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Early growth response 1 is an early signal inducing Cav3.2 T-type calcium channels during cardiac hypertrophy

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Aims The $Ca_v3.2$ T-channel plays a pivotal role in inducing calcineurin/nuclear factor of activated T cell (NFAT) signalling during cardiac hypertrophy. Because calcineurin/NFAT signalling is induced early after pressure overload, we hypothesized that $Ca_v3.2$ is induced by an early signal. Our aim is to investigate when and how $Ca_v3.2$ is induced during cardiac hypertrophy.

Methods and results The evolutionary conserved promoter $Ca_v3.2$-3500 from mouse genome was validated to express the reporter gene as endogenous $Ca_v3.2$ in cell lines and transgenic (Tg; $Ca_v3.2$-3500-Luc) mice. The early induction of luciferase in Tg mice and $Ca_v3.2$ mRNA in wild-type mice after transverse aortic banding (TAB) surgery supported our hypothesis that $Ca_v3.2$ is induced early during cardiac hypertrophy. The TAB-responding element [−81 to −41 bp upstream of the transcription start site (TSS) of mouse $Ca_v3.2$] was identified by *in vivo* gene transfer by injecting reporter constructs into the left ventricle followed by TAB surgery. Electrophoresis mobility shift assay and chromatin immunoprecipitation assays revealed that Egr1 bound to the TAB-responding element of $Ca_v3.2$. Egr1 level was increased with increased $Ca_v3.2$ mRNA level at 3 days after TAB. To demonstrate that Egr1 indeed regulates $Ca_v3.2$ expression after hypertrophic stimulation, knockdown of Egr1 with short hairpin RNA prevented the phenylephrine-induced up-regulation of $Ca_v3.2$ expression and cellular hypertrophy in neonatal rat ventricular myocytes (NRVMs) and H9c2 cells. Furthermore, overexpression of $Ca_v3.2$ in Egr1-knockdown cells restored the phenylephrine-induced hypertrophy.

Conclusion $Ca_v3.2$ is induced early by Egr1 during cardiac hypertrophy and $Ca_v3.2$ is an important mediator of Egr1 in regulating cardiac hypertrophy.

Keywords Egr1 • $Ca_v3.2$ • T-type calcium channel • Cardiac hypertrophy • Transcriptional regulation

1. Introduction

Adult hearts undergo hypertrophic remodelling as a compensatory response to various stimulations, including exercise, pregnancy, chronic hypertension, myocardial infarction, and other heart diseases.[1] Although considered a beneficial response in the beginning, prolonged hypertrophy will progress into heart failure under pathological conditions. Hallmarks of cardiac hypertrophy include increased cell size, actin reorganization, increased protein synthesis, and foetal gene re-expression. The foetal genes re-expressed in hypertrophic hearts include atrial and brain natriuretic peptide (ANP and BNP, respectively), foetal isoforms of contractile proteins (α-skeletal actin and β-myosin heavy chain), and ion channels typically expressed in the foetal heart, such as hyperpolarization-activated cyclic nucleotide-gated channel and T-type calcium channel (T-channel).[2] Although the transcriptional regulation of ANP and BNP during cardiac hypertrophy was well studied, the regulation of other foetal genes including T-channel is still unclear.

Two types of voltage-gated Ca^{2+} channels are present in hearts, namely L- and T-channel. Ca^{2+} current through the L-channel plays a key role in excitation–contraction coupling and Ca^{2+} current through the T-channel is thought to be involved in the intracellular calcium signal pathway.[3] T-channels are encoded by two genes, $Ca_v3.1$ and $Ca_v3.2$, in cardiac myocytes.[4,5] In murine sinoatrial node, $Ca_v3.1$ was expressed more prominent than $Ca_v3.2$.[6] Mice lacking $Ca_v3.1$ developed bradycardia and slower atrioventricular conduction.[7,8] In contrast, mice

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lacking $Ca_v3.2$ showed normal heart rate.[9] In myocardium, T-channel is normally expressed in foetal and absent in adult ventricles, but re-expressed in adult ventricular myocytes in different cardiac myopathy models.[10–12] T-current is found induced along with $Ca_v3.1$ or $Ca_v3.2$ up-regulation in different models of cardiac dysfunction[11–15]; however, the roles of the genes in the progression of cardiac hypertrophy differ. Cardiac-specific overexpression of $Ca_v3.1$ attenuated cardiac hypertrophy through a nitric oxide synthase 3-dependent signalling mechanism,[15] whereas $Ca_v3.2$ deletion attenuated pressure overload-induced cardiac hypertrophy by reducing the calcineurin/NFAT signalling pathway.[12] These studies suggest that $Ca_v3.1$ is anti-hypertrophic and $Ca_v3.2$ is pro-hypertrophic in response to pressure overload stress.

$Ca_v3.2$ T-channel is required for angiotensin II- and pressure overload-induced cardiac hypertrophy in mice.[12] The re-expression of ventricular T-channels observed in wild-type hypertrophic hearts was absent in $Ca_v3.2$-null mice, which suggests that re-expressed T-channel is encoded by $Ca_v3.2$. Moreover, a pharmacological blockade of T-channel reduced arrhythmias during dilated cardiomyopathy and prevented sudden death due to myocardial infarction in mice.[16] The mouse model of dilated cardiomyopathy in their study involved transgenic mice with a cardiac-specific dominant-negative form of neuron-restrictive silencer factor (dnNRSF-Tg) showing cardiac up-regulation of $Ca_v3.2$ but not $Ca_v3.1$.[17] These studies suggest that targeting $Ca_v3.2$ could be beneficial for heart function.

Although re-expressed $Ca_v3.2$ plays a vital role in the development of cardiac hypertrophy, how $Ca_v3.2$ is re-expressed during the early stage of cardiac hypertrophy is still unclear. $Ca_v3.2$ is induced by overexpressing transcription factor (TF) Csx/Nkx2.5 in cardiomyocytes; however, direct evidence showing the binding of Csx/Nkx2.5 on the promoter of $Ca_v3.2$ is lacking.[18] The transcription repressor NRSF is another transcriptional regulator of cardiac $Ca_v3.2$,[17] but whether NRSF regulates early induction of $Ca_v3.2$ in the hypertrophic heart is unknown. Here, we used mice carrying different $Ca_v3.2$ promoters to answer this question *in vivo* and demonstrated that the TF early growth response 1 (Egr1) was the early signal to induce $Ca_v3.2$ during cardiac hypertrophy.

2. Methods

2.1 Generation of transgenic mice

All research performed conformed to National Institutes of Health guidelines in accordance with the guidelines specified by the Institutional Animal Care and Utilization Committee, Academia Sinica (Taipei, Taiwan). The expression cassette containing the mouse $Ca_v3.2$-3500, firefly luciferase, and SV40 poly A signal was injected in oocytes from C57BL/6J mice to generate transgenic mice.

2.2 *In vivo* direct gene transfer method

The injection procedure was modified from method described previously.[19–21] In brief, 8- to 10-week-old mice (C57BL/6J) were anaesthetized (vaporized 1% isoflurane) and pectoralis muscle was cut and flipped up to visualize the beating heart underneath intercostal muscle. DNA-saline solution (40 μL containing 5 μg testing firefly luciferase and 5 μg control *Renilla* luciferase) was injected through intercostal muscle into the left ventricle at three to four sites using a 27-gauge needle. After injection, the pectoralis minor muscle was adhered to the oblique muscle by the use of tissue glue. Immediately after gene transfer, mice underwent TAB or sham operation.

2.3 Animal model of cardiac hypertrophy

Eight-week-old adult male mice (C57BL/6J) weighing 20–25 g were anaesthetized (vaporized 1% isoflurane) and subjected to pressure overload by transverse aortic banding (TAB) as described.[12]

2.4 Establishing the stable line of H9c2

RNAi reagents were from the National RNAi Core Facility at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan. The vectors (pLKO TRC005) carrying the short hairpin RNA (shRNA) with the target sequence of Egr1 (Egr1 KD) 5′-CACTCCACTATCCACTATTAA-3′ were electroporated into H9c2 cells. After recovery overnight, cells were cultured and maintained with complete medium with 0.125 μg/mL puromycine (Sigma). The control knock down (KD) construct carried the shRNA targeting the EGFP sequence 5′-CCTACGGCGTGCAGTGCTTCA-3′.

2.5 Electrophoresis mobility shift assay

Binding reactions involved use of a digoxigenin (DIG)-labelled probe containing the sequence from −81 to −41 of the mouse $Ca_v3.2$ promoter (5′-CCGCCCCGGCCGCGTTGGCCCCGCCCCATGGGCCGCCCCCG-3′) and transfected HEK293 nuclear extracts. In competition experiments, the nuclear extract was incubated with a 100-fold molar excess of appropriate unlabelled competitor oligonucleotides. Oligonucleotide sequences used in competition assays were as follows (underlines indicate mutated sites):

WT, 5′-CCGCCCCGGCCGCGTTGGCCCCGCCCCATGGGCCGCCCCCG-3′;

M1 mutant, 5′-CAAAATAAGCCGCGTTGGCCCCGCCCCATGGGCCGCCCCCG-3′;

M2 mutant, 5′-CCGCCCCGGCCGCGTTGGCCTTATTTCATGGGCCGCCCCCG-3′;

M3 mutant, 5′-CCGCCCCGGCCGCGTTGGCCCCGCCCCATGGGCAAAAATAG-3′.

2.6 Chromatin immunoprecipitation assay

Transfected H9c2 cells were crosslinked and chromatins were prepared as the instruction of the Pierce agarose chromatin immunoprecipitation (ChIP) kit (Thermo). For immuno-precipitation, 10 μL anti-Flag M2 affinity gel (Sigma) or an equal amount of murine IgG was added. After purification, DNA was amplified by real-time quantitative PCR with primer sequences corresponding to the rat $Ca_v3.2$ promoter region (−180 to −1, 5′ primer: 5′-gatgtcttgggatagcttacgt-3′; 3′ primer: 5′-aggaaacttcggagtgagc-3′).

2.7 *In situ* hybridization

The fragment of $Ca_v3.2$ 3′-UTR was cloned from a mouse testis cDNA pool with the primer sequences forward with a BamHI site, 5′-GGATCCAGGGCTTTGGCATTGAGGTTGT-3′ and reverse with an EcoRI site, 5′-GAATTCACGTCTTATTGCATGGGCCAGT-3′ and subcloned into the vector pBluescript II KS (Promega). With *in vitro* transcription (DIG RNA Labeling Kit, Roche), single-stranded DIG-labelled antisense RNA probe was obtained with T7 RNA polymerase. DIG-labelled RNA probe was allowed to hybridize overnight with deparaffinized mouse heart sections at 65°C, and bound RNAs were visualized by use of anti-DIG conjugated with alkaline phosphatase with NBT/BCIP substrate (Roche).

2.8 Immunostaining

Immunofluoresence staining for Egr1 was conducted using anti-Egr1 antibody (1 : 250, SC-110, Santa Cruz) and Alexa-594-conjugated anti-rabbit IgG was used as the secondary antibody.

2.9 Image capture and analysis

Images of *in situ* hybridization and immunostaining were obtained by the use of the digital camera attached to an Olympus upright microscope. To measure cell size, H9c2 cells were incubated with 2.5 μM CFSE (Invitrogen) following the manufacturer's instructions. After fixation with 4% paraformaldehyde, DAPI was used to stain nuclei. Images were taken by use of High Content Screening (Molecular Devices).

2.10 Statistical analysis

Data are expressed as means ± SD. Statistical comparisons were performed with independent-samples Mann–Whitney rank sum test for two sample groups, one-way ANOVA for more than two sample groups and followed by Dunnett's test for the *post hoc* analysis with control groups in different experiment. A $P < 0.05$ was considered statistically significant.

3. Results

3.1 Proximal 3.5 kb of the mouse $Ca_v3.2$ 5′-flanking sequence carries the regulatory elements for $Ca_v3.2$ gene expression in hearts

The genomic sequences of $Ca_v3.2$ from different species were aligned by the use of the online ECR browser to obtain the conserved region.[22] 5′-flanking sequences (FSs) of $Ca_v3.2$ from dog, rat, mouse, and human showed two highly conserved regions located at −1 to −1100 and −2200 to −3500 bp relative to the transcription start site (Supplementary material online, *Figure S1*). The proximal 3.5 kb ($Ca_v3.2$-3500) sequence of mouse $Ca_v3.2$ 5′-FS was then fused with firefly luciferase and transfected into heart-derived cells expressing $Ca_v3.2$. When compared with HEK293 cells, which do not express $Ca_v3.2$, $Ca_v3.2$-expressing cells, NRVMs and mouse atrial HL-1 cells, showed significantly greater luciferase activity (*Figure 1A*). To confirm that $Ca_v3.2$-3500 is the regulatory promoter of $Ca_v3.2$ *in vivo*, we generated Tg mice expressing firefly luciferase driven by $Ca_v3.2$-3500 (Supplementary material online, *Figure S2A*). Sixteen founder lines were established, and one line was chosen for analysis on the basis of tissue distribution of luciferase activity (Supplementary material online, *Figure S2B*) as the endogenous $Ca_v3.2$ expression spectrum (Supplementary material online, *Figure S2C*). During the heart development, the luciferase activity of $Ca_v3.2$-3500-Luc was high in embryonic and neonatal ventricles and greatly reduced in mature ventricles (*Figure 1B*) as reported $Ca_v3.2$ expression pattern.[5] These results suggest that $Ca_v3.2$-3500 is sufficient to drive reporter expression as endogenous $Ca_v3.2$ expression in hearts.

To investigate whether $Ca_v3.2$-3500 is activated by hypertrophic stimulation, we induced cardiac hypertrophy in Tg($Ca_v3.2$-3500-Luc) mice by TAB. In left ventricles, $Ca_v3.2$-3500-Luc luciferase activity was significantly increased from Day 3 (2.11 ± 0.59-fold) and further increased to 4.02 ± 1.56-fold at Day 14 after TAB when compared with sham operation (*Figure 1C*). In contrast, in atria, luciferase activity was not induced after TAB (Supplementary material online, *Figure S3*). Therefore, $Ca_v3.2$-3500 contains the TAB-responding elements. In addition, transcriptional up-regulation of $Ca_v3.2$ expression was an early event during cardiac hypertrophy, because $Ca_v3.2$-3500-Luc was induced before detectable cardiac hypertrophy (*Figure 1D*).

3.2 The TF Egr1 induced $Ca_v3.2$ expression and its putative binding site $Ca_v3.2$ −81 to −41 was the TAB-responding element

To identify the TAB-responding element within $Ca_v3.2$-3500, we performed *in vivo* gene transfer in mouse hearts as described previously.[19,21] Serial 5′-deleted reporter constructs were created from $Ca_v3.2$-3500-Luc and injected into mouse left ventricular wall directly. Immediately after injection, the mice underwent TAB or sham surgery, and ventricular luciferase activity was analysed 5 days later. Consistent with results from Tg mice, the luciferase activity of $Ca_v3.2$-3500-Luc was induced 4.48 ± 2.25-fold

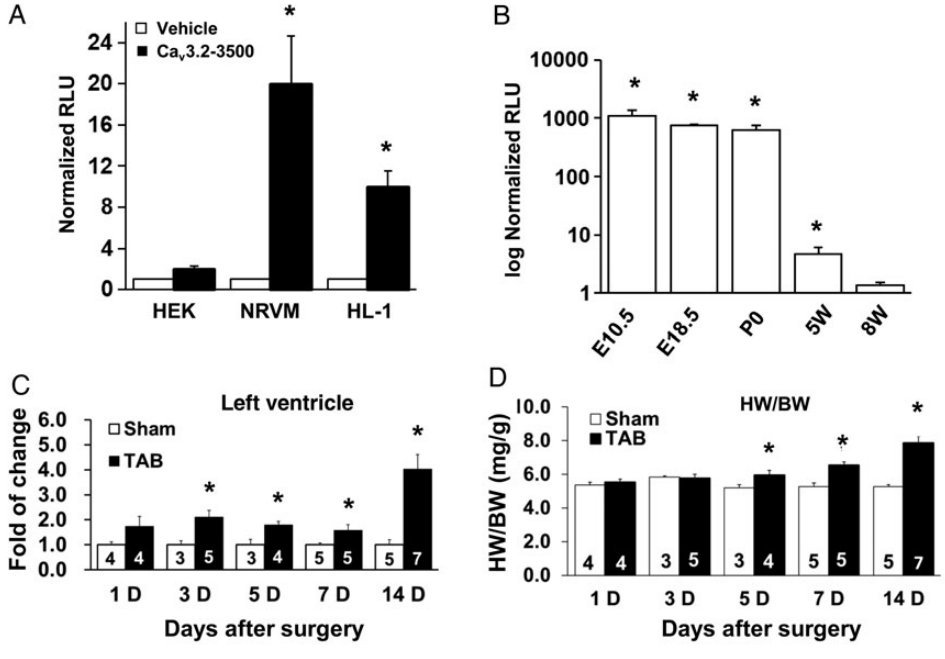

Figure 1 Proximal 3.5 kb of mouse $Ca_v3.2$ 5′-flanking sequence carries the regulatory elements for $Ca_v3.2$ gene expression in hearts. (*A*) Normalized relative luciferase activity (RLU) driven by $Ca_v3.2$-3500 in different cells. $n = 3$ for each group. *$P < 0.05$ vs. vehicle. (*B*) Normalized RLU of cardiac ventricles isolated from different ages of transgenic mice Tg($Ca_v3.2$-3500-Luc). $n = 8, 8, 7, 6, 6$ for each group, respectively. *$P < 0.05$ vs. 8-week-old group. (*C*) Normalized RLU of left ventricles isolated from Tg mice at different times after surgery. (*D*) The hypertrophy index heart weight to body weight of each harvested heart. Number of mice in each group are shown. *$P < 0.05$, TAB vs. Sham surgery.

after TAB (*Figure 2A*). Because TAB-induced luciferase activity was similar in hearts injected with Cav3.2-2000-Luc, Cav3.2−1000-Luc, and Cav3.2-420-Luc (*Figure 2A*); TAB-responding element is located within the proximal 420 bp of mouse $Ca_v3.2$ 5′-FS.

To determine the potential TFs that regulate $Ca_v3.2$ expression, we searched the putative TF binding sites (BSs) on $Ca_v3.2$-420 using TFSEARCH.[23] $Ca_v3.2$-420 contains TF BSs for E2F, nuclear factor-kB (NF-kB), CP2, heat shock factor (HSF), Sp1, Egr1, and GATAs (*Figure 2B*). $Ca_v3.2$-420-Luc constructs with individual deletion in each predicted BS were generated and expressed in HL-1 cells. Compared with $Ca_v3.2$-420-Luc, deletion constructs contained the BSs for NFkB, Sp1, Egr1, and HSF significantly reduced promoter activity (Supplementary material online, *Figure S4*). We coexpressed these TFs individually with $Ca_v3.2$-3500-Luc to test their ability to induce luciferase activity. Egr1 was the only TF that could induce $Ca_v3.2$-3500 luciferase activity in a dose-dependent manner (*Figure 2C*). Furthermore, overexpression of Egr1 could up-regulate the endogenous $Ca_v3.2$ mRNA level by 1.57 ± 0.07-fold in a cardiac-derived cell line, H9c2 (*Figure 2D*). These results suggest that Egr1 enhances $Ca_v3.2$ transcription. We also generated a reporter construct lacking the predicted Egr1 BSs ($Ca_v3.2$-420 **Δ** −81 to −41-Luc) and showed that this construct could not respond to TAB *in vivo* (*Figure 2A*). These results suggest that the Egr1 putative BS $Ca_v3.2$−81 to −41 is the TAB-responding element.

3.3 Egr1 bound to the TAB-responding element ($Ca_v3.2$−81 to −41)

To confirm the interaction between Egr1 and the TAB-responding element, we used EMSA with a DIG-labelled oligonucleotide ($Ca_v3.2$−

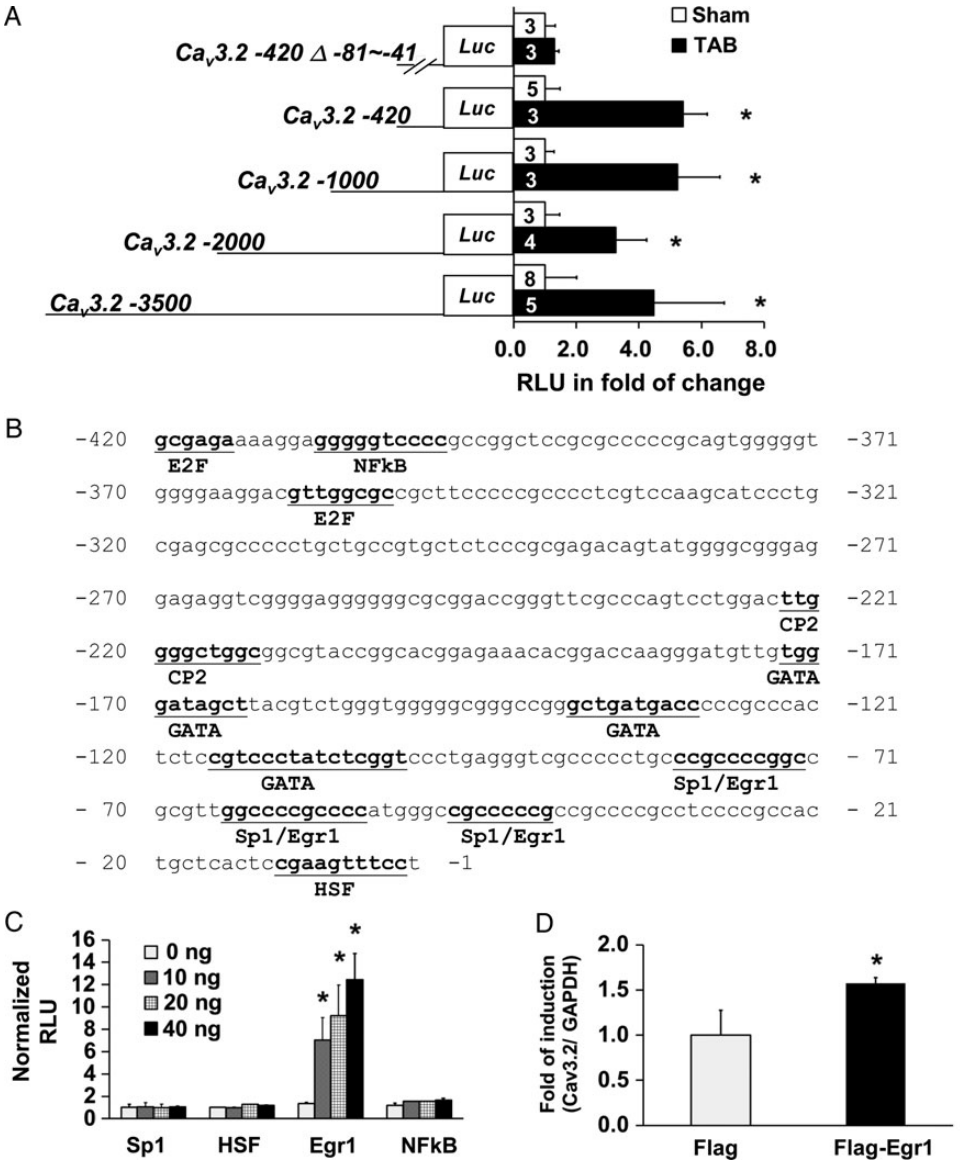

Figure 2 Egr1 induces $Ca_v3.2$ expression and its putative BS is the TAB-responding element. (*A*) Normalized RLU of direct gene transferred hearts $^*P < 0.05$, TAB vs. Sham surgery. (*B*) The sequence of $Ca_v3.2$-420 and the putative BSs for transcription factors are bold and underlined. The TFs E2F, CP2, nuclear factor-kB (NF-kB), GATA, Sp1, early growth response 1 (Egr1), and heat shock factor (HSF) are predicted to bind on $Ca_v3.2$-420. (*C*) Normalized RLU in HEK293 cells transfected with $Ca_v3.2$-3500-Luc and different amounts of TFs. $^*P < 0.05$ vs. 0 ng. $n = 3$ for each group. (*D*) Quantitative RT–PCR analysis of $Ca_v3.2$ mRNA expression in H9c2 cells transfected with Flag-tagged Egr1 or Flag plasmids. $^*P < 0.05$ vs. Flag. $n = 3$ for each group.

81 to −41) as a probe. Lysates from Flag-Egr1 but not Flag-transfected cells showed three shifted bands (lanes 2 and 3 in *Figure 3A*). The shifted bands a and c completely disappeared in the presence of excess unlabelled wild-type probe (lane 8 in *Figure 3A*). Thus, only bands a and c were specific complexes of Egr1-Ca_v3.2−81 to −41. Because three Egr1 recognition sites were predicted in the TAB-responding element Ca_v3.2−81 to −41 (*Figure 3B*), we investigated the binding ability of each site using unlabelled competitors carrying the mutation in each site. The shifted bands a and c remained with the addition of excess unlabelled triple mutated probe (Trp m) but disappeared when the unlabelled probes carrying mutations in BS 1 and 3 were used (m1 and m3) (lanes 4,5,7 in *Figure 3A*). In the presence of cold competitor with mutation in BS2 (m2), the shifted bands a and c were reduced but not completely disappeared (lane 6 in *Figure 3A*). The m2 partially abolished the competition ability of the cold probe; therefore, some hot probe with normal sequence can still form the complex with Egr1. These results suggest that all three putative Egr1 BSs can be recognized by Egr1 and the BS2 binds Egr1 better than BS1 and BS3.

To demonstrate that Egr1 binds to the Ca_v3.2 promoter *in vivo*, we used the ChIP assay to examine enrichment of the proximal 180 bp in Ca_v3.2 5′-FS pulled down with Flag-Egr1 in H9c2 cells. Compared with the Flag control, the precipitated product with Flag-Egr1 showed significantly enriched proximal 180 bp of Ca_v3.2 5′-FS (*Figure 3C*). Combined with the fact that overexpressing Egr1 increased Ca_v3.2 expression (*Figure 2D*), these results suggest that Egr1 induced Ca_v3.2 expression by binding to the TAB-responding element Ca_v3.2−81 to −41.

3.4 Egr1 is required to induce Ca_v3.2 gene expression during cardiac hypertrophy

Because the luciferase activity of Tg(Ca_v3.2-3500-Luc) can be induced as early as 3 days after TAB (*Figure 1C*), we should be able to detect the expression of Egr1 early after TAB if Egr1 is involved in regulation of Ca_v3.2 during cardiac hypertrophy. We examined the protein level of Egr1 in mouse left ventricles after surgery and found that Egr1 was significantly increased after TAB when compared with sham operation (*Figure 4A* and *B*). The expression of Ca_v3.2 mRNA was also induced (1.33 ± 0.08-fold) 3 day after TAB (*Figure 4C*). To confirm our hypothesis that up-regulated Egr1 induces Ca_v3.2 in the progression of the pressure overload, the expression pattern of Ca_v3.2 and Egr1 were examined by *in situ* hybridization and immunostaining in adjacent slides, respectively. We confirmed the specificity of the Ca_v3.2 probe by no positive signals in the TAB Ca_v3.2-knockout (KO) heart. We detected increased mRNA expression of Ca_v3.2 in TAB but not sham-operated hearts 3 days after TAB (upper panels, *Figure 4D*). Interestingly, induced Ca_v3.2 was not observed in every myocyte but rather in a mosaic pattern. Egr1 was also not expressed in every myocyte (lower panels, *Figure 4D*). Most of the Ca_v3.2-expressing myocytes appeared to be Egr1-positive in the WT TAB hearts (middle panels). The early induction and similar expression pattern of Egr1 and Ca_v3.2 after hypertrophic stimulation supports our hypothesis that Egr1 is the early regulator of Ca_v3.2 during cardiac hypertrophy.

To demonstrate that Egr1 indeed regulated Ca_v3.2 expression during hypertrophic stimulation, we used RNAi technique to KD Egr1 in H9c2 cells. At 24 h after treatment, phenylephrine (PE) increased Ca_v3.2 gene expression (2.88 ± 0.02-fold), luciferase activity of Ca_v3.2-3500 (4.19 ± 1.37-fold), and the protein level of Egr1 in H9c2 cells (*Figure 5A–C*). ShEgr1 (Egr1 KD) repressed the basal and PE-induced Egr1 when compared with the shEGFP (control KD) (*Figure 5D*). More importantly, PE-induced Ca_v3.2 expression was absent in shEgr1-transfected cells when compared with the control (*Figure 5E*). Similar results were observed in NRVMs. At 45 min after PE treatment, Egr1 KD but not control KD reduced the PE-induced expression of Ca_v3.2

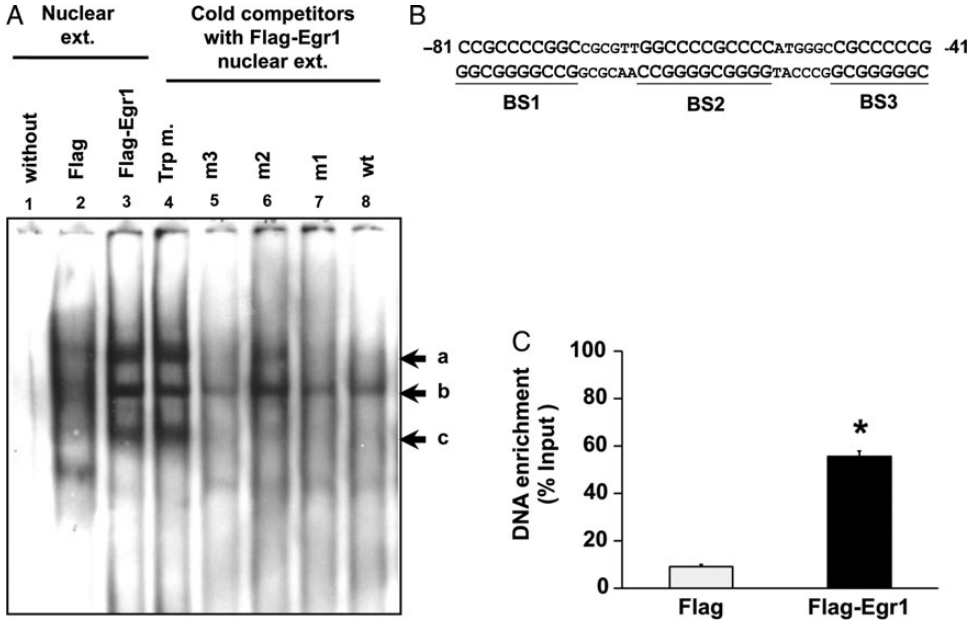

Figure 3 Egr1 binds to the TAB-responding element Ca_v3.2−81 to −41. (*A*) Electrophoretic mobility shift assay with DIG-labelled Ca_v3.2−81 to −41 as a probe detecting Egr1-Ca_v3.2 DNA complexes in nuclear extracts from Flag-Egr1- but not Flag-transfected cells (lanes 1–3). Cold and excessive wild-type or mutated competitors were incubated with nuclear extract from Flag-Egr1 transfected HEK293 cells before adding DIG-labelled Ca_v3.2−81 to −41 (lanes 4–8). Potential Egr1-Ca_v3.2 DNA complexes are indicated by arrows. (*B*) Three putative BSs for Egr1 in the TAB-responding element Ca_v3.2−81 to −41. (*C*) Chromatin immunoprecipitation assay of anti-Flag antibody in H9c2 cells transfected with Flag-Egr1 or Flag plasmids. DNA immunoprecipitated by anti-Flag antibody was amplified by real-time PCR to detect Ca_v3.2 fragment containing Egr1 BSs. $n = 3$ for each group. *$P < 0.05$ vs. Flag.

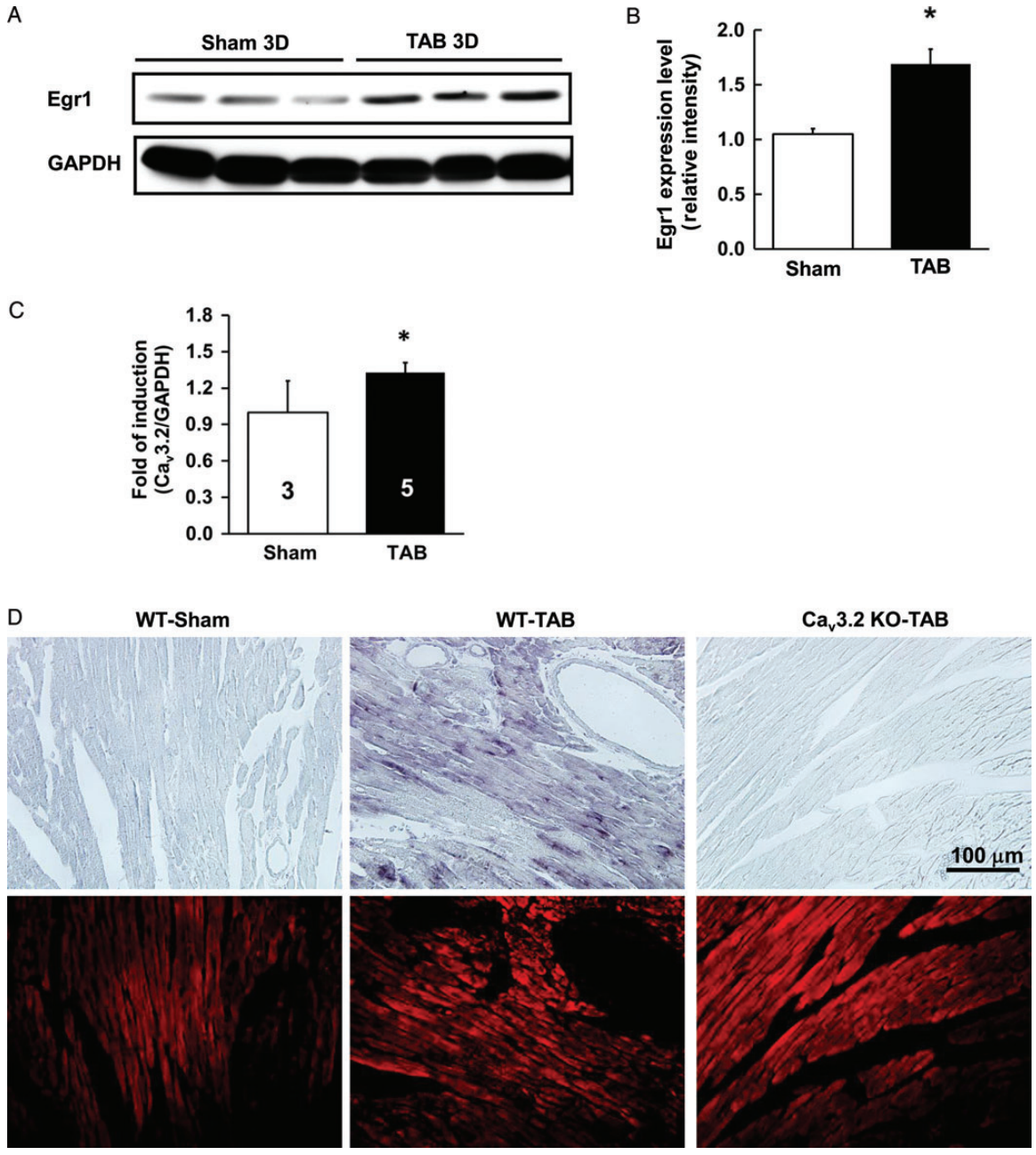

Figure 4 Egr1 and $Ca_v3.2$ are early induced by the pressure overload in wild-type mice. (*A*) Western blot analysis of Egr1 expression in 3-day sham- or TAB-operated left ventricles. (*B*) Quantification of Egr1 expression in (*A*) $n = 3$ for each group. $^*P < 0.05$ vs. Sham. (*C*) Fold of induction of $Ca_v3.2$ mRNA in left ventricles isolated from surgery-subjected mice at Day 3 after surgery. $^*P < 0.05$ vs. Sham. (*D*) (upper) *In situ* hybridization of $Ca_v3.2$ transcript (DIG-labelled antisense probe) in left ventricles after 3 days of surgery. (lower) The adjacent slide of those used in *in situ* hybridization was used for Egr1 immunostaining. (KO, knockout.)

in NRVMs (*Figure 5F*). Thus, we concluded that the up-regulation of $Ca_v3.2$ expression by PE requires Egr1.

3.5 $Ca_v3.2$ is an important effector of Egr1 contributing to the progression of cardiac hypertrophy

Egr1 is essential for the progression of cardiac hypertrophy because Egr1 KO mice showed attenuated cardiac hypertrophy induced by the pressure overload.[21] Although many foetal genes with up-regulated expression after cardiac hypertrophy contain putative Egr1 BSs, the direct downstream effector of Egr1 contributing to the cardiac hypertrophy is still unknown.[21] Because both $Ca_v3.2$- and Egr1-KO mice showed attenuated cardiac hypertrophy and $Ca_v3.2$ expression was up-regulated by Egr1 after hypertrophic stimulation, $Ca_v3.2$ is likely an important downstream effector of Egr1 in contributing to cardiac hypertrophy. To test this possibility, we used PE-induced hypertrophy in H9c2 cells as our cellular model.[24,25] PE significantly increased the cell surface area in control

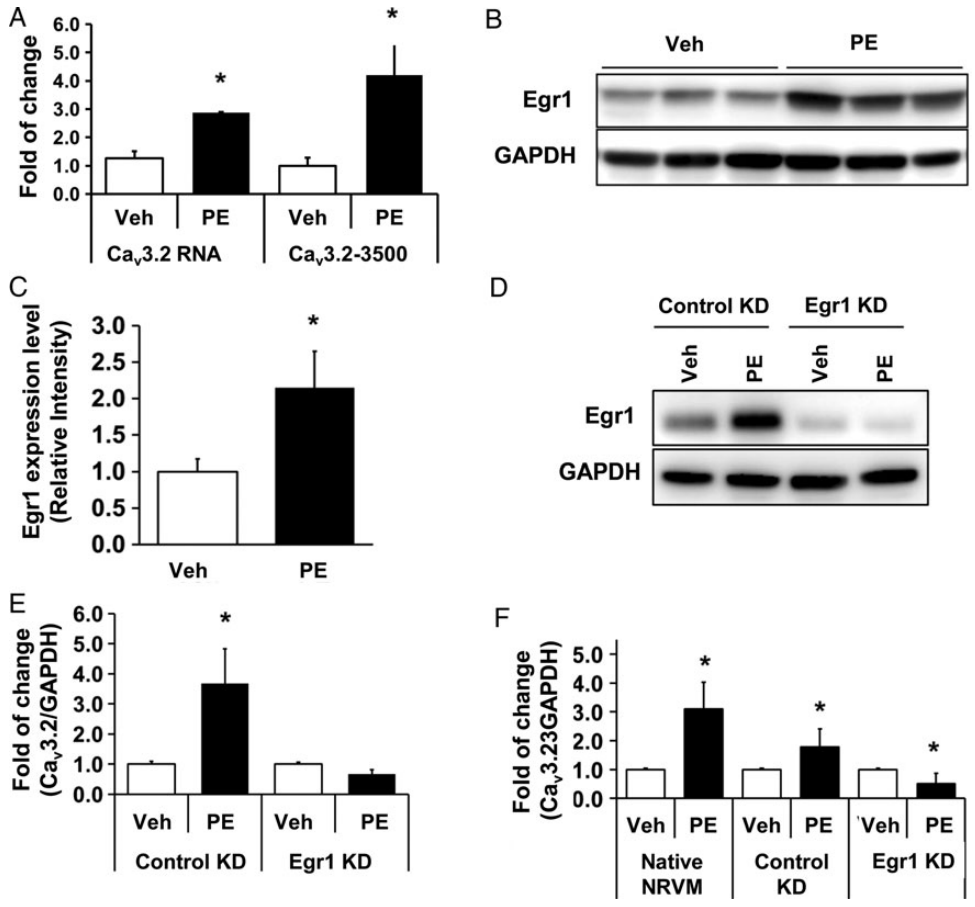

Figure 5 Egr1 is required for PE-induced $Ca_v3.2$ expression in H9c2 and NRVM cells. (*A*) Induction of $Ca_v3.2$ mRNA ($Ca_v3.2$/GAPDH) and luciferase activity of $Ca_v3.2$-3500-Luc by phenylephrine (PE) treatment (50 μM) in H9c2 cells. (*B*) Western blot analysis of Egr1 protein expression in H9c2 cells treated with PE or vehicle (Veh). (*C*) Quantification of Egr1 expression in (*B*). (*D*) Western blot analysis of Egr1 protein expression in shEGFP (control KD) or shEgr1 (Egr1 KD) H9c2 cells treated with PE or Veh. (*E*) Quantitative RT–PCR analysis of $Ca_v3.2$ mRNA expression in control KD or Egr1 KD H9c2 cells treated with PE or Veh for 30 hr. (*F*) Quantitative RT–PCR analysis of $Ca_v3.2$ mRNA in control KD or Egr1 KD NRVMs treated with PE or Veh for 45 min. $n = 3$ for each group in this figure. $^*P < 0.05$ vs. Veh.

but not Egr1 KD cells (*Figure 6A* and *B*). This finding verified the importance of Egr1 in the cardiac hypertrophy. More importantly, overexpression of $Ca_v3.2$ in Egr1 KD cells restored the PE-induced hypertrophy (*Figure 6A* and *B*). The finding suggests that $Ca_v3.2$ plays a vital role in Egr1-mediated cardiac hypertrophy.

4. Discussion

Although $Ca_v3.2$ plays a pivotal role in cardiac hypertrophy, how it is regulated is difficult to study because of its low expression and poor quality of available antibodies. To overcome this problem, we validated the evolutionary conserved promoter of $Ca_v3.2$ ($Ca_v3.2$-3500) and generated $Ca_v3.2$ reporter mice (Tg[Cav3.2-3500-Luc]) to monitor its expression *in vivo*. The early induction of luciferase activity in the reporter mice and $Ca_v3.2$ mRNA in the wild-type mice by pressure overload suggests that the up-regulation of $Ca_v3.2$ precedes morphological changes of hearts with cardiac hypertrophy (*Figures 1* and *4*). Combined with our previous findings of blunted angiotensin II- and pressure overload-induced cardiac hypertrophy in $Ca_v3.2$-null mice,[12] these results suggest that early induced $Ca_v3.2$ is required for the development of cardiac hypertrophy.

Egr1 is an immediate early gene rapidly responding to different stimulations in various cells including cardiac myocytes.[26,27] The mRNA of Egr1 can be induced early by hypertrophy-inducing hormones such as endothelin 1, angiotensin II, and PE in NRVMs, neonatal rat cardiac fibroblasts, isolated heart tissues or mice.[26–32] Previously, overexpression of Egr1-specific repressor NGF1A-binding protein (NAB1) or deletion of Egr1 was shown to blunt pathological cardiac hypertrophy induced by pressure overload or adrenergic stimulation.[21] These studies demonstrate that Egr1 is an important regulator of pathological cardiac hypertrophy. However, the downstream mediator of Egr1 contributing to the regulation of pathological cardiac hypertrophy is not completely understood. Our results showed that Egr1 was up-regulated in the left ventricles at the Day 3 after TAB surgery (*Figure 4A* and *B*). At the same time, $Ca_v3.2$ mRNA was induced in most of Egr1-up-regulated cardiomyocytes (*Figure 4C* and *D*). Our results showed that PE-induced Egr1 and $Ca_v3.2$ were blunted by Egr1 knockdown (*Figure 5*). Egr1 knockdown also blunted the PE-induced cellular hypertrophy in H9c2 cells and this phenomenon

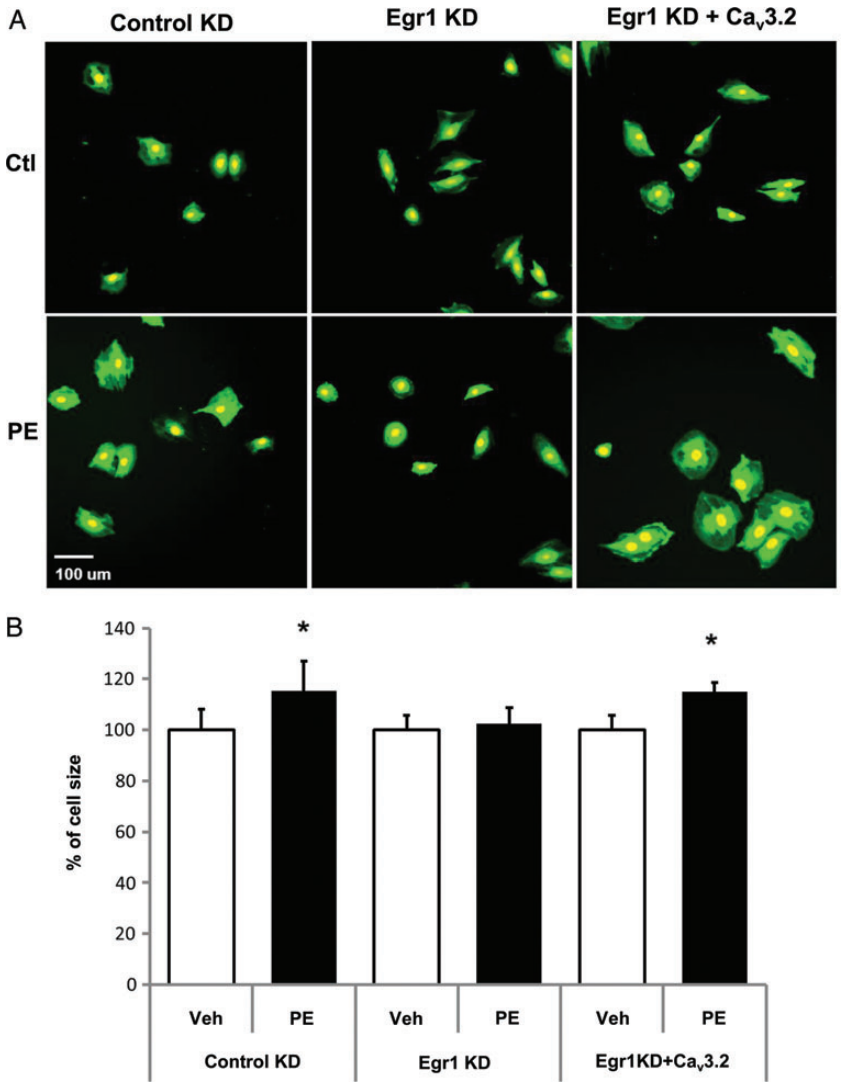

Figure 6 $Ca_v3.2$ is an important effector of Egr1 in PE-induced hypertrophy. (*A*) Immuofluoresent images of H9c2 cells treated with PE (50 μM) or Veh for 4 days. Cells were stained with CFSE (green) dye and nuclei were stained with DAPI (pseudocolor yellow). (*B*) Increase of the cell surface area in PE or vehicle-treated control KD, Egr1 KD, or Egr1 KD H9c2 cells transfected with $Ca_v3.2$ plasmids. The data were from three independent experiments and >200 cells were counted in each experiment. *$P < 0.05$ vs. Veh in each group.

could be rescued by the overexpression of $Ca_v3.2$ (*Figure 6*). Taken together, these results suggest that $Ca_v3.2$ is an important downstream target of Egr1 mediating pathological cardiac hypertrophy.

Egr1 was recently shown to bind to the region between −1426 and −1188 bp upstream of rat $Ca_v3.2$ and induce the expression of $Ca_v3.2$ in neuronal cells.[33] The corresponding region of rat $Ca_v3.2$−1426 to −1188 bp is −850 to −612 bp in mouse $Ca_v3.2$ and the sequence similarity of this region between rat and mouse is 89% (Supplementary material online, *Figure S5*). In this region, one conserved Egr1 BS was predicted in both rat and mouse genomes and an additional Egr1 BS was present in the rat genome. Interestingly, TAB-induced luciferase activity did not differ between $Ca_v3.2$-420 and $Ca_v3.2$-1000 reporter constructs in mouse hearts (*Figure 2A*). It suggests that the region between −1000 and −420 bp of mouse $Ca_v3.2$ including the conserved Egr1 BS is not involved in TAB-induced $Ca_v3.2$ gene expression.

In addition to Egr1, the TFs Sp1, HSF, and NFkB are potential regulators of $Ca_v3.2$ expression because deletion of their putative BSs reduced the basal expression of $Ca_v3.2$-420-Luc in HL-1 cells (Supplementary material online, *Figure S4*). The lack of $Ca_v3.2$-3500-Luc induction by these TFs in HEK293 cells may be due to the lack of cofactors or improper post-translational modification of these TFs. For example, coactivator-associated arginine methyltransferase (CARM1/PRMT4) directly binds to NF-kB subunit p65 and synergistically activate NF-kB target genes through histone H3-R17 methylation.[34] CARM1 is required for some NF-kB target gene expression, because a subset of NF-kB-dependent gene expression was impaired in CARM1 knock out cells.[35] Therefore, solely expression of Sp1, HSF, and NFkB may not be enough to induce $Ca_v3.2$-3500 luciferase activity. Their potential regulatory function in $Ca_v3.2$ expression *in vivo* need to be further examined.

In summary, we provided evidence that Egr1 induced $Ca_v3.2$ mRNA expression by interacting with the TAB-responding element $Ca_v3.2$−81 to −41. We further demonstrate that Egr1 is required for PE-induced cellular hypertrophy and $Ca_v3.2$ is an important downstream target of Egr1 mediating this phenomenon.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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