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Authors

Awad, Salma
Kunhi, Muhammad
Little, Gillian H
[et al.](#)

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Nuclear CaMKII enhances histone H3 phosphorylation and remodels chromatin during cardiac hypertrophy

Salma Awad¹, Muhammad Kunhi¹, Gillian H. Little², Yan Bai², Woojin An³, Donald Bers⁴, Larry Kedes² and Coralie Poizat^{1,5,*}

¹Cardiovascular Research Program, King Faisal Specialist Hospital and Research Centre, PO Box 3354, Riyadh 11211, Kingdom of Saudi Arabia, ²Institute for Genetic Medicine, University of Southern California 2250 Alcazar Street, Los Angeles, CA 90033, USA, ³Department of Biochemistry and Molecular Biology, University of Southern California 2250 Alcazar Street, Los Angeles, CA 90089, USA, ⁴Department of Pharmacology, University of California at Davis, Davis, CA 95616, USA and ⁵Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182, USA

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ABSTRACT

Calcium/calmodulin-dependent protein kinase II (CaMKII) plays a central role in pathological cardiac hypertrophy, but the mechanisms by which it modulates gene activity in the nucleus to mediate hypertrophic signaling remain unclear. Here, we report that nuclear CaMKII activates cardiac transcription by directly binding to chromatin and regulating the phosphorylation of histone H3 at serine-10. These specific activities are demonstrated both *in vitro* and in primary neonatal rat cardiomyocytes. Activation of CaMKII signaling by hypertrophic agonists increases H3 phosphorylation in primary cardiac cells and is accompanied by concomitant cellular hypertrophy. Conversely, specific silencing of nuclear CaMKII using RNA interference reduces both H3 phosphorylation and cellular hypertrophy. The hyper-phosphorylation of H3 associated with increased chromatin binding of CaMKII occurs at specific gene loci reactivated during cardiac hypertrophy. Importantly, H3 Ser-10 phosphorylation and CaMKII recruitment are associated with increased chromatin accessibility and are required for chromatin-mediated transcription of the Mef2 transcription factor. Unlike phosphorylation of H3 by other kinases, which regulates cellular proliferation and immediate early gene activation, CaMKII-mediated signaling to H3 is associated with hypertrophic growth. These observations reveal a previously unrecognized function of CaMKII as a kinase signaling to histone H3 and

remodeling chromatin. They suggest a new epigenetic mechanism controlling cardiac hypertrophy.

INTRODUCTION

Histones are major components of chromatin and assemble with DNA to form nucleosomes, the basic unit of chromatin in all eukaryotic cells (1). The N-terminal ‘tail’ domain of histones modulates chromatin architecture through flexible contacts with DNA and adjacent nucleosomes. These ‘tails’ are subjected to a variety of post-translational modifications, including acetylation, methylation, ubiquitination, SUMOylation and phosphorylation (2,3). These modifications are reversible and take place sequentially or in combination to form a set of indicators that provide a linkage between signal transduction and gene expression (2,4). Although the vast majority of studies have established a central role of histone acetylation and methylation in the control of gene transcription, histone phosphorylation is much less studied and understood (5,6). The identification of the kinases responsible for histone modifications is critical to understand regulatory processes governing gene expression in all cells. It has become apparent that protein kinases not only target cellular proteins, transcription factors and components of the transcriptional machinery but also signal the chromatin to regulate various cellular processes, such as transcription, mitosis, DNA damage and apoptosis (7) (for review). For instance, histone H3 phosphorylation at serine-10 (Ser-10) was originally associated with chromosome condensation during mitosis (7–9). Subsequently, H3 Ser-10 phosphorylation was observed after growth factor stimulation. Kinases phosphorylating H3 Ser-10 discovered so far include Rsk2, IKK α , Msk1, PIM1 and

*To whom correspondence should be addressed. Tel: +966 1 464 7272 (ext. 32984); Fax: +966 1 464 7858; Email: coralie.poizat@gmail.com or cpoizat99@kfshrc.edu.sa

Present address:

Larry Kedes, Medical Genetics Institute, Cedars Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, CA 90048, USA.

Akt (10–14) [for review (6)]. The rapid and transient H3 phosphorylation event correlates with activation of immediate early genes, such as *c-jun* and *c-fos*, cytokine gene activation and Myc-dependent transcription. Increased accessibility to nuclear factors and remodeling of chromatin were suggested as critical events linking H3 Ser-10 phosphorylation and transcription activation. Whether additional enzymes such as calcium-regulated kinases act as physiological histone H3 kinases to regulate gene expression through chromatin modification remains unknown.

Calcium/calmodulin-dependent protein kinases (CaMKs) are ubiquitous serine/threonine protein kinases that phosphorylate specific substrates in different sub-cellular compartments (15–17). There they serve as mediators of calcium signals to produce a multitude of cellular responses ranging from gene regulation to synaptic function, muscle contraction and apoptosis (15,16,18) (for reviews). CaMKII, the major CaMK isoform in the adult heart, is produced as a holo-enzyme after alternative splicing of four genes α , β , γ and δ (15) that assemble as homo- or heteromultimer of 8–12 subunits (16–18). Some of the alternatively spliced isoforms such as the δ B contain a functional nuclear localization signal that allows expression of the enzyme in the nucleus, whereas other isoforms such as the δ C are strictly cytoplasmic. These CaMKII δ isoforms have established roles in cardiac pathologies (19–21). Increased CaMKII activity has been detected in human failing hearts (20), and protection against structural cardiac disease has been observed after CaMKII inhibition (22). Mice expressing high-cardiac levels of CaMKII δ develop hypertrophy and dilated cardiomyopathy (22,24), whereas deletion of CaMKII δ in mouse heart results in attenuated pathological cardiac hypertrophy (21). One mechanism involves specific signaling to the class II histone deacetylase HDAC4 (25,26), which leads to dissociation of HDAC4 from Mef2 factors, subsequent nuclear export of HDAC4/HDAC5 complexes and relief of Mef2 repression resulting in hypertrophic growth (27–29). Although CaMKII δ B has been shown to phosphorylate HDAC4, it is currently unknown whether CaMK enzymes have histones as direct substrates.

Here, we report a new function of nuclear CaMKII that directly signals to histone H3 and increases its phosphorylation at Ser-10 during cardiac hypertrophy. This modification increases chromatin accessibility and is a pre-requisite for the hypertrophic transcriptional response. This reveals a previously unrecognized function of CaMKII enzyme as histone H3 kinase and a new mechanism of transcriptional control in the heart. Our findings also have important implications for the treatment of cardiac disorders and other pathologies involving CaMK enzymes.

MATERIALS AND METHODS

Primary neonatal rat cardiomyocytes isolation and treatment

Primary neonatal rat cardiomyocytes were isolated using Worthington Neonatal Cardiomyocytes System (Worthington, USA) according to the manufacturer's

instructions, which are described in detail in the Supplementary Data.

Production of recombinant adenoviruses, infection and purification of CaMKII δ and CaMKIV

Recombinant adenoviruses expressing green fluorescent protein (GFP) (Ad-GFP), wild-type CaMKII δ B (Ad-CaMKII δ B), inactive CaMKII δ B-K43A (Ad-CaMKII δ B-K43A) and constitutively active CaMKII δ B-T287D (Ad-CaMKII δ B-T287D) were produced using the AdEASY Vector System as described previously (26,30). Flag-HDAC4 and Flag-HDAC5 were described before (26). Details of the protocols can be found in the Supplementary Data. Constitutively 'active' CaMKIV truncated at Leu 317 carrying a flag epitope was purified using the same approach after transfection of COS7 cells.

Antibodies

Antibodies used for western blot, immunoprecipitation, chromatin immunoprecipitation and immunofluorescence were as follows: Anti-HA probe (Y-11), anti-p-Histone H3 (Ser28), anti-Histone H2B (N-20), anti-p-CaMKII δ and anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were from Santa Cruz. Anti-p-Histone H3 (Ser10) was from Santa Cruz, Sigma and Invitrogen. Anti- α actinin was from Sigma. Anti-Histone H3, anti-H3 (phospho T3) and (phospho T11) antibodies were from Abcam. Anti-p-CaMKII (T286) was from Cell Signaling. Anti-CaMKII δ -specific antibody was a gift from Dr Donald Bers (University of California Davis, California). Ki67 and BrdU were from Abcam, UK.

Generation of mutant histone H3 S10A and CaMKII δ B- Δ 360-530

Mammalian expression vector for histone H3 was described before (31). H3 cDNA was inserted in the NotI/EcoRI site of pIRESneo (Clontech) also carrying a Flag and an HA tag. Mutant histone H3 carrying S10A substitution was generated by site-directed mutagenesis using the GENEART system (Invitrogen). The mutation was confirmed by restriction digestion and by DNA sequencing. Three individual clones carrying the S10A mutation were selected for further analysis. CaMKII δ B- Δ 360-530 lacking the association domain was generated by polymerase chain reaction (PCR) and was verified by restriction digest and by sequencing. Briefly, CaMKII δ B- Δ 360-530 was created by PCR performed from HA-CaMKII δ B inserted into SR-alpha vector between PstI and KpnI restriction sites. The primers were designed to delete the association domain and retain the HA tag (forward primer: TAC CGA AGC TGG TGG TGG ACG TGG GCC AAG TGG, reverse primer: GAG CAT CGG TCG TAT CAA CAG). The PCR product generated was then inserted into SR-alpha vector.

Mass spectrometry

Large-scale primary neonatal rat cardiomyocyte cultures were infected with inactive Ad-CaMKII δ B-K43A or control Ad-GFP. Nuclear extracts were prepared, and

after binding of cellular proteins to HA resin and washing, proteins ‘trapped’ on CaMKII δ B-K43A were eluted with HA peptide. The identity of the proteins that form a complex with CaMKII δ B-K43A was revealed by mass spectrometry by comparing mass values with theoretical values from protein sequence databases. The analysis was done at the Scripps Center for Mass Spectrometry, CA, USA.

Immunofluorescence, immunoprecipitation, protein extraction and immunoblotting

Indirect immunofluorescence studies were performed using standard methods. After adenoviral infection or siRNA transfection, nuclear or whole-cell extracts were prepared from primary neonatal rat cardiomyocytes as described previously (26). Details of the procedures can be found in the Supplementary Data.

BrdU incorporation and immunocytochemistry

Primary neonatal rat cardiomyocytes were transfected with siControl or siRNA against CaMKII δ B (siCaMKII δ B). Twenty-four hours post-transfection, cells were treated with 100 μ M BrdU for 4 h and then treated with 100 μ M phenylephrine (PE) for 24 h followed by standard immunocytochemistry. Briefly, cells were fixed using pre-cooled 70% ethanol and then treated with 4 M HCl for 40 min at 37°C. In total, 0.1 M borax buffer, pH 8.5, was then added for 5 min at room temperature to neutralize the reaction. After blocking with 5% fetal calf serum, rabbit anti-BrdU IgG (1/100) was incubated overnight at 4°C. After washing, cells were incubated in biotin-conjugated secondary antibody (Vector, USA) at room temperature for 1 h. Slides were developed with 3,3'-diaminobenzidine tetrahydrochloride (Vector, USA) and lightly counterstained with hematoxylin before mounting in vectashield (Vector, USA). Slides were visualized with a Nikon Ti-E microscope.

Chromatin preparation and immunoprecipitation

Chromatin was prepared as originally described (32). Details of the procedure can be found in the Supplementary Data. Briefly, primary cardiomyocytes or confluent HeLa cells were washed in hypotonic buffer, and released nuclei were centrifuged through a sucrose cushion, and the pellet was re-suspended in hypotonic buffer. Chromatin DNA was treated with micrococcal nuclease, and after stopping the reaction, insoluble material was removed by centrifugation. Fragmented chromatin in the supernatant was analyzed by centrifugation through glycerol gradients. DNA fragments were analyzed after de-proteinization of chromatin fragments by agarose gel electrophoresis.

0W0 and 0W47 nucleosome assembly

Nucleosome arrays were assembled using the 601 Widom nucleosome positioning DNA sequence with either no linker DNA (0W0 template) or with 47 bp of linker sequence on one side (0W47), or with histone octamers purified from HeLa cells or primary cardiomyocytes.

After high- to low-salt dialysis, reconstituted nucleosomes were analyzed by native polyacrylamide gel electrophoresis (PAGE). Electromobility shift assays (EMSA) were performed with labeled 0W0 or 0W47 nucleosomes and increasing concentrations of purified CaMKII δ B or purified ISWI used as positive control. The procedures are described in detail in the Supplementary Data.

In vitro phosphorylation assay

The 15 μ M CaMKII δ B wild-type, kinase ‘dead’ CaMKII δ B-k43A, ‘active’ CaMKIV or Aurora B kinase and 20 ng/ μ l histone octamers, dephosphorylated chromatin, HDAC4 or HDAC5 were incubated in a kinase reaction buffer containing 50 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 7.0, 20 mM MgCl₂, 0.2 mg/ml bovine serum albumin, 50 μ M adenosine triphosphate (ATP), 5 μ Ci/ml (1 Ci = 37 GBq) of [γ -³²P]ATP (3000–6000 Ci/mmol) and 1 mM CaCl₂. The reaction was carried out at room temperature for 30 min and terminated by addition of cold 15% trichloroacetic acid. Reactions were then analyzed by sodium dodecyl sulfate (SDS)–PAGE followed by autoradiography or western blotting using anti-H3 Ser-10 antibody.

Q-PCR

Q-PCR reactions were performed from chromatin templates after immunoprecipitation. Primers used for fetal cardiac genes were specific for atrial natriuretic factor (ANF) and β -myosin heavy chain (β -MHC). Amplification was also carried out for cardiac α -actin and GAPDH. Details of the protocol are available in the Supplementary Data. Chromatin immunoprecipitation (ChIP)–Q-PCR data were calculated based on the standard per cent input method (Life Technologies, USA). Briefly, 1% of the input fraction was subjected to immunoprecipitation followed by Q-PCR with the indicated gene primers. The adjusted input was calculated by subtracting the raw Ct to log₂ of 100 (6.644). The samples were expressed as per cent input by applying the following formula: $100 \times 2^{-(\text{adjusted input} - \text{sample Ct})}$. IgG values were calculated in the same way and subtracted from the per cent input of the samples. Results were expressed either corrected or non-corrected over basal condition (serum-free).

siRNA and shRNA

siRNA and shRNA experiments were described before (26). Briefly, primary neonatal rat cardiomyocytes treated with 100 μ M PE were transfected with siControl (siCt) or siRNA against CaMKII δ B (si δ B) from Dharmacon (ThermoScientific, USA). Seventy-two hours post-transfection, total cell lysates were prepared and analyzed by western blot using anti-histone H3 Ser-10-specific antibody. Duplicate of the cell extracts were analyzed with anti-CaMKII δ B and GAPDH antibodies. For immunofluorescence experiments, primary cardiomyocytes were transfected with siControl or siCaMKII δ B and treated with PE. Double immunostaining was performed with H3 Ser-10 and α -actinin antibodies to visualize H3 phosphorylation in ventricular myocytes. For

ChIP-Q-PCR experiments, primary cardiomyocytes were infected with a mock lentivirus or the vector carrying sh-CaMKII δ B for 24 h followed by PE treatment for additional 24 h and standard ChIP-Q-PCR assay.

MNase digestion

Long oligo nucleosomes isolated from Cos-7 cells transected with a eukaryotic expression vector for either Flag-H3 or Flag-H3 S10A were incubated with MNase at a final concentration of (60 U/ml) on ice for 15 min. After stopping the reaction by addition of stop buffer [250 mM ethylenediaminetetraacetic acid (EDTA) and 5% SDS], the MNase-digested DNA was analyzed on 1.5% agarose gel.

Restriction enzyme accessibility assay

Octamers were reconstituted onto nucleosomes using 601 DNA template synthesized by PCR using Cy5 labeled primers corresponding to 0W0 (upstream primer: CTGCAGAAGCTTGGTCCC, downstream: ACAGGATGTA TATATCTG) (33,34). Reconstitutions were performed at 1 μ M concentration and pH 7.5 by stepwise dialysis from 2 M NaCl or KCl to 0.85, 0.65, 0.5 and finally 0 M. Equimolar amounts (~20 nM) of ISWI, CaMKIITMB and CaMKII δ B-K43A were added to ~10 ng of the cy5-0W0 nucleosome template in the presence or absence of 2 mM ATP in reaction buffer [13 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 3 mM Tris-HCl, pH 8.0, 60 mM KCl, 9 mM NaCl, 3 mM MgCl₂, 6% glycerol, 0.6 mM dithiothreitol (DTT) and 10 mM CaCl₂]. The binding reactions were then treated with StyI for 30 min at 30°C. The proportion of DNA cleaved assessed by electrophoresis on 6% urea polyacrylamide gel was quantified, and the values of three independent experiments of the remodeling assay measuring the digested band intensity were calculated.

Gel shift assay

ISWI and CaMKIITMB were serially diluted to the concentrations indicated in the figures in 1 \times HEPES-magnesium-acetate (HMA) buffer (20 mM HEPES, pH 7.6, 25 mM KOAc and 5 mM MgAc) containing 0.1% Tween-20. Binding reactions were done in a final volume of 10 μ l containing 0.5 \times HMA buffer, 0.1 mg/ml bovine serum albumin, 1 mM DTT, 5 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride and 30 nM Cy5-labeled nucleosome or DNA. Reactions were allowed to equilibrate on ice for 30 min before electrophoresis on 0.5 \times Tris-Borate-EDTA native polyacrylamide (49:1) gels at 150 V for 60 min at 4°C. Gels were scanned on a Typhoon laser scanner for the Cy5 signal and the nucleosome/DNA bands quantified in ImageQuant image analysis software. Quantification of EMSA gels was performed using Image Quant software. The signal of nucleosome/CaMKII δ B or nucleosome/ISW-bound fraction (AB) and the signal of total nucleosome (A_{tot}) were quantified and the fraction bound (AB/A_{tot}) calculated. Dissociation constants (K_d) were calculated using the standard formula: $K_d = [A] \times [B] / [AB]$, where K_d is the affinity constant, and [A], [B] and [AB] are the

concentrations of nucleosomes, enzymes and nucleosome/enzyme complex, respectively.

Core histone-binding assays

Individual core histones or assembled octamers (4 μ g) were incubated with 10 μ l of wild-type CaMKII δ B, kinase 'dead' CaMKII δ B-K43A or mutant CaMKII δ B- Δ 360-530 bound to HA beads, in 60 μ l binding buffer (10 mM Tris, pH 7.5, 1 mM MgCl₂, 3 mM DTT and 1% Ficoll) containing 75 mM NaCl. The beads were pelleted, and the supernatant was added to SDS sample buffer for electrophoretic analysis. The beads representing the bound fraction were re-suspended in 30 μ l of SDS sample buffer. The bound and supernatant fractions were analyzed on 4–12% SDS-PAGE and revealed by Coomassie staining.

In vitro transcription assay with assembled chromatin

Transcription assay with reconstituted chromatin was carried out as described previously (35). Mutant histone H3 carrying Ser-10 to alanine substitution was produced by using the GENEART site-directed mutagenesis system (Invitrogen). Three colonies carrying the mutation were selected and confirmed by sequencing. Histone H3 wild-type or mutant H3 S10A were purified by Flag resin after transfection in HeLa cells. Wild-type or mutant histone H3 S10A was reconstituted as octamers with the other core histones as described before. The DNA template for the *in vitro* transcription assay contained multimerized Mef2-response elements and was generated by excising the 3xMef2-binding sites from the 3xMef2-luciferase vector by Nhe/SmaI-blunt double digestion. The Gal4BD was excised from the pS2-G5ML vector by NheI/NcoI-blunt double digestion and the 3xMef2 linearized fragment was then inserted in pS2-G5ML to generate the pS2-3xMef2 vector. Chromatin reconstituted with wild-type or mutant histone H3 S10A was incubated with CaMKII δ B and HeLa nuclear extracts in the presence of γ [³²P]-ATP. Transcription was measured by the formation of RNA corresponding to the transcribed Mef2 from the DNA template alone and from the DNA template reconstituted with chromatin. In parallel, transcription was also measured from endogenous HeLa reconstituted chromatin.

Animals

Procedures to isolate primary neonatal rat cardiomyocytes were performed under approved protocol (RAC# 2100 024) from the Office of Research Affairs at King Faisal Specialist Hospital & Research Centre.

Statistical analysis

Statistical analyses were performed using Student *t*-Test. *P*-values <0.05 were considered significant.

RESULTS

Nuclear CaMKII δ B binds to chromatin

To understand the final steps of the transcriptional reprogramming occurring during cardiac hypertrophy,

we precipitated proteins that form a complex with the most abundant cardiac nuclear CaMKII, the δB isoform. CaMKII δB was made inactive by K43A substitution to favor kinase:substrate interaction and was overexpressed in primary cardiac cells using a recombinant adenovirus (Supplementary Figure S1). Cellular proteins binding to CaMKII δB -K43A were purified by immunochromatography on hemagglutinin (HA) affinity gels. Analysis of the complex revealed the presence of several proteins interacting with CaMKII δB not present in the control sample, whose identities were revealed by mass spectrometry. Surprisingly, among the peptides previously not known to interact with CaMKII were histones (Figure 1A). Western blot analysis performed from the eluate used in the mass spectrometric analysis confirmed these interactions of CaMKII δB -K43A with several histone proteins (Figure 1B). Subsequently, binding of endogenous CaMKII δB with histones was also validated by immunoprecipitating endogenous histone H3 from cardiac nuclear extracts followed by western analysis using anti-CaMKII δ antibody (Figure 1C and 1D). Thus, CaMKII δB forms a protein complex with histone proteins in cardiac cells.

To further establish CaMKII δB binding to histones, we assessed *in vitro* binding of the kinase with chromatin isolated from primary neonatal rat cardiomyocytes. The integrity of cardiac chromatin was verified by MNase digestion, which showed the expected laddering pattern of fragmented nucleosomes on the DNA fiber (Supplementary Figure S2A), and the four core histones when chromatin was analyzed by SDS-PAGE (Supplementary Figure S2B). After incubation of cardiac chromatin with CaMKII δB bound to HA beads, both the supernatant and the eluted beads were analyzed by western blot (Supplementary Figure S2C). The majority of CaMKII δB were detected in the 'bound fraction' (Figure 1E, lanes 2 versus 3), whereas chromatin incubated with the beads alone showed minimal binding and mostly remained in the supernatant (Figure 1E, lanes 4 versus 5).

To fully establish the binding of CaMKII δB to chromatin, we performed EMSA using reconstituted 0W47 mononucleosomes as substrates, in which the 601 nucleosome positioning sequence directs assembly of a nucleosome such that it is flanked by 47 bp of linker DNA on one side (Supplementary Figure S3A). [γ - ^{32}P]-DNA and Cy5-labelled 0W47 mononucleosome templates were incubated with increasing amounts of HA-bound CaMKII δB or ISWI, a known chromatin remodeler that binds nucleosomes (34). As expected, ISWI bound free DNA and 0W47 nucleosomes in a dose-dependent manner (Supplementary Figure S3B, lanes 1–4 and 9–11). CaMKII δB was unable to bind free DNA (Supplementary Figure S3B, lanes 5–8) but bound 0W47 nucleosomes in a dose-dependent manner (Supplementary Figure S3B, lanes 12–14). CaMKII δB also associated with 0W0 nucleosomes, which lack any linker DNA flanking sequence (Figure 1F, lanes 4–6), whereas ISWI binding was specific for 0W47 nucleosomes (Figure 1F, lanes 1–3 versus 7–9) as reported before (34). Next, we studied the stoichiometry of CaMKII δB binding to nucleosomes by performing

binding assays using 0W47 nucleosomes and a wide range of enzyme concentration (0–80 nM). Increased CaMKII δB binding to nucleosomes was observed with increasing amounts of CaMKII δB (Supplementary Figure S4A). Generation of a binding curve after quantitation of the bound enzyme showed that ISWI followed a standard Michaelis–Menten curve, whereas CaMKII δB binding to 0W47 showed a sigmoidal pattern, consistent with a cooperative binding. CaMKII δB showed a slightly lower affinity toward 0W47 compared with ISW ($K_d = 37.5 \pm 1.74$ nM for CaMKII δB versus 23.33 ± 3.15 nM for ISWI) (Supplementary Figure S4A and B). The observation that CaMKII δB failed to bind DNA (Supplementary Figure S3) and had no preference toward the linker DNA of nucleosomes (Figure 1F) suggests that CaMKII δB association with chromatin is mediated by histones rather than by flanking DNA. Thus, CaMKII δB directly binds to mononucleosomes and nucleosome arrays *in vitro*. The strong association between CaMKII δB and chromatin *in vitro* suggests that CaMKII δB may play a role in chromatin architecture in the heart.

The association domain of CaMKII δB is required for its interaction to nucleosomes

Next, we mapped the region of interaction between CaMKII δB and chromatin. For this, we generated a CaMKII deletion mutant lacking the association domain (CaMKII δB - Δ 360-530) carrying an HA tag and performed immunoprecipitation with the purified deletion construct immobilized on HA agarose and individual histones or histone octamers. We observed binding of wild-type CaMKII δB and kinase 'dead' CaMKII δB -K43A to free H3, with a minimal binding to H2A/H2B (Supplementary Figure S5B, lanes 5–8). When histone octamers were used in the binding reaction, wild-type CaMKII δB and kinase 'dead' CaMKII δB -K43A bound preferentially to H3 and minimally to H4. (Supplementary Figure S5C, lanes 2–5). Mutant CaMKII δB - Δ 360-530 failed to bind free histones (Supplementary Figure S5B, lanes 9–10) or histones reconstituted as octamers (Supplementary Figure S5C, lanes 6–7). These results indicate that CaMKII δB preferentially binds H3, and that the association domain is critical for this interaction.

CaMKII δB preferentially phosphorylates histone H3

The observation that CaMKII δB interacts with chromatin *in vitro* and in primary cardiomyocytes suggested to us that histones maybe novel substrates of the kinase in cardiac muscle. To test this hypothesis, we performed *in vitro* kinase assays with purified constitutively active CaMKII δB -T287D (Supplementary Figure S1A–C) and histones reconstituted as octamers in the presence of [γ - ^{32}P] ATP. Among the four core histones, CaMKII δB preferentially phosphorylated histone H3 (Figure 2A). The specificity of CaMKII δB for histone H3 in the context of chromatin was next assessed by performing *in vitro* kinase reactions with CaMKII δB and chromatin isolated from primary neonatal rat cardiomyocytes subjected to de-phosphorylation. Again, we observed that CaMKII δB preferably phosphorylates histone H3

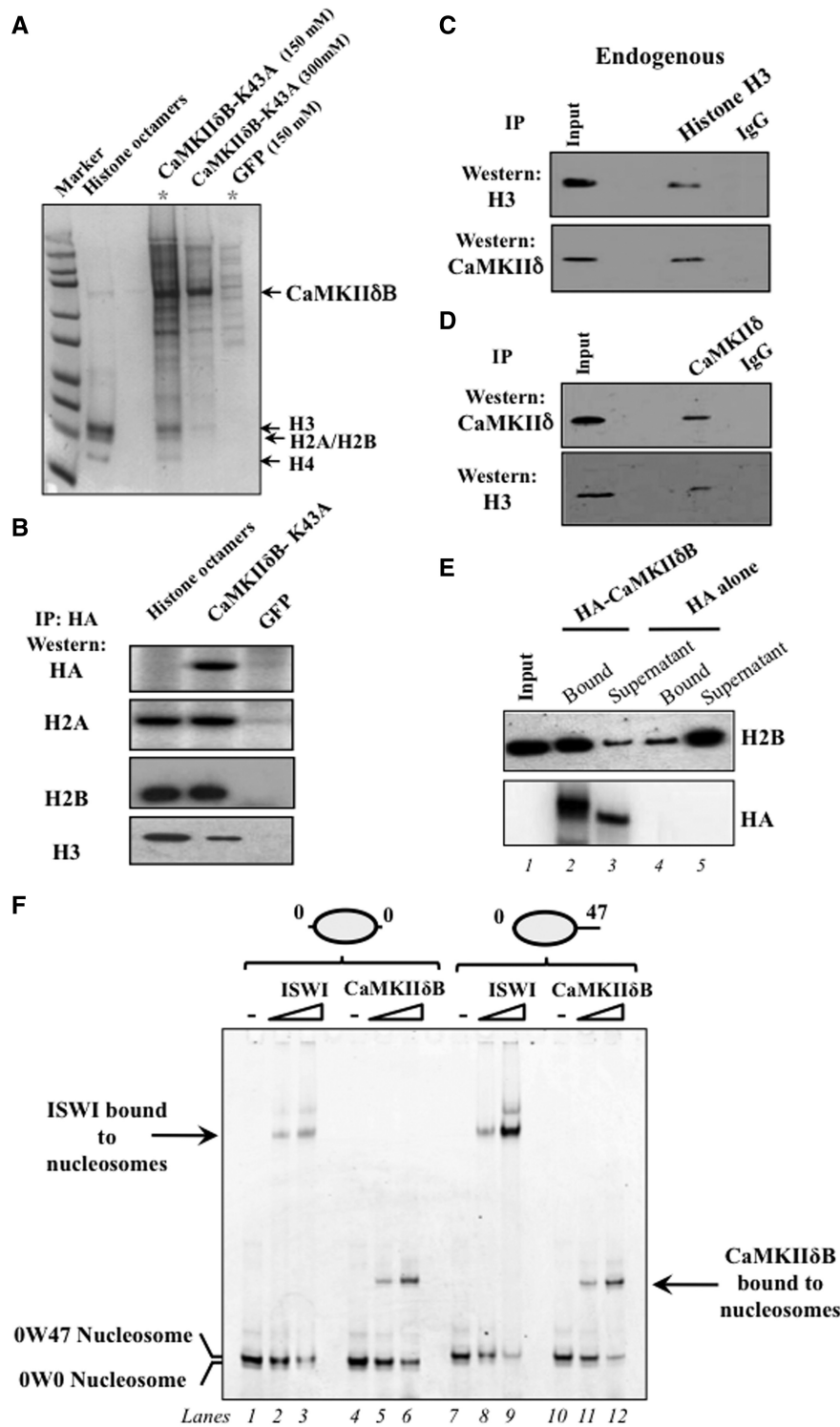


Figure 1. CaMKII δ B binds to cardiac chromatin *in vitro* and in primary cardiomyocytes. (A) Coomassie staining of proteins that interact with inactive HA-tagged CaMKII δ B-K43A in cardiac cells, after immunoprecipitation of nuclear extracts using HA agarose affinity gels and analysis by SDS-PAGE. Arrows indicate CaMKII δ B and the histone proteins later identified by mass spectrometry that co-migrate with purified histone octamers. Asterisks show immunoprecipitated proteins at 150 mM salt concentration in cells expressing CaMKII δ B-K43A or GFP. (B) Western blot analysis showing interaction of CaMKII δ B-K43A with histone proteins after immunoprecipitation with HA affinity gels ($n = 3$). Purified histone octamers were run in parallel as a positive control. (C) Interaction of endogenous histone proteins with endogenous CaMKII δ B shown after immunoprecipitation of cardiac nuclear extracts with α -H3 or control IgG antibodies and immunoblotting using a CaMKII δ -specific antibody. (D) Reverse experiment where CaMKII δ was immunoprecipitated first with anti-CaMKII δ antibody, and the precipitated proteins were analyzed by SDS-PAGE and immunoblotted with α -H3-specific antibody. Blots in C and D are representative of two independent experiments. (E) Western blot analysis showing the fraction of cardio-chromatin that binds to CaMKII δ B attached to HA agarose or that remains in the supernatant ($n = 4$). Immunoblotting with anti-H2B and -HA antibodies from 1: input chromatin, 2: chromatin bound to HA-CaMKII δ B, 3: chromatin remaining in the supernatant not bound to HA-CaMKII δ B, 4: chromatin bound to HA beads alone, 5: chromatin remaining in the supernatant not attached to HA beads. (F) Representative EMSA with increasing concentration of purified CaMKII δ B and 0W0 or 0W47 mononucleosome templates followed by native PAGE, $n = 3$. Arrows indicates the position of ISWI or CaMKII δ B bound to mononucleosomes.

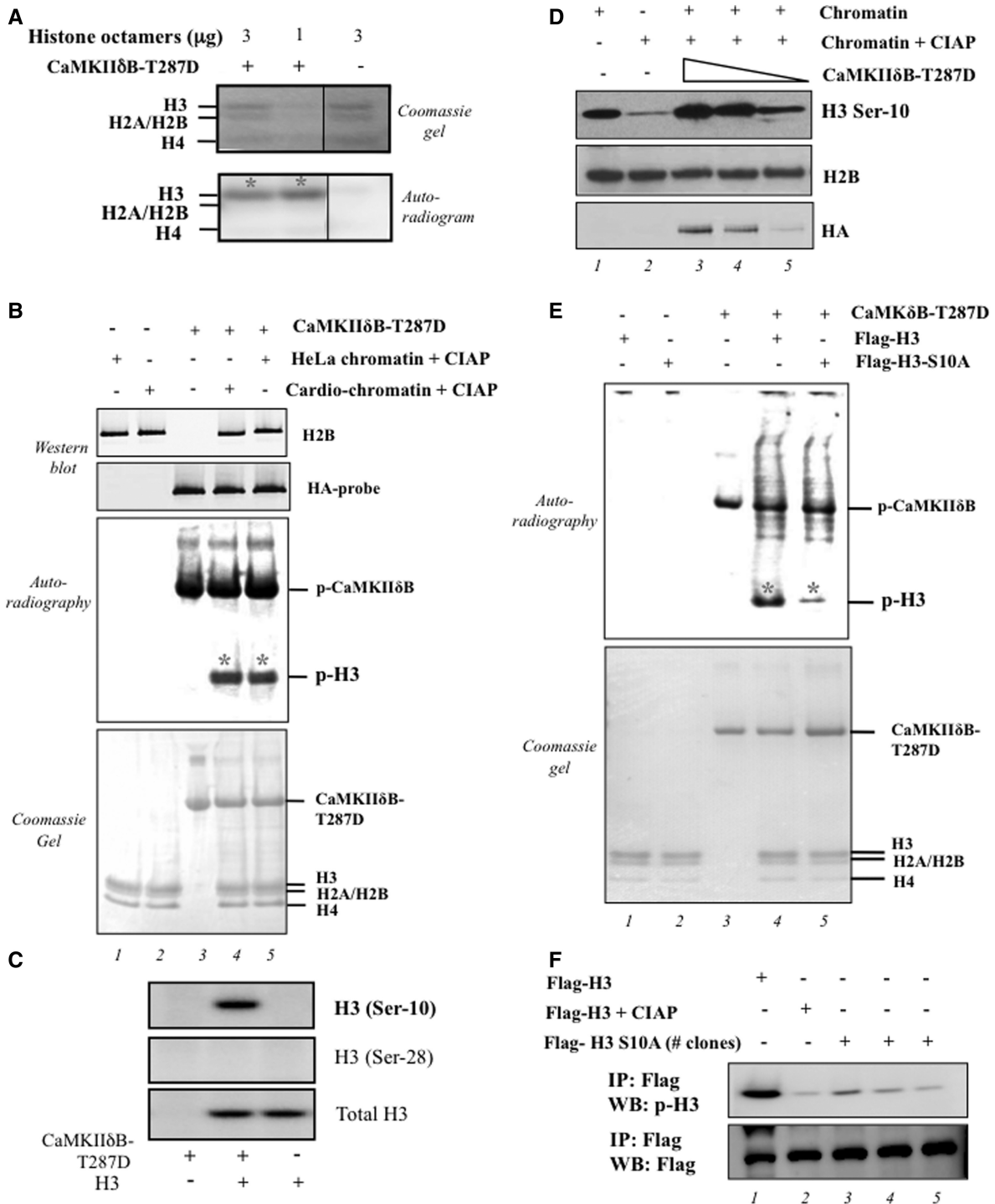


Figure 2. CaMKIIδB specifically phosphorylates histone H3 in histone octamers and in chromatin. (A) *In vitro* kinase assay ($n = 3$) with purified constitutively active CaMKIIδB-T287D and reconstituted histone octamers in the presence of [γ^{32} P] ATP followed by SDS-PAGE and autoradiography. Asterisks indicate phosphorylation of histone H3 by CaMKIIδB. (B) *In vitro* kinase assays with constitutively active CaMKIIδB-T287D and de-phosphorylated chromatin after treatment with calf intestine alkaline phosphatase (CIAP), purified from neonatal rat cardiomyocytes and HeLa cells in the presence of [γ^{32} P] ATP followed by SDS-PAGE ($n = 5$). Asterisks indicate phosphorylated histone H3. Lane 1: input de-phosphorylated cardio-chromatin, 2: input de-phosphorylated HeLa chromatin, 3: CaMKIIδB-T287D alone, 4: CaMKIIδB-T287D and de-phosphorylated cardio-chromatin, 5: CaMKIIδB-T287D and de-phosphorylated HeLa chromatin. (C) Western blot analysis with phospho-H3 (Ser-10), phospho-H3 (Ser-28) or total H3-specific antibodies after *in vitro* kinase assay with recombinant histone H3 and active CaMKIIδB-T287D ($n = 3$). (D) Immunoblots after *in vitro* phosphorylation assay with endogenous cardio-chromatin or chromatin de-phosphorylated by CIAP treatment in the presence of increasing amount of CaMKIIδB-T287D enzyme, using anti-H3 Ser-10, -H2B and -HA antibodies ($n = 3$). Lane 1: cardio-chromatin alone, 2: cardio-chromatin treated with CIAP, 3–5: cardio-chromatin treated with CIAP incubated with different amounts of CaMKIIδB-T287D. (E) *In vitro* kinase assay in the presence of [γ^{32} P] ATP after transfection of HeLa cells with CaMKIIδB-T287D and wild-type Flag-histone H3 or mutant histone H3 S10A and immunoprecipitation of isolated chromatin using Flag resins ($n = 3$). Lane 1: Flag-H3 wild-type alone, 2: Flag-H3-S10A alone, 3: CaMKIIδB-T287D alone, 4: Flag-H3 wild-type+CaMKIIδB-T287D, 5: Flag-H3-S10A+CaMKIIδB-T287D. (F) Immunoblots showing phosphorylation at Ser-10 of wild-type Flag-histone H3 and three different H3 S10A mutants after transfection in HeLa cells and immunoprecipitation using Flag resins ($n = 3$). Lane 1: Flag H3 wild-type, 2: Flag-H3 wild-type treated with CIAP, 3–5: Flag-H3-S10A from three separate clones.

indicating specificity of CaMKII δ B toward H3 and not toward other histones (Figure 2B, lanes 4 and 5). Next, we compared the kinetics of H3 phosphorylation with a bona fide H3 kinase, Aurora B. *In vitro* kinase assay was performed using increasing amounts of purified Aurora B or CaMKII δ B and reconstituted nucleosomes. H3 was phosphorylated by both kinases, although phosphorylation by Aurora B was more efficient than CaMKII δ B (Supplementary Figure S6A–C). Next, we compared H3 phosphorylation by CaMKII δ B with a known CaMKII δ B substrate, the histone deacetylase HDAC4. HDAC5, which is related to HDAC4 but is a much weaker CaMKII δ B target (26), was also used in parallel for the phosphorylation reaction. *In vitro* phosphorylation assay revealed that CaMKII δ B phosphorylate H3 to a level similar to that observed when using HDAC4. As reported before, CaMKII δ B did not potently phosphorylate HDAC5 (Supplementary Figure S6E). These results indicate that CaMKII δ B has specificity toward H3.

As CaMKII δ B is a serine/threonine kinase, we surveyed serines and threonines in the histone H3 ‘tail’ to identify potential CaMKII δ B phosphorylation sites (Supplementary Figure S7A). *In vitro* kinase reaction performed with recombinant histone H3 and constitutively active CaMKII δ B-T287D followed by western blot showed preferentially intense phosphorylation of histone H3 at serine-10 (Ser-10) (Figure 2C), a well-characterized site that plays a role in mitosis (7–9) and in growth factor stimulation in non-cardiac cells (6,11–14,36–38). Adjacent Ser-28 showed no detectable signal (Figure 2C). Next, we investigated whether CaMKII δ B targets Ser-10 in chromatin itself. Chromatin was heavily phosphorylated at Ser-10 in primary cardiac cells and was first subjected to de-phosphorylation reaction (Figure 2D, lane 1 versus 2). Addition of constitutively active CaMKII δ B-T287D increased phosphorylation of histone H3 at Ser-10, in a dose-dependent manner (Figure 2D, lanes 3–5). The robust phosphorylation signal observed in the presence of the kinase suggests that Ser-10 is a major phosphorylation site. To confirm this, we generated mutant histone H3 with Ser-10 to Ala substitution carrying a Flag epitope and performed *in vitro* kinase assay in the presence of [γ - 32 P] ATP and CaMKII δ B-T287D. Wild-type H3 was heavily phosphorylated by CaMKII δ B-T287D as expected, whereas mutant H3 S10A showed significantly lower phosphorylation (Figure 2E). Next, we confirmed that the S10A mutation effects phosphorylation by CaMKII δ B by immunoblotting. HeLa cells were transfected with Flag-H3 or Flag-H3 S10A, and western analysis was carried out after immunoprecipitation with Flag resin and *in vitro* phosphorylation in the presence of CaMKII δ B. We observed intense phosphorylation of Flag-H3, whereas mutant H3 S10A phosphorylation was severely impaired (Figure 2F). To address the specificity of CaMKII δ B for H3 and to exclude the possibility that H3 phosphorylation by CaMKII δ B is due to other kinases purified in the immunoprecipitate, we compared H3 phosphorylation by wild-type CaMKII δ B, kinase ‘dead’ CaMKII δ B-K43A and by ‘active’ CaMKIV. H3 phosphorylation by wild-type CaMKII δ B was intense, whereas phosphorylation by CaMKII δ B-K43A was

minimal as shown before. No phosphorylation of H3 was detected in the presence of ‘active’ CaMKIV, suggesting specificity of H3 for CaMKII (Supplementary Figure S6D). Thus, histone H3 is a novel bona fide CaMKII substrate, and Ser-10 seems to be a predominant phosphorylation site.

Histone H3 phosphorylation is increased after hypertrophic stimulation

CaMKII δ is central to pathological hypertrophic signaling pathways (21,23). Rapid (30 min) and transient H3 Ser-10 phosphorylation has been reported in response to mitogens in non-cardiac cells (10). Thus, we examined H3 phosphorylation level after hypertrophic stimulation in primary neonatal rat cardiomyocytes by testing the impact of short-term treatment with the hypertrophic agonist PE. Increased H3 Ser-10 phosphorylation was detected as early as 1 h after PE treatment and paralleled increased expression of α -actinin, a hallmark of sarcomere re-organization during hypertrophic growth (Supplementary Figure S7B). Temporal analysis of H3 phosphorylation by immunoblotting showed further increase of H3 Ser-10 levels beyond 6 h of treatment with PE or its close analog xylometazolin (XY). The phosphorylation signal reached its maximum at 24 h of stimulation and coincided with increased CaMKII δ activity and cellular hypertrophy evidenced by enhanced α -actinin expression (Figure 3A and B and Supplementary Figure S8). Phosphorylation of other amino acids present in the histone H3 ‘tail’ such as Thr-3 and Thr-11 was very low in cardiac cells and did not increase after treatment with PE or XY further supporting that Ser-10 is a major phosphorylation site (Supplementary Figure S7C).

To establish the link between CaMKII δ B and H3 phosphorylation during cardiac hypertrophy, we reduced CaMKII δ B expression in primary cardiac cells treated with PE using siRNA oligonucleotides specific for the δ B isoform (26) and assessed H3 Ser-10 levels by immunoblotting. Downregulation of CaMKII δ B significantly reduced PE-mediated phosphorylation of H3 Ser-10, whereas GAPDH levels did not change (Figure 3C). We then investigated whether specific reduction of CaMKII δ B decreases H3 Ser-10 in ventricular myocytes. Double-immunostaining with α -actinin and H3 Ser-10 antibodies in ventricular myocytes transfected with siControl showed H3 Ser-10-positive cells (~35%), whereas signal was strongly reduced (~5%) in siCaMKII δ B transfected cells (Figure 3D and E). As histone H3 phosphorylation at Ser-10 correlates with mitotic activity, we investigated whether the increase in H3 phosphorylation after hypertrophic agonist treatment could reflect an increase in cellular proliferation. Levels of several cell cycle-regulated proteins including Cdk4, cyclin E and Cdc2 were relatively low and remained mostly unchanged in primary cardiomyocytes treated with PE and XY (Figure 4A). Thus, H3 Ser-10 phosphorylation in primary cardiomyocytes stimulated with hypertrophic agonists seems to be linked to cellular hypertrophy rather than mitotic activity. To confirm this, we measured BrdU incorporation in cells transfected with siControl or siCaMKII δ B using

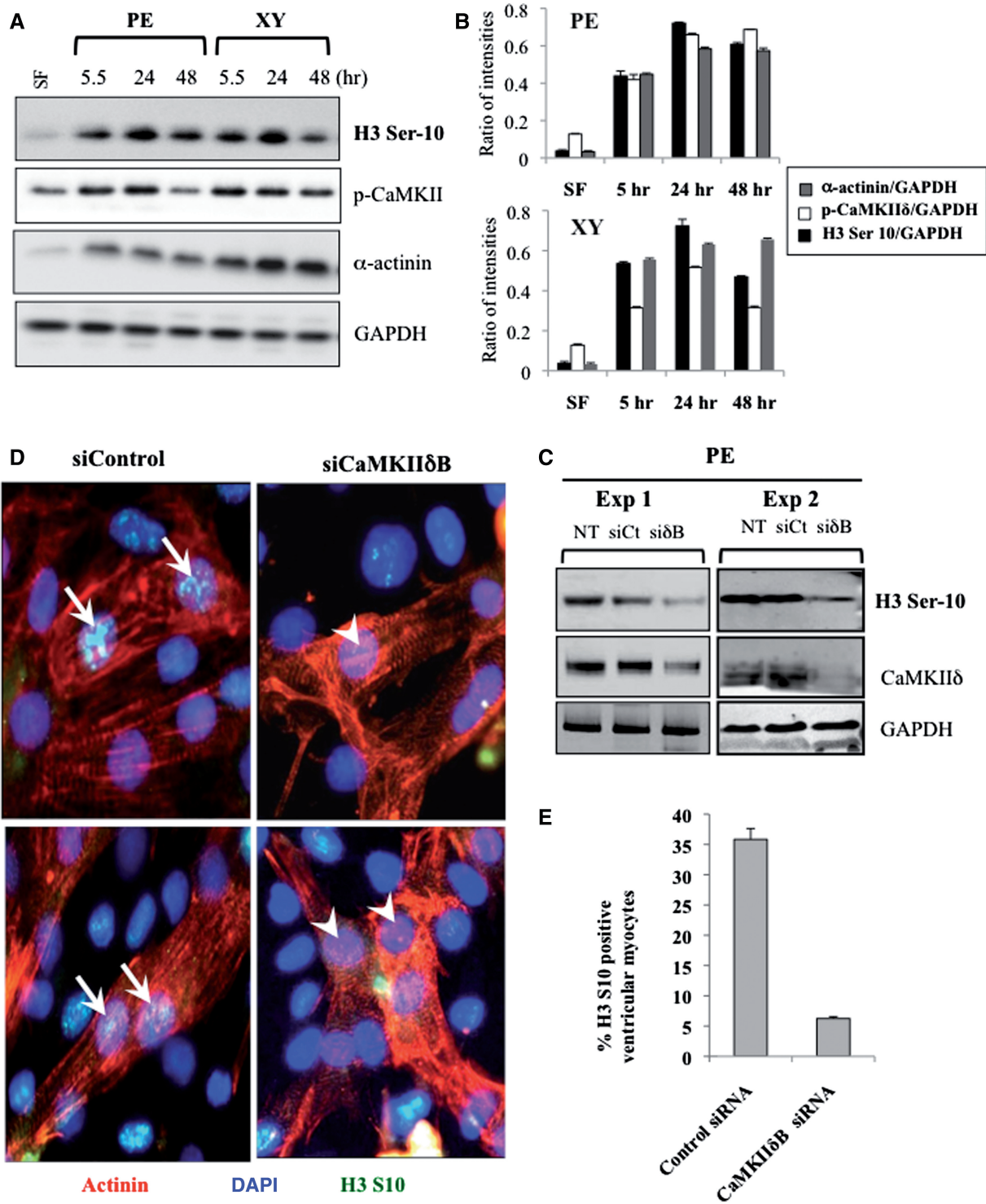


Figure 3. Increased histone H3 phosphorylation during hypertrophic stimulation. (A) Western analysis from primary cardiac cells stimulated with PE and XY using anti-H3 Ser-10, p-CaMKII, α -actinin and GAPDH antibodies. This experiment was repeated three times with three independent cardiomyocyte preparations. (B) Quantitation of A. SF: cells maintained in serum-free conditions. (C) Western blot analysis of cell extracts prepared from primary cardiomyocytes treated with PE and transfected with control siRNA (siCt) or siRNA against CaMKII δ B (si δ B), using α -histone H3 Ser-10 antibody. NT: non-transfected cells ($n = 3$). (D) Specific silencing of CaMKII δ B by siRNA decreases H3 Ser-10 in ventricular myocytes undergoing hypertrophy. Merge image showing decreased histone H3 phosphorylation in ventricular myocytes stimulated with PE for 24h transfected with siCaMKII δ B compared with siControl using indirect immunofluorescence. Nuclei are shown in blue with 4',6-diamidino-2-phenylindole (DAPI) staining, histone H3 Ser-10 is in green. Ventricular cardiomyocytes stained with α -actinin antibody are shown in red. Arrows indicate the nuclei of myocytes positive for H3 Ser-10 in siControl myocytes and arrowheads nuclei which are negative for H3 Ser-10 in ventricular myocytes after CaMKII δ B knock-down. (E) Percentage of ventricular myocytes positive for H3 Ser-10. Quantitative analysis of B was done in five different fields. * $P < 0.05$ using Student t -test.

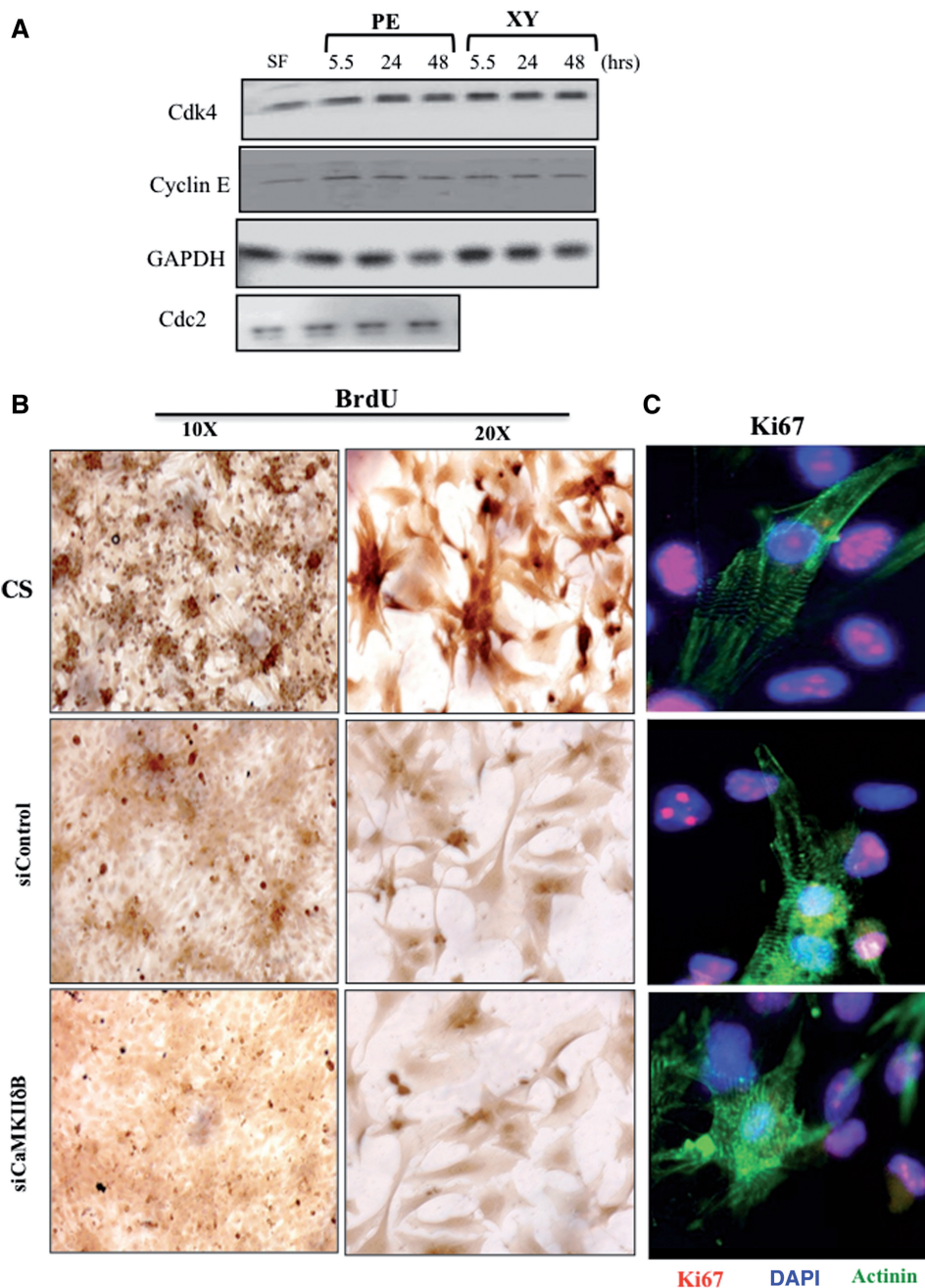


Figure 4. H3 Ser-10 phosphorylation in ventricular myocytes is not associated with cellular proliferation. (A) Expression level of cell cycle proteins and cycle-dependent kinases during hypertrophic stimulation. Immunoblots from primary cardiac cells stimulated with PE and XY using anti-Cdk4, cyclin E, Cdc2 and GAPDH antibodies. This experiment was repeated twice in two independent cardiomyocyte preparations. SF: serum-free conditions. (B) Immunocytochemistry for BrdU and immunofluorescence for Ki67 in primary neonatal rat cardiomyocytes treated with PE for 24 h after transfection with siControl or siCaMKIIδB. CS: cardiomyocytes treated with serum. For Ki67, nuclei are shown in blue with DAPI staining, Ki67 is shown in red. Ventricular cardiomyocytes stained with α -actinin antibody are shown in green.

immunocytochemistry. Low BrdU staining was observed in both siControl- and siCaMKIIδB-transfected cells. Cardiac cells treated with serum showed a stronger BrdU staining, which was used as a positive control (Figure 4B). Expression of Ki67 proliferation marker using immunofluorescence was low in PE-treated ventricular myocytes transfected with siControl or siCaMKIIδB, indicating a lack of cellular proliferation in these cells (Figure 4C). These results show H3 Ser-10

phosphorylation in ventricular myocytes with normal CaMKIIδB expression undergoing hypertrophy, although these cells have limited proliferative capacity. They also show that CaMKIIδB knock-down reduces H3 Ser-10 phosphorylation in ventricular myocytes. Together, our results show that CaMKIIδB is required for H3 phosphorylation at Ser-10. Our results suggest that CaMKIIδB signaling to histone H3 may be a new underlying mechanism controlling the transcriptional

reprogramming of fetal cardiac genes occurring during pathological cardiac hypertrophy.

Hyper-phosphorylation of histone H3 and recruitment of CaMKII δ B at fetal cardiac genes during hypertrophic stimulation

The hypertrophic response is characterized by the re-activation of cardiac genes normally expressed during embryonic development, which results in ventricular remodeling and growth of the heart. To determine whether the phosphorylation status of histone H3 is consistent with the transcriptional reprogramming of fetal cardiac genes during cardiac hypertrophy, we performed ChIP followed by Q-PCR to evaluate H3 phosphorylation and CaMKII δ B occupancy at regulatory regions of known hypertrophy-related genes. Cardiac chromatin isolated from primary cardiomyocytes either unstimulated or stimulated with PE was immunoprecipitated with H3 Ser-10 or CaMKII δ antibodies, and associated genomic DNA was analyzed by Q-PCR using primers for two genes re-expressed during cardiac hypertrophy, the ANF and β -MHC and for two genes not regulated during hypertrophic stimulation, cardiac α -actin and GAPDH. Under basal condition, phosphorylation of H3 and CaMKII δ B binding was detected at the α -actin and GAPDH genes, whereas a weak signal was detected at the two hypertrophic loci, ANF and β -MHC (Supplementary Figure S9). After PE stimulation, H3 phosphorylation and CaMKII δ B binding remained the same at the α -actin and GAPDH genes, whereas signals increased dramatically at the ANF and β -MHC promoter, reaching a maximum at 24 h. The marked increased signal at hypertrophic gene loci was obvious when results were expressed over basal condition (Figure 5B and C). Thus, hypertrophic stimulation increases H3 phosphorylation and CaMKII δ B binding at hypertrophic-sensitive genes, whereas genes not regulated during cardiac hypertrophy remain unchanged. To determine whether CaMKII δ B plays a major role in H3 Ser-10 phosphorylation, we reduced CaMKII δ B expression using a lentivirus expressing CaMKII δ B shRNA (Figure 5D) and performed ChIP-Q-PCR after hypertrophic stimulation with PE. Reduction of CaMKII δ B resulted in a marked decrease in H3 Ser-10 phosphorylation at the ANF and β -MHC promoters, whereas α -actin and GAPDH remained unchanged (Figure 5E). Consistent with the downregulation of CaMKII δ B, the kinase was not detected at hypertrophic genes (data not shown). These results indicate that H3 hyper-phosphorylation and increased CaMKII binding are mostly detected at hypertrophic gene loci actively transcribed during cardiac hypertrophy, and that CaMKII δ B plays an important role in H3 Ser-10 phosphorylation. All together, these results suggest a functional link between CaMKII recruitment, H3 phosphorylation and transcriptional activation.

Nuclear CaMKII increases chromatin accessibility and remodeling

The extent of DNA packaging into nucleosomes determines the condensation state of chromatin, which can be

‘opened/active’ or ‘condensed/inactive’. Transcriptional activation often depends on remodeling of chromatin by remodeling complexes that change the local chromatin architecture to a more ‘open’ stage conducive for recruitment of nuclear factors. To further define the mechanism by which CaMKII-mediated signaling to H3 activates cardiac transcription, we investigated whether chromatin structure is altered by the S10 mutation in H3. For this, we examined MNase-cleaved patterns of chromatin reconstituted with Flag-H3 or mutant Flag-H3 S10A. Both ectopic Flag-H3 and Flag-H3 S10A incorporated into chromatin as shown by western blot analysis using anti-Flag or anti-H3 antibodies (Figure 6A). We observed efficient MNase digestion indicated by the release of small fragments on Flag-H3-digested chromatin, whereas chromatin harboring mutant H3 S10A remained compact and unaffected by MNase digestion as shown by the production of fragments of mostly larger sizes (Figure 6B). These results indicate that Ser-10 phosphorylation is crucial for chromatin re-structuring. To investigate the importance of CaMKII δ B in chromatin remodeling, we performed a second assay, restriction enzyme accessibility assay. In all, 601 Widom nucleosomal DNA were reconstituted *in vitro* (Figure 6C) and treated with restriction endonuclease StyI in the presence or absence of CaMKII δ B. Naked DNA was cut effectively by StyI as expected, whereas the enzyme failed to cut DNA in the absence of any remodeler (Figure 6D, lanes 1–4). As anticipated, ISWI remodeling complex remodeled chromatin efficiently in the presence of ATP as shown by the presence of a partially digested fragment after incubation with StyI (Figure 6D, lanes 5 versus 6). We observed significant nucleosome remodeling in the presence of CaMKII δ B, but not with inactive CaMKII δ B-K43A (Figure 6D, lanes 7–10 and Figure 6E). These results indicate that CaMKII δ B is able to remodel chromatin, and that this remodeling is dependent on its kinase activity. Together, these results indicate that CaMKII δ B alters chromatin conformation, and that its remodeling activity requires its kinase domain.

Impaired Mef2-dependent transcription from chromatin reconstituted with mutant H3 S10A

To establish the functional and causal link between H3 Ser-10 phosphorylation by CaMKII δ B, chromatin remodeling and transcription activation, we developed an *in vitro* transcription system to measure transcription rates using either DNA or chromatin templates that were reconstituted with wild-type histone H3 or mutant H3 S10A. The Mef2 transcription factor is a point of conversion for many hypertrophic signaling pathways (39), and CaMKII δ B activates Mef2-dependent transcription in cardiac cells (26). Therefore, we generated DNA templates harboring Mef2-response elements and measured rates of transcription with wild-type and mutant reconstituted chromatin in the presence of CaMKII δ B and nuclear extracts (Figure 7A and B). Transcription from endogenous chromatin or chromatin reconstituted with wild-type Flag-H3 was high in the presence of CaMKII δ B, calcium and ATP, whereas transcription from native DNA templates was dependent on the

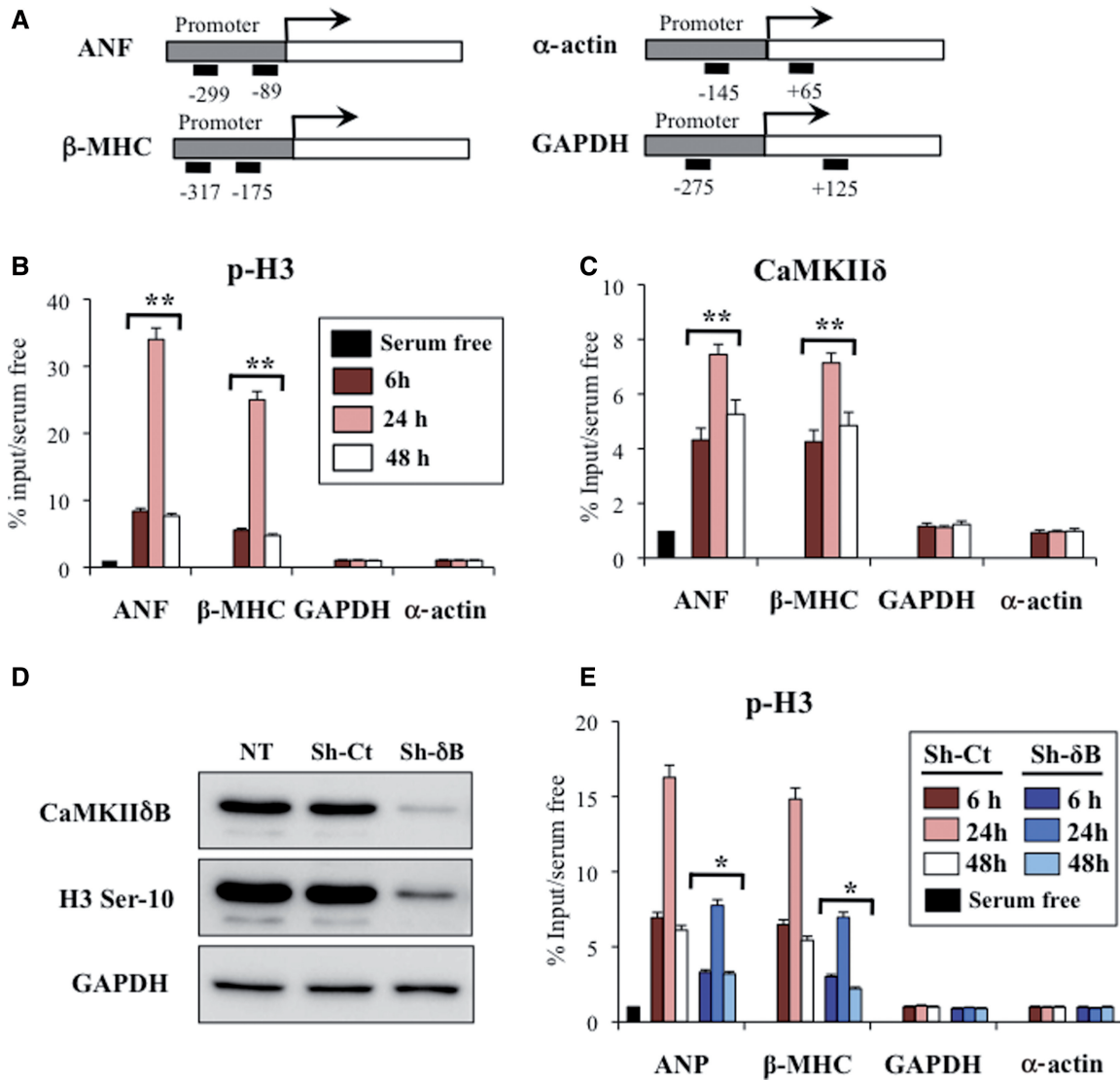


Figure 5. Increased histone H3 Ser-10 and CaMKIIδB recruitment at fetal-cardiac promoters during hypertrophic stimulation. (A) Primers used for the amplification of hypertrophic genes after ChIP from primary cardiomyocytes stimulated with PE. Primer sets are specific for the ANF, β-MHC, α-cardiac actin and GAPDH genes. Black boxes represent the position of the primers relative to the start of transcription. ChIP-Q-PCR assay using (B) α-histone H3 Ser-10 or (C) CaMKIIδ antibodies to precipitate chromatin from primary neonatal rat cardiomyocytes maintained in serum-free conditions or treated with PE for 6, 24 and 48 h. Results are expressed as % input over basal condition (serum-free treatment). Error bars represent means ± SD (*n* = 3). ***P* < 0.001 PE versus serum free. (D) Western blot showing downregulation of CaMKIIδB using a lentivirus expressing CaMKIIδB shRNA (sh-δB) compared with control GFP lentivirus (sh-Ct). NT: primary cardiomyocytes non-transduced. H3 Ser-10 expression and GAPDH control are shown in parallel. (E) ChIP-Q-PCR performed with H3 Ser-10 antibody from primary cardiomyocytes expressing CaMKIIδB shRNA (sh-δB) or a mock GFP lentivirus and stimulated with PE for 6, 24 and 48 h. Results represent the average of three independent experiments and are expressed as % input over basal condition (serum-free-treated cells). **P* < 0.05. Comparison was made between Sh-Ct and Sh-δB for each time point.

presence of CaMKIIδB (Figure 7C). When chromatin was reconstituted with mutant H3 S10A, CaMKIIδB almost failed to transcribe Mef2 (Figure 7D, lane 4 versus 6). These results demonstrate that CaMKIIδB is critical for Mef2 transcription from chromatin templates. They also show that CaMKIIδB-mediated Mef2 transcription requires phosphorylation at Ser-10. Together, these results show that CaMKIIδB remodels chromatin through phosphorylation of Ser-10, which is required for the transcriptional activation of hypertrophic genes.

DISCUSSION

In cardiac muscle, CaMKIIδB is central to pathological hypertrophic pathways (20,21,23). In this study, we demonstrate a novel function of nuclear CaMKIIδB that increases histone H3 phosphorylation and remodels chromatin. We show that CaMKIIδB directly signals to chromatin to regulate the transcription of genes required for the hypertrophic response (Figure 8). This shows that nuclear calcium regulates gene activity by direct signaling to chromatin.

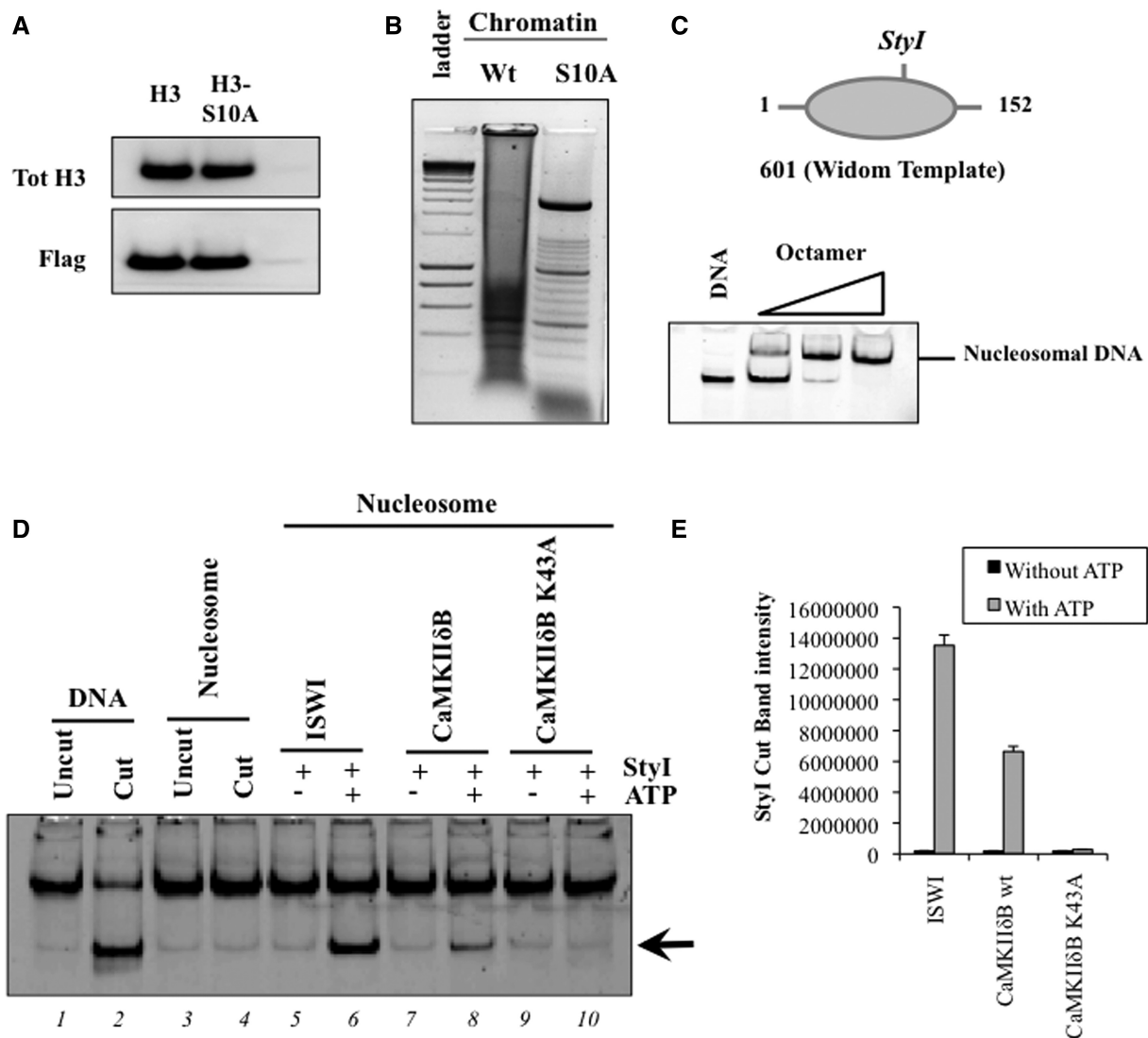


Figure 6. CaMKII δ B increases chromatin accessibility. (A) Flag-H3 and Flag-H3 S10A are efficiently incorporated into chromatin as measured by western blot using anti-Flag or anti-H3 antibodies. (B) MNase digestion of chromatin reconstituted with Flag-H3 or mutant H3 S10A separated on agarose gel by electrophoresis. The DNA fragments obtained after digestion show that chromatin reconstituted with H3 S10A is more compact than wild-type H3. (C) Schematic illustration of the 601 Widom template used after reconstitution for restriction enzyme accessibility assays. Analysis of nucleosomal DNA by native polyacrylamide gel electrophoresis showing successful reconstitution *in vitro*. (D) Restriction enzyme accessibility assay. Similar amounts (~ 20 nM) of ISWI (lanes 5–6), CaMKII δ B (lanes 7–8) and CaMKII δ B K43A were added to ~ 10 ng of the 0W0 template in the presence or absence 2 mM ATP. The binding reactions were then treated with 10 U of StyI for 30 min at 30°C, and the proportion of DNA cleaved was assessed by electrophoresis. (E) Quantification of three independent experiments of the remodeling assay measuring the digested band intensity.

To understand the transcriptional reprogramming taking place during cardiac hypertrophy, we purified proteins that form a complex with CaMKII δ B, the most abundant nuclear CaMKII in the adult heart. Histones were found to precipitate with the kinase. Direct interaction between exogenous CaMKII δ B and reconstituted histone octamers, and chromatin was demonstrated *in vitro*. Subsequently, we showed a strong association of endogenous or ectopically expressed CaMKII δ B with chromatin in primary cardiac cells. Among the four core histones, CaMKII δ B seems to markedly phosphorylate histone H3 with minimal phosphorylation of H2A/H2B, suggesting specificity of the kinase. Ser-10 is a major phosphorylation site, as mutation of this residue to alanine

abolishes phosphorylation by CaMKII δ B. Our results also show that specific signaling of CaMKII δ B to histone H3 at Ser-10 is critical for the activation of fetal cardiac genes after hypertrophic stimulation. Activation of cardiac hypertrophy by α -adrenergic agonists increases global H3 phosphorylation at Ser-10. Conversely, specific reduction of CaMKII δ B using RNA interference reduces H3 Ser-10 level with concomitant inhibition of cellular hypertrophy.

Unexpectedly, we found that Ser-10 is a critical amino acid targeted by the kinase and that H3 Ser-10 phosphorylation by CaMKII δ B is not associated with cellular proliferation. Rather, H3 Ser-10 phosphorylation by CaMKII δ B regulates growth by hypertrophy. H3 Ser-10

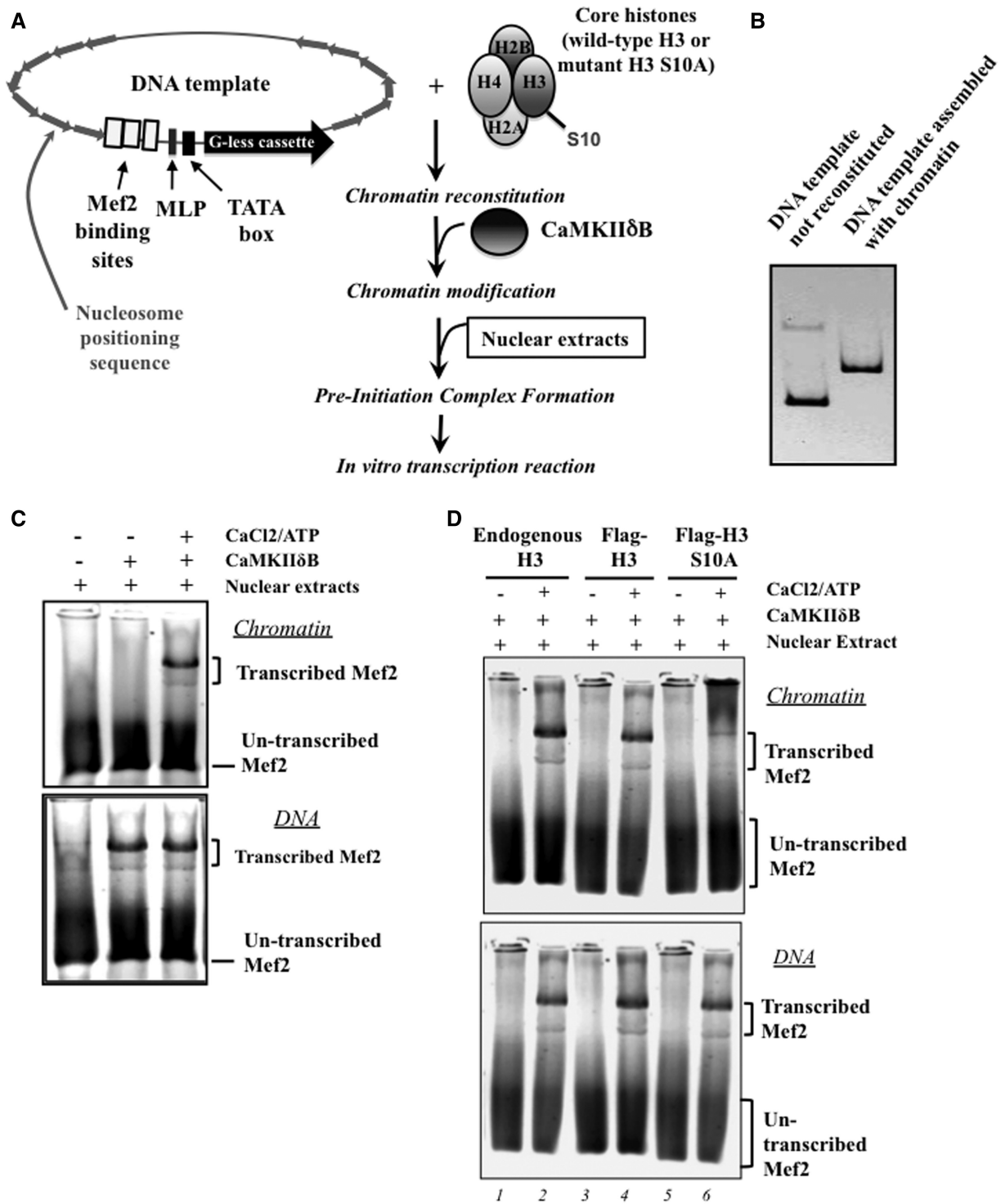


Figure 7. *In vitro* transcription assay with reconstituted chromatin and DNA templates with multimerized Mef2-binding sites. (A) DNA templates harboring upstream multimerized Mef2-binding sites, the adenovirus major late promoter (MLP) in front of a G-less cassette, and 5' and 3' flanking regions repeats of nucleosome positioning sequences. *In vitro* transcription was measured from chromatin templates reconstituted *in vitro* with wild-type histone H3 or mutant histone H3 S10A after addition of active CaMKIIδB-T287D and nuclear extracts. (B) Agarose gel showing DNA templates with multimerized Mef2-binding sites and chromatin reconstitution *in vitro*. (C) Transcription from chromatin reconstituted with histone octamers and DNA templates harboring 3xMef2-binding sites in the presence of CaMKIIδB and nuclear extracts. (D) *In vitro* transcription assay showing that CaMKIIδB-mediated chromatin transcription requires phosphorylation of histone H3 at Ser-10. Transcription from free Mef2 DNA templates and endogenous HeLa chromatin or chromatin reconstituted with wild-type Flag-H3 or mutant Flag-H3 S10A was measured in the presence of active CaMKIIδB-T287D, ATP, calcium and nuclear extracts.

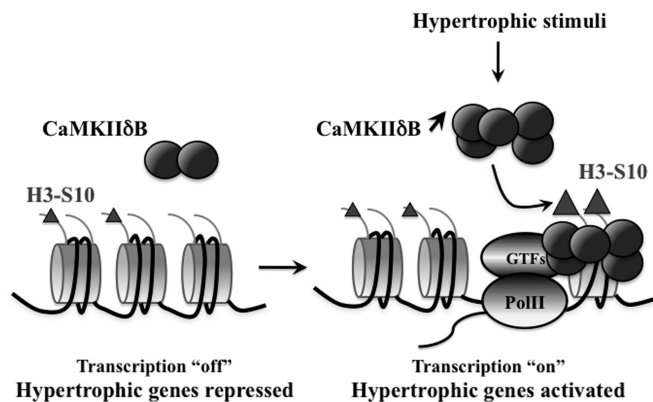


Figure 8. Model of CaMKII δ B signaling to H3 and remodeling of chromatin with subsequent transcriptional activation. Hypertrophic stimulation increases CaMKII δ B expression in cardiac cells, which is then recruited to hypertrophic gene loci and phosphorylates H3 at Ser-10. This phosphorylation event remodels chromatin and allows the activation of fetal cardiac genes. GTFs: general transcription factors, PolII: RNA polymerase II.

phosphorylation was previously shown to play a dual role; in mitosis where chromatin condenses and becomes silent, and in interphase where chromatin decondenses for transcriptional activation. Mitotic kinases that phosphorylate histone H3 at Ser-10 include Aurora B (40) and NIMA (7,9,41). Also, Snf1 (42), Jil-1 (43), Rsk2 (10), Msk1/2 (44), protein kinase B (PKB)/Akt and PIM1 are mitogenic kinases inducing a rapid and transient phosphorylation of H3 in non-cardiac cells with an effect on a limited number of immediate early genes. Based on these reports, we investigated whether the marked increase in H3 phosphorylation in hypertrophic cardiomyocytes could reflect an increase in cellular proliferation. Expression of one proliferation marker and several cell cycle-regulated proteins was unchanged after hypertrophic challenge, whereas histone H3 phosphorylation increased dramatically. BrdU incorporation and expression of the Ki67 remained very low in ventricular myocytes treated with PE expressing normal or reduced CaMKII δ B. Furthermore, phosphorylation of histone H3 by CaMKII δ B was not as transient as H3 phosphorylation by other H3 kinases. These observations underscore that H3 Ser-10 has another function than the 'classical' mitotic marker. Thus, it seems that the same 'mark' on histone H3 can drive both cellular proliferation and growth by increase in cell size. The implication is that these processes are controlled by distinct kinases. Another explanation is that specificity of the signal comes from the assembly of large transcriptional complexes that are different in various cell types. These possibilities are currently under investigation.

CaMKII is used as a non-specific H3 kinase *in vitro*. To ascertain the efficiency of CaMKII δ B for H3, we performed kinetic experiments with increasing amounts of CaMKII δ B and compared H3 phosphorylation with an established H3 kinase, Aurora B. CaMKII δ B was able to phosphorylate H3, although not as efficiently as Aurora B. We also compared H3 Ser-10 phosphorylation

by kinase 'dead' CaMKII δ B-K43A and CaMKIV. Significant H3 Ser-10 phosphorylation was observed with CaMKII δ B but not by kinase 'dead' CaMKII δ B nor by CaMKIV. Specificity of CaMKII δ B for H3 in cardiac cells was established by siRNA experiments and by comparing phosphorylation of H3 to an established CaMKII δ B substrate, HDAC4. H3 phosphorylation by CaMKII δ B was similar to CaMKII δ B-mediated phosphorylation of HDAC4. Marked H3 phosphorylation was observed in ventricular myocytes undergoing hypertrophy and expressing CaMKII δ B, whereas H3 Ser-10 was severely reduced in ventricular myocytes where CaMKII δ B is knocked down. Thus, although phosphorylation of H3 is not as high as with Aurora kinase, CaMKII δ B specifically signals to H3 in cardiac cells undergoing hypertrophy.

Mechanistically, we demonstrate a functional interaction between CaMKII-mediated signaling to H3 and transcription activation. Increased occupancy of CaMKII δ B and hyper-phosphorylation of histone H3 at Ser-10 are detected at the promoter region of fetal cardiac genes after hypertrophic stimulation. A number of transcriptional units examined showed binding of CaMKII δ B and phosphorylation signal under basal conditions but these remained unchanged after hypertrophic stimulation. Specific elimination of CaMKII δ B severely impaired H3 Ser-10 at hypertrophic promoter regions, whereas genes non-responsive to hypertrophy were minimally affected. Therefore, CaMKII δ B targets histone H3 at specific genomic loci, mostly those involved in regulating the expression of fetal cardiac genes. Thus, it seems that CaMKII δ B not only signals to chromatin remodeling enzymes like HDAC4 (25,26) but also to chromatin itself. Our results show that signaling of CaMKII δ B to H3 modulates chromatin architecture, as indicated by restriction enzyme digestion assay that shows increased chromatin accessibility by CaMKII δ B. Importantly, the effect of CaMKII δ B on chromatin structure depends on its kinase activity, as a 'kinase dead' mutant enzyme has no effect on chromatin conformation. Also, MNase digestion of chromatin reconstituted with mutant H3 S10A shows its high degree of condensation compared with chromatin reconstituted with wild-type H3. One remaining challenge in the 'histone field' is to establish the direct link between histone modifications and their functional consequences *in vivo*. In particular, these mutations are usually lethal, and histone genes are represented as large copy numbers in the genome making it a technical challenge to introduce specific mutations in selected organs to study their impact *in vivo*. To establish the relationship between H3 Ser-10 phosphorylation and transcriptional activation, we developed an *in vitro* transcription assay and measured transcription of Mef2 from chromatin reconstituted with wild-type or mutant H3 in the presence of CaMKII δ B. Strikingly, transcription rates were severely reduced with chromatin reconstituted with mutant H3 S10A compared with wild-type H3, indicating that the Ser-10 site is critical for CaMKII δ B to properly activate transcription. As Mef2 is a nodal point for many hypertrophic signaling pathways (39), our results overall

strongly suggest a causal link between H3 Ser-10 phosphorylation by CaMKII δ B and cardiac hypertrophy.

With the recent interest and rapid development of large-scale genomic and epigenetic studies, ChIP experiments followed by sequencing are underway and should provide a clear insight on functional consequences genome wide of chromatin remodeling and transcriptional events controlled by CaMKII δ B. The study of CaMKII δ B interaction with other chromatin modifiers, together with experiments addressing the relationship between the various histone modifications (i.e. methylation and acetylation) should also provide a deeper understanding of underlying mechanisms regulating cardiac hypertrophy. Considering the broad tissue distribution of CaMK enzymes and their role in a variety of cellular functions, our study provides a novel conceptual framework on the functional role of CaMK enzymes as epigenetic modifiers.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–8, Supplementary Methods and Supplementary References [26,30,32].

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Conflict of interest statement. None declared.

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