21):14590-14599 (in eng).
5. Ha CH, Jhun BS, Kao HY, & Jin ZG (2008) VEGF stimulates HDAC7 phosphorylation and cytoplasmic accumulation modulating matrix

metalloproteinase expression and angiogenesis. (Translated from eng) Arterioscler Thromb Vasc Biol 28(10):1782-1788 (in eng).
6. Wong C & Jin ZG (2005) Protein kinase C-dependent protein kinase D activation modulates ERK signal pathway and endothelial cell proliferation by vascular endothelial growth factor. J Biol Chem 280(39):33262-33269.

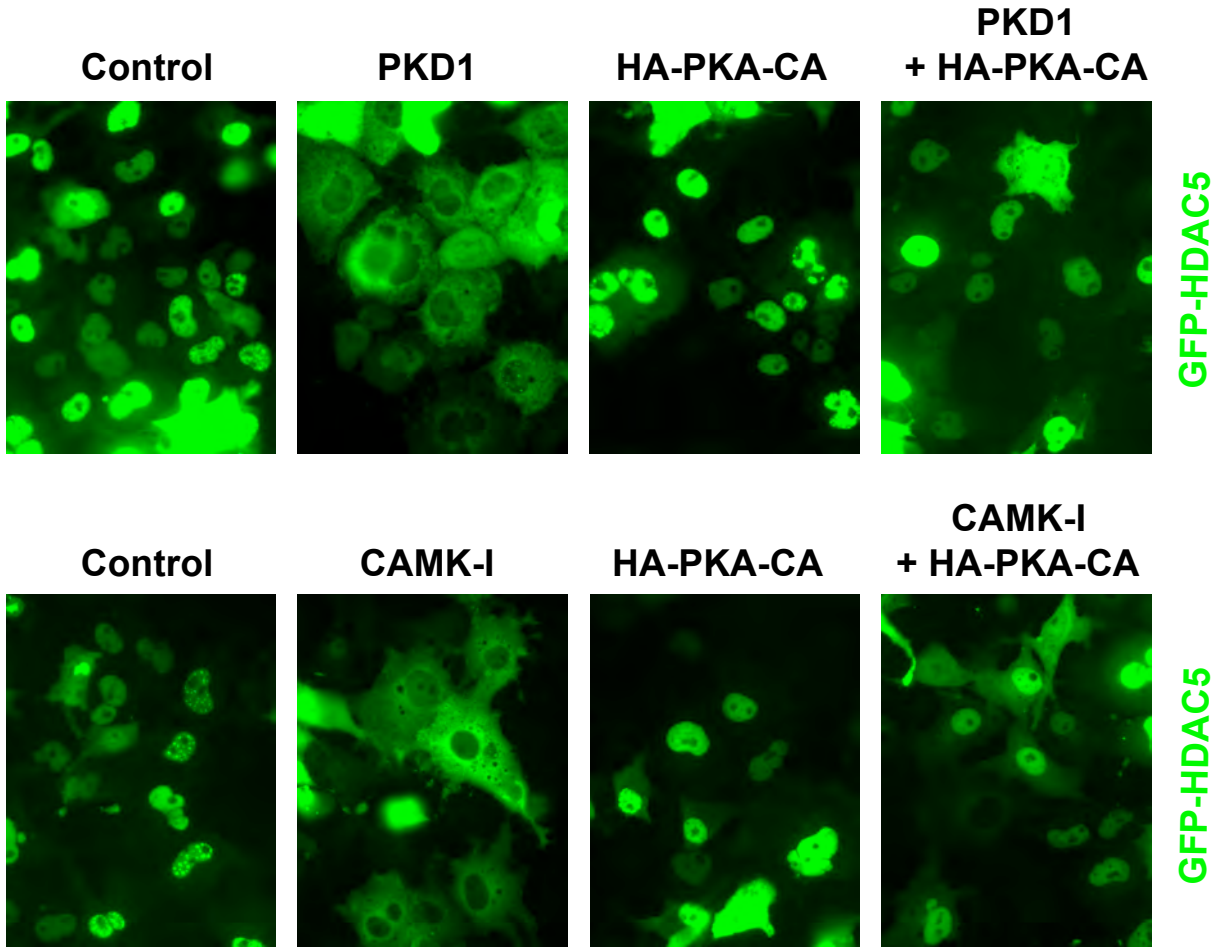


Figure S1. PKA inhibits PKD/CaMK-induced HDAC5 nuclear export in COS7 cells. Cos7 cells were co-transfected with expression vectors encoding GFP-tagged HDAC5 and the constitutively active form of PKD1, CaMK-I, and HA-PKA-CA.

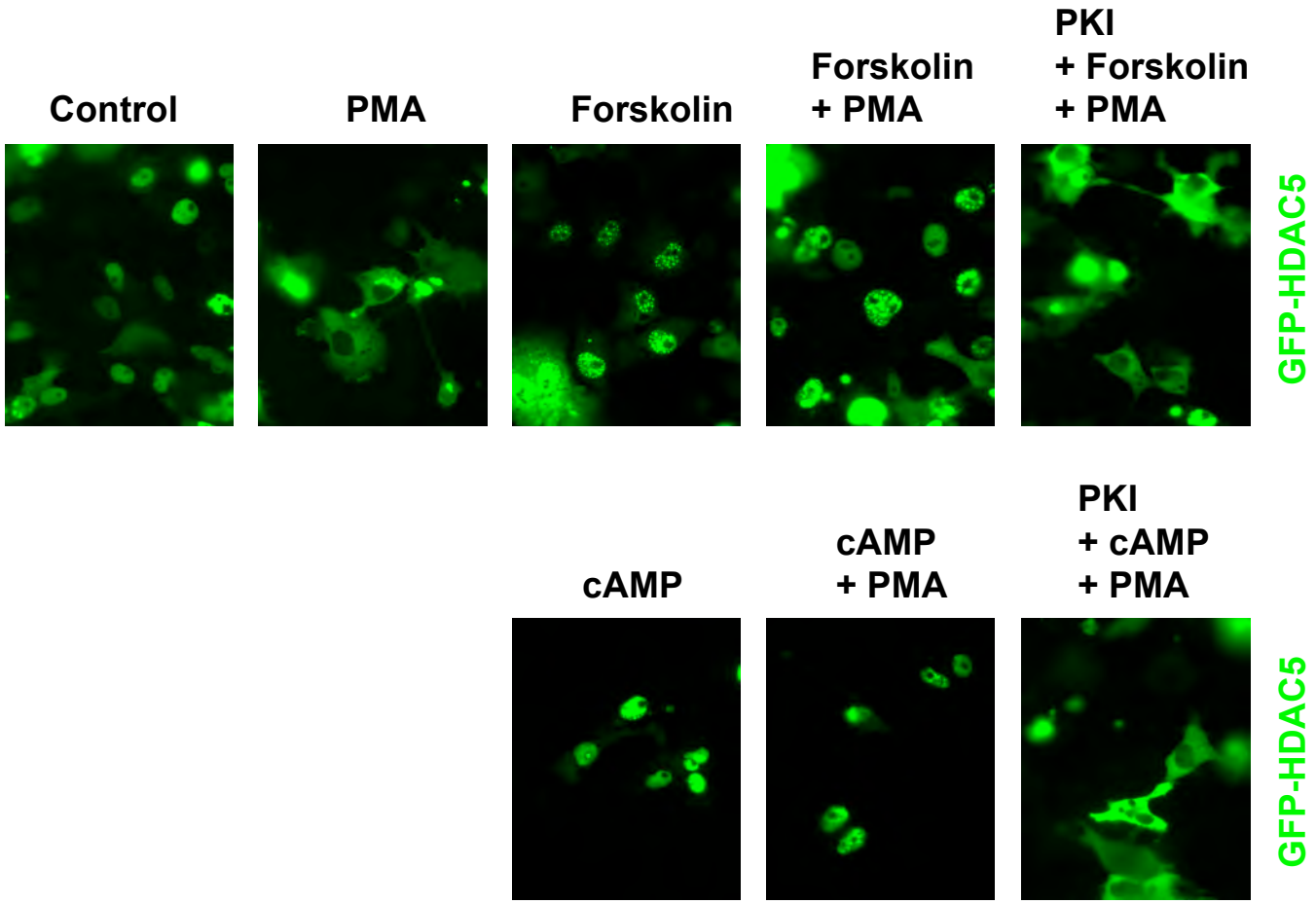


Figure S2. PKI inhibits forskolin/cAMP effects on PMA-induced HDAC5 Nuclear Export in Cos7 cells. Cos7 cells were transfected with plasmids GFP-tagged HDAC5 and then pretreated with the vehicle (DMSO as control) and PKA inhibitor 14-22 amide (PKI 14-22 Amide), and then treated with forskolin or cAMP, followed by the exposure of PMA for 3 hours. The cells were fixed and GFP-HDAC5 localization was analyzed by fluorescence microscopy.

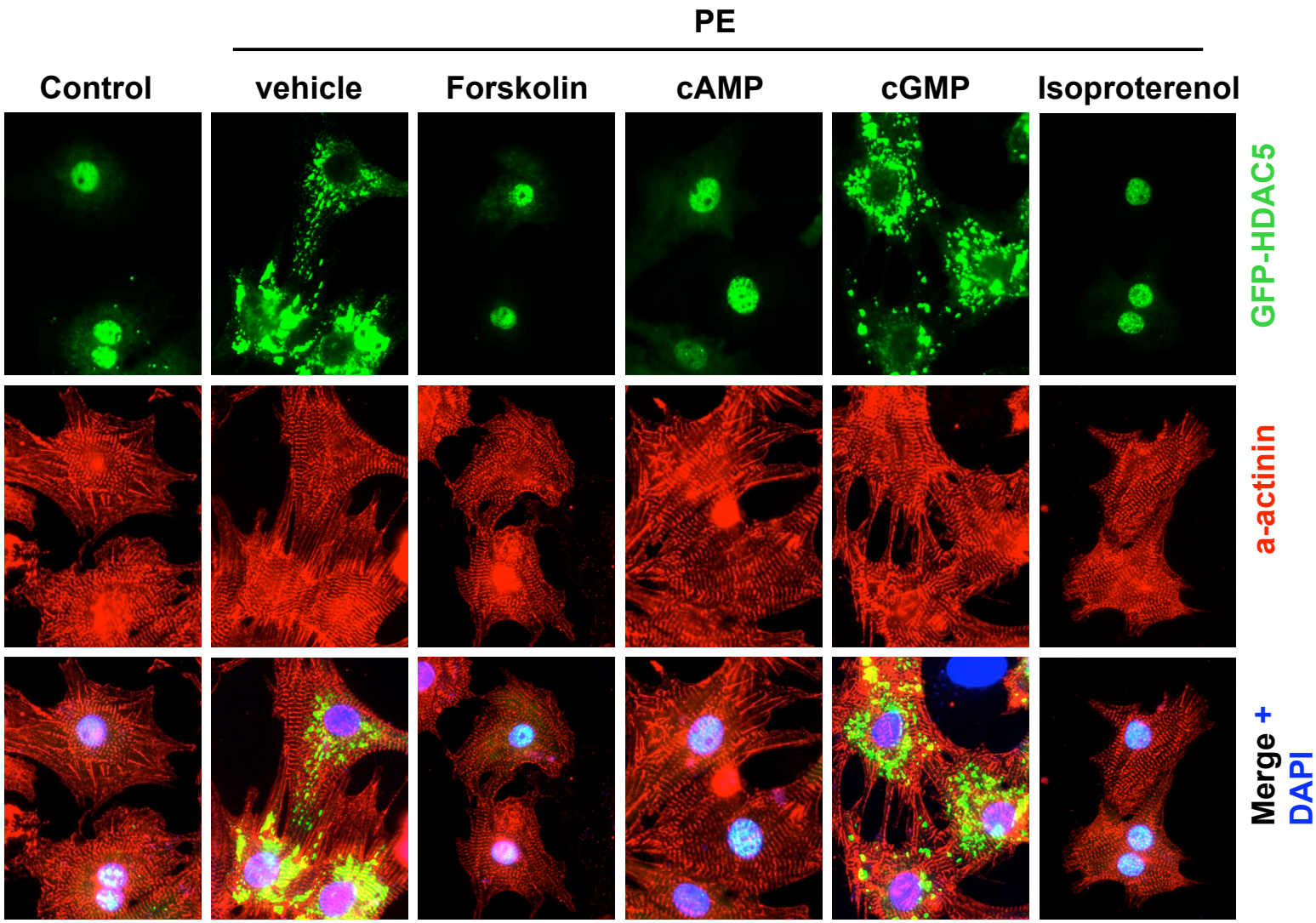


Figure S3. High resolution images of Figure 1E (see the text in detail).

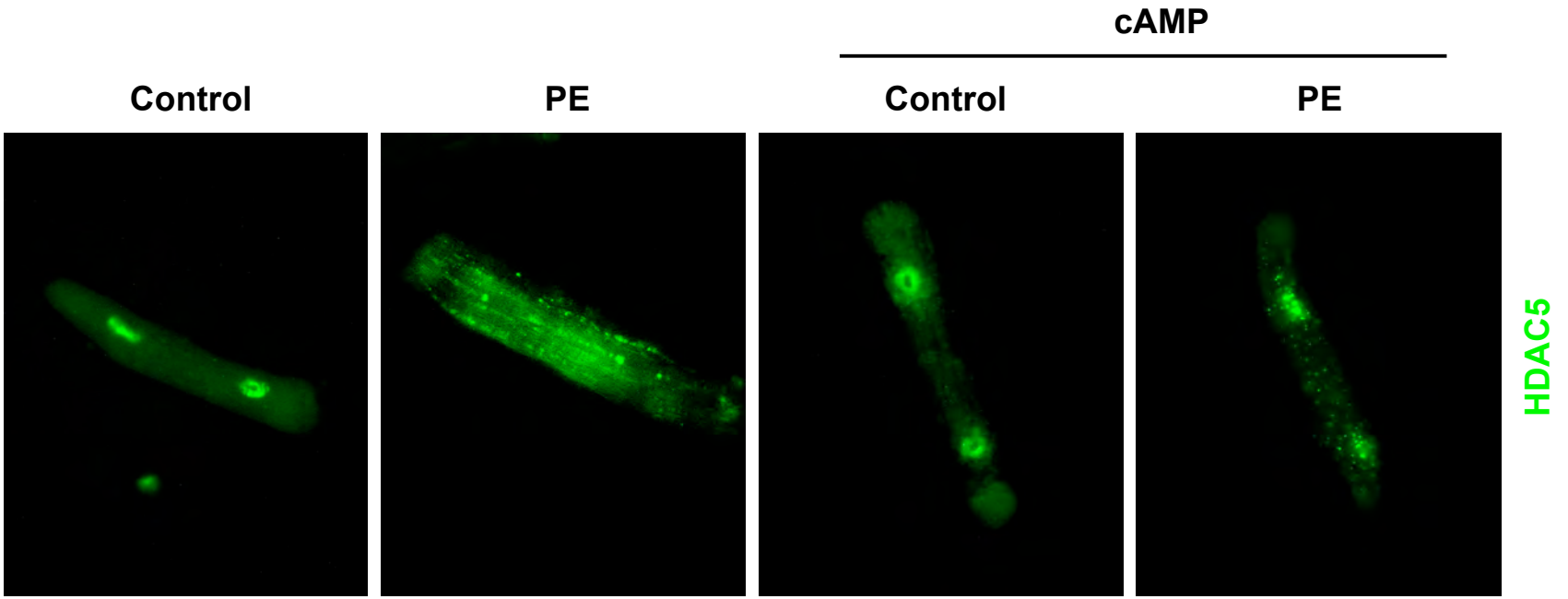


Figure S4. PKA inhibits stress signal-regulated HDAC5 nuclear export in ARVMs. Adult rat ventricular cardiomyocytes (ARVMs) were pretreated with the vehicle (DMSO as control) and cAMP (500 μM) for 30 min, followed by the exposure of α-adrenergic agonist phenylephrine hydrochloride (PE, 10 μM) for 3 hours. The cells were fixed and endogenous HDAC5 localization was analyzed by fluorescence microscopy. HDAC5 was detected by immunofluorescence using an antibody against HDAC5 (Santa Cruz sc-11419) and a fluorescein-conjugated secondary antibody (Green).

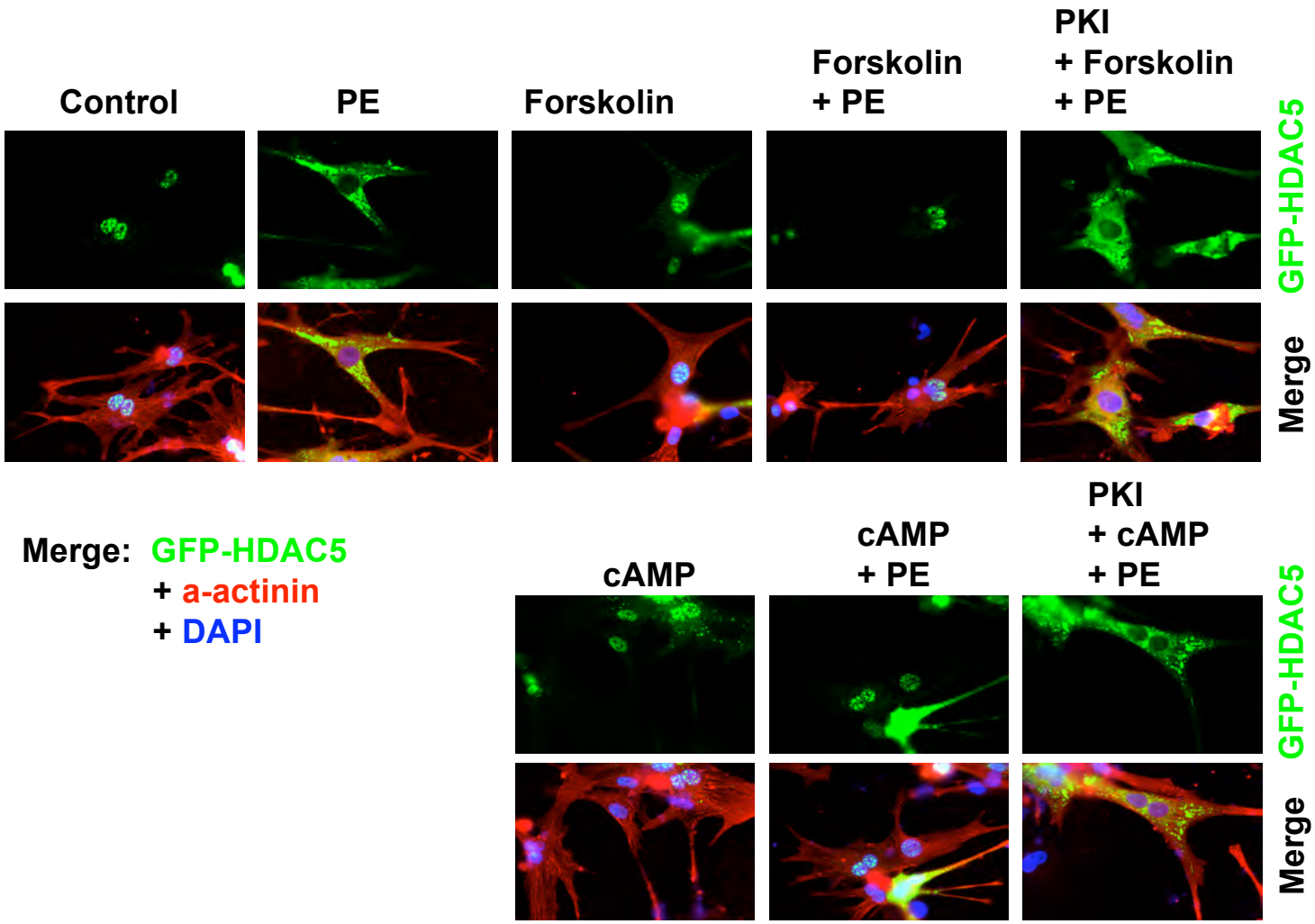


Figure S5. PKI inhibits forskolin/cAMP effects on PE-induced HDAC5 Nuclear Export in NRVMs. NRVMs were infected with adenoviral expression vector encoding GFP-tagged HDAC5 and then pretreated with the vehicle (DMSO as control) and PKI 14-22 Amide, and then treated with forskolin or cAMP, following by the exposure with phenylephrine hydrochloride (PE) for 3 hous. The cells were fixed and GFP-HDAC5 localization was analyzed by fluorescence microscopy. -actinin was detected by immunofluorescence using an antibody against -actinin and a fluorescein-conjugated secondary antibody (red). The nuclei were stained with DAPI (blue).

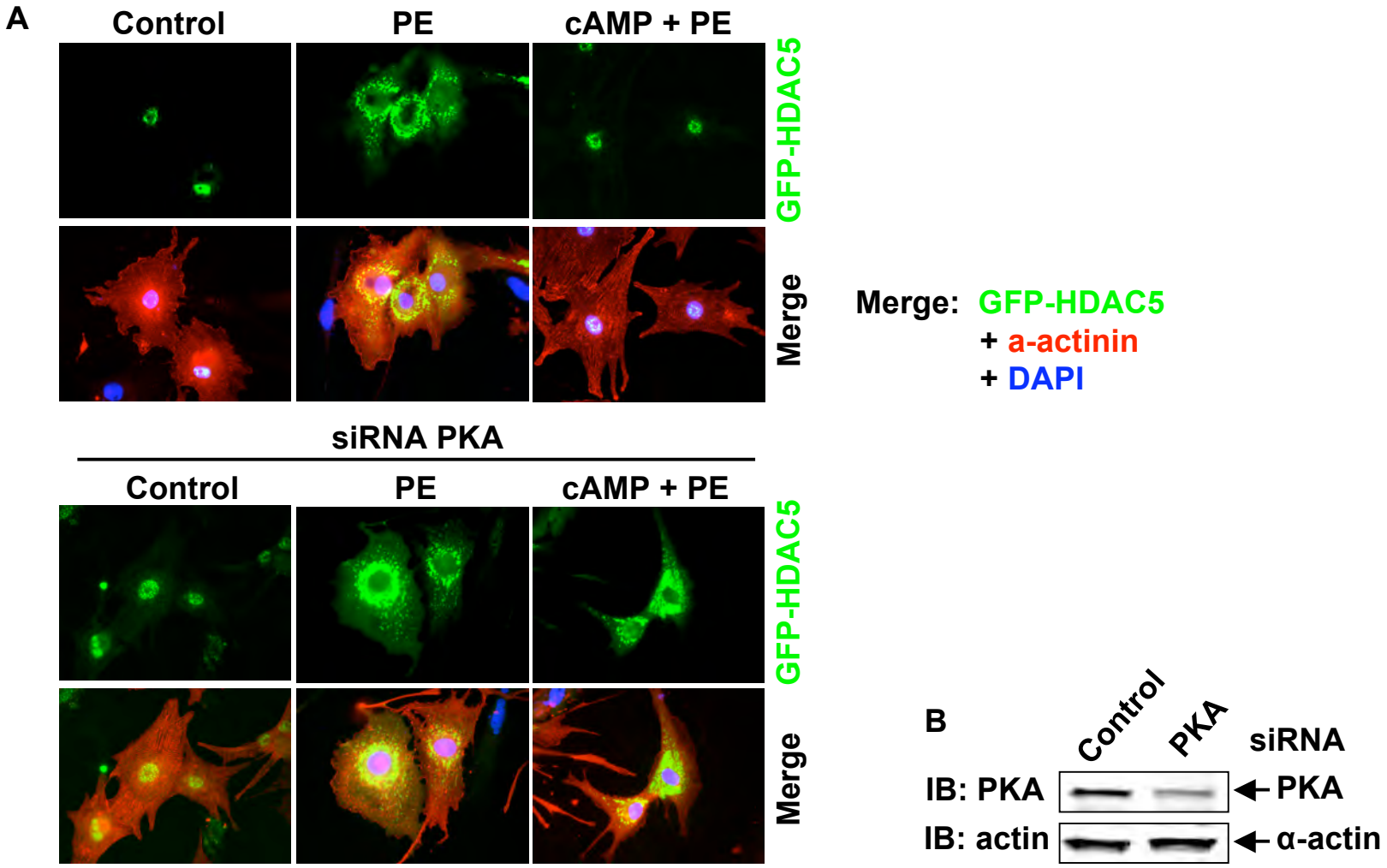


Figure S6. PKACA siRNA inhibits cAMP effects on PE-induced HDAC5 Nuclear Export in NRVMs. A, NRVMs were transfected with PKACA siRNA and then infected with adenoviral expression vector encoding GFP-tagged HDAC5, and then pretreated with the vehicle (DMSO as control) and cAMP, following by the exposure with phenylephrine hydrochloride (PE) for 3 hous. The cells were fixed and GFP-HDAC5 localization was analyzed by fluorescence microscopy. α-actinin was detected by immunofluorescence using an antibody against -actinin and a fluorescein-conjugated secondary antibody (red). The nuclei were stained with DAPI (blue). **B,** PKA expression in cell lysates were determined by Western blot using PKA antibody.

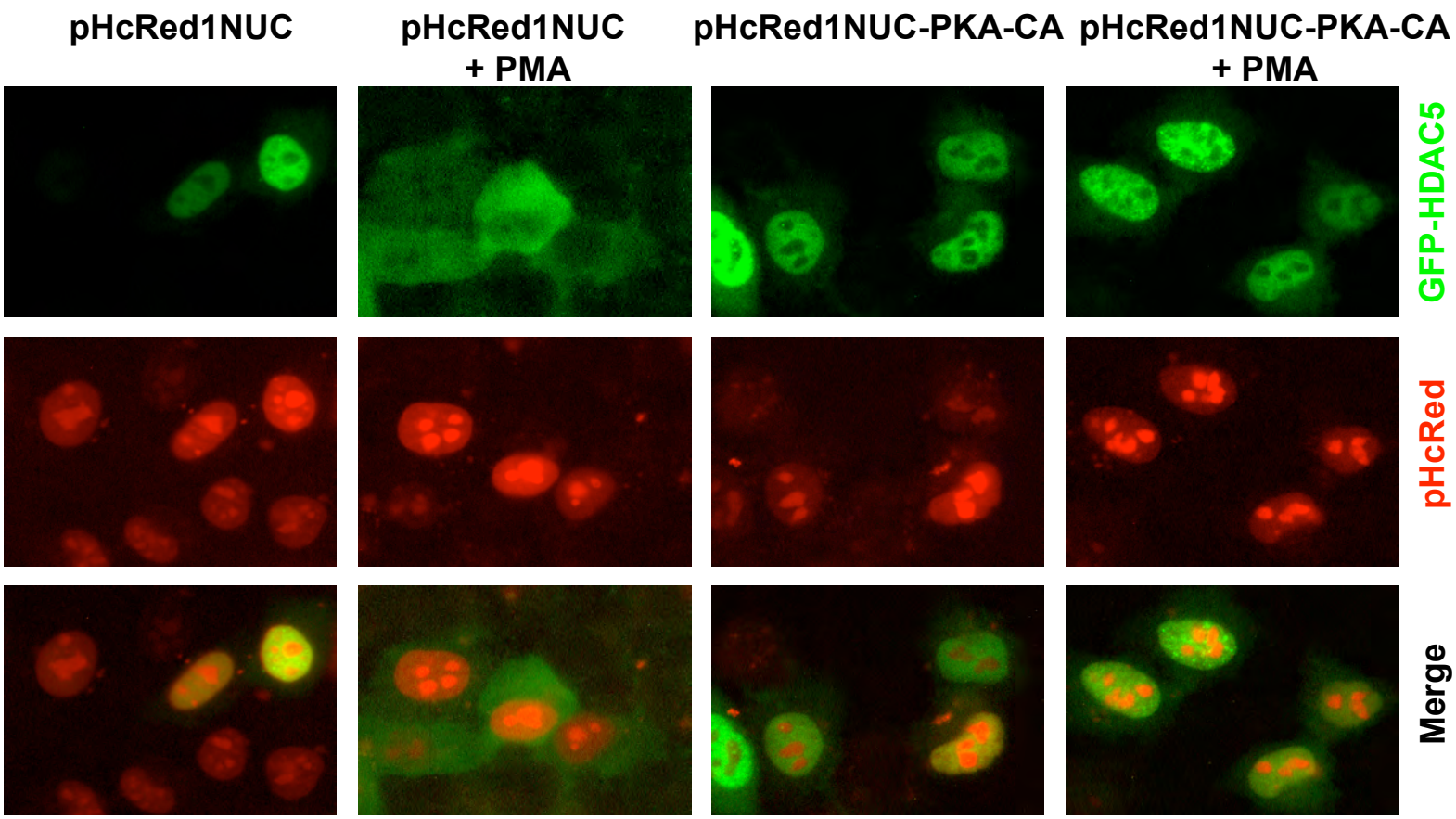


Figure S7. Nuclear localized PKA inhibits HDAC5 nuclear export in COS7 cells. CoS7 cells were cotransfected with expression vectors encoding GFP-tagged HDAC5 and HcRed1-NUC, or HcRed1-NUC-PKA-CA in the absence or presence of PMA.

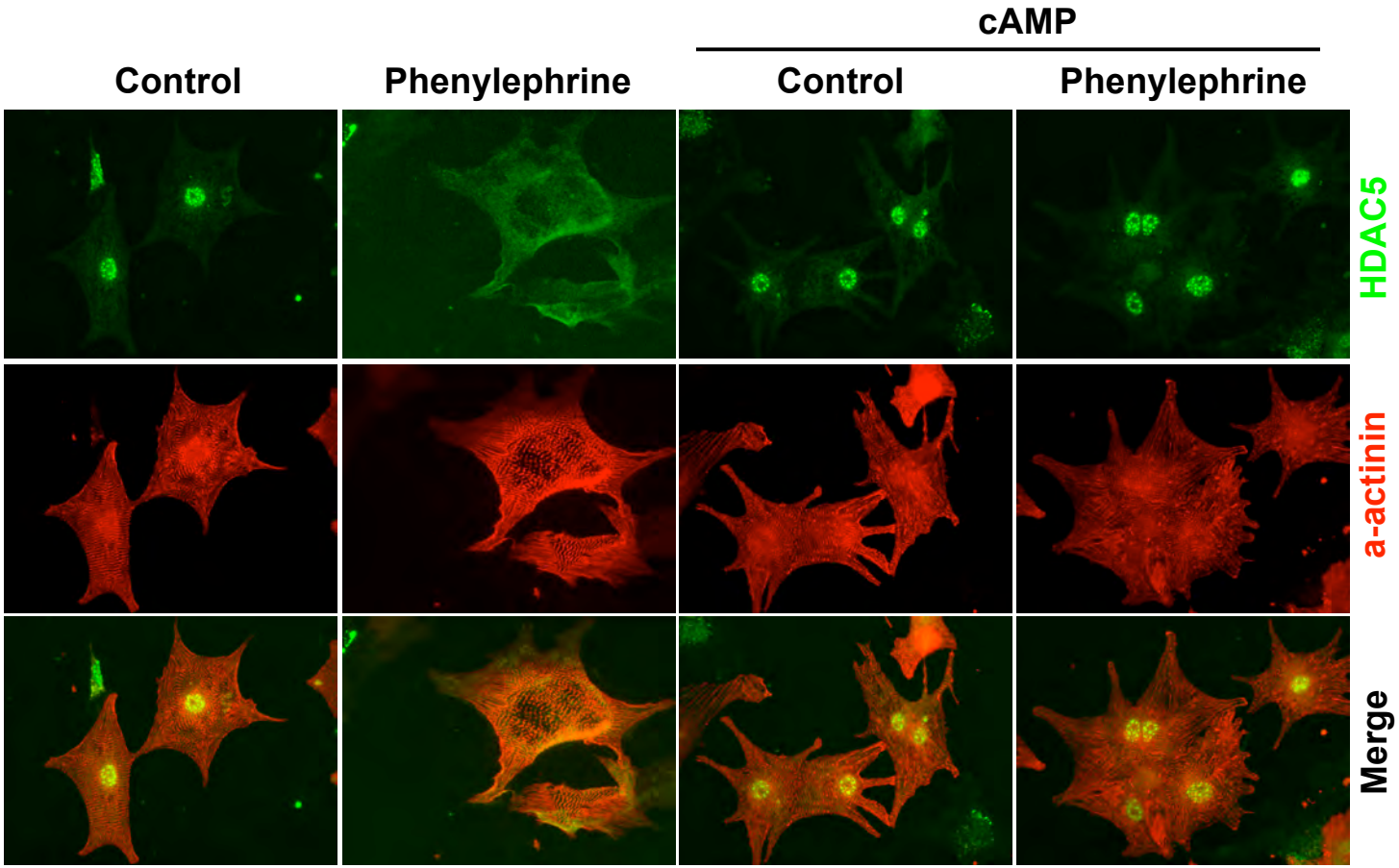


Figure S8. PKA inhibits endogenous HDAC5 nuclear export in NRVMs. Neonatal rat ventricular cardiomyocytes (NRVMs) were pretreated with the vehicle (DMSO as control) and cAMP (500 μM) for 30 min, followed by the exposure of α-adrenergic agonist phenylephrine hydrochloride (PE, 10 μM) for 3 hours. The cells were fixed and endogenous HDAC5 localization was analyzed by fluorescence microscopy. HDAC5 and α-actinin was detected by immunocytochemistry with antibody against HDAC5 and α-actinin.

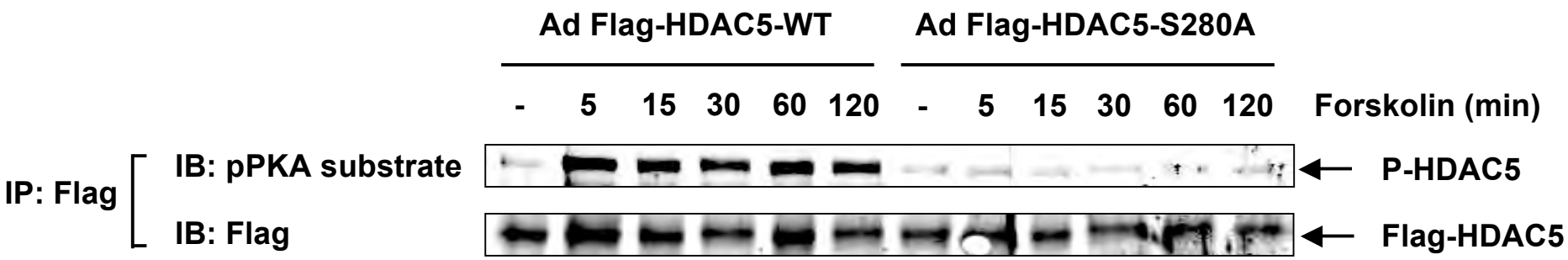


Figure S9. Forskolin stimulated phosphorylation of HDAC5-WT but not HDAC5-S280A mutant.
Cos7 cells were transfected with Flag-tagged HDAC5-WT or Flag-tagged HDAC5-S280A and then treated with forskolin (10 μM) at different time. Phosphorylation of HDAC5 in cell lysates was detected by immunoblotting with PKA phospho-substrate antibodies after immunoprecipitation with anti-Flag antibodies.

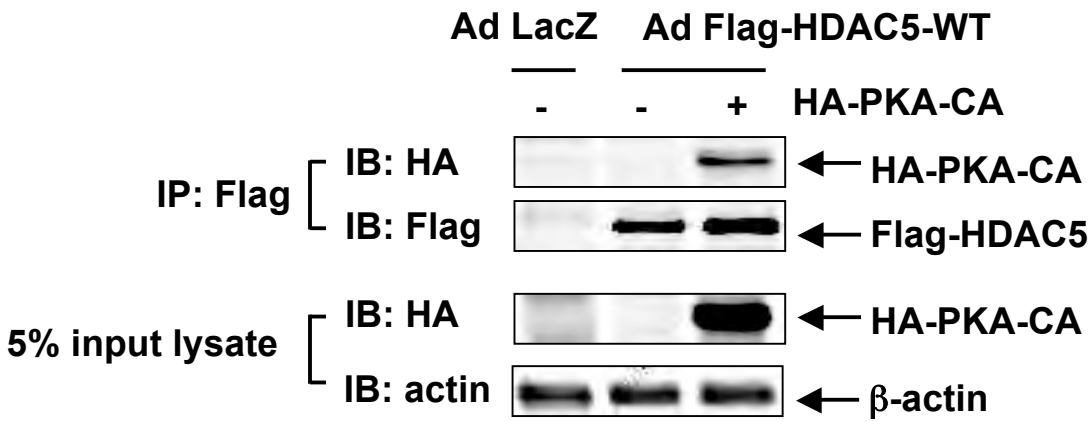


Figure S10. The association of PKA and HDAC5. Cos7 cells were co-transfected with Flag-HDAC5 and HA-PKA-CA. Co-immunoprecipitation with Flag-HDAC5 antibodies in total cell lysates and then immmunobloting with anti-HA and anti-Flag. The levels of HA-PKA-CA and β-actin in total cell lysates were detected by immmunobloting with anti-HA and anti-β-actin antibodies, respectively.

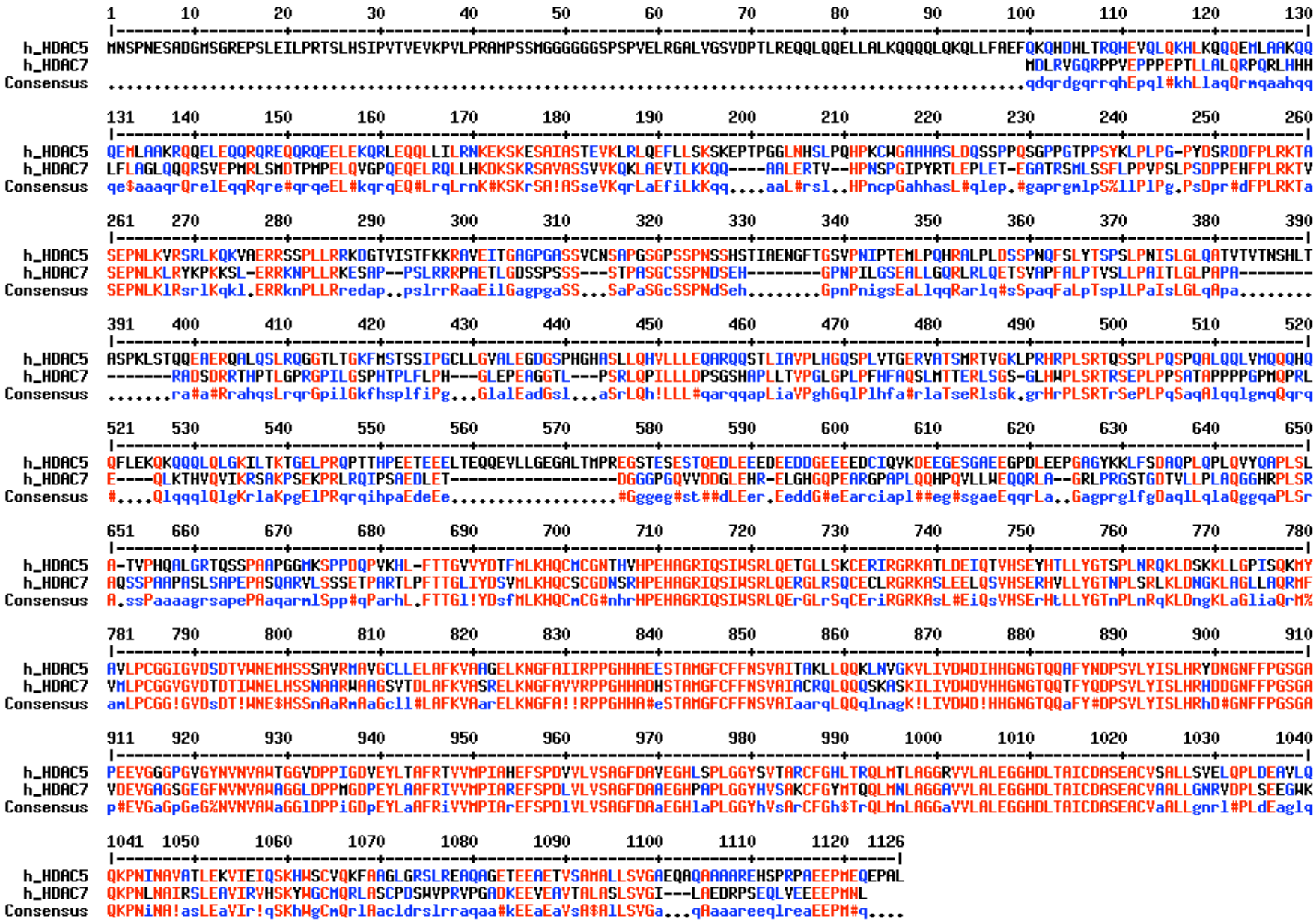


Figure S11. Alignment of human HDAC5 and human HDAC7. There are only 50% identities between amino acids of HDAC5 and HDAC7. “RRSS” motif in HDAC5 is replaced by “RRKN” in HDAC7.

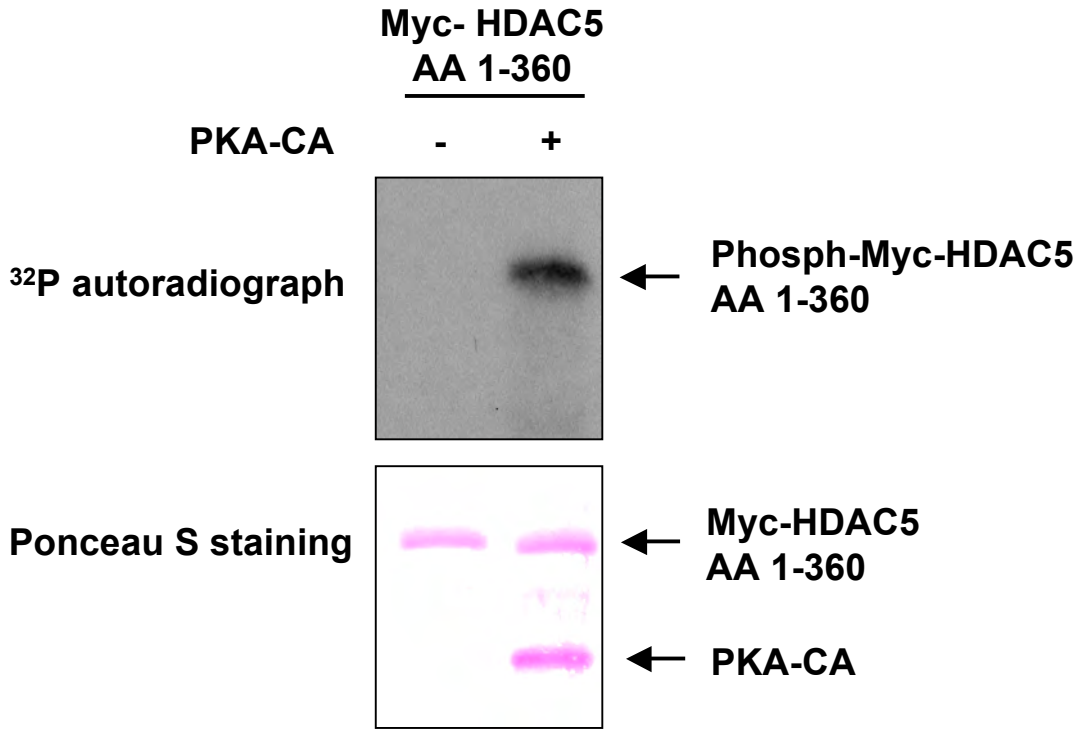


Figure S12. PKACA phosphorylates HDAC5 at Serine 280. ^{32}P autoradiograph image from an *in vitro* kinase assays performed with recombinant PKA-CA and Myc-HDAC5 AA 1-360. Myc-HDAC5 AA 1-360 were transfected into Cos7 cells, and then immunoprecipitated with Myc antibody. The equal loading of Myc-proteins was shown by Ponceau S staining.

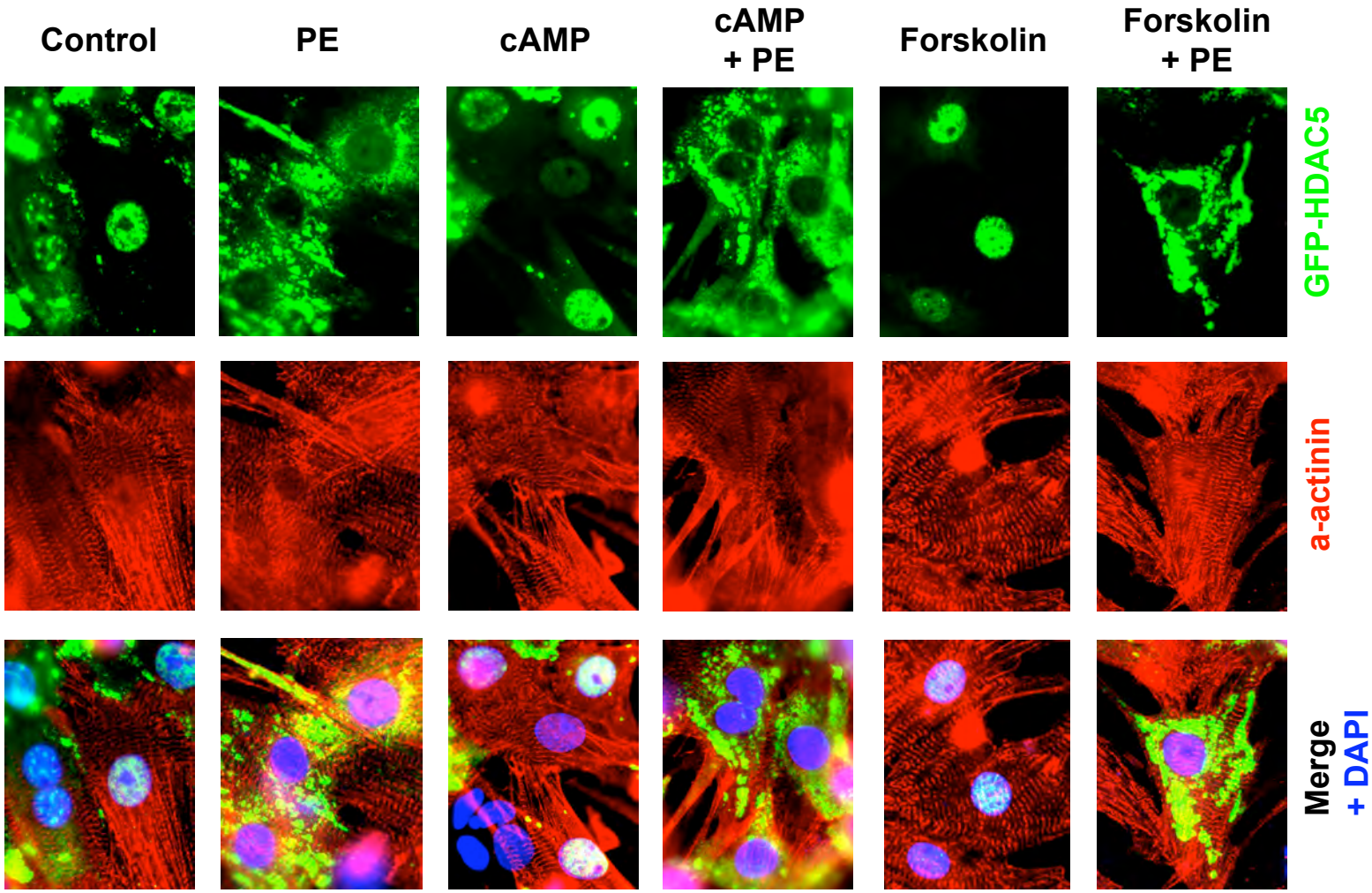


Figure S13. Nuclear export of HDAC5-S280A mutant is resistant to PKA inhibition in cardiomyocytes. NRVMs were infected adenoviral expression vector encoding GFP-tagged HDAC5 S280A and then pretreated with the vehicle (DMSO as control), cAMP (500 μM), and forskolin (10 μM) for 30 min, followed by the exposure of PE for 3 hours. The cells were fixed and GFP-HDAC5 subcellular localization was analyzed by fluorescence microscopy and stained with an -actinin antibody (red) and DAPI (blue).

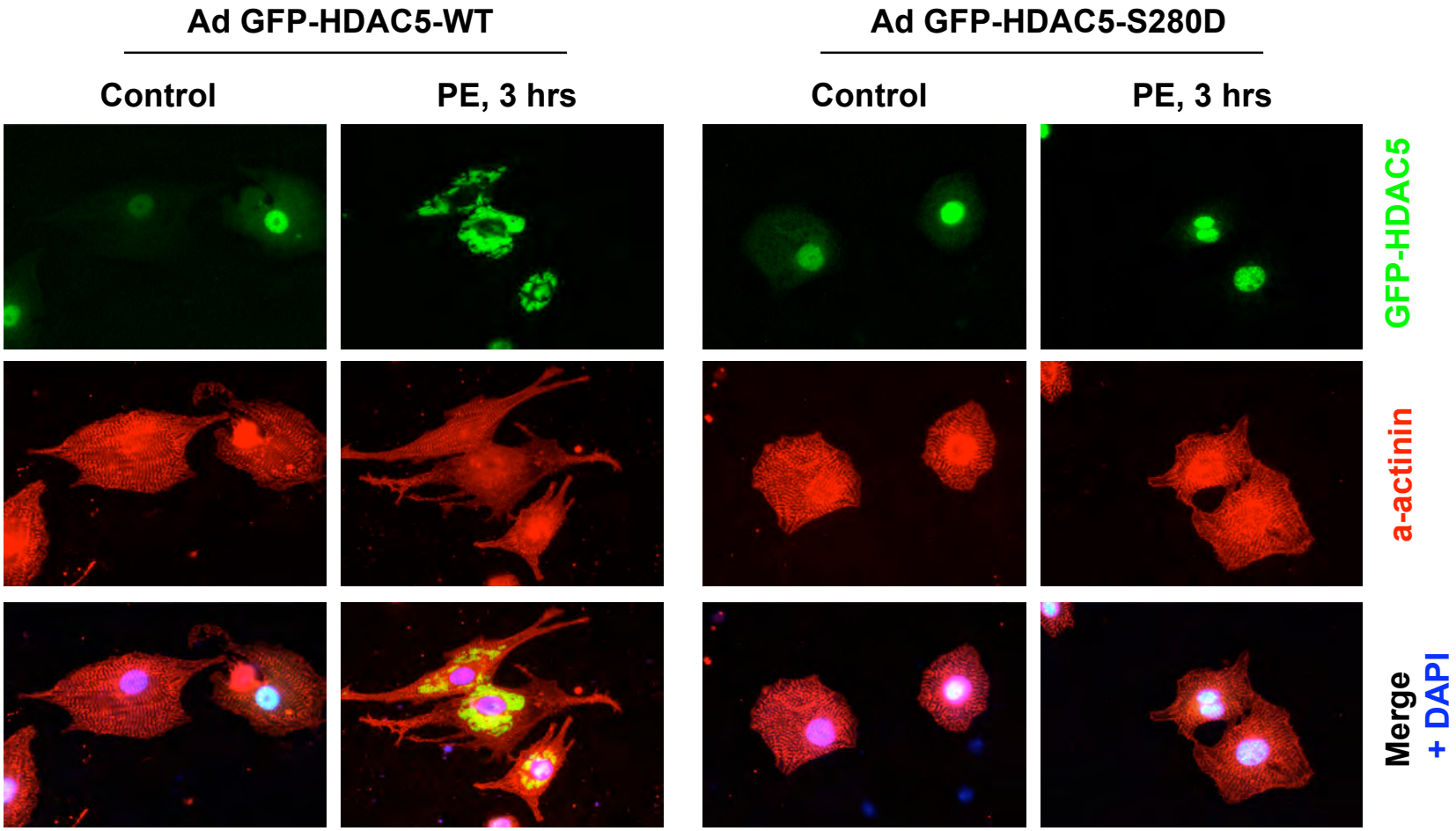


Figure S14. HDAC5-S280D mutant mimics PKA effect on HDAC5 nuclear export in cardiomyocytes. NRVMs were infected with an adenoviral expression vector encoding GFP-tagged HDAC5 WT or GFP-tagged HDAC5 S280D, and then treated with PE for 3 hours. The cells were fixed and GFP-HDAC5 localization was analyzed by fluorescence microscopy and stained with an -actinin antibody (red) and DAPI (blue).

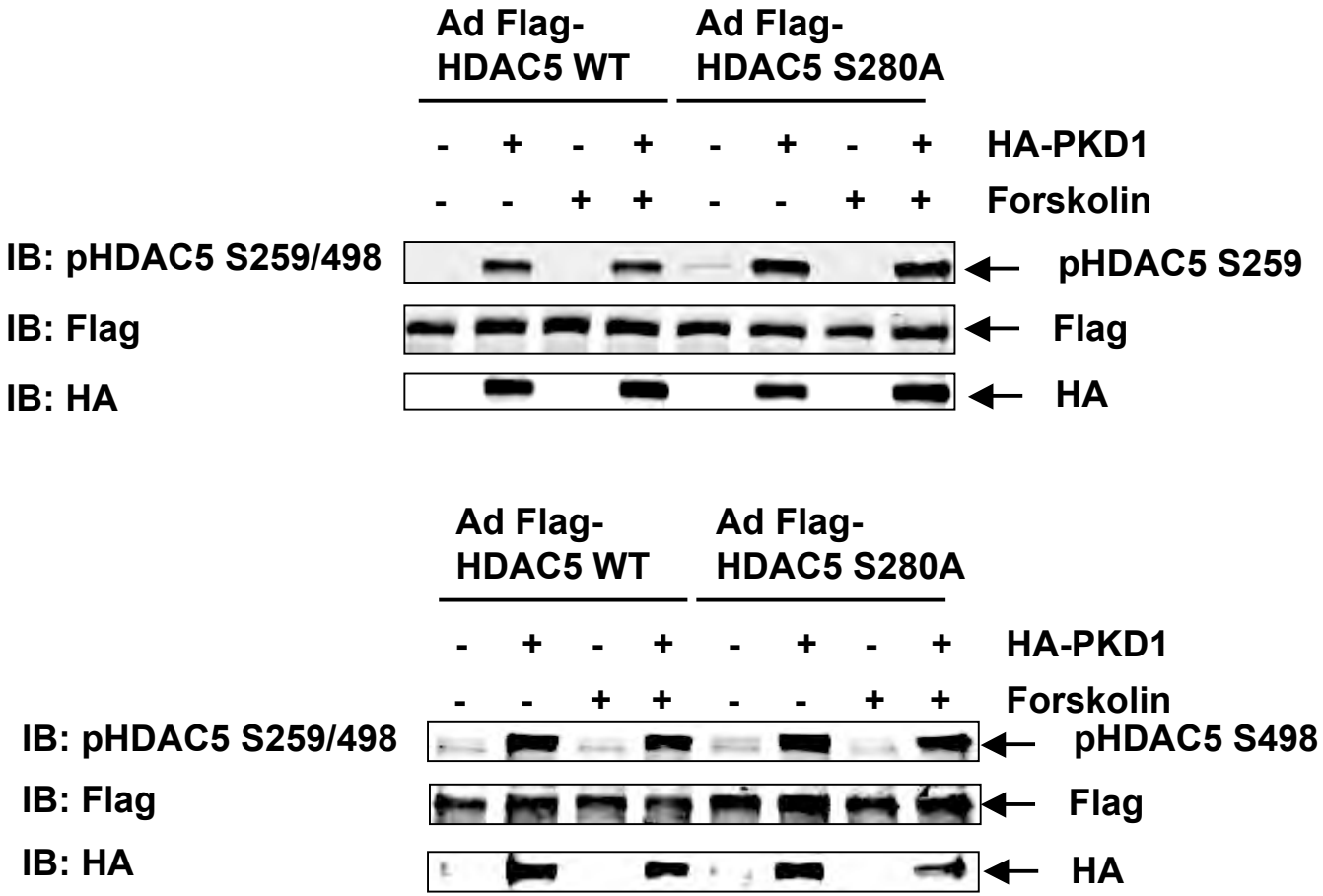


Figure S15. Forskolin (PKA activator) did not affect PKD1-dependent HDAC5 phosphorylation at Ser259 and Ser498 sites. Cos7 cells were co-transfected with plasmids Flag-HDAC5-WT or Flag-HDAC5-S280A and constitutively active HA-PKD1, and then treated with forskolin. Immunoblots of HDAC5 phosphorylation at Ser259 and Ser498 sites was shown.

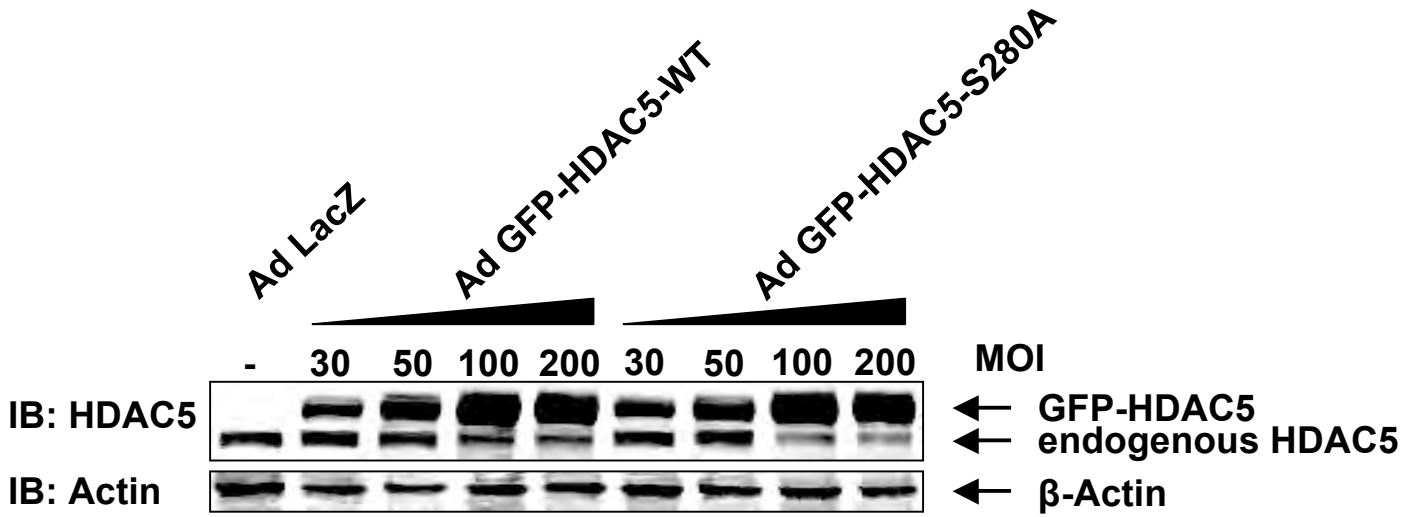


Figure S16. Overexpression of HDAC5 dose-dependently decreases the level of endogenous HDAC5. NRVMs were infected adenoviral expression vector encoding GFP-tagged HDAC5 WT or S280A at different doses. The endogenous HDAC5 and exogenous GFP-HDAC5s were analyzed by immunoblotting.

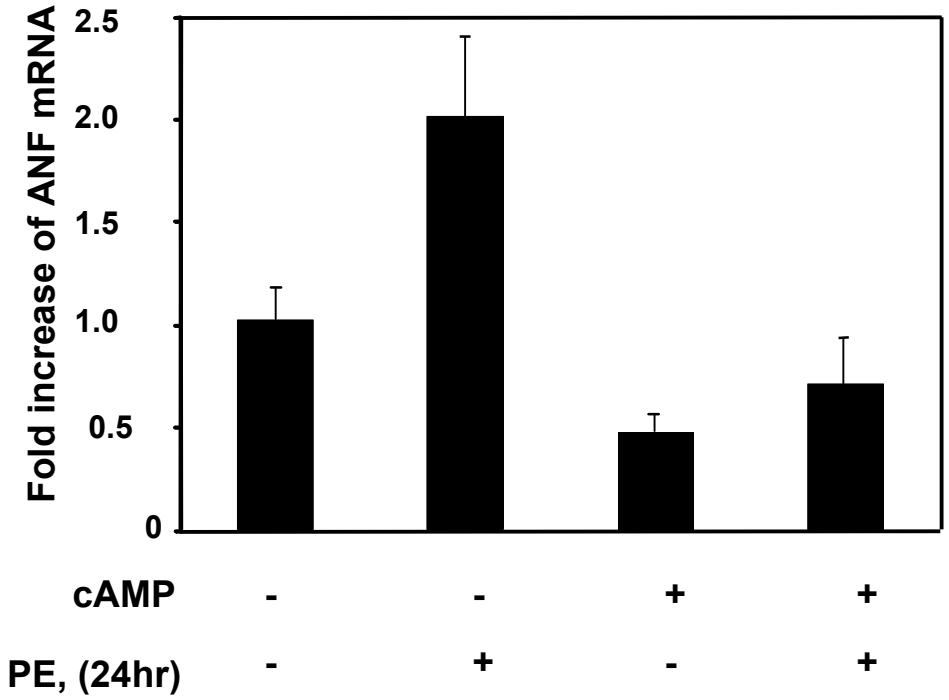


Figure S17. cAMP decreases PE-induced ANF expression in cardiomyocytes. NRVMs were pretreated with cAMP for 30 min, followed by stimulating with PE for additional 24 hours. The mRNA was extracted from the cell lysates, and quantitative RT-PCR with the primers for ANF and GADPH (internal control) were performed as described in Materials and Methods. Quantitative data are shown (n=4).

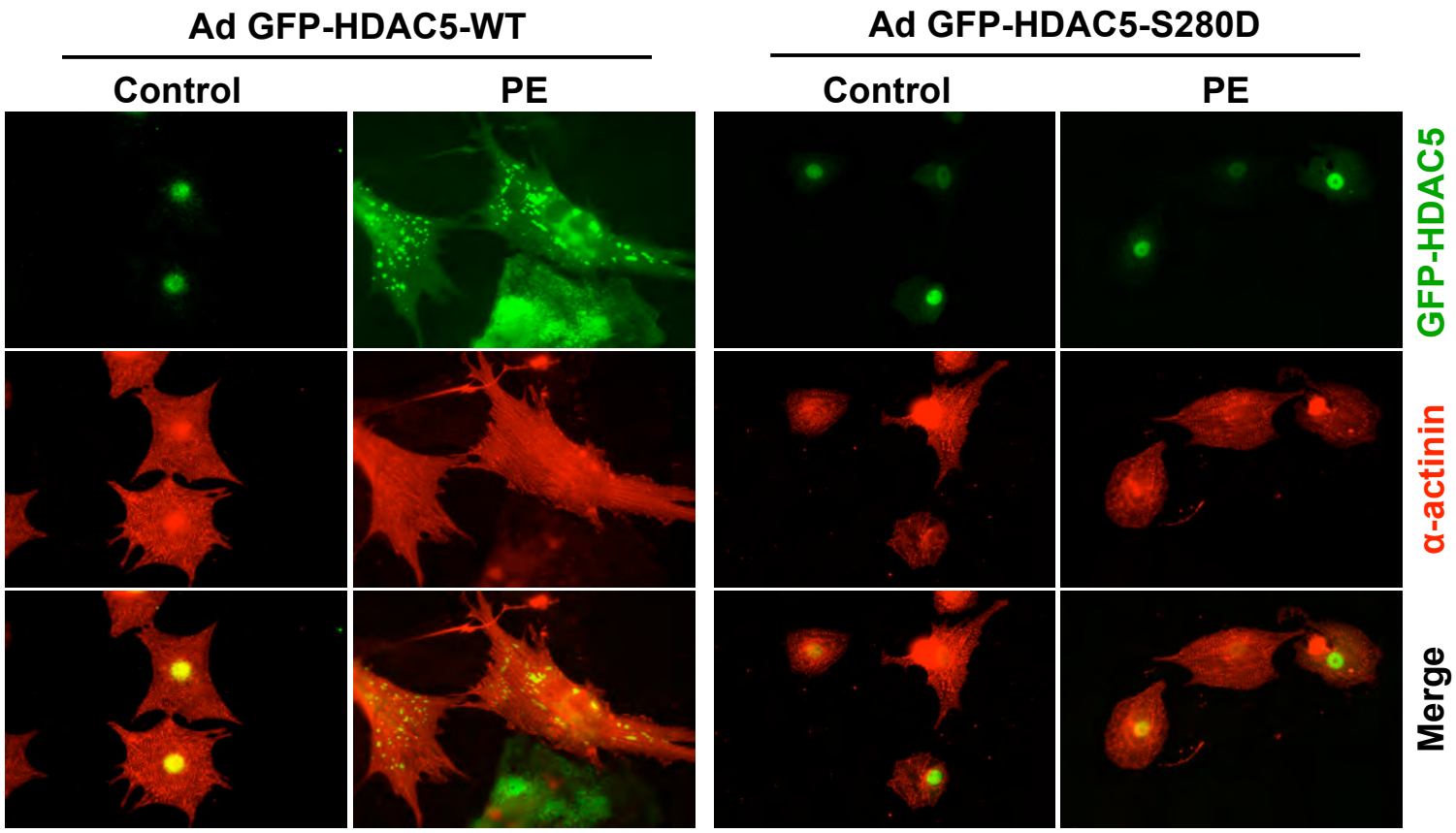


Figure S18. Overexpression of HDAC5-S280D inhibits cardiomyocyte hyerptrophy in NRVMs. Cardiomyocytes size detected by immunostaining with anti-α-actinin antibody. NRVMs were treated with PE for 24 hours (A, B, C). NRVMs were infected adenoviruses expressing GFP-HDAC5-WT, GFP-HDAC5-S280D and then treated with the vehicle (DMSO as control) and PE for 24 hours. The cells were fixed and analyzed for GFP-HDAC5 localization, α-actinin staining (red).

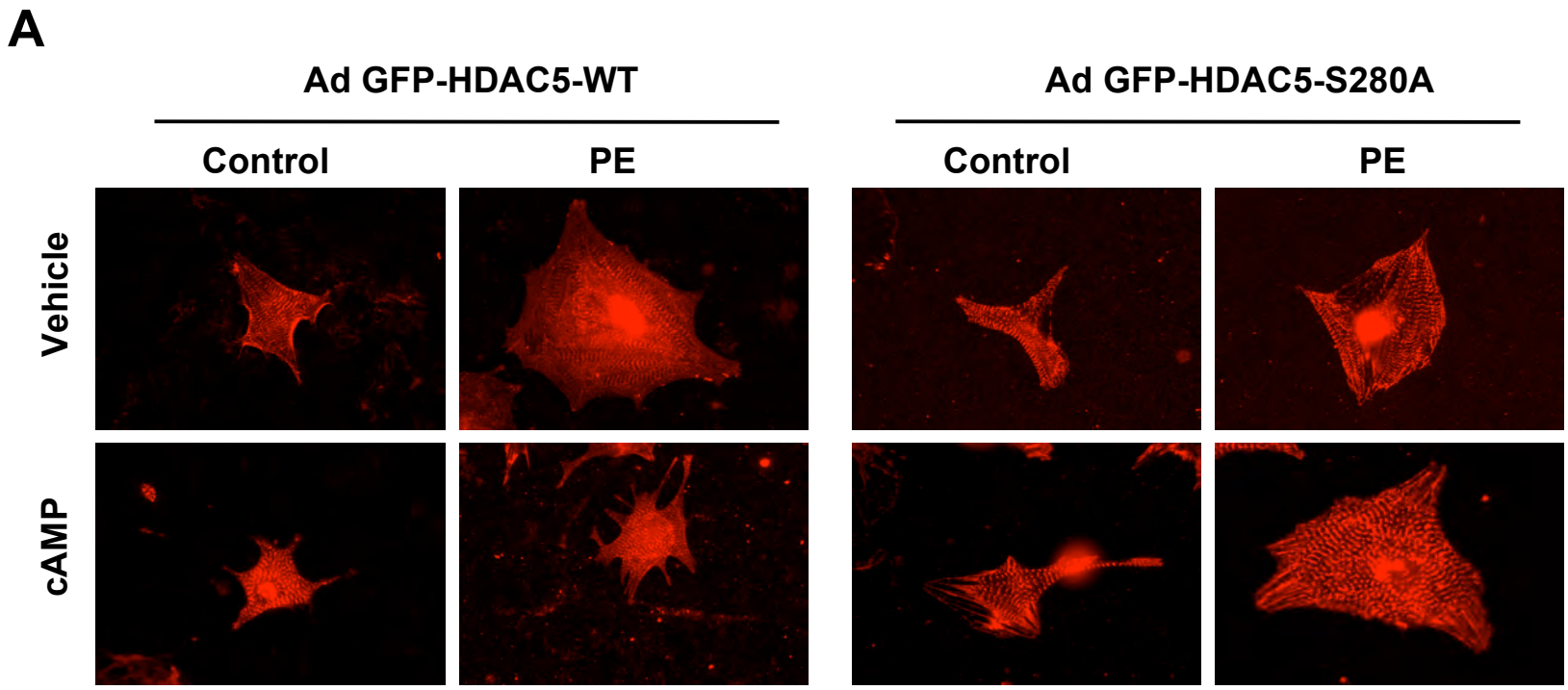


Figure S19. PKA-dependent HDAC5 phosphorylation and nuclear retention inhibits cardiomyocyte hyerptrophy. NRVMs were infected adenoviruses expressing GFP-HDAC5-WT or GFP-HDAC5-S280A, and then pretreated with the vehicle (DMSO as control), cAMP for 30 min, followed by the exposure of PE for 24 hours. The cells were fixed and analyzed for α-actinin staining (red)*.

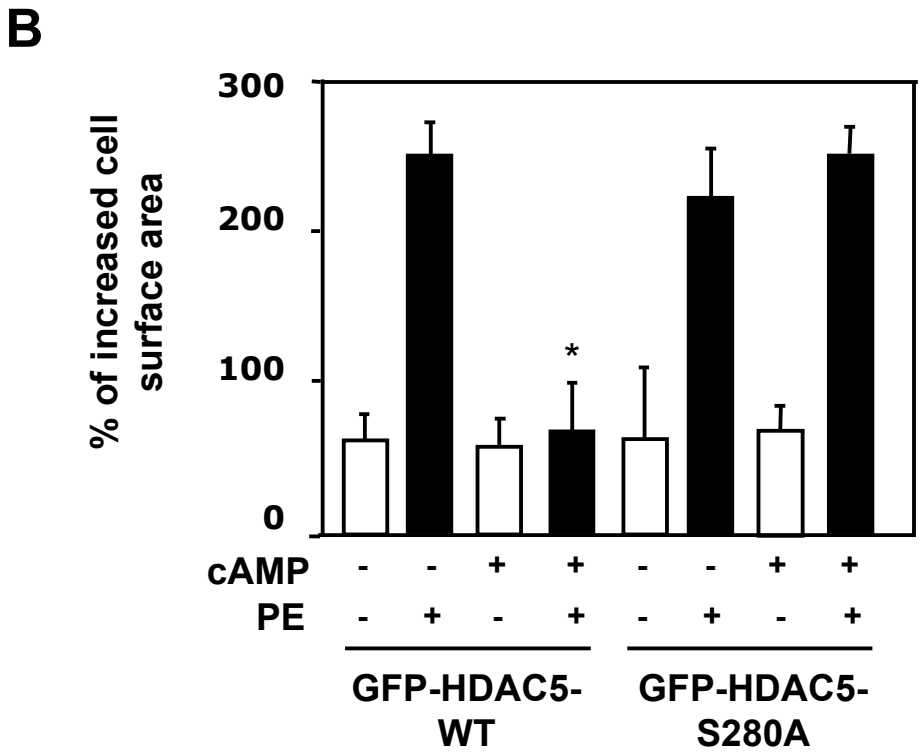


Figure S19 (B). PKA-dependent HDAC5 phosphorylation and nuclear retention inhibits cardiomyocyte hyerptrophy. NRVMs were infected adenoviruses expressing GFP-HDAC5-WT or GFP-HDAC5-S280A, and then pretreated with the vehicle (DMSO as control), cAMP for 30 min, followed by the exposure of PE for 24 hours. The cells were fixed and analyzed for α-actinin staining (red). * $p<0.05$ versus with PE, n = 4.

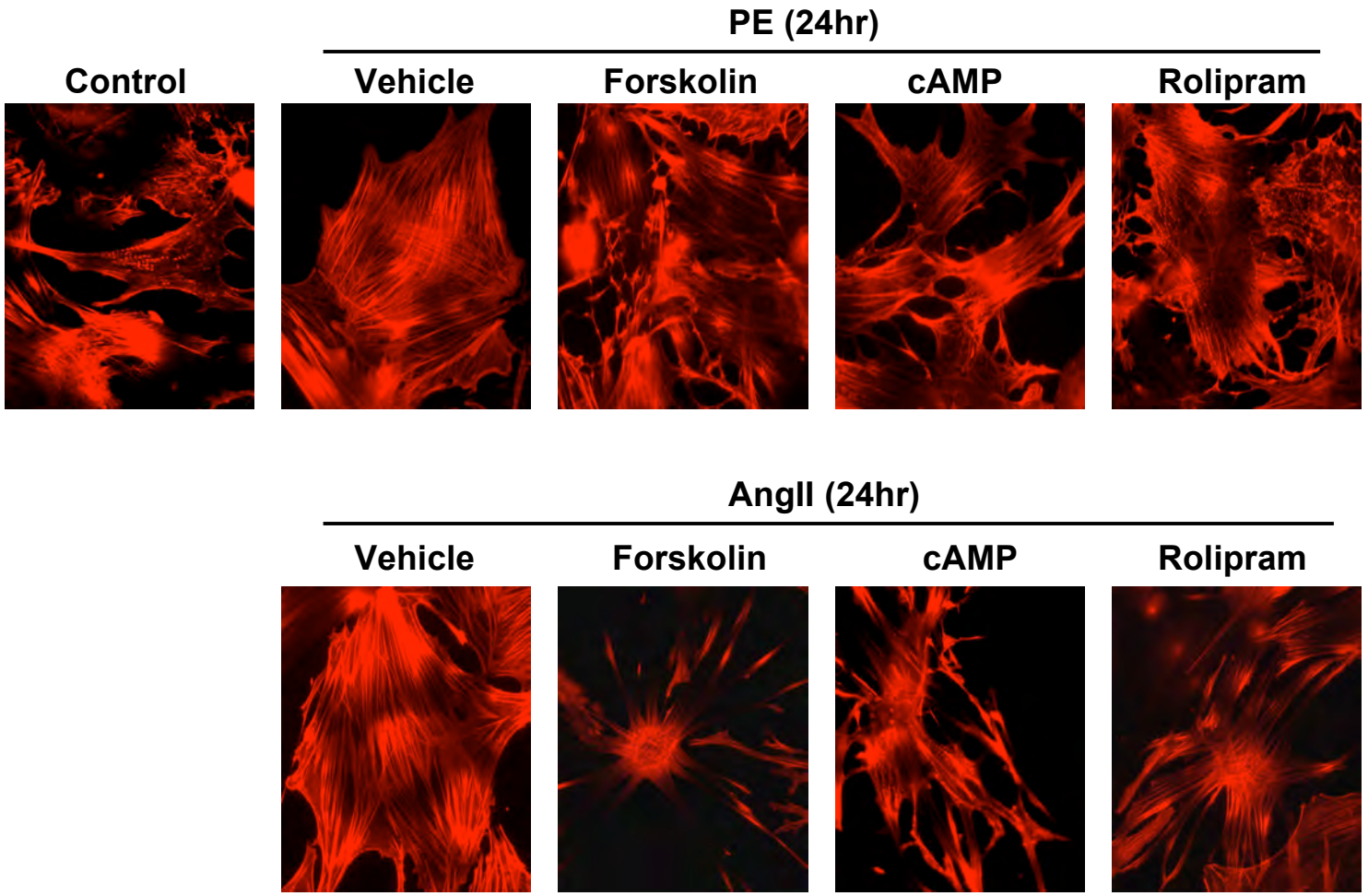
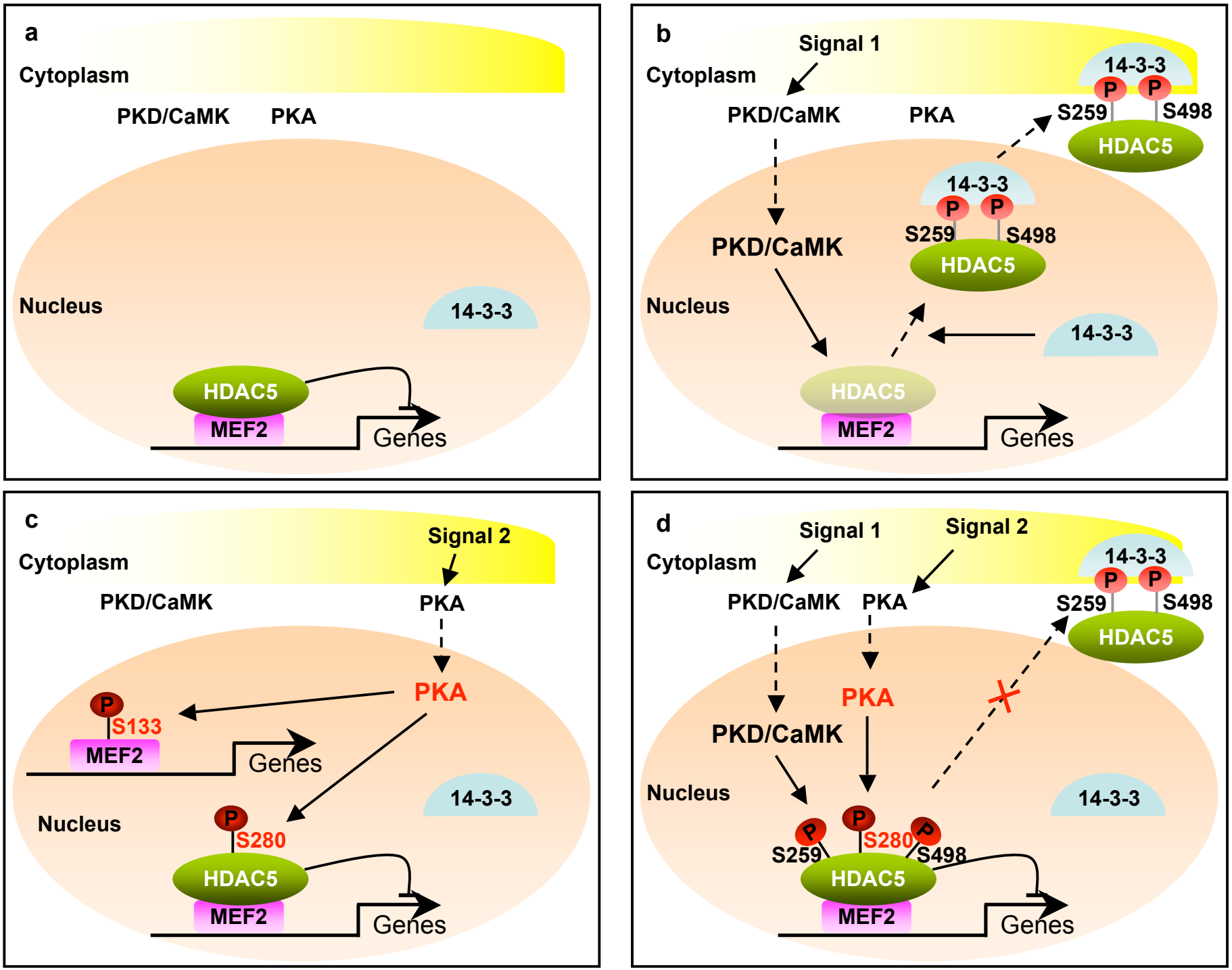


Figure S20. PKA activators inhibit PE / Angiotensin II-induced cardiomyocyte hypertrophy. NRVMs were pretreated with the vehicle (DMSO as control), PKA activator forskolin (10 μM), cAMP (500 μM), and Rolipram (10 μM) for 30 min, followed by the exposure of PE or Angiotensin II for 24 hours. Cardiomyocyte protein marker -actinin was detected by immunofluorescence using antibody against -actin and fluorescein-conjugated secondary antibody (red).

Figure S21. Schematic model for PKA inhibition on HDAC5 nucleocytoplasmic shuttling and gene expression. a, HDAC5 represses transcriptional expression of target genes by binding with MEF2. **b**, Activated PKD or CaMK phosphorylates HDAC5 at serine 259 and serine 498 resulting in export of HDAC5 to the cytoplasm as a complex with 14-3-3. The displacement of HDAC5 from MEF2 allows for transcriptional expression of the MEF2-dependent genes. **c**, Activated PKA translocates to the nucleus, and then phosphorylates HDAC5 at serine 280 and CREB at serine 133. **d**, Phosphorylations of HDAC5 by PKA blocks PKD/CaMK-dependent HDAC5 association with 14-3-3 resulting in HDAC5 nuclear retention and consequent repression of MEF2-dependent genes.


a
Cytoplasm
PKD/CaMK
PKA
Nucleus
14-3-3
HDAC5
MEF2
Genes
b
Signal 1
Cytoplasm
PKD/CaMK
PKA
14-3-3
P
P
S259
S498
HDAC5
PKD/CaMK
14-3-3
P
P
S259
S498
HDAC5
Nucleus
14-3-3
HDAC5
MEF2
Genes
c
Signal 2
Cytoplasm
PKD/CaMK
PKA
PKA
P
S133
MEF2
Genes
Nucleus
14-3-3
P
S280
HDAC5
MEF2
Genes
d
Signal 1
Signal 2
Cytoplasm
PKD/CaMK
PKA
14-3-3
P
P
S259
S498
HDAC5
PKA
PKD/CaMK
Nucleus
14-3-3
P
P
P
S259
S280
S498
HDAC5
MEF2
Genes

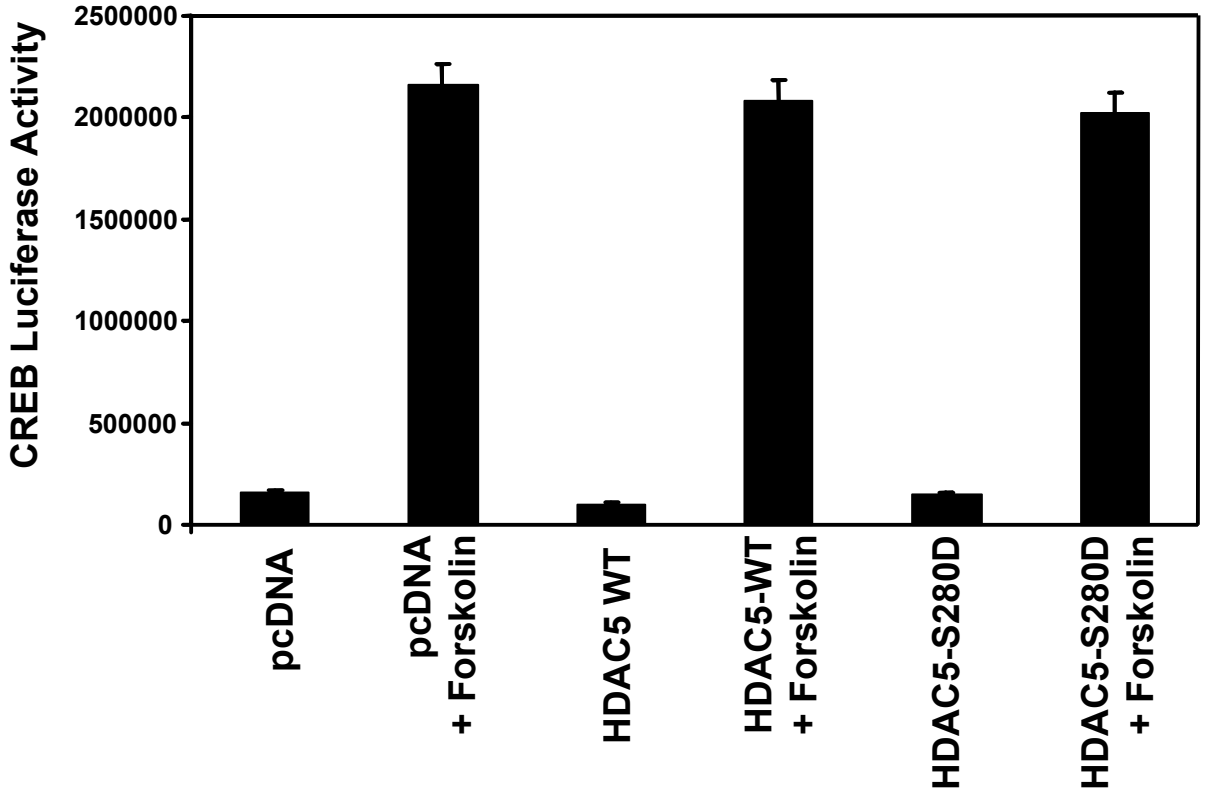
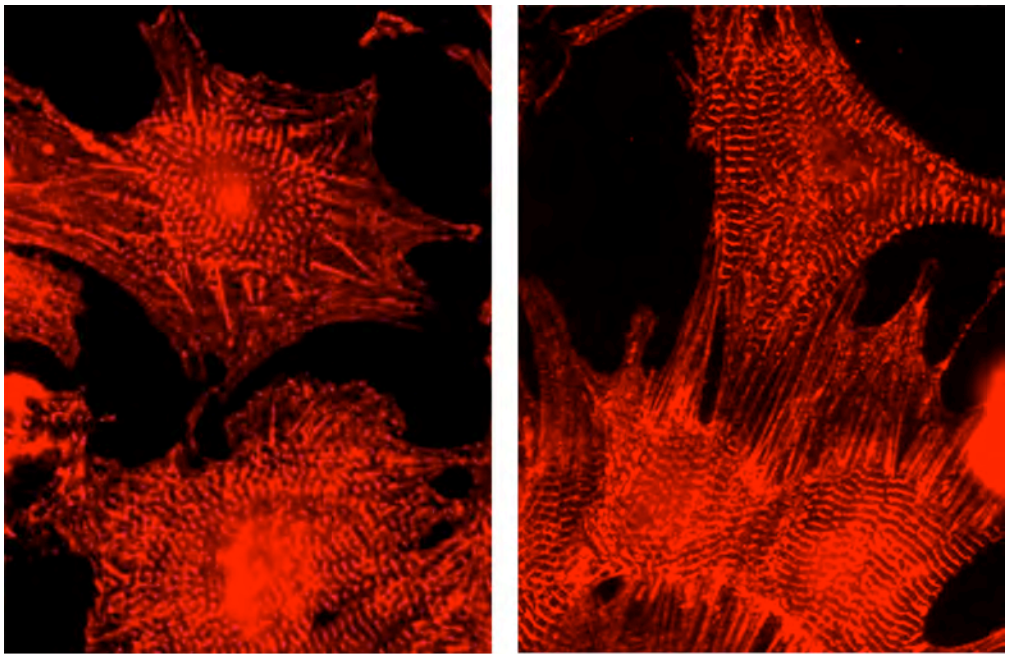
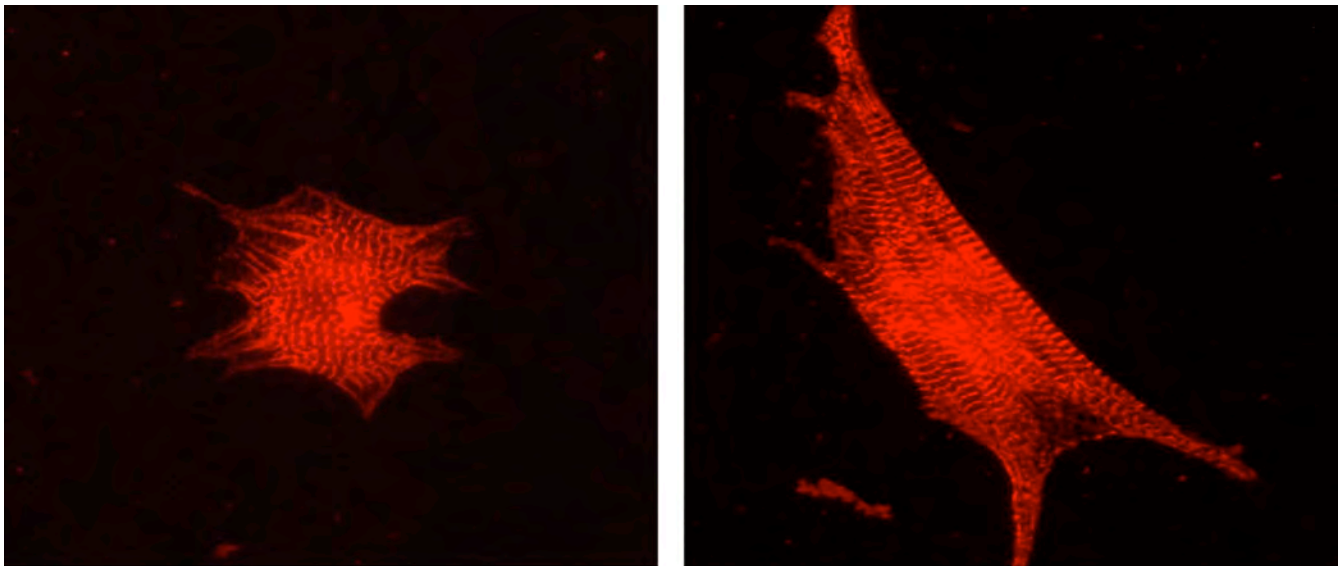


Figure S22. HDAC5 does not inhibit CREB transcriptional activity in COS7 cells. NRVMs were co-transfected with expression vector encoding CREB-luciferase reporter gene along with expression vector encoding Flag-HDAC5-WT or Flag-HDAC5-S280D for 48 h and then treated with forskolin. Error bars indicate standard errors (n=3).

High resolution actinin staining images of Figure 1E



High resolution actinin staining images of Figure 5D



High resolution actinin staining images of Figure 5G