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Calcium Channel Blockers Act through Nuclear Factor Y to Control Transcription of Key Cardiac Genes[S]

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ABSTRACT

First-generation calcium channel blockers such as verapamil are a widely used class of antihypertensive drugs that block L-type calcium channels. We recently discovered that they also reduce cardiac expression of proapoptotic thioredoxin-interacting protein (TXNIP), suggesting that they may have unappreciated transcriptional effects. By use of TXNIP promoter deletion and mutation studies, we found that a CCAAT element was mediating verapamil-induced transcriptional repression and identified nuclear factor Y (NFY) to be the responsible transcription factor as assessed by overexpression/knockdown and luciferase and chromatin immunoprecipitation assays in cardiomyocytes and in vivo in diabetic mice receiving oral verapamil. We further discovered that increased NFY-DNA binding was associated with histone H4 deacetylation and transcriptional repression and mediated by inhibition of calcineurin signaling. It is noteworthy that the transcriptional control conferred by this newly identified verapamil-calcineurin-NFY signaling cascade was not limited to TXNIP, suggesting that it may modulate the expression of other NFY targets. Thus, verapamil induces a calcineurin-NFY signaling pathway that controls cardiac gene transcription and apoptosis and thereby may affect cardiac biology in previously unrecognized ways.

Introduction

Verapamil is a first-generation calcium channel blocker of the phenylalkylamine class and as an oral medication widely used to treat hypertension and angina pectoris. Verapamil functions through inhibition of L-type calcium channels, which are abundantly expressed in the cardiovascular system, and thereby blocks influx of calcium ions into myocardial and vascular smooth muscle cells during depolarization, resulting in relaxation and vasodilation. We recently discovered that calcium channel blockers reduce cardiac expression of thioredoxin-interacting protein (TXNIP) (Chen et al., 2009). TXNIP is an important regulator of the cellular redox state and binds to and inhibits thioredoxin, resulting in increased oxidative stress (Nishiyama et al., 1999, 2001; Junn et al., 2000; Yamanaka et al., 2000; Patwari et al., 2006). It is noteworthy that cardiac TXNIP expression is strongly upregulated in diabetes (Chen et al., 2009) and acute myocardial ischemia (Xiang et al., 2005) and that increased TXNIP expression induces cardiomyocyte apoptosis and has major detrimental effects on the cardiovascular system, including vascular inflammation and atherosclerosis (Wang et al., 2002; Schulze et al., 2004, 2006; Yoshioka et al., 2004; Yamawaki et al., 2005). In contrast, we found that inhibition of TXNIP has pronounced protective effects and enhances cardiomyocyte survival, even in the face of severe diabetes (Chen et al., 2009). This indicates that TXNIP plays a key role in cardiomyocyte biology and that inhibition of its expression by calcium channel blockers may represent a novel approach for the treatment of diabetic cardiomyopathy, a potentially fatal complication of diabetes characterized by cardiomyocyte injury, apoptosis, and ultimately heart failure (Boudina and Abel, 2007). However, the mechanisms by which calcium channel blockers inhibit TXNIP expression

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ABBREVIATIONS: TXNIP; thioredoxin-interacting protein; NFY, nuclear factor Y; CyA, cyclosporine A; FK506, tacrolimus; STZ, streptozotocin; ChIP, chromatin immunoprecipitation; RT, reverse transcriptase; PCR, polymerase chain reaction; si, small interfering; TUNEL, transferase-mediated dUTP nick-end labeling; NFAT, nuclear factor of activated T cells; bp, base pair(s).

have remained elusive. Moreover, these recent findings also raise the possibility that, despite its wide use, verapamil has yet unappreciated effects reaching beyond its well known functions and may modulate the expression of critical cardiac genes such as TXNIP. Therefore, by use of TXNIP as an example, the present study was aimed at identifying the factor(s) and signaling pathways conferring verapamil-mediated cardiac gene regulation and indeed led to the discovery of a novel calcium-nuclear factor Y (NFY)-TXNIP signaling cascade in cardiomyocytes.

Materials and Methods

Cell Culture. H9C2 rat cardiomyocytes (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium modified to contain 4 mM L-glutamine, 4500 mg/l glucose, 1 mM sodium pyruvate, 1500 mg/l sodium bicarbonate, 1.8 mM $CaCl_2$, and 0.8 mM $MgCl_2$, pH 7.3, and supplemented with 10% FBS. Primary adult rat cardiomyocytes were isolated, cultured on laminin-coated plates as described previously (Belke et al., 2002; Stavinoha et al., 2004), and maintained in Dulbecco's modified Eagle's medium as detailed above. Cells were treated with verapamil at various concentrations and different time points as indicated in the figure legends. To modulate the signaling pathway that mediates the verapamil effects, cells were treated with the calcium-dependent protein phosphatase inhibitors FK506 (tacrolimus) and cyclosporine A (CyA) (two specific protein phosphatase 2B/calcineurin inhibitors) (Sigma-Aldrich, St. Louis, MO).

Animal Studies. All mouse studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee under animal protocol numbers 09259 and 09258 and conformed to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). Wild-type, 6- to 8-week-old male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) received verapamil in their drinking water (1 mg/ml) for 3 weeks, resulting in an average dose of 100 mg/kg per day, whereas control mice were housed under identical conditions without verapamil as described previously (Chen et al., 2009). This dose of verapamil has been shown previously to be well tolerated and adequate for mice (Cohn et al., 2001). Mice were rendered diabetic by a single intraperitoneal injection of streptozotocin (STZ; 200 mg/kg) as described previously (Chen et al., 2009). This protocol reliably results in diabetes with hyperglycemia of >300 mg/dl within 48 h (Chen et al., 2009) and induces cardiomyocyte alterations reminiscent of diabetic cardiomyopathy (Ghosh et al., 2004). Mice were euthanized 4 days after the injection, and their hearts were collected and the ventricles isolated for RNA extraction and chromatin immunoprecipitation (ChIP) assays.

Plasmid Construction and Transient Transfection Assays. The two subunits of NFY, NFYA and NFYB, were cloned from rat cardiomyocytes using primers 15 and 16 and 17 and 18, respectively (Supplemental Table S1). Amplicons were then subcloned into the pEF6/V5-His TOPO vector (Invitrogen, Carlsbad, CA) under the control of the constitutively active elongation factor 1α promoter, and correct constructs were confirmed by sequencing.

Construction of the TXNIP promoter reporter and control LacZ plasmids have been described previously (Minn et al., 2005). Mutations at the CCAAT box, altering it to GTCGA as described previously (Le Flem et al., 1999), were introduced into the full-length TXNIP promoter by fusion PCR using primers 21 to 24 (Supplemental Table S1), and the resulting mutCCAAT-FL construct was verified by sequencing.

Transfection experiments were performed as described previously (Chen et al., 2009), and cotransfected pRL-TK control plasmid (Promega, Madison, WI) was used to correct for transfection efficiency. Firefly and *Renilla reniformis* luciferase activities were determined by Dual Luciferase Assays (Promega).

RNA Interference and Western Blotting. For NFY knockdown, H9C2 cells were grown in six-well plates and transfected with specific siRNA oligonucleotides, siNFYA (0.1 μM), siNFYB (0.1 μM), or scrambled oligonucleotides (0.1 μM), using DharmaFECT Duo transfection reagent (Dharmacon RNA Technologies/Thermo Fisher Scientific, Waltham, MA) (5 μl/well), and after 48 h, appropriate NFY knockdown was confirmed by Western blotting. Whole-cell and nuclear protein extracts were prepared as described previously (Saxena et al., 2010). Antibodies used were: NFYA (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), NFYB (1:200), and TXNIP (1:400; MBL International Co., Woburn, MA). Bands were visualized by ECL plus (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and quantified by ImageQuant.

Transferase-Mediated dUTP Nick-End Labeling assay. For transferase-mediated dUTP nick-end labeling (TUNEL) the DeadEnd Fluorometric TUNEL System Kit (Promega) was used as described previously (Chen et al., 2008). Cardiomyocytes were visualized by desmin staining using desmin antibody (ab15200, 1:200; Abcam, Cambridge, MA), biotinylated anti-rabbit IgG 1:200, and streptavidin-conjugated Alexa Fluor 594 (Invitrogen). The VectaShield with 4,6-diamidino-2-phenylindole mounting solution (Vector Laboratories, Burlingame, CA) was used for visualization of nuclei (Scarabelli et al., 1999).

Chromatin Immunoprecipitation. ChIP assays were performed as described previously (Cha-Molstad et al., 2009). In brief, 500 μg of cross-linked H9C2 protein extracts (by BCA protein assay) were incubated overnight at 4°C with 10 μg of NFYA, NFYB, CDP (CCAAT displacement protein), YB-1, and nuclear factor of activated T cells (NFAT) antibodies (Santa Cruz Biotechnology) or specific antibodies against acetylated histone H3 (acetylated histone H4; Millipore, Billerica, MA), or an affinity-purified polyclonal IgG as a negative control (β-galactosidase; Abcam).

Mouse ventricles were chopped into fine pieces with a razor blade and transferred into a 50-ml tube containing 20 ml of 1% formaldehyde solution and incubated for 15 min at room temperature for cross-linking. To stop cross-linking, glycine (2.5 M) was added to a final concentration of 1.25 μM and incubated at 4°C for 10 min. The tissue was then centrifuged at 100*g*, washed twice with ice-cold phosphate-buffered saline (1×), and homogenized using a Dounce homogenizer. Homogenates were centrifuged at 1000 rpm at 4°C for 5 min and stored at −80°C for future immunoprecipitation, which was performed as for H9C2 cells, except that 750 μg of protein were used. Bound DNA was quantified by quantitative real-time PCR.

Reverse Transcription and Quantitative PCR. Total RNA was extracted using an RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. RNA (1 μg) was reverse-transcribed to cDNA using the first strand cDNA synthesis kit (Roche Diagnostics, Indianapolis, IN).

Quantitative real-time PCR was performed on a Prism 7000 Sequence Detection System using SYBER Green (Applied Biosystems, Foster City, CA). Primers were designed using primer express 3.0 (Applied Biosystems) with default parameters. All samples were analyzed in triplicate and corrected for the 18S ribosomal subunit. For analysis of ChIP samples, primers flanking the CCAAT element were selected. Primer sequences are provided in Supplemental Table S1.

Statistical Analysis. *P* values were calculated by Student's *t* test or by one-way analysis of variance for data sets of more than two groups.

Results

Verapamil Inhibits TXNIP mRNA and Protein Expression in H9C2 Cells. To assess the effects of verapamil on cardiomyocyte TXNIP expression, we first performed dose- and time-course experiments measuring endogenous TXNIP in the H9C2 cardiomyocyte cell line in response to verapamil. The results demonstrate that verapamil inhibits TXNIP mRNA expression in a dose- and time-dependent

manner (Fig. 1, A and B). Analysis of TXNIP protein levels also revealed a significant time-dependent down-regulation in response to verapamil (Fig. 1C). These findings are consistent with our previously reported dose-dependent decrease in TXNIP protein levels in H9C2 cardiomyocytes (Chen et al., 2009).

Verapamil Inhibits TXNIP mRNA and Protein Expression in Primary Adult Rat Cardiomyocytes. In addition, we determined the effects of verapamil on isolated adult rat cardiomyocytes. The results showed an even more dramatic >30-fold decrease in TXNIP mRNA and protein levels in response to verapamil (Fig. 2, A and B), thereby confirming and validating the H9C2 data and underlining the physiological relevance of these observations for cardiomyocyte biology.

Verapamil-Mediated Inhibition of TXNIP Expression Occurs at the Transcriptional Level and Requires a Conserved CCAAT Box in the Proximal TXNIP Promoter. To further determine the mechanism of verapamil action, we performed luciferase assays to assess TXNIP promoter activity. Indeed, verapamil inhibited TXNIP promoter activity, which (together with the observed reduction in TXNIP mRNA levels observed) indicates that verapamil inhibits TXNIP expression at the transcriptional level. Moreover, TXNIP promoter deletion studies suggested that a 20-bp region in the proximal promoter containing a CCAAT box was responsible for the verapamil-mediated TXNIP repression (Fig. 3A). Indeed, a separate set of experiments again revealed a highly significant inhibition of TXNIP transcription in response to verapamil (Fig. 3B), and mutation of just the CCAAT box within the full-length TXNIP promoter was sufficient to blunt this effect (Fig. 3C). Although we cannot exclude the possibility that additional elements upstream of the identified 20-bp promoter region are also involved, these findings strongly support the key role this CCAAT box plays in mediating the observed verapamil effects.

The CCAAT Box-Binding Transcription Factor NFY Represses TXNIP Promoter Activity. Analysis of this conserved CCAAT box containing a 20-bp region (Fig. 4A) with the MatInspector program (Genomatix Software GmbH, Munich, Germany) revealed three putative binding factors, CDP, YB-1 (Y box-binding protein), and NFY, whereby NFY had the highest matrix score. Indeed, ChIP studies using specific antibodies against each of these proteins showed that only NFY, a heteromeric metalloprotein, was capable of binding to the TXNIP promoter (Supplemental Fig. S1).

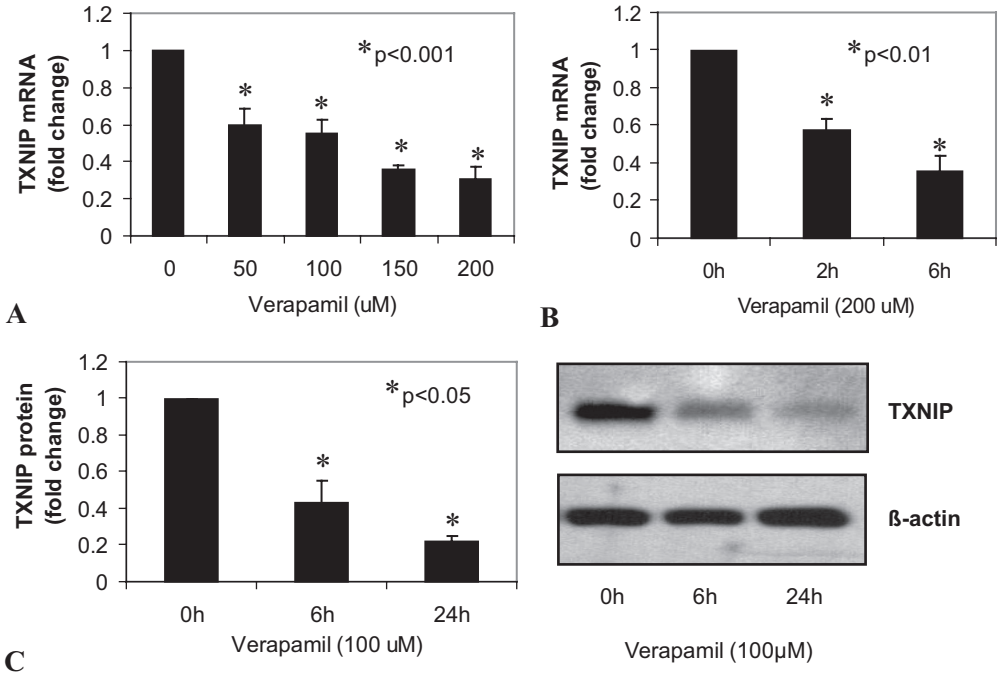

Fig. 1. Verapamil effects on TXNIP mRNA and protein expression in H9C2 cells. H9C2 cardiomyocytes were treated with the designated doses of verapamil for 24 h (A) or for various lengths of time as indicated (B), and TXNIP mRNA was measured by quantitative RT-PCR using the ΔΔCt method. C, TXNIP protein levels as assessed by immunoblotting and corrected for β-actin in response to various lengths of verapamil treatment. (The quantification and a representative immunoblot are shown.) Bars represent means ± S.E.M. of the average fold change of at least three independent experiments.

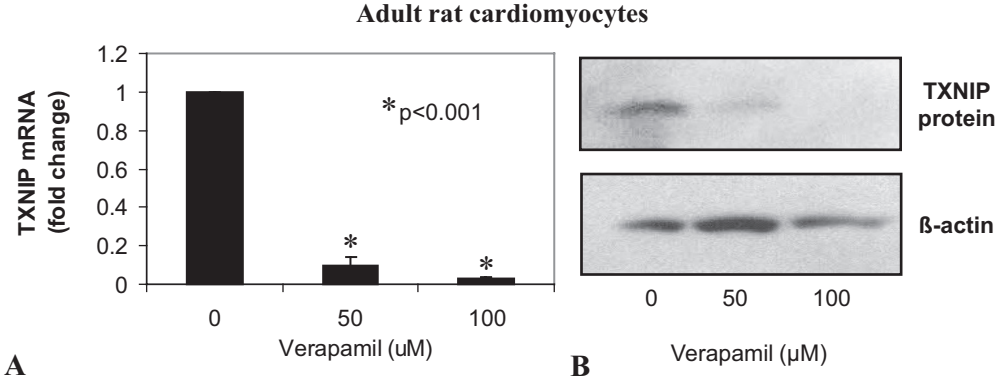

Fig. 2. Verapamil effects on adult rat cardiomyocyte TXNIP expression. A, primary adult cardiomyocytes were isolated as described under *Materials and Methods* and incubated for 24 h with verapamil at the designated doses before assessment of TXNIP mRNA by quantitative RT-PCR. B, isolated primary adult cardiomyocytes were treated with increasing doses of verapamil for 48 h, and TXNIP protein levels were assessed by immunoblotting. Bars represent means ± S.E.M. of the average fold change of three independent experiments.

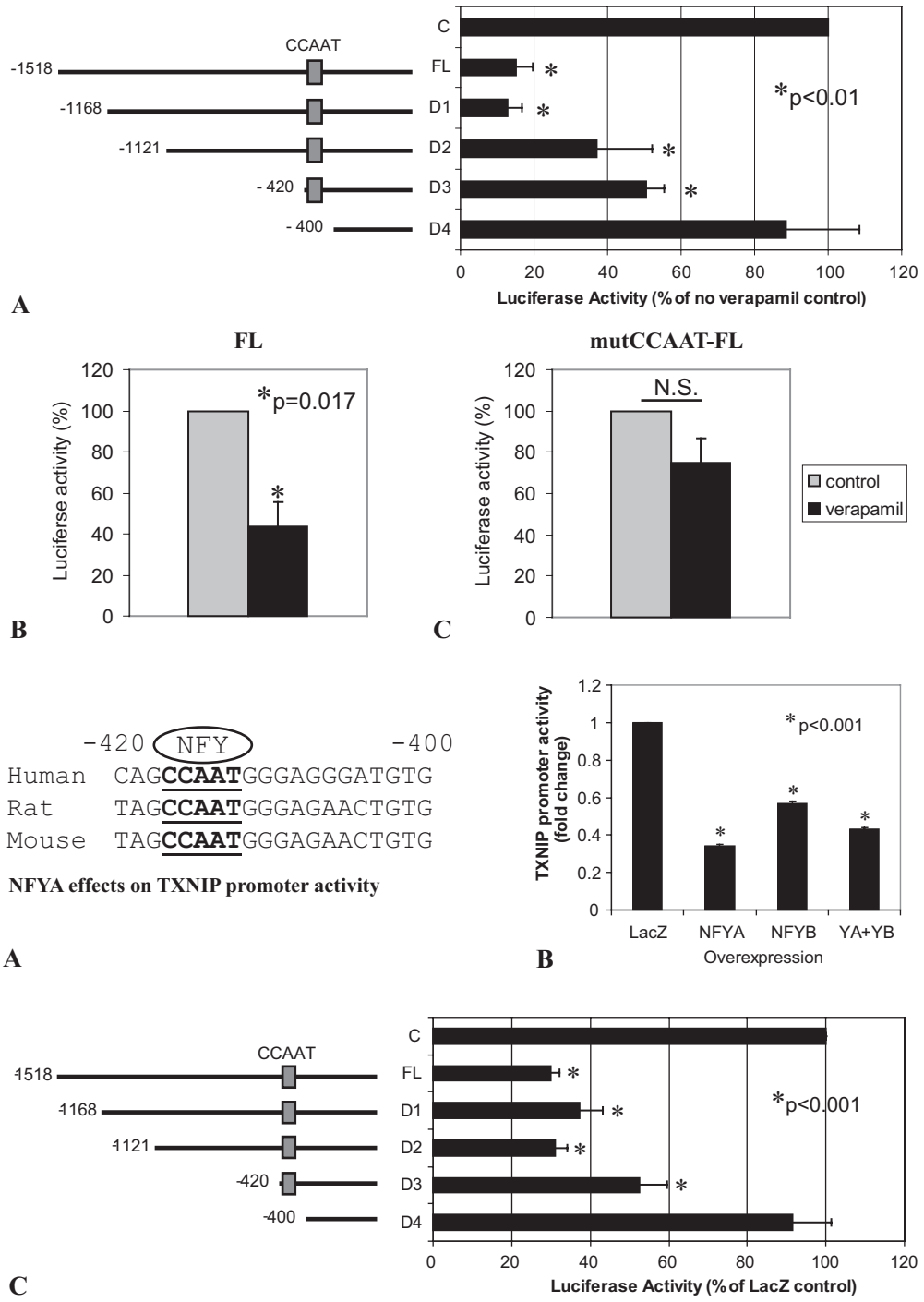

Fig. 3. A, verapamil effects on TXNIP promoter activity. H9C2 cardiomyocytes were transfected with different TXNIP-promoter deletion reporter constructs and incubated with or without 100 μM verapamil for 24 h. H9C2 cells were transfected with the wild-type full-length TXNIP-promoter construct (FL) (B) or with a full-length construct with mutated CCAAT box (mutCCAAT-FL) (C), and the effects of a 24-h incubation with verapamil (100 μM) were assessed. Promoter activities were measured by dual luciferase assays. Bars represent means ± S.E.M. of at least three independent experiments.

Fig. 4. NFY effects on TXNIP promoter activity. A, conserved CCAAT elements in human, rat and mouse. B, H9C2 cardiomyocytes were transfected with the TXNIP promoter reporter construct and with expression plasmids of NFYA and/or NFYB at a 1:2 ratio, and after 72 h promoter activity was assessed by luciferase assays. C, H9C2 cells were cotransfected with TXNIP-promoter deletion constructs and NFYA-expression or LacZ-control plasmids, and luciferase activity was compared. Bars represent means ± S.E.M. of at least three independent experiments.

To determine whether NFY was indeed controlling TXNIP transcription, we performed transfection studies overexpressing NFY in H9C2 cardiomyocytes and found that both subunits, NFYA and NFYB, reduced TXNIP promoter-driven luciferase activity (Fig. 4B). Moreover, this repression was again dependent on the identified CCAAT box as shown by the loss of inhibition with the D4 deletion construct (Fig. 4C). NFY has been described as a ubiquitous, bifunctional transcription factor that can act as an activator or as a repressor (Ceribelli et al., 2008), consistent with our present findings of TXNIP repression. Thus, these results indicate that NFY can mimic the verapamil effects on TXNIP expression, suggesting that it may be the responsible transacting factor. In addition, NFY knockdown by siRNA significantly increased TXNIP promoter activity and TXNIP protein levels ($P < 0.01$) (Supplemental Fig. S2), further supporting the regulatory role of NFY in the control of TXNIP expression.

Verapamil Stimulates NFY Binding to the TXNIP Promoter and Decreases Histone H4 Acetylation and TXNIP Transcription. To further prove that verapamil acts through NFY, we performed ChIP assays using H9C2 cardiomyocytes incubated with/without verapamil and found that verapamil results in a significantly higher oc-

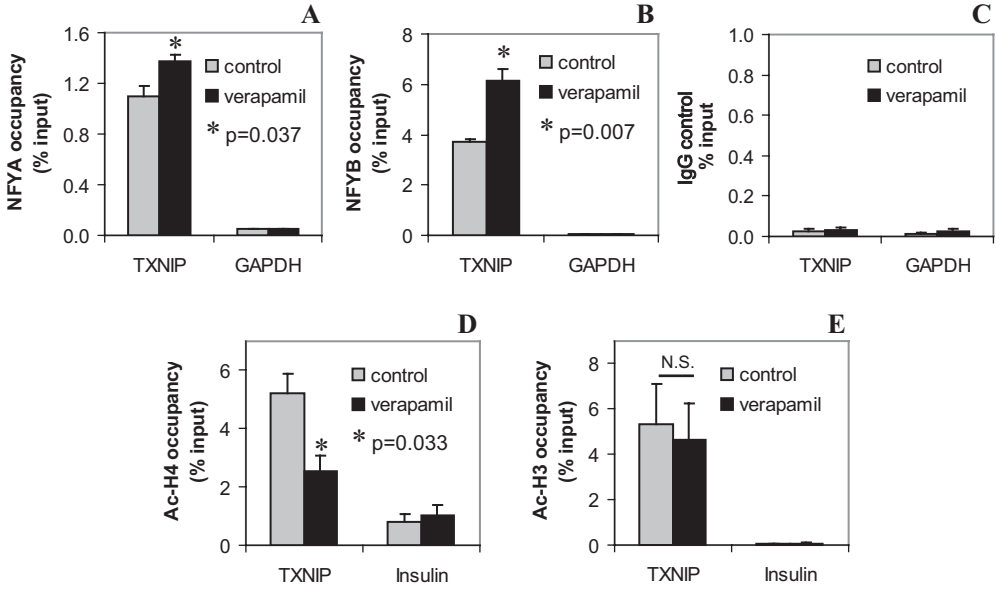

Fig. 5. ChIP analysis of the verapamil effects on NFY DNA-binding and histone acetylation. H9C2 cardiomyocytes were treated with or without verapamil (200 μM) for 1 h, and ChIP analysis was performed using antibodies directed against NFYA (A), NFYB (B), or normal IgG (C) as a negative control or against acetylated histone H4 (D) and acetylated histone H3 (E) to assess chromatin modification. The CCAAT regions (for NFYA and NFYB) or 5′-coding regions (for H4 and H3) of rat TXNIP were amplified by quantitative real-time PCR. (Because of the high basal level of histone acetylation at the GAPDH promoter, we used insulin as a negative internal control for the modified histone ChIP experiments.) Bars represent means ± S.E.M. of at least three independent ChIP assays.

cupancy of NFYA and NFYB on the TXNIP promoter (Fig. 5, A and B). The negligible binding with the IgG and internal GAPDH controls confirmed the specificity of these ChIP experiments (Fig. 5C). Since we recently showed that activation of TXNIP transcription also involves chromatin modification and in particular H4 acetylation (Cha-Molstad et al., 2009), we also performed modified histone ChIP assays. Consistent with the important role of H4 acetylation in TXNIP transactivation, we found that verapamil-mediated repression was associated with a significant and highly specific decrease in H4 acetylation, whereas H3 acetylation was not affected (Fig. 5, D and E).

Verapamil-Mediated TXNIP Repression Is Conferred by Inhibition of the Calcineurin Signaling Pathway. Down-regulation of TXNIP expression by calcium channel blockers is not class-specific and was observed with both phenylalkylamines (e.g., verapamil and benzothiazepines) and diltiazem as well as with the calcium chelator EGTA, indicating that it is conferred by decreased intracellular calcium levels (Chen et al., 2009). Calcium regulates gene transcription mainly through two major pathways, the calcium-dependent serine-threonine protein phosphatase 2B/calcineurin and the calcium/calmodulin-dependent protein kinase. It is noteworthy that we recently discovered that in pancreatic β-cells, verapamil inhibits TXNIP transcription through the calcineurin rather than the calcium/calmodulin-dependent protein kinase pathway (Xu et al., 2012). It did so, however, by phosphorylating carbohydrate response element-binding protein, a transcription factor not expressed in the heart that binds to an E-box repeat within the proximal -400 bp of the TXNIP promoter (Cha-Molstad et al., 2009). Nevertheless, this raised the possibility that calcineurin signaling might also mediate verapamil-induced repression of TXNIP in cardiomyocytes. Indeed, two specific calcineurin inhibitors, FK506 and CyA, significantly reduced TXNIP promoter activity in H9C2 cardiomyocytes (Fig. 6, A and B). We also assessed the effects of CyA on endogenous TXNIP mRNA levels and observed a significant dose-dependent decline in TXNIP expression, further confirming the results of the reporter assay (Fig. 6C). To determine whether the CyA effect is also mediated by NFY, we performed

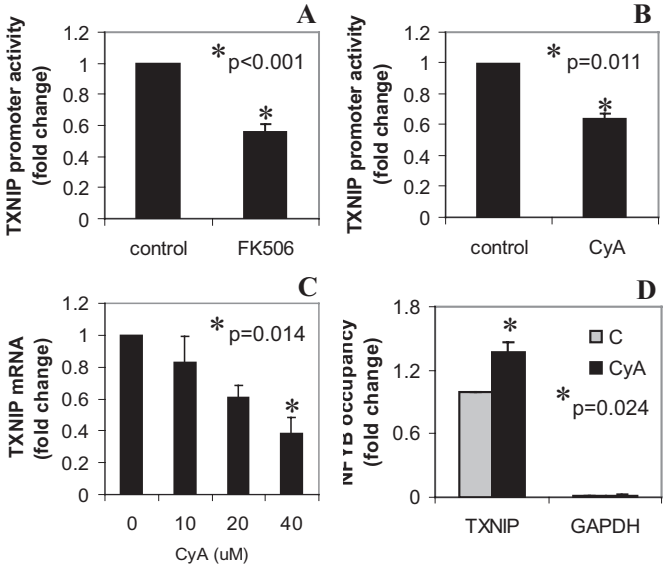

Fig. 6. Effects of calcium-dependent protein phosphatase/calcineurin inhibitors on TXNIP transcription. H9C2 cardiomyocytes were transfected with full-length TXNIP promoter reporter construct and treated with FK506 (10 μM) (A) or CyA (5 μM) (B), and after 24 h TXNIP promoter activity was assessed by luciferase assays. C, H9C2 cardiomyocytes were treated with the indicated doses of CyA for 2 h, and endogenous TXNP mRNA levels were measured by quantitative real-time RT-PCR. D, H9C2 cardiomyocytes were treated with CyA (40 μM) for 1 h, and ChIP analysis was performed to determine NFYB binding to the TXNIP promoter. Bars represent means ± S.E.M. of at least three independent experiments.

ChIP assays again and found that, similar to verapamil, CyA led to increased NFY binding to the TXNIP promoter (Fig. 6D). Although we cannot exclude some contribution from other pathways, these results suggest that verapamil blocks cardiomyocyte TXNIP expression by inhibiting the calcineurin signaling pathway, which promotes NFY binding to the TXNIP promoter and results in transcriptional repression. These findings also raise the possibility that, by acting through NFY, verapamil might affect the expression of other target genes.

The Verapamil-Regulated Calcineurin-NFY Pathway Inhibits the Transcription of Other Proapoptotic Cardiac Genes. Therefore, we analyzed the list of putative

NFY target genes identified in a previous liver cell ChIP-chip study (ChIP combined with genome tiling array) (Reed et al., 2008), and Shc1 caught our attention. Similar to TXNIP, Shc1 is highly expressed in cardiomyocytes, contains a conserved CCAAT element, modulates oxidative stress, promotes cell cycle arrest, and induces mitochondria-mediated apoptosis. Moreover, Shc1 has been implicated in the pathogenesis of diabetic cardiomyopathy (Rota et al., 2006; Francia et al., 2009). These facts prompted us to examine whether cardiomyocyte expression of Shc1 is also regulated by verapamil and CyA. Indeed, incubation of H9C2 cardiomyocytes with verapamil or with CyA led to a significant time- and dose-dependent decline in endogenous Shc1 mRNA expression (Fig. 7, A and B). To examine whether the verapamil effect was mediated by NFY, we performed ChIP assays again and found that verapamil dramatically increased NFY binding to the Shc1 promoter (Fig. 7C), although no enrichment was observed with IgG and GAPDH internal controls (Fig. 7D). These results demonstrate that, aside from its repression of TXNIP, the verapamil-regulated calcineurin-NFY pathway controls the expression of other important proapoptotic cardiac genes, such as Shc1.

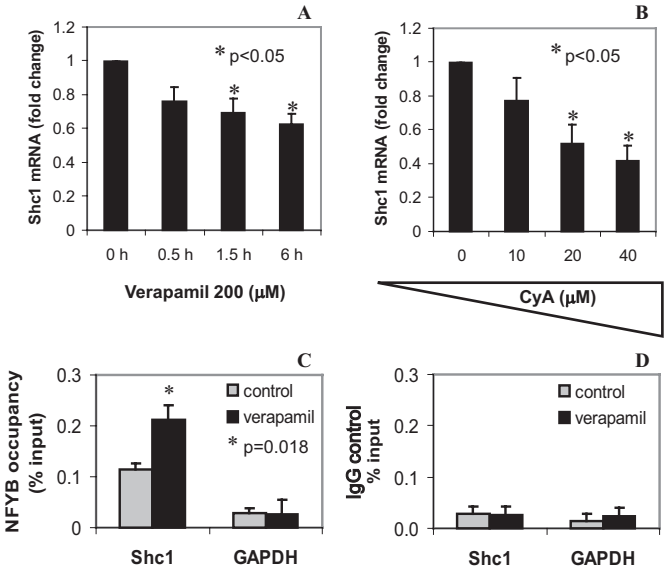

Fig. 7. Regulation of Shc1 expression and NFY-Shc1-promoter binding by verapamil and CyA. H9C2 cardiomyocytes were treated with verapamil for indicated times (A) or CyA for 2 h at indicated concentrations (B). Bars represent mean changes ± S.E.M. in Shc1 mRNA expression as measured by quantitative real-time RT-PCR. H9C2 cardiomyocytes were treated with verapamil (200 μM) for 1 h, and ChIP analysis was performed using antibodies against NFYB (C) or normal control IgG (D). Bars represent means ± S.E.M. of at least three independent ChIP assays.

In Vivo TXNIP Transcription and Apoptosis in the Diabetic Heart Are Repressed by Verapamil and NFY. These in vitro experiments revealed the mechanisms of verapamil-mediated transcriptional control and identified NFY as the main transcription factor involved. However, we wanted to further investigate the in vivo role of NFY and determine whether it mediates the down-regulation of proapoptotic TXNIP expression in the diabetic heart in response to verapamil. To this end, we rendered mice diabetic with a single dose of STZ, and half of the animals received verapamil in their drinking water for a total of 3 weeks. This regimen rendered mice of both groups overtly diabetic within 48 h (blood glucose >300 mg/dL), but did not affect body weight or blood pressure as described previously (Chen et al., 2009). At sacrifice, mouse hearts were harvested, and the ventricles were used for cross-linking, ChIP, RNA extraction, and quantitative real-time RT-PCR or TUNEL. The results revealed that oral verapamil administration significantly induced NFY binding to the TXNIP promoter (Fig. 8A), and this was associated with a significant decrease in TXNIP expression (Fig. 8C), consistent with our findings in H9C2 cardiomyocytes. Furthermore, oral verapamil administration also dramatically reduced apoptosis in the cardiac ventricles of STZ-diabetic mice (Fig. 8, D and E). Taken together, these

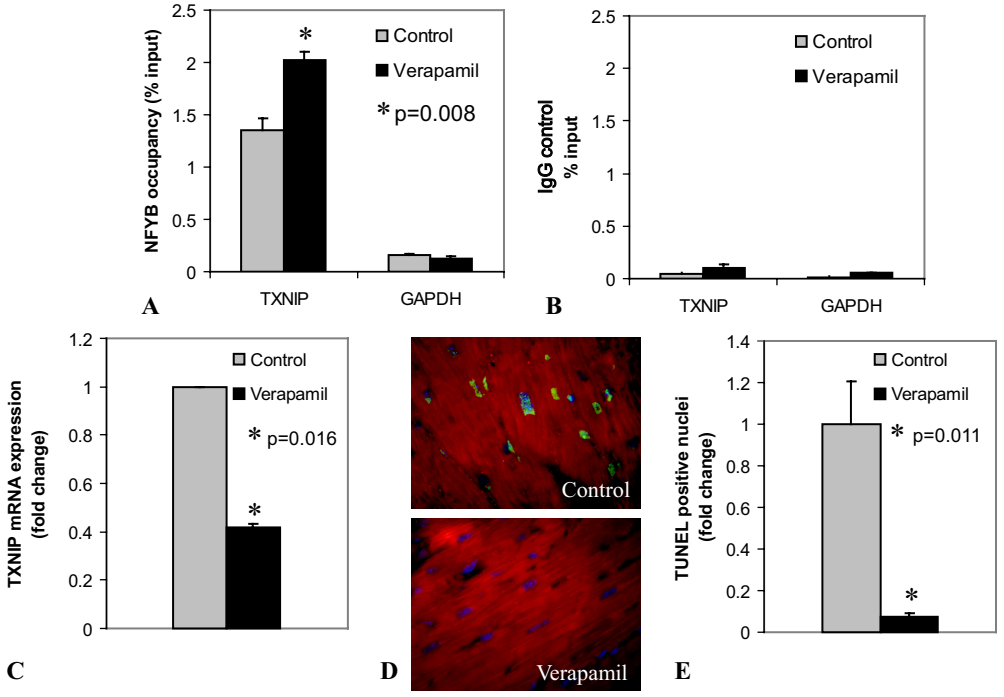

Fig. 8. In vivo verapamil effects on NFY occupancy of the TXNIP promoter in the diabetic heart. Wild-type mice were divided into two groups and treated with verapamil (100 mg/kg) (Verapamil) or without (Control) in their drinking water for a total of 3 weeks. Four days before euthanasia, mice of both groups were rendered diabetic by STZ injection (200 mg/kg). After euthanasia, their hearts were harvested, and the ventricles used for ChIP analysis using antibodies against NFYB (A) or normal control IgG (B) to assess NFYB binding to the TXNIP promoter and changes in TXNIP mRNA expression were determined by real-time RT-PCR (C). Apoptosis was assessed by TUNEL (green) (cardiomyocytes: desmin = red; nuclei: 4,6-diamidino-2-phenylindole = blue; 100×) (D) and quantified by analyzing over 2000 cardiomyocyte nuclei per group (E). Bars represent means ± S.E.M. from at least three mice per group.

findings suggest that the verapamil-mediated decrease in intracellular calcium leads to inactivation of calcineurin, an event that results in the increase of NFY-DNA binding, repression of TXNIP transcription, and inhibition of cardiac apoptosis, thereby revealing a novel signaling pathway (Fig. 9).

Discussion

The results of this study reveal for the first time that verapamil, a widely used blood pressure medication and calcium channel blocker, controls cardiac gene transcription by inhibiting the calcineurin signaling pathway, leading to increased DNA binding of NFY and resulting in transcriptional repression of proapoptotic genes such as TXNIP and Shc1.

Verapamil has been approved by the U.S. Food and Drug Administration for almost 30 years and, in its oral formulation, has been used extensively for the treatment of hypertension and angina pectoris, yet the present findings demonstrate that it has additional effects regulating cardiomyocyte gene expression. It is obvious that this deserves consideration when prescribing verapamil (or any other first-generation calcium channel blockers) especially given the impaired cardiovascular health of the patient population receiving this class of drugs. However, so far we have found that this new verapamil-induced signaling pathway leads to down-regulation of proapoptotic genes implicated in diabetic cardiomyopathy, suggesting that it actually may have beneficial effects especially in the context of diabetes. Consistent with this notion, we have found that oral administration of verapamil to diabetic mice is sufficient to promote cardiomyocyte survival. It is noteworthy that verapamil also had beneficial effects in a clinical study of patients with diabetic cardiomyopathy (Afzal et al., 1988).

Diabetic cardiomyopathy (defined as contractile dysfunction independent of coronary artery disease and hypertension) is believed to be the result of microvascular pathology, interstitial fibrosis, oxidative stress, and progressive cardiomyocyte damage and apoptosis and ultimately leads to heart failure and often fatal outcome (Yoon et al., 2005; Boudina and Abel, 2007). Despite the worldwide diabetes epidemic and the increasing number of patients affected, no effective therapies addressing the underlying cardiomyocyte damage are currently available. Taking into account that diabetes increases cardiac TXNIP expression and that TXNIP in turn promotes cardiomyocyte apoptosis (Chen et al., 2009), verapamil-induced down-regulation of TXNIP (and Shc1) may represent such a novel approach for the treatment of diabetic cardiomyopathy. In fact, 3 weeks of oral verapamil administration were sufficient to dramatically reduce apoptosis in the diabetic heart. These results are consistent with our previous findings of calcium channel blocker-induced decrease in TXNIP levels and reduced expression of apoptosis and fibrosis markers in normal and diabetic hearts (Chen et al., 2009). The current studies have now uncovered a molecular mechanism explaining these protective verapamil effects and revealed a previously unappreciated signaling pathway capable of controlling the transcription of critical cardiac genes even in the face of severe diabetes. Whereas other processes might also be involved, a simplified schematic model of this novel signaling pathway is shown in Fig. 9. It is obvious that further studies will be necessary to determine the role of this pathway in additional models and in human diabetic cardiomyopathy.

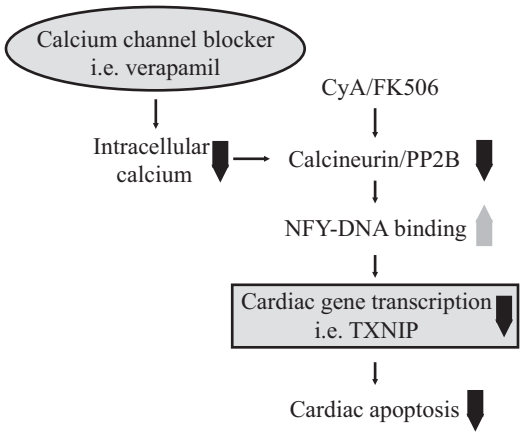

Fig. 9. Schematic model of calcium channel blocker-mediated control of cardiac gene transcription.

In addition, the identified signaling pathway may have implications beyond diabetic cardiomyopathy, especially given the important roles that TXNIP and Shc1 play in other cardiovascular pathologies, such as myocardial infarction, cardiac hypertrophy, and atherosclerosis (Wang et al., 2002; Schulze et al., 2004, 2006; Yoshioka et al., 2004; Xiang et al., 2005; Yamawaki et al., 2005). In fact, a recent study further revealed the protective effects of genetic deletion of TXNIP in ischemia/reperfusion injury (Yoshioka et al., 2012), underlining the need for effective pharmacological approaches to inhibit cardiac TXNIP expression. Moreover, considering that the CCAAT motif found to mediate the verapamil effect is a fundamental promoter element present in 25 to 30% of eukaryotic genes, it is tempting to speculate that the discovered verapamil-induced signaling cascade can regulate the expression of many more genes.

The established role of verapamil as a calcium channel blocker that reduces intracellular calcium and the finding that other calcium channel blockers as well as calcium chelators have similar effects on TXNIP transcription suggested that the observed decrease in TXNIP expression in response to verapamil was mediated by decreased intracellular calcium concentrations (Chen et al., 2009). Calcium is a critical second messenger and has recently been shown to regulate a number of transcription factors, including NFAT (Mellström et al., 2008). Although MatInspector analysis revealed that TXNIP and Shc1 promoters contain conserved putative NFAT binding sites and that a calcineurin-NFAT signaling pathway has been established in cardiac hypertrophy and cardiomyopathy (Molkentin, 2004), initial ChIP studies did not detect any significant NFAT binding to the TXNIP promoter (data not shown). It is obvious that this does not exclude the possibility of any indirect involvement of NFAT but makes a major direct effect rather unlikely. Moreover, detailed promoter deletion studies revealed that a proximal 20-bp region containing a CCAAT box was essential for verapamil-induced repression. Mutation of this CCAAT box within the full-length promoter further confirmed the importance of this element in mediating the observed verapamil effects. However, while still conferring significant inhibition of TXNIP transcription in response to verapamil or NFY, some upstream deletions (-1168 to -420) seemed to exhibit a slightly weaker response than the full-length TXNIP pro-

moter, raising the possibility that additional upstream elements might contribute to the effects observed, but an inverted CCAAT box (ATTGG) 30 bp upstream did not seem to be involved in the verapamil effects and indeed has been described to increase transcription (Butler et al., 2002; Yu and Luo, 2009).

Systematic analysis of putative CCAAT box binding factors led to the identification of NFY as the transacting factor conferring verapamil-induced TXNIP repression as confirmed by NFY overexpression, RNA interference, and in vitro and in vivo ChIP studies. It is noteworthy that this is the first demonstration of NFY conferring calcineurin-regulated control of gene transcription. In the case of NFAT, calcineurin regulates the phosphorylation status and transcriptional activity of NFAT through direct binding to a distinct PxIxIT motif (whereby x denotes any amino acid) (Rodriguez et al., 2009). Furthermore, NFY contains a PxIxIT-like motif (PxGxIT/PAGLIT) that is well conserved across species. Because even less well conserved NFAT-like docking sites have been described to allow for calcineurin binding to target proteins (Czirják and Enyedi, 2006), it is very possible that this PAGLIT motif may be involved in the observed calcineurin effect on NFY activity. In any case, our studies revealed for the first time that NFY acts as a downstream target of verapamil and mediates its effects in vitro in H9C2 cardiomyocytes as well as in vivo in the diabetic heart. Taking into account our recent findings in pancreatic β-cells (Xu et al., 2012), we have now discovered that verapamil inhibits TXNIP transcription in cardiomyocytes and in pancreatic β-cells, but although calcineurin signaling is involved in both cases, the transcription factors mediating the effects are distinct, i.e., NFY and carbohydrate-response element-binding protein, respectively.

Increased NFY binding to the promoter of grp78/BiP (a calcium-binding endoplasmic reticulum chaperone) has been observed with calcium depletion but was attributed to a slower dissociation rate and a change in the calcium ionic environment (Roy and Lee, 1995). However, considering our findings of calcineurin inhibitors mimicking the verapamil effect and CyA stimulating NFY binding to the TXNIP promoter (Fig. 6D), it is tempting to speculate that post-translational modification may be involved in the observed increase in NFY-DNA binding, especially given the lack of a verapamil-induced increase in nuclear NFY (Supplemental Fig. S3). In fact, NFY has been shown to be post-translationally modified, and phosphorylation of certain serine residues of the NFYA subunit are required for DNA binding (Yun et al., 2003), raising the possibility that inhibition of the calcineurin serine-threonine protein phosphatase by CyA or verapamil may promote NFY phosphorylation and thereby regulate its binding to target gene promoters such as TXNIP and Shc1.

NFY acts as a bifunctional transcription factor (Ceribelli et al., 2008), whereby DNA binding can be associated with transactivation (e.g., grp78/BiP gene) (Roy and Lee, 1995) and procollagen α1 gene (COL1A1) (Lindahl et al., 2002) or lead to repression (e.g., proline-rich nuclear receptor coactivator gene) (Zhang et al., 2008) or both (e.g., topoisomerase IIα and von Willebrand factor genes), demonstrating that the context of promoter structure, CCAAT element, and DNA binding sequence determines NFY function (Coustry et al., 2001; Peng and Jahroudi, 2002). This is consistent with our results where NFY clearly led to transcriptional repression of TXNIP and Shc1, whereas other reports suggested NFY involvement in TXNIP transactivation through the inverted CCAAT element or as a prerequisite for inducible expression (Butler et al., 2002; Yu and Luo, 2009).

In the present study, we focused on the effects of NFY on TXNIP transcription and its role in mediating verapamil-induced repression. However, our findings that NFY also controlled the transcription of Shc1 through the same signaling pathway demonstrate that NFY function is not limited to TXNIP and suggest that the newly discovered verapamil-calcineurin-NFY pathway may regulate a large number of other important cardiac target genes. In fact, NFY has been shown to control the transcription of cell cycle control genes such as cdc2 in cardiac muscle (Liu et al., 1998) (consistent with its regulation of proapoptotic genes observed in our study) and was identified as a regulator of clock-controlled genes and circadian transcription in a large-scale promoter analysis (Bozek et al., 2009).

NFY contains histone 2A/2B-like subunits, binds to H3 and H4, and can interfere with nucleosome formation (Caretti et al., 1999; Ceribelli et al., 2008). NFY has also been implicated in histone methylation (Ceribelli et al., 2008; Donati et al., 2008). In addition, NFY has been shown to associate with histone deacetylases and thereby mediate some of its repressive functions (Peng and Jahroudi, 2003; Takahashi et al., 2006; Peng et al., 2007). In fact, we found that verapamil-induced binding of NFY and TXNIP repression was associated with marked histone H4 deacetylation. This suggests that our verapamil-calcineurin-NFY signaling pathway may exert its transcriptional repression not only through transcription factor recruitment but also through histone modification and chromatin remodeling.

In summary, we have identified a novel verapamil-induced calcineurin-NFY signaling pathway that controls chromatin modification and cardiac gene transcription. Given the wide use of calcium channel blockers and verapamil, these findings also have major medical implications and suggest that this class of drugs may affect cardiac pathology in ways previously not appreciated. In addition, the results shed new light on the regulation of TXNIP and Shc1, both of which play critical roles in diabetic cardiomyopathy and cardiovascular health and disease.

Authorship Contributions

Participated in research design: Cha-Molstad and Shalev.

Conducted experiments: Cha-Molstad, Xu, Chen, Jing, Young, and Chatham.

Contributed new reagents or analytic tools: Jing, Young, and Chatham.

Performed data analysis: Cha-Molstad, Xu, Chen, and Shalev.

Wrote or contributed to the writing of the manuscript: Cha-Molstad and Shalev.

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