GRK5-Mediated Exacerbation of Pathological Cardiac Hypertrophy Involves Facilitation of Nuclear NFAT Activity

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Abstract

Rationale—G protein-coupled receptor (GPCR) kinases (GRKs) acting in the cardiomyocyte regulate important signaling events that control cardiac function. Both GRK2 and GRK5, the predominant GRKs expressed in the heart, have been shown to be up-regulated in failing human myocardium. While the canonical role of GRKs is to desensitize GPCRs via phosphorylation, it has been demonstrated that GRK5, unlike GRK2, can reside in the nucleus of myocytes and exert GPCR-independent effects that promote maladaptive cardiac hypertrophy and heart failure (HF).

Objective—To explore novel mechanisms by which GRK5 acting in the nucleus of cardiomyocytes participates in pathological cardiac hypertrophy.

Methods and Results—In this study, we have found that GRK5-mediated pathological cardiac hypertrophy involves the activation of nuclear factor of activated T-cells (NFAT) as GRK5 causes enhancement of NFAT-mediated hypertrophic gene transcription. Transgenic mice with cardiomyocyte-specific GRK5 overexpression activate an NFAT-reporter in mice basally and after hypertrophic stimuli including transverse aortic constriction (TAC) and phenylephrine treatment. Complimentary to this, GRK5 null mice exhibit less NFAT transcriptional activity after TAC. Further, loss of NFATc3 expression in the heart protected GRK5 overexpressing transgenic mice from the exaggerated hypertrophy and early progression to HF seen after TAC. Molecular studies suggest that GRK5 acts in concert with NFAT to increase hypertrophic gene transcription in the nucleus via GRK5's ability to bind DNA directly without a phosphorylation event.

Conclusions—GRK5, acting in a kinase-independent manner, is a facilitator of NFAT activity and part of a DNA binding complex responsible for pathological hypertrophic gene transcription.

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Keywords

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INTRODUCTION

Heart failure (HF) is a clinical end point defined by the heart's inability to adequately perfuse the body with blood. This condition effects over 5 million Americans with 825,000 new cases annually [1]. Although HF can be the result of many diverse etiologies there seems to be common underlying molecular mechanism including the dysfunction of the β-adrenergic receptor (β-AR) system, dysregulation of myocyte calcium handling and activation of the fetal gene program among which are genes that can lead to pathological cardiac hypertrophy [2]. β-ARs act to drive the contractile function but become dysfunctional following chronic catecholamine stimulation, which occurs in HF. G protein-coupled receptor (GPCR) kinases (GRKs) phosphorylate these receptors leading to their desensitization and down-regulation [3]. GRK2 and GRK5, the two major GRKs in the heart, are in fact up-regulated in HF leading to a loss of the heart's inotropic reserve [4-6]. In fact, GRK2 inhibition and the improved resensitization of β-AR signaling in the failing heart has led to HF reversal and a potential therapeutic strategy [7]. The role of GRK5 on cardiac β-AR signaling is less understood although GRK5 can also desensitize these GPCRs [8].

Recently, many non-GPCR functions of GRKs have been discovered and some of these appear physiologically important. For example, GRK2 is a pro-death kinase in myocytes acting at the level of mitochondria in a non-GPCR fashion mediated by oxidative stress [9]. While GRK5 is not found in the mitochondria it does contain a nuclear localization sequence (NLS) homologous to homeobox-containing transcription factors, which allows it to translocate to the nucleus where it has been shown to have DNA binding properties in some cells [10, 11]. In cardiomyocytes, it has been demonstrated that GRK5 can accumulate in the nucleus in a Gq-dependent manner either through pharmacologic hypertrophic stimulation or left ventricular (LV) pressure-overload [12-14]. Once in the nucleus GRK5 has been shown to phosphorylate histone deacetylase-5 (HDAC5) leading to derepression of MEF2-mediated hypertrophic gene transcription [14]. This was best shown in transgenic mice with cardiac GRK5 overexpression as these mice, but not mice overexpressing a NLS-mutant GRK5 that cannot accumulate in the nucleus, displayed exaggerated cardiac hypertrophy and early-onset HF following pressure-overload [14]. Not only did the NLS-GRK5 mutant mice not have maladaptive cardiac hypertrophy, but GRK5 knockout (KO) mice are also protected against pressure-overload stress [12].

In addition to the phosphorylation of HDAC5, nuclear GRK5 has been shown to activate the NF-κB pathway through phosphorylation of Iκ-Bα as well as regulate cell cycle progression through direct phosphorylation of nucleophosmin [15-17]. Thus, there could be additional targets in the nucleus of hypertrophic cardiomyocytes. One of the key transcription factors involved with myocardial hypertrophy and subsequent HF is the nuclear factor of activated T cells (NFAT). NFAT resides in the cytoplasm in a hyper-phosphorylated state and then upon increased calcium entry, NFAT is de-phosphorylated by the calcium sensitive

phosphatase calcineurin and then it can translocate to the nucleus where it controls transcription of several hypertrophic and maladaptive genes [18]. The NFAT pathway is an interesting therapeutic target because it is involved in pathological, but not physiological hypertrophy and several drugs exist that can regulate this pathway including cyclosporine [19, 20].

Herein, we provide evidence that GRK5 is able to activate the NFAT transcriptional pathway following hypertrophic stress. Our data reveal that the GRK5 localization in the nucleus and not cytosol facilitates the transcriptional activity of NFAT in vitro and in vivo. Further, NFATc3 expression is needed for the induction of early pathology after hypertrophic stress with enhanced GRK5 expression. Molecular studies suggest that GRK5 acts in concert with NFAT at the level of chromatin to facilitate hypertrophic gene transcription in a kinase-independent manner. Thus, nuclear GRK5 appears to be a key player and determinant of pathological cardiac hypertrophy and limiting its accumulation in the nucleus may allow for the development of novel therapeutics and strategies to prevent and reverse the progression of maladaptive cardiac hypertrophy and HF.

METHODS

Cell culture

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 2-day old rats as previously described [21]. H9c2 cells, a rat myoblast cell line, were cultured in DMEM from ATCC supplemented with 10% bovine calf serum and penicillin-streptomycin in a humidified chamber with 5% CO_2 at 37°C.

Adenovirus, plasmids and transfection

NRVMs were infected with recombinant, replication-deficient adenoviruses at an MOI from 1-10 viral particles per cell. Plasmids encoding wild-type full length bovine GRK5, GRK5 K215R kinase dead and the GRK5 NES nuclear exclusion sequence mutant (a kind gift of Dr. Julie Pitcher at University College London) in the pRK5 vector were transiently transfected into H9c2 cells using Lipofectamine 2000 as described [11, 22-24].

Luciferase assay

Cells were harvested 48 hrs after infection in lysis buffer according to manufacturer's protocol (Promega)[14].

Real-time PCR

Total RNA was extracted by the TRIzol method and RT-PCR was performed as described [12].

Experimental animals

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University School of Medicine.

Echocardiography

To measure global cardiac function, echocardiography was performed with the VisualSonics VeVo 2100 imaging system in anesthetized animals as described [12].

Transverse aortic constriction

Transverse aortic constriction (TAC) was performed as described previously [14].

Mini-osmotic pumps

Chronic infusion of phenylephrine (PE, purchased from Sigma) was done using Alzet 3-day mini-osmotic pumps (model 1003D, DURECT Corporation) following the manufacturer's specifications.

Cardiomyocyte cross-sectional area

Axial cut tissue sections were stained with Alexa Fluor 594 conjugated of wheat germ agglutinin. Cell borders were planimetered manually by an operator who was blinded to treatment group.

Electrophoretic Mobility Shift Assay (EMSA)

IRDye 700 end labeled NFAT consensus oligonucleotides were incubated with 2.5 μg NRVM nuclear extracts (NE). For antibody mediated super shift assay, 1 ug antibody was incubated with 5 μg NE in reaction mixture. Protein-DNA complexes were seperated on 4% non-denaturing polyacrylamide gel and visualized using an Odyssey infrared imaging system[17].

Statistics

All the values in the text and figures are presented as mean +/− SEM. Statistical significance was determined by Student's t-test or ANOVA. P values of <0.05 were considered significant.

RESULTS

GRK5 enhances cardiac NFAT transcriptional activity in vitro

Recent data from our laboratory have shown that GRK5 acting in the nucleus is a key mediator of pathological cardiac hypertrophy and this is due, in part, to its actions as a HDAC kinase [12-14]. In this study we sought other actions of GRK5 in the nucleus since in addition to its kinase activity in the nucleus, GRK5 has been shown to have potential DNA binding capabilities [10, 11] and to interact with other nuclear proteins [25]. It is well known that in addition to hypertrophic gene transcription occurring through the HDAC-regulated MEF2 transcription factor, NFAT is also a critical regulator of hypertrophic gene regulation [26]. To investigate whether GRK5 may influence NFAT activity we first used an NFAT reporter assay in myocytes. In NRVMs expressing an NFAT-luciferase reporter adenovirus we overexpressed GRK5 and found significantly increased luciferase levels after stimulating the cells with the hypertrophic α1-adrenergic agonist, phenylephrine (PE) (Fig. 1A).

In order to confirm that GRK5-mediated increases in NFAT luciferase activity post-PE was due to a true induction in NFAT transcriptional activity we performed an additional and complementary experiment where we measured expression levels of the NFAT target gene RCAN1.4 (henceforth referred to only as RCAN) in myocytes after PE stimulation with varying levels of GRK5. NRVMs were subjected to viral over-expression of GRK5 or LacZ as a control followed by stimulation with 50 µM PE for 16 hrs and RCAN levels were assessed by quantitative RT-PCR. While PE was able to increase RCAN expression in myocytes, we found a significant increase in RCAN mRNA levels when GRK5 levels were enhanced (Fig. 1B). This confirmed that GRK5 overexpression is able to induce increased NFAT transcriptional activity with RCAN expression serving as an endogenous reporter of NFAT activity.

Finally, PE is known to activate the α1-AR and hypertrophic signaling occurs down-stream of Gα_q activation. In order to confirm that GRK5's actions on NFAT after PE were due to Gα_q activation, we performed RCAN expression assays in NRVMs expressing a constitutively active mutant of Gα_q (CAM-Gq) and found that when GRK5 is elevated there is significant more RCAN expression induced with CAM-Gq (Fig. 1C).

In separate experiments using immunofluorescence and confocal microscopy we found that GRK5 overexpression in myocytes does not cause more NFAT to accumulate in the nucleus following PE stimulation. This indicates that the induction of NFAT activity by GRK5 is not due to more NFAT translocating to the nucleus, but rather somehow enhancing activity within the nucleus (Supplemental Fig. I). Supporting this are studies where overexpressing GRK5 had no effect on cytoplasmic calcineurin activity (Supplemental Fig. IC, ID).

GRK5 enhances NFAT transcriptional activity in vivo in models of cardiac pathology

After demonstrating in vitro that increased GRK5 expression in myocytes can enhanced NFAT activity and hypertrophic gene transcription we sought to confirm these findings in vivo. To do so we crossed cardiac-specific NFAT luciferase reporter mice (LUC) previously characterized [20] with mice that our lab previously created with cardiac-specific transgenic overexpression of GRK5 (TgGRK5) [14]. Using in vivo bioluminescence imaging techniques we find that cardiac GRK5 overexpresssion causes increased NFAT activity in the upper thoracic cavity consistent with the heart (Supplemental Fig. II). Taking hearts from TgGRK5 mice, LUC mice and LUC/TgGRK5 hybrid mice we assessed the ex vivo luciferase activity and found approximately twice as much NFAT activity in the TgGRK5 mice compared to those with endogenous levels of GRK5 (Fig. 2A). Somewhat surprisingly, these results indicate that only by overexpressing GRK5 in the myocytes is NFAT activity enhanced. Overall, this activity, although significant, must not be robust enough to drive hypertrophy since the LUC/TgGRK5 mice do not exhibit altered cardiac mass at this age in the absence of hypertrophic stress.

To determine whether the increase in NFAT luciferase activity seen in TgGRK5 mice was relevant in the context of pathology, these mice were subjected to left ventricular (LV) pressure-overload through surgical transverse aortic constriction (TAC). Cardiac function in these animals was assessed 14 days after TAC by echocardiography. The ejection fraction (EF) in the TgGRK5/LUC mice that were subjected to TAC was significantly decreased

(Fig. 2B), consistent with early post-TAC HF seen with TgGRK5 mice previously [14]. Importantly, after 14 days of pressure-overload an ex vivo NFAT luciferase assay was performed on heart tissue and NFAT activity via luminescence readings was significantly higher in LUC mice as expected, however when GRK5 was elevated in these mice (LUC/TgGRK5) there was significantly more NFAT activity (Fig. 2C).

To confirm that this increase in luciferase activity was indeed a result of increased NFAT transcriptional activity we performed RT-PCR from the hearts of these mice for the NFAT target gene RCAN. Following TAC, animals with endogenous levels of GRK5 (LUC mice) showed an increase in RCAN expression while LUC/TgGRK5 hybrid animals demonstrated a significant increase in RCAN expression compared to all other groups (Fig. 2D). Therefore, using an endogenous gene targeted readout we were able to confirm that NFAT transcriptional activity is increased in TgGRK5 mice in a model of pathology.

It is known that TAC causes hypertrophy through the $G\alpha_q$ pathway and has been shown to drive GRK5 into the nucleus [14, 27]; therefore, we wanted to test if GRK5 overexpression would enhance NFAT activity following stimulation of the $G\alpha_q$ pathway via the $\alpha 1$-adrenergic hypertrophic agonist PE in these animals. Mini-osmotic pumps were used to deliver a sub-pressor dose of PE subcutaneously for 24 hrs in LUC and LUC/TgGRK5 mice. As seen previously, TgGRK5 mice alone pumped with vehicle control have an increase in NFAT luciferase activity as compared to LUC-alone mice with endogenous levels of GRK5 (Fig 2E). After PE treatment, LUC/TgGRK5 mice demonstrate a significant increase in NFAT activity over all other groups (Fig. 2E). Thus, in this in vivo mouse model, we demonstrate that GRK5 is able to increase NFAT transcriptional activity basally and down-stream of the $G\alpha_q$ hypertrophic signaling pathway.

GRK5 KO mice demonstrate attenuated NFAT transcriptional activity following hypertrophic stress

In order to determine that GRK5 mediated activation of the NFAT pathway is physiologically relevant, we subjected GRK5 null (GRK5 KO) mice to LV pressure-overload via TAC. At baseline and 2 and 4 weeks post-TAC echocardiography was performed to assess LV posterior wall thickness (LVPWT) of these animals as a marker of hypertrophy. At baseline we saw no difference in wall thickness; however, at 4 weeks post-TAC wild-type mice showed a significant increase in wall thickness compared to all other groups (Fig. 3A). As expected from previous work in our laboratory [12], the increase in wall thickness seen following TAC was significantly attenuated in GRK5 KO mice (Fig. 3A). After 4 weeks hearts were harvested and RT-PCR was performed on heart tissue to determine the level of transcription of the NFAT target gene RCAN and we found a significant increase in RCAN transcription in wild-type mice subjected to TAC as compared to Sham groups (Fig. 3B). Interestingly, we found less RCAN transcription in GRK5 KO mice subjected to TAC as compared with wild-type littermates that also underwent TAC surgery (Fig. 3B) indicating that endogenous levels of GRK5 are required to get a normal hypertrophic response that includes NFAT-mediated RCAN expression. These results are consistent with GRK5 being relevant for normal NFAT activation following pressure-overload.

NFATc3 KO attenuates GRK5-mediated cardiac pathology after pressure-overload stress

To determine whether NFAT activity is required for GRK5-mediated cardiac pathology after TAC we bred TgGRK5 mice with mice null for NFATc3 (NFATc3 KO). We found significant differences in mice overexpressing GRK5 in the hearts with and without NFATc3 as early as 1 week post-TAC as determined initially by simple heart weight to body weight (HW:BW) ratio (Fig. 4A and Supplemental Fig. III). As expected, we saw a significant increase in HW:BW in TgGRK5 mice subjected to TAC compared to control non-transgenic, wild-type animals that have endogenous levels of GRK5 (Fig. 4A). Interestingly, NFATc3 deletion significantly attenuated the observed increase in heart weight seen in the TgGRK5 mice following TAC (Fig. 4A). We also assessed cardiac function in these animals by echocardiography at 1 week post-TAC and found EF% significantly decreased in the TgGRK5 mice compared to control mice supporting early onset HF (Fig. 4B and Supplemental Fig. IV). However, importantly, this LV dysfunction that was present in TgGRK5 mice at one week was not present in TgGRK5 mice where NFATc3 is deleted, including LV dilatation (Fig. 4B and 4C). Thus, the loss of NFATc3 can prevent early progression to HF after TAC when GRK5 is overexpressed in myocytes. Chronically, at 4 weeks post-TAC, these TgGRK5/NFATc3 KO mice were dysfunctional consistent with GRK5 overexpression exerting HDAC kinase effects (data not shown). We also assessed cardiomyocyte cross-sectional area in these animals to confirm that hypertrophy is occurring at the cellular level using wheat germ agglutinin staining and indeed TgGRK5 mice display a significantly increased cell size after pressure-overload compared to wild-type littermates while NFATc3 deletion leads to an attenuation of cardiomyocyte hypertrophy as shown by a significant decrease in cross-sectional area following TAC in TgGRK5 mice (Fig. 4D).

Finally, we assessed the transcription of select genes by quantitative RT-PCR to determine on a molecular level if NFATc3 deletion protects the TgGRK5 mice from transcriptional changes associated with the post-TAC GRK5-mediated HF phenotype (Fig. 4E). Induction of the fetal gene program was assessed by transcription of genes for the contractile proteins skeletal muscle actin (Acta-1) and β-myosin heavy chain (β-MHC). To assess remodeling and fibrosis of the heart transcription of the connective tissue growth factor (CTGF) gene was also determined. Finally, transcription of the NFAT target gene RCAN was analyzed to show specificity for NFAT. All of these transcripts were similarly up-regulated in TgGRK5 mice compared to wild-type mice following TAC, however expression of all these genes is attenuated when NFATc3 is deleted (Fig. 4E). Therefore, NFATc3 deletion in cardiac GRK5 overexpressing mice leads to a more favorable genetic profile following LV pressure-overload.

GRK5 interacts with NFAT in a kinase-independent manner through DNA binding

To assess the mechanism by which GRK5 activates the NFAT pathway, we used H9c2 myoblasts in order to introduce plasmids carrying wild-type (WT) GRK5 and mutants that render GRK5 kinase-dead (K215R) or incapable of being exported from the nucleus (NES) (Supplemental Fig. VII). All of these GRK5 proteins can accumulate in the nucleus after hypertrophic stimuli and we tested whether kinase activity and nuclear localization is required for NFAT activation via GRK5. These cells were also infected with the CAM-Gq

mutant adenovirus to activate hypertrophic signaling and RT-PCR was performed for the NFAT target gene RCAN. We found that the NES mutant which becomes trapped in the nucleus caused the greatest activation of NFAT (Fig. 5A). This is not surprising as nuclear GRK5 is responsible for the exaggerated cardiac pathology seen in TgGRK5 mice after pressure-overload [14]. Surprisingly, this experiment also demonstrates that the kinase dead K215R GRK5 was able to activate NFAT to a similar degree as WT GRK5 (Fig. 5A). This proves that the activation of NFAT by GRK5 does not involve a phosphorylation event and, therefore, segregates the activation of MEF2 by GRK5 via HDAC5 phosphorylation from the activation of the NFAT pathway.

As the activation of NFAT by GRK5 appears to occur within the nucleus (Fig. 5A) we decided to perform an electrophoretic mobility shift assay (EMSA) to determine if GRK5 was able to alter DNA binding by NFAT. The assay was performed by incubating NFAT specific DNA probes with nuclear lysates from NRVMs overexpressing GRK5 or a LacZ control and stimulated with PE for 5, 15 or 30 min (Fig. 5B). We found that GRK5 was able to potentiate NFAT:DNA binding as GRK5 overexpression led to an increase in the amount of NFAT specific DNA probe that was bound following PE stimulation (Fig. 5B).

Next, we hypothesized that GRK5 may be interacting with NFAT at the level of DNA as GRK5 has previously been shown to bind DNA in a kinase-independent manner [10, 11]. Accordingly, we performed an EMSA with antibody mediated supershift to determine if GRK5 was present in a complex with NFAT at the level of DNA (Fig. 5C). Nuclear lysates from NRVMs overexpressing GRK5 and stimulated with PE for 30 min were incubated with IgG, GRK2 or GRK5 antibodies. Importantly, a shift band was observed in the lysate incubated with GRK5 antibody, but not with IgG or GRK2 negative controls (Fig. 5C). This suggests that GRK5 is interacting with NFAT at the level of DNA in order to potentiate the binding of the NFAT:DNA complex and adds a new mechanism for GRK5 in the facilitation of hypertrophic gene transcription (Fig. 6).

DISCUSSION

While the canonical role of GRKs is to phosphorylate activated seven transmembrane receptors leading to their desensitization and down-regulation, a growing non-GPCR 'interactome' is emerging [25]. GRK5, as well as GRK6, have the unique properties among GRK family members to translocate and localize to the nucleus where in myocytes it has been shown GRK5 has the non-GPCR activity of acting as a Class II HDAC kinase facilitating maladaptive cardiac hypertrophy [12-14]. This novel nuclear activity of GRK5 was confirmed as playing a role in the normal hypertrophic response of the heart as GRK5 KO mice (global as well as cardiomyocyte specific) have less growth after TAC as well delayed HF [12]. Further, simply keeping overexpressed GRK5 out of the nucleus prevents the pathological growth of the heart after hypertrophic stimuli via lower HDAC kinase activity and less MEF2 activation [14]. Since GRK5 has been shown to bind DNA in the nucleus of non-myocytes [10] and shown to interact with other nuclear proteins such as Iκ-Bα, p53 and nucleophosmin [15, 16, 28], we explored whether GRK5's role in pathological cardiac hypertrophy, in addition to MEF2 regulation through phosphorylation of HDAC5, may involve other targets and mechanisms.

Here we identify the NFAT pathway as another target of GRK5 within the nucleus. While GRK5 is able to activate MEF2 in myocytes after hypertrophic stress, it appears that NFAT is a critical pathway through which GRK5 causes pathology after pressure-overload as NFATc3 deletion in GRK5 overexpressing mice is protective after TAC. The HDAC activity appears still in play after hypertrophic stress since chronic hypertrophy and HF still occurs in TgGRK5/NFATc3 KO hybrid mice following longer periods of TAC. This result could also be explained by an up-regulation in other NFAT isoforms which are able to partially compensate for the loss of the c3 isoform.

In this study we utilized mutant constructs of GRK5 to determine a possible mechanism for the regulation of the NFAT pathway by GRK5 and indeed confirmed a nuclear-dependent effect that happens to be kinase-independent. We found that the NES mutant of GRK5, which lacks a nuclear export sequence and is therefore trapped in the nucleus, leads to the greatest activation of NFAT activity. This is logical as in vivo experiments performed previously in our lab found nuclear GRK5 to be the cause of cardiac pathology after TAC [14]. Surprisingly, the kinase-dead GRK5 K215R mutant was able to activate NFAT as well as WT GRK5 providing the first hint of kinase-independent regulation of this transcription factor by GRK5. Previously, GRKs have been known to exert kinase-independent effects including GRK5, which was shown recently to promote filamentous actin bundling through a kinase-independent manner by interacting with F-actin and PIP_2 [25, 29]. This finding with GRK5-K215R limits the possibility of NFAT regulation through HDAC kinase activity or other phosphorylation events, which is different than how GRK5 can regulate MEF2 hypertrophic gene transcription. The kinase-independent activation of NFAT by GRK5 seems logical when one considers that NFAT is negatively regulated by phosphorylation yet GRK5, a kinase, is able to increase its activity. Other kinases such as ERK, casein kinase II, JNK, p38, PKA, and GSK3-β negatively regulate the NFAT pathway through phosphorylation of NFAT [26, 30-34]. Additionally, CAMKII is able to oppose the NFAT pathway by direct phosphorylation of calcineurin [35].

Contrary to the above kinases which oppose NFAT activity, p90 ribosomal S6 kinase (RSK) has been shown to potentiate NFAT:DNA binding through interaction with the NFAT:DNA complex [36]. This is of particular interest to this study as both RSK and GRK5 belong to the AGC protein kinase subfamily [37]. Overall, our findings add significantly to the understanding of GRK5 in cardiac hypertrophic gene transcription through the two most important pathways (NFAT and MEF2). Importantly, these two transcriptional regulation pathways influenced by nuclear GRK5 occur due to either kinase-dependent effects (HDAC5 kinase) as well as kinase-independent actions (via NFAT:DNA binding and induction of NFAT transcriptional activity). These mechanisms are illustrated in Fig. 6.

While we are not the first to suggest that GRK5 can exert effects through its DNA binding property we are the first to show that this can lead to positive regulation of transcription [10, 11, 38]. It has been demonstrated previously that GRK5 and GRK4 subfamily member GRK6 can bind directly to DNA in vitro [10, 11]. In vivo Liu, et al. found that GRK5 binds directly to the Bcl-2 promoter and inhibits transcription of Bcl-2 [38]. As NFAT is known to have weak DNA binding ability and cooperates with other transcription factors in a complex [39], we believe our data are consistent with GRK5 being present in this

transcriptional complex and is, therefore, able to enhance the transcription of NFAT target genes (Fig. 6). This is interesting because NF-κB, which shares the DNA binding Rel homology domain with NFAT, is also regulated by GRK5 although controversy surrounds the mechanism by which this occurs [40, 41]. Sorriento, et al. believe that GRK5 is a negative regulator of NF-κB through forced nuclear accumulation of NF-κB inhibitor Iκ-Bα [42, 43]. Islam, et al. and Patial, et al. find that GRK5 is a positive regulator of NF-κB signaling through either up-regulation of protein levels of NF-κB subunits p50 and p65 or phosphorylation of Iκ-Bα [17, 44]. It is possible that GRK5 is able to further regulate the NF-κB pathway through interaction with p50 and p65 at the level of chromatin.

It remains to be seen if GRK5 is binding directly to DNA in order to potentiate NFAT:DNA binding and transcription or if GRK5 is simply present in a transcriptional complex with NFAT. While we cannot rule out a role for HDACs in the regulation of NFAT activity by GRK5 we find that GRK5 is acting in a kinase-independent fashion and therefore the phosphorylation of HDAC5 by GRK5 is not the mechanism at work (Fig. 6). Future experiments involving chromatin immunoprecipitation (ChIP) for GRK5 would allow for the identification of novel GRK5 targets and interactions. This data could then be used along with gene expression to determine which promoters are positively or negatively regulated by GRK5.

In summary, we provide evidence that GRK5 is able to activate the NFAT transcriptional pathway leading to the up-regulation of hypertrophic genes. We find that cardiac-specific NFAT luciferase reporter mice crossed with mice that overexpress wild-type GRK5 in a cardiomyocyte specific manner exhibit an increase in NFAT activity both in the basal state as well as after the hypertrophic stressors TAC and phenylephrine administration. Complimentary to this, GRK5 null mice exhibit less NFAT transcriptional activity after left ventricular pressure-overload as shown by the expression of the NFAT target gene RCAN. Importantly, loss of NFATc3 expression protected GRK5 over-expressing mice from the exaggerated hypertrophy and early progression to HF seen acutely after TAC. Molecular studies suggest that GRK5 acts in concert with NFAT to increase hypertrophic gene transcription in the nucleus and this is a kinase-independent action of GRK5 that involves its known property of DNA binding. The overall translational significance of these finding are substantial as simply finding a kinase inhibitor of GRK5 as a potential therapeutic for maladaptive cardiac hypertrophy would block its HDAC kinase activity in the nucleus, but it would not prevent the activation of the NFAT pathway as our data shows that GRK5 acts in a kinase-independent manner. We suggest that a more effective strategy would be to develop a therapy that is capable of inhibiting the nuclear accumulation of GRK5 allowing this enzyme to exert protective effects at the sarcolemma through transactivation of the β-AR and EGF receptor while preventing its activation of MEF2 and NFAT in the nucleus [45]. By developing novel therapeutics to inhibit GRK5 nuclear accumulation it may be possible to prevent and reverse the progression of HF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

βAR	β-adrenergic receptor
CaM	Calmodulin
CAM-Gq	Constitutively active Gαq
CaMK	Calmodulin-dependent kinase
Cn	Calcineurin
CTGF	Connective tissue growth factor
EF	Ejection fraction
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GSK-3β	Glycogen synthase kinase 3β
HDAC	Histone deacetylase
HF	Heart failure
HW/BW	Heart weight / Body weight
JNK	c-Jun N-terminal kinase
KO	Knockout
LVID	Left ventricular internal diameter
LVPWT	Left ventricular wall thickness
PBS	Phosphate buffered saline
PE	Phenylephrine
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
MEF2	Myocyte enhancer factor 2

MHC	Myosin heavy chain
NE	Nuclear extract
NES	Nuclear export signal
NFAT	Nuclear factor of activated T cells
NLC	Non-transgenic littermate control
NLS	Nuclear localization signal
NRVM	Neonatal rat ventricular myocyte
RCAN	Regulator of calcineurin
RSK	p90 ribosomal S6 kinase
TAC	Transverse aortic constriction
Tg	Transgenic

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Novelty and Significance

What Is Known?

- G protein-coupled receptor kinase 5 (GRK5) is up-regulated in models of heart failure (HF), as well as in the failing human heart.

- GRK5 enters the nucleus of cardiomyocytes via a functional nuclear localization sequence in response to hypertrophic stimuli where it can act in a non-GPCR manner as a histone deacetylase 5 (HDAC5) kinase enhancing MEF2-dependent hypertrophic gene transcription.

- Increased nuclear GRK5 is pathological in the setting of chronic pressure-overload while GRK5 ablation significantly delays the onset of HF after pressure-overload.

What New Information Does This Article Contribute?

- GRK5-mediated pathological cardiac hypertrophy involves the activation of nuclear factor of activated T-cells (NFAT) within the nucleus in a kinase-independent manner.

- Molecular studies suggest that GRK5 acts in concert with NFAT to facilitate and increase hypertrophic gene transcription in the nucleus via GRK5's ability to directly bind DNA.

- GRK5-dependent cardiac maladaptive hypertrophy and early onset HF after pressure overload are dependent on NFAT activity as loss of NFATc3 expression in the heart protected GRK5 overexpressing transgenic mice from early progression to HF.

GRK5 can localize to the nucleus of cardiomyocytes and this localization increases under conditions of hypertrophic stress. Nuclear accumulation of GRK5 promotes maladaptive cardiac hypertrophy and HF at least in part due to novel HDAC kinase activity. However, in this study we sought additional mechanisms involved in GRK5-mediated cardiac pathology. We found that GRK5 can also facilitate activation of the hypertrophic transcription factor, NFAT. Studies in NFATc3 knockout mice with cardiac GRK5 overexpression demonstrate that GRK5-mediated cardiac pathology after pressure-overload requires NFAT expression. Molecular studies demonstrate that GRK5 acts in concert with NFAT to increase hypertrophic gene transcription in the nucleus in a non-canonical, kinase-independent manner via GRK5's ability to directly bind DNA. The overall translational significance of these findings are substantial since GRK5 is elevated in the failing human heart, which would also lead to nuclear accumulation (has this been shown, or is it assumed? Clarify). Moreover, our results suggest that a kinase inhibitor of GRK5 would not prevent the activation of NFAT and not be effective in HF. We suggest that a more effective strategy would be to develop a therapy that is capable of inhibiting the nuclear accumulation of GRK5.
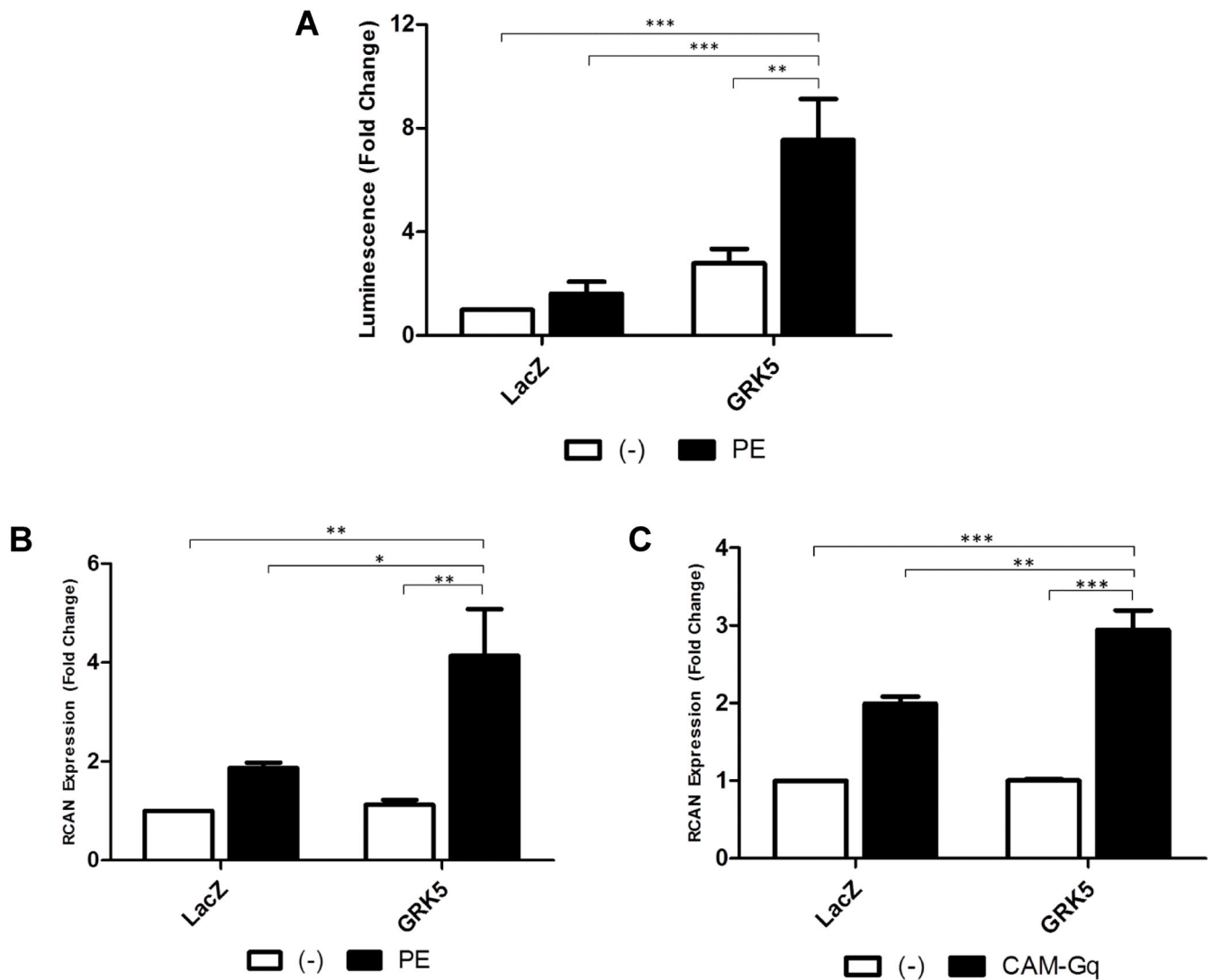
Figure 1. GRK5 Enhances Cardiac NFAT Transcriptional Activity In Vitro
(A) NFAT luciferase activity in neonatal rat ventricular myocytes (NRVMs) infected with an NFAT luciferase reporter virus as well as a GRK5 or LacZ control virus. Cells were stimulated for 24 hours with 50µmol/L phenylephrine (PE). (n=6. **,p<0.01; ***, p<0.001 by ANOVA). (B) Quantitative RT-PCR for NFAT target gene RCAN1.4 (RCAN) in NRVMs overexpressing GRK5 or LacZ control for 48 hours and stimulated with 50µmol/L PE for 16 hours. (n=3. *,p<0.05; **, p<0.01 by ANOVA). (C) Quantitative RT-PCR for NFAT target gene RCAN in NRVMs overexpressing GRK5 or LacZ control adenovirus with or without CAM-Gq adenovirus 48 hours after adenoviral infection. (n=3. **, p<0.01; ***, p<0.001 by ANOVA).
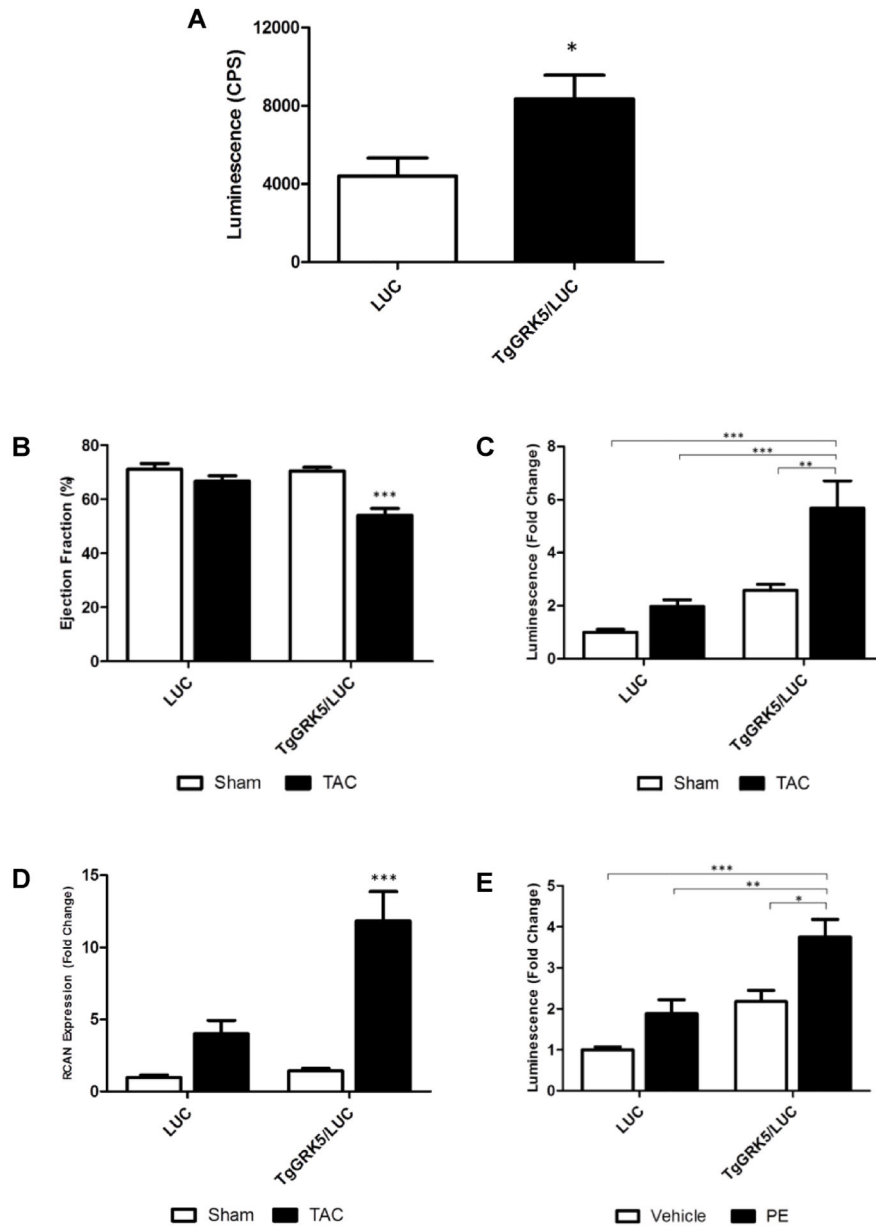
Figure 2. GRK5 Enhances NFAT Transcriptional Activity In Vivo in Models of Cardiac Pathology

(A) Ex vivo NFAT luciferase assay from whole heart of NFAT-luciferase reporter mice with concomitant GRK5 overexpression (TgGRK5/LUC) or littermates with endogenous levels of GRK5 (LUC). Hearts were removed from 8-12 week old mice. (LUC n=6, TgGRK5/LUC n=8. *,p<0.05 by t-test). (B) Ejection fraction was determined by echocardiography in TgGRK5/LUC or LUC littermates with endogenous levels of GRK5 2 weeks after transaortic constriction (TAC) or sham surgery (SHAM). (C) Ex vivo NFAT luciferase assay from whole heart of TgGRK5/LUC or LUC littermates after TAC. (D) Quantitative RT-PCR for NFAT target gene RCAN from whole heart of NFAT luciferase reporter mice. (LUC SHAM n=10, LUC TAC n=13, TgGRK5/LUC SHAM n=17, TgGRK5/LUC TAC n=17. **,p<0.01; ***,p<0.001 by ANOVA). (E) Ex vivo NFAT luciferase assay from whole

heart of TgGRK5/LUC mice or LUC littermates after 24 hours of PE administration (35 mg/kg/day). (LUC PBS n=9, LUC PE n=7, TgGRK5/LUC PBS n=11, TgGRK5/LUC PE n=15. **,$p<0.01$; ***,$p<0.001$ by ANOVA).
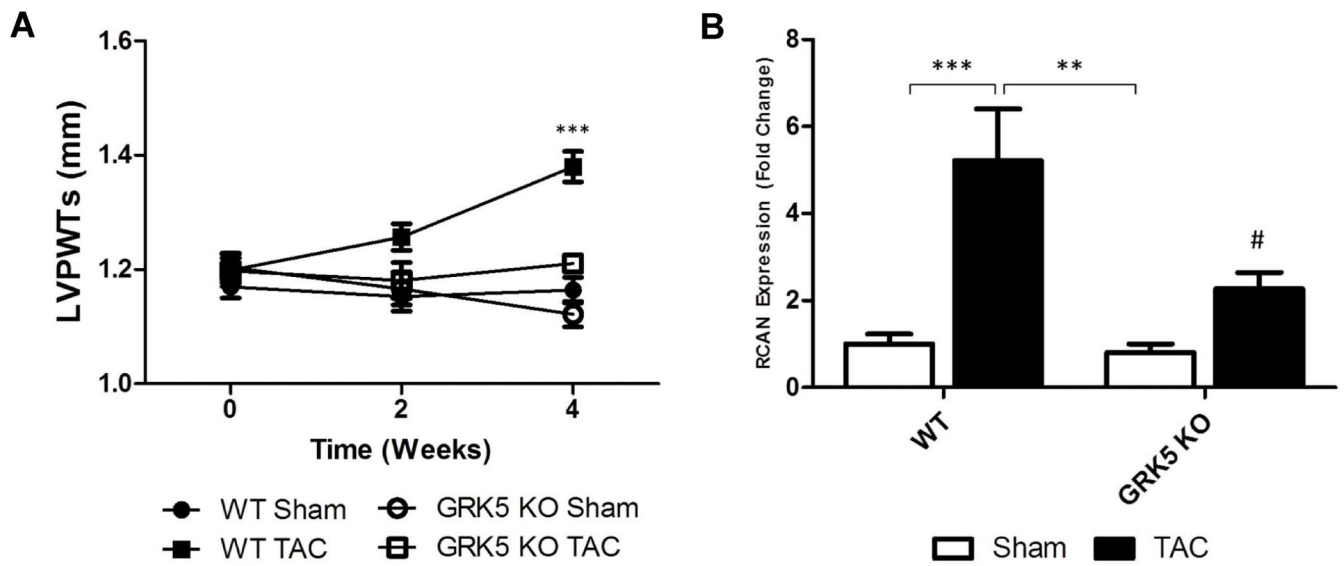
Figure 3. GRK5 KO Mice Demonstrate Attenuated NFAT Transcriptional Activity Following Hypertrophic Stress

*(*A) Left ventricular wall thickness during systole (LVPWTs) as measured by echocardiography in GRK5 null mice (GRK5 KO) at baseline, 2 and 4 weeks after transaortic constriction (TAC) or sham surgery (SHAM) as compared to wildtype (WT) controls. (WT SHAM, *closed circle,* n=10; WT TAC, *closed square,* n=10; GRK5 KO SHAM, *open circle,* n=6; GRK5 KO TAC, *open square,* n=8. ***,$p<0.001$ by ANOVA). (B) Quantitative RT-PCR for NFAT target gene RCAN from whole heart of GRK5 KO mice 4 weeks after TAC or SHAM as compared to WT. (**,$p<0.01$; ***,$p<0.001$; #,$p<0.05$ vs WT TAC by ANOVA).
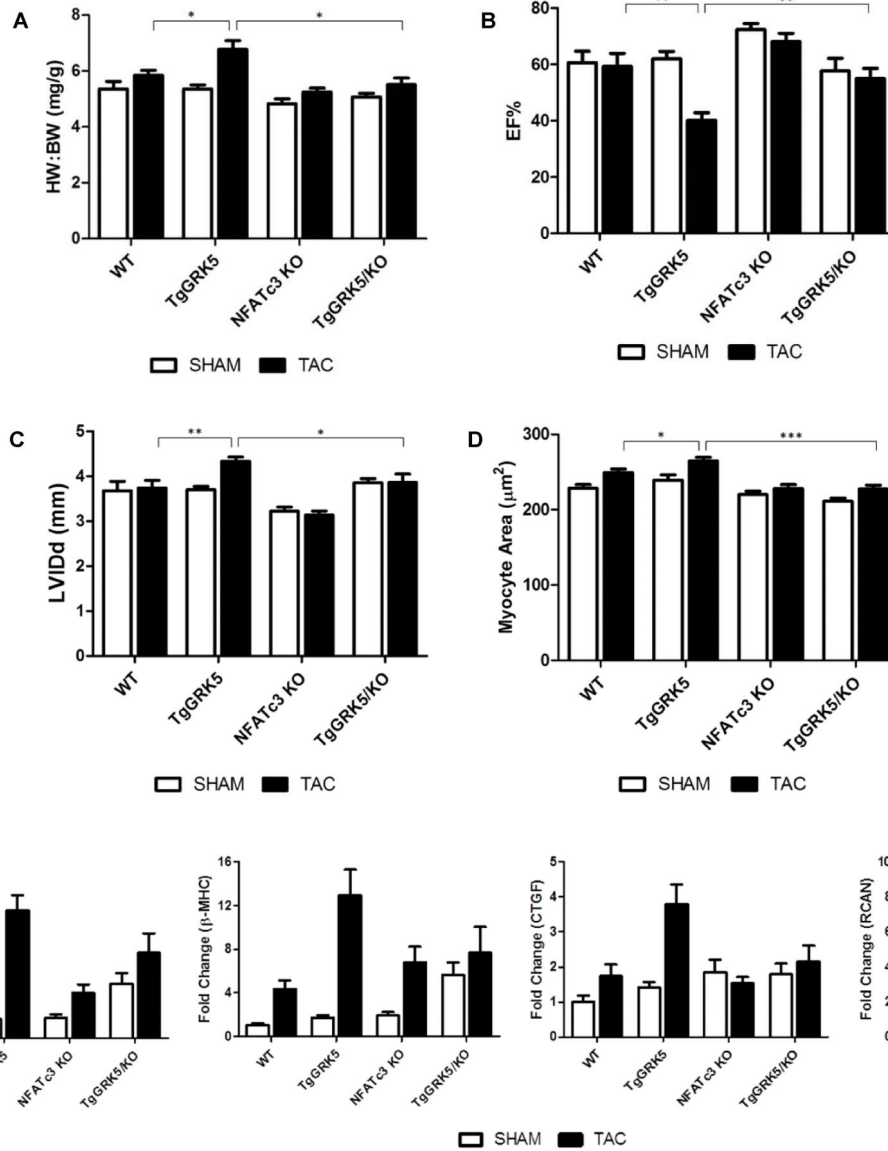
Figure 4. NFATc3 KO Attenuates GRK5-Mediated Cardiac Pathology After Pressure-Overload Stress

(A) Heart weight to body weight ratio (B) ejection fraction as determine by echocardiography (C) left ventricular internal chamber dimension during diastole (LVIDd) as determined by echocardiography of WT, TgGRK5, NFATc3 KO, TgGRK5/NFATc3 KO mice 1 week after TAC or SHAM. (WT SHAM n=8, WT TAC n=9, TgGRK5 SHAM n=11, TgGRK5 TAC n=11, NFATc3 KO SHAM n=10, NFATc3 KO TAC n=9, TgGRK5xNFATc3 KO SHAM n=7, TgGRK5xNFATc3 KO TAC n=7. *, p<0.05; ** p<0.01; ***, p<0.001 by t test). (D) Cardiomyocyte cross-sectional area. (n=3 hearts per group with a minimum of 50 cells measured per heart. *, p<0.05; ***, p<0.001 by t test). (E) Quantitative RT-PCR for genes Acta-1, β-MHC, CTGF, RCAN from whole heart of the above double transgenic mice.
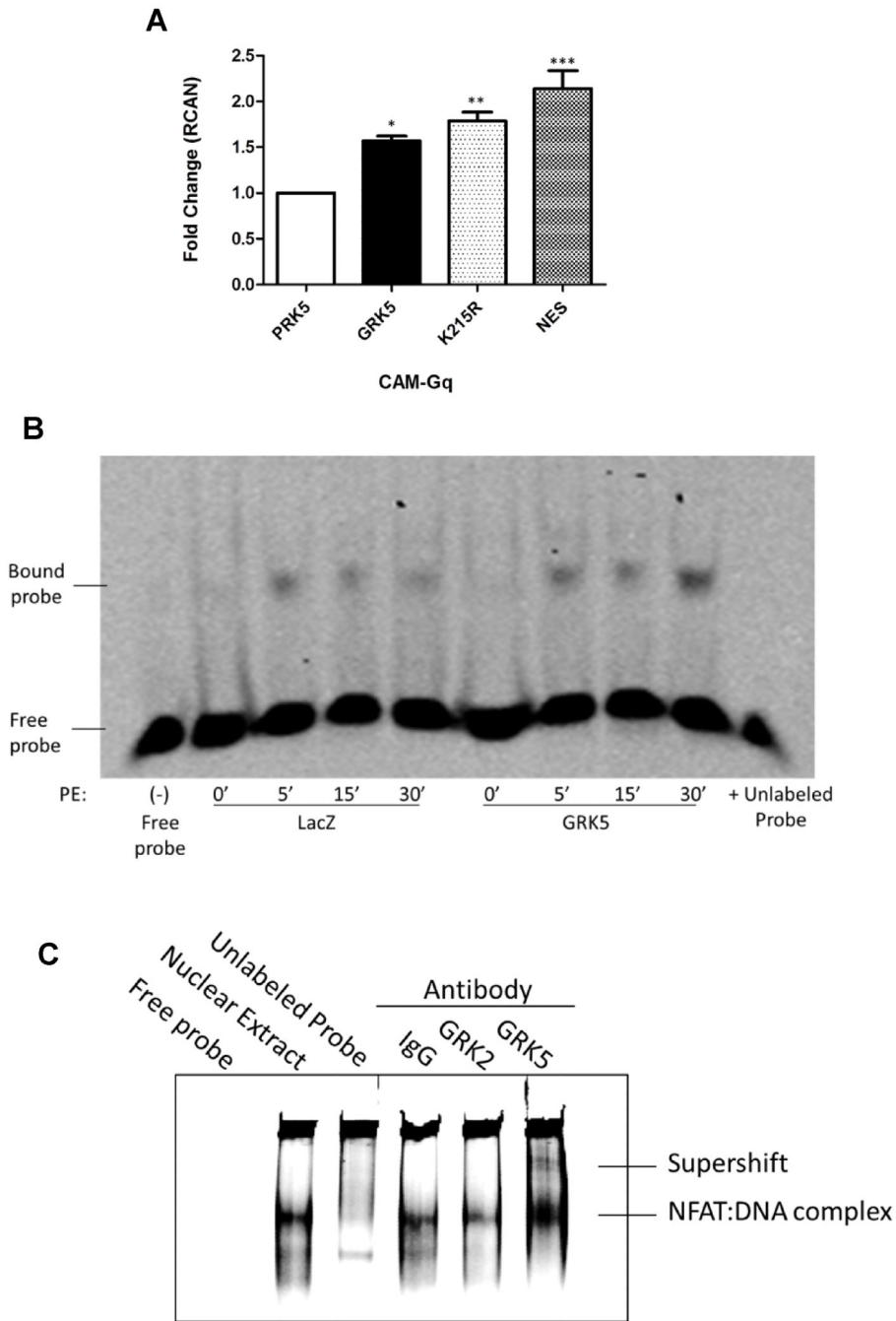
A

B

C

Figure 5. GRK5 Interacts with NFAT in a Kinase-independent Manner Through DNA Binding
(A) Quantitative RT-PCR for NFAT target gene RCAN in H9c2 cells transfected with wild-type GRK5, kinase dead (K215R) GRK5 mutant, nuclear export sequence (NES) GRK5 mutant or vector (pRK5) control for 72 hours and infected with CAM-Gq virus for 24 hours. (n=3; *, p<0.05; ** p<0.01; ***, p<0.01 by ANOVA). (B) Electrophoretic mobility shift assay was carried out with 2 μg of nuclear extract (NE) from cultured NRVMs infected with GRK5 or LacZ control and stimulated with hypertrophic agonist phenylephrine (PE) for 0, 5, 15 or 30 minutes to analyze DNA binding activity of NFAT using IR dye-labeled

oligonucleotides containing the consensus NFAT binding sequence. Lane 1 is free probe no NE; lanes 2 to 9 GRK5 overexpression and 50μmol/L PE treatment as indicated; lane 10 NE from GRK5 overexpressing myocytes stimulated with 50μmol/L PE for 30 minutes and 200-fold excess non-labeled consensus oligonucleotides. Shown is a representative image from 3 independent experiments. (C) Antibody-mediated super-shift EMSA using 5μg NE from AdGRK5 infected (NRVMs) stimulated with PE for 30 minutes. Samples were incubated with 1 μg specific antibodies to GRK2, GRK5 or rabbit IgG. Shown is a representative image from 3 independent experiments.
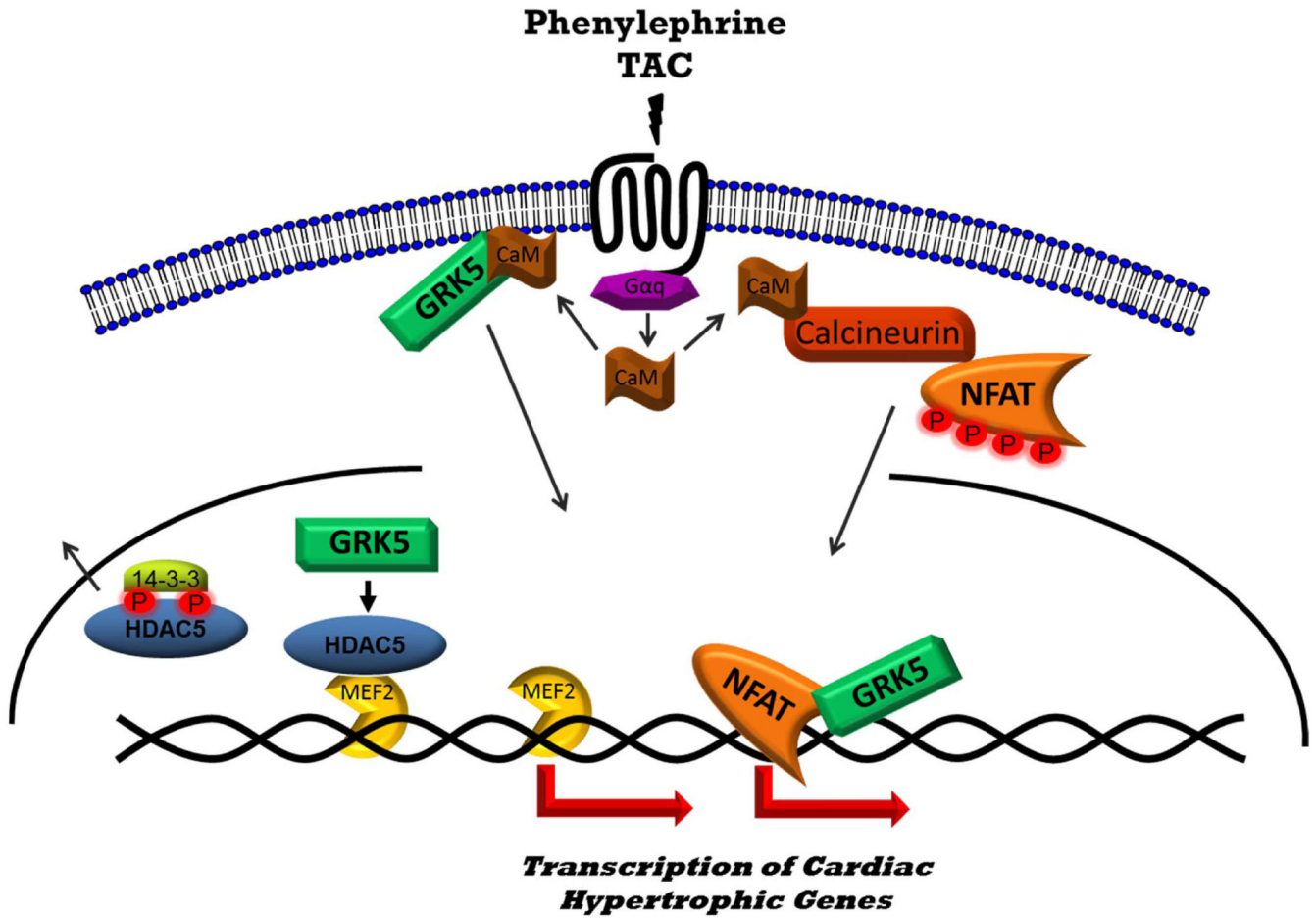
Figure 6. Schematic Depicting the Facilitation of Hypertrophic Transcription by GRK5
Nuclear translocation of GRK5 occurs via stimulation of the Gq pathway following transaortic constriction (TAC) or phenylephrine stimulation via activated calmodulin (CaM) binding the N-terminus of GRK5. CaM binding causes GRK5 to dissociate from the plasma membrane and translocate to the nucleus [13]. Once in the nucleus, GRK5 phosphorylates HDAC5 leading to its nuclear export and derepression of the transcription factor MEF2. In parallel, CaM also binds to and activates the phosphatase calcineurin which dephosphorylates NFAT leading to its nuclear translocation. At the level of the DNA, GRK5 potentiates NFAT:DNA binding and enhances the transcription of hypertrophic genes and subsequent maladaptive cardiac hypertrophy.