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“The Expanding GRK Interactome: Implications in Cardiovascular Disease and Potential for Therapeutic Development”

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Abstract

Heart failure (HF) is a global epidemic with the highest degree of mortality and morbidity of any disease presently studied. G protein-coupled receptors (GPCRs) are prominent regulators of cardiovascular function. Activated GPCRs are “turned off” by GPCR kinases (GRKs) in a process known as “desensitization”. GRKs 2 and 5 are highly expressed in the heart, and known to be upregulated in HF.

Over the last 20 years, both GRK2 and GRK5 have been demonstrated to be critical mediators of the molecular alterations that occur in the failing heart. In the present review, we will highlight recent findings that further characterize “non-canonical” GRK signaling observed in HF. Further, we will also present potential therapeutic strategies (i.e. small molecule inhibition, microRNAs, gene therapy) that may have potential in combating the deleterious effects of GRKs in HF.

Graphical abstract

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1. Introduction

1.1 GPCR Signaling

Cellular receptors allow for the transduction and amplification of external environmental stimuli into intracellular signals which initiate various cellular functions. Many classes of receptors exist, with the G protein-coupled receptors (GPCRs) or seven-transmembrane receptors comprising the largest group with over 800 members [1]. GPCRs comprise the largest protein superfamily in the mammalian genome and are able to respond to a wide variety of extracellular signals including photons, small molecules, peptides and proteins [2]. This family of receptors is a major target of therapeutic intervention as most physiological processes require signaling through GPCRs including vision, smell, taste as well as neurologic, cardiovascular, endocrine and reproductive function [3].

Upon ligand binding, GPCRs signal intracellularly via downstream activation of heterotrimeric G-proteins. Activated GPCRs are effectively “turned off” in a process known as “receptor desensitization”, whereby the GPCR is phosphorylated by GPCR kinases (GRKs) leading to β -arrestin binding (Figure 1). GPCR phosphorylation by GRKs increases the affinity of the interaction between the GPCR and β -arrestins ultimately leading to either receptor recycling, degradation or β -arrestin mediated signaling [4, 5].

1.2. The GRK family

The seven GRKs are further classified into 3 subfamilies based on sequence and structural similarity: the rhodopsin kinase subfamily (GRK1 and GRK7), the β ARK subfamily (GRK2 and GRK3) and the GRK4-like subfamily (GRK4, GRK5, GRK6) [6]. The rhodopsin kinase subfamily is primarily expressed in the retina and regulates opsins [7]. GRKs 2, 3, 5 and 6 are expressed ubiquitously and are known to play a role in GPCR phosphorylation in the cardiovascular system [8]. Alternatively, GRK4 is found primarily in the testes [9].

GRKs exhibit a tri-domain structure with a strongly conserved, centrally located catalytic domain (Figure 2). Approximately, the first 30 N-terminal amino acids are highly conserved within the GRK superfamily and are necessary for recognition of the activated GPCR [10]. The N-terminal third also contains a regulator of G protein signaling (RGS) homology (RH) domain. It has been demonstrated that the RH domain of GRK2 and GRK3 is able to interact with $G\alpha_q$, while the RH domain of GRK5 and GRK6 has not been shown to interact with G-proteins [11, 12].

The carboxyl (C)-terminal third of GRKs is responsible for membrane localization and is divergent between GRK subfamilies [13]. The ocular GRKs, 1 and 7, contain a short sequence which can be prenylated and this lipid group is responsible for interacting with the plasma membrane. Alternatively, GRK2 and 3 contain a C-terminal domain that binds to free $G\beta\gamma$ subunits after receptor activation [14]. This domain overlaps with a pleckstrin homology (PH) domain, which interacts with membrane phospholipids after $G\beta\gamma$ binding [14]. Meanwhile, GRK4, 5 and 6 cellular localization is mediated by the interaction with phosphatidylinositol 4, 5 bisphosphate (PIP2) within the plasma membrane [15]. GRK4 and GRK6 contain a palmitoylation site that does this while GRK5 is dependent on a positively charged lipid-binding element in the form of an amphipathic helix for membrane association [16, 17].

1.3. The Role of GRKs in the Heart

The first GRK identified in the heart was GRK2, originally named β ARK (or β ARK 1) since it was purified with the β 2-adrenergic receptor (AR) and found to phosphorylate this GPCR as well as other receptors [18]. β ARK2, now known as GRK3, was then cloned and found to be very highly homologous to GRK2 with a very similar tissue distribution; however, expression levels of GRK3 were found to be approximately 10–20% that of GRK2 [19]. GRK5 was later discovered and found to be most highly expressed in the heart and muscle but it is also ubiquitously expressed [20, 21]. Finally, GRK6, which shares a 70% amino acid identity with GRK5, was found to be expressed in the heart only weakly, but with greater expression in tissues such as the brain and skeletal muscle [22].

The importance of GRKs in the heart and in particular during cardiac development has been shown via genetic deletion in animal models. For example, GRK2 homozygous knockout (KO) is lethal in mouse models due to its role in embryonic development of the heart [23, 24]. These animals die by gestational day 15.5, presumably due to heart failure (HF), as they exhibit hypoplasia of the ventricular myocardium and a 70% decrease in ejection fraction [23]. However, the embryonic lethality of GRK2 KO mice is not cardiomyocyte autonomous as early myocyte deletion does not lead to an abnormal heart or early death [24]. Conversely, the GRK5 homozygous KO mouse is viable, and no adverse effects have been attributed to this gene deletion [9]. GRK5 has been found to fine tune cardiac development in the zebrafish through the mTOR pathway. Zebrafish lacking the GRK5 homologue Grk51 develop hearts with incorrect symmetry [25]. Further, this study demonstrated for the first time embryonic lethality in GRK5/GRK6 double KO mice [25]. Previously, it was assumed that GRK5 was dispensable for embryonic development; however, this finding suggested that GRK6 is able to compensate for the loss of GRK5 in the mouse embryo [25].

As the heart transitions to disease, various cellular processes become dysregulated leading to diminished cardiac function that usually occurs after significant myocyte cell death. The failing heart (both human and animal models) has certain consistent molecular characteristics including desensitization and down-regulation of β -ARs, dysregulation of calcium (Ca^{2+}) handling and activation of the fetal gene program leading to the transcription of hypertrophic genes [26]. In health, catecholamines (i.e. norepinephrine and epinephrine) produced by sympathetic nervous system (SNS) neurons and the adrenal medulla act on α - and β -ARs in the heart to maintain homeostatic growth, function, and contractility. Sympathetic catecholamines can rapidly increase the rate and force of myocardial contractions in response to stress and exercise in the so-called “fight-or-flight” response. This crucial homeostatic process becomes abnormal in HF as the SNS is activated after cardiac injury as it senses a loss of cardiac output. However, over time augmented catecholamine production causes the desensitization and down-regulation of β -ARs through induction of GRK2 expression leading to insensitivity to the fight-or-flight response (Figure 3A). This creates and perpetuates the vicious cycle of neurohormonal bombardment of the injured heart that cause increased cell death and further HF progression [27]. GRK5 is also up-regulated in the failing heart, and GRK's appear to play a crucial role in deconstruction of the fight-or-flight response in HF [28].

The loss of inotropic reserve in HF was the impetus for the use of β -AR agonist therapy. Although this strategy can be effective acutely, chronic administration has been shown to increase mortality [29]. Somewhat paradoxically, β -AR antagonists are the first line therapy for HF patients since they block the aforementioned catecholamine bombardment and rescue myocytes from the noxious effects of these neurohormones [30]. Although β -blockers reduce hospitalization and improve HF care, they leave a lot to be desired with respect to effective reversal of disease; further, animal models show only modest effects to increasing failing heart function [31–33]. Thus, new therapies are needed and data from our lab has shown that GRK inhibition appears to be a novel therapeutic strategy capable of restoring the fight-or-flight response, inducing enhanced cardiac function, and reversal of disease in various animal models [28]. This was first shown over 20 years ago through the discovery that inhibition of GRK2 by a C-terminal peptide known as β ARKct, could prevent GRK2 from binding to $\text{G}\beta\gamma$ and the membrane leading to increased cardiac contractile function [34]. Over the last two decades, this research has expanded to clearly define GRK2 inhibition as an innovative emerging HF treatment, and additional studies have also identified GRK5 as a potential therapeutic target.

2. GRK2 Regulation of Cardiac β -ARs in Health and HF

As described above, β -ARs are the most powerful means by which the heart can increase its contractile function in response to SNS activity and catecholamine production [28]. Further, β -AR dysregulation is a hallmark of the failing heart's molecular signature such that these receptors are significantly down-regulated with remaining receptors desensitized due to elevated GRK2 and GRK5 expression [28]. In healthy myocardium, the predominant β -ARs present in the heart are the β 1 and β 2 subtypes, which are expressed in an approximate 80:20 ratio, respectively with only a relatively minor contribution of β 3-ARs [35, 36]. The ratios of each β -AR are important with respect to function, but this becomes increasingly relevant in

disease. Of note, although all 3 β -AR subtypes can activate Gs and adenylate cyclase leading to increased cAMP production and promotion of myocyte contractility, they have significant individual and unique signaling processes that become critical in HF. For example, β 1-ARs can promote myocyte cell death, while β 2-ARs actually activate cell survival pathways and can even protect against β 1-AR mediated cell death [37–39]. β 3-ARs have also been shown to be up-regulated in HF, but are not regulated by GRKs. Interestingly, these receptors are protective in the heart presumably due to the fact that they couple to nitric oxide (NO) pathways [40]

The overall significance of the various signaling pathways associated with each β -AR becomes critical during HF progression since loss of β -AR density has been known for the last 40 years to be selective for the β 1-AR [41–42]. Consequently, the ratio of β 1: β 2 AR's is closer to 50:50 [43] and since β 3-ARs are also elevated [36], there is a significant alteration of signaling especially in terms of cell survival. In fact, it has been suggested that β 3-ARs can act as a “physiological brake” to reduce the effects of SNS overstimulation at the level of the β 1-AR [44]. However, it still should be emphasized that the overall loss of β -AR density severely cripples the contractile response of the failing heart.

The central importance of β -AR dysregulation in HF was first found to involve GRK2 in 1993 [45] as this GRK was found to be significantly up-regulated at mRNA, protein, and activity level in explanted failing human hearts. This filled in a mechanistic gap to explain why remaining β -ARs in failing myocardium were largely desensitized and uncoupled from G-proteins, thus leaving no doubt that increased GRK2 expression was responsible for this phenomena [46]. Over the last 20 years several animal models as well as further human studies have demonstrated that GRK2 is indeed elevated in cardiac disease and HF [28]. Accordingly, activity and expression of GRK2 in HF is generally inversely correlated with β -AR expression [47].

The exact mechanism responsible for inducing GRK2 elevation in the compromised myocyte is not clear yet as it can occur rapidly after the heart is injured [48]. It is presumed that GRK2 expression is regulated by increased catecholamine production as a result of the fight-or-flight response's attempt to stimulate contraction of the weakened myocardium [28]. This is supported by studies where GRK2 levels are elevated in the heart after chronic β -AR agonist administration [49]. This hypothesis extends not only to cardiomyocytes that are flooded with norepinephrine after injury but also to circulating white blood cells exposed to increased plasma epinephrine levels [50]. Interestingly, studies have shown that GRK2 levels in peripheral lymphocytes mirror those found in the failing heart [50–52], and these correlate strongly with increased lymphocyte GRK2 levels inversely correlating with cardiac function such that lower levels are associated with improved myocardial function in HF [50–51]. These findings have led to a hypothesis that GRK2 levels in blood can be used as a biomarker in HF, which is quickly becoming an exciting reality as a recent study in over 200 patients demonstrated that GRK2 levels had independent power as a prognostic marker of mortality and events in HF patients [53].

The concept that GRK2 may be a nodal regulator of cardiac dysfunction after injury has been validated over the last two decades using genetically engineered mouse models. In

1995, the first transgenic mouse was generated [34] with cardiac-specific overexpression of GRK2 similar to levels observed in HF, and these mice presented with a loss of β -AR inotropic reserve [34]. Within this study, mice were generated that expressed a 194 amino acid carboxy-terminal fragment of GRK2, β ARKct, as a presumed GRK2 inhibitor in myocytes, which resulted in a mouse with chronic elevation of baseline contractile function and super-sensitivity to β -AR stimulation [34]. A subsequent study demonstrated that β ARKct expression could reverse the β -AR dysregulation induced by GRK2 overexpression (Figure 3B) [54]. Thus, the field of investigating GRK2 in the normal and failing heart was established. Over the last 20 years, several studies have solidified and supported GRK2 inhibition as a novel therapeutic target in HF.

Most studies to test the hypothesis that GRK2 may be a target for HF therapy have been conducted using β ARKct. The first study to support this hypothesis was the prevention of HF in the Muscle Lim Protein (MLP) KO mouse model, which is an established model of dilated cardiomyopathy (DCM) from birth wherein myocardial GRK2 levels were found to be elevated [55]. Importantly, MLP KO mice expressing β ARKct in cardiomyocytes produced a mouse model that never developed cardiomyopathy and demonstrated the prevention of HF when GRK2 is inhibited in the heart [55]. Similarly, this finding was reaffirmed in a mouse model of caldesmon (CSQ) overexpression, which is HF characterized by decompensated hypertrophic cardiomyopathy and reduced survival [56]. Expression of β ARKct increased contractile function, prevented adverse remodeling, and enhanced lifespan in these mice [56]. In this study, the beneficial effects of β ARKct were potentiated by co-administration of metoprolol, a β -AR blocker, suggesting that clinically, GRK2 inhibition may be able to complement established HF treatments [56]. In addition, transgenic β ARKct expression has been shown to prevent systolic dysfunction and exercise intolerance while also decreasing cardiac remodeling and hypertrophic gene expression in a model of hypertrophic cardiomyopathy, wherein a missense mutation was created in myosin heavy chain [57]. All of these models demonstrated that β ARKct normalized all β -AR signaling abnormalities including normalizing β -AR density and functional responses, which were characteristic of HF phenotypes.

It is likely that inhibition of GRK2 with β ARKct will have off-target effects due to the fact that it can inhibit $G\beta\gamma$ dependent cellular interactions. Thus, further studies have been performed to evaluate GRK2 inhibition as a viable therapeutic treatment in mice with genetic ablation of GRK2. As detailed above, homozygous deletion of GRK2 is embryonic lethal [23], therefore two conditional models of GRK2 ablation were generated (α MHC-Cre/GRK2^{flox/flox} and MerCreMer/GRK2^{flox/flox}), which allow for targeted KO of GRK2 only in cardiomyocytes either at birth (α MHC-Cre) or induced cardiac deletion after tamoxifen administration, respectively [32]. Of note, adult global heterozygous GRK2 KO mice do present with significantly enhanced cardiac contractile function to a similar level as observed in β ARKct transgenic mice [58]. Importantly, both models of cardiac-specific GRK2 KO resulted in positive outcomes after myocardial infarction (MI) [32]. Constitutive (at birth) cardiac GRK2 KO in mice prevented HF development chronically after MI and inducible GRK2 KO mice where GRK2 was ablated in myocytes after left ventricle (LV) dysfunction was apparent post-MI significantly improved cardiac function and induced positive reverse remodeling [32]. In fact following GRK2 KO post-MI, mice exhibited reduced mortality

demonstrating a robust cardioprotective effect of lowering GRK2 levels and supporting the hypothesis that elevated myocardial GRK2 post-injury is pathological. These effects were significantly better than results when the β -blocker metoprolol was used to treat post-MI wild-type (WT) mice over the same time period [32].

Overall, studies in cardiac GRK2 KO mice mirror results with β ARKct expression supporting the fact that this peptide is inhibiting GRK2 and independent $G\beta\gamma$ inhibition is not a major mechanism of action. However, some subtle molecular differences have been found including differences in how the L-type Ca^{2+} channel is regulated as β ARKct inhibits $G\beta\gamma$ -mediated inhibition while loss of GRK2 increases Ca^{2+} current by a distinct mechanism [59, 60]. At present, no further potential mechanisms have been identified for the beneficial effects of GRK2 KO, but it is likely that more molecular pathways are involved in this signaling paradigm.

2.1 GRK2 Manipulation in Acute Myocardial Injury

As described above, it is clear that GRK2 is detrimental to contractile function in chronic HF models; however more recently, data has demonstrated that having lowered GRK2 activity or expression at the time of injury leads to beneficial effects and outcomes. Accordingly, mechanistic data has shown that GRK2 up-regulation is one of the first cardiac adaptations observed after acute ischemic injury (Figure 4) [48]. Thus, studies have been conducted to investigate GRK2 in ischemia/reperfusion injury (I/R) injury. The first indication that GRK2 was critical in acute ischemic injury was data obtained in cardiac GRK2 overexpressing and β ARKct transgenic mice [61]. First, mice with cardiac-specific overexpression of GRK2 exhibited diminished cardiac function, and increased cell death in a model of I/R induced injury [61]. Interestingly, mice expressing β ARKct, are protected from I/R induced injury such that cardiac function was preserved, and cell death was significantly diminished post I/R [61]. Mechanistically, cardiac-specific expression of β ARKct promoted Akt activation, and stimulation of nitric oxide (NO) production via increased endothelial nitric oxide synthase (eNOS) activity in ischemia [61]. Conversely, GRK2 overexpression negated these beneficial effects, in agreement with a prior finding that GRK2 binds Akt leading to its inhibition [61, 62]. β 2-AR signaling was shown to be critical to this signaling paradigm such that ICI-118,551, a β 2-AR antagonist abrogated the beneficial effects of β ARKct, while fenoterol, a β 2-AR agonist, led to cardioprotection in GRK2 overexpressing mice [61]. These findings correlate with the fact that β 2-AR signaling is considered to be cardioprotective in HF [38].

Data concerning I/R injury, GRK2, NO and the Akt signaling pathway have uncovered an interesting and novel mechanistic picture. Studies have shown that NO production can lead to Akt activation [63], however, increased NO bioavailability also can lead to GRK2 inhibition via direct S-nitrosylation at Cys340 [64]. Since GRK2 activity can decrease eNOS activation after I/R [61], there appears to be a dynamic negative interplay between GRK2 and eNOS-NO signaling. Interestingly, S-nitrosylation (SNO) of proteins has been postulated as a strategy to reduce I/R induced injury in the heart [65]. A recent study has directly demonstrated that SNO of GRK2 is critical to the myocardial response to ischemic injury [66]. In this study it was found that an interaction between GRK2 and eNOS

increased with ischemic injury [66], further cardiomyocyte specific overexpression of eNOS protected against the increased I/R injury and myocyte cell death observed with GRK2 overexpression [66]. Consistent with this interplay, eNOS KO mice with cardiac GRK2 overexpression had an exacerbation of cell death and injury post I/R [66]. Interestingly, the interplay between GRK2 and eNOS was also evident in β ARKct transgenic mice as the cardioprotection afforded by β ARKct after I/R injury was significantly reduced in the background of eNOS KO, thus demonstrating the importance of NO in this mechanism of GRK2 inhibition [66]. The direct relevance of GRK2 S-nitrosylation in ischemia was shown in a novel knock-in mouse model where Cys340 of GRK2 was mutated to a serine (GRK2-C340S), thereby preventing GRK2-SNO [66]. GRK2-C340S mice displayed higher GRK2 activity, increased susceptibility to I/R induced injury, and reduced activation of Akt signaling [66]. Taken together, it is clear that the interaction of GRK2 with eNOS is crucial for ischemic cardioprotection, and NO is protective in the heart with GRK2 being a nodal target via its inhibition by SNO at Cys340 [66].

Studies detailed above showing the cardioprotective nature of β ARKct after I/R injury have been recapitulated in cardiac-specific GRK2 KO mice [67]. This validates the pro-death and pathological nature of GRK2, and the fact lowering its expression in cardiomyocytes even before injury is beneficial. Cardiac-specific GRK2 KO mice have significantly less cell death after I/R in part due to increased release of the anti-apoptotic molecules, Bcl-XL and Bcl-2 [67]. Further, as seen in β ARKct mice, GRK2 KO mice also exhibited increased phosphorylation of Akt, a key pro-survival pathway within the myocyte [67]. Thus, both lowering GRK2 and inhibiting its membrane targeting leads to robust cardioprotection adding acute benefits to chronic HF benefits associated with GRK2 targeting.

Recent studies have revealed novel mechanistic insight into GRK2's role in I/R injury as a pro-death kinase in the heart [67, 68]. Taken together, these findings greatly add to the pathology of acute ischemic injury, and implicate mitochondrial localization of GRK2 in this process [67, 68]. Data generated recently has found GRK2 to exist within the mitochondria [68–70], and in fact, GRK2 is found in all mitochondrial compartments including the matrix [69]. Mitochondria are important in both regulation of myocyte cell metabolism and cell death [69–71]. Interestingly, GRK2 KO mice post I/R displayed less cytochrome C release from mitochondria demonstrating that GRK2 levels within the mitochondria can control the “apoptosome complex” [67].

Even though GRK2 is localized within mitochondria basally, our lab has uncovered the novel finding that under conditions of increased oxidative stress such as those observed in I/R injury, GRK2 can translocate and accumulate in mitochondria leading to cell death [68]. This is due to stress-mediated phosphorylation of GRK2 at Ser670 by MAP kinases [68]. This phosphorylation event stimulates GRK2 interaction with a chaperone protein, Heat Shock Protein 90 (Hsp90) promoting translocation to mitochondria and eliciting the non-canonical pro-death activity of GRK2 [68]. These data also revealed that the cardioprotection inferred by β ARKct expression is not due solely to its G β γ binding at the plasma membrane [68]. Rather, the β ARKct peptide contains Ser670 and can also be phosphorylated by MAP kinases resulting in inhibition of full-length GRK2 binding to Hsp90 and ultimately mitochondrial localization [68]. Accordingly, the localization of

β ARKct to mitochondria does not promote cell death. Of note, a recent study has also found that chronic hyper-aldoosteronism, a potent activator of myocyte oxidative stress, induces GRK2 mitochondrial accumulation and this appears to be a major mechanism of cardiac pathology for this neurohormone [72].

Importantly, GRK2 elevation in myocytes itself has recently been shown to promote reactive oxygen species (ROS) generation, including induction of mitochondrial-based superoxide formation [69, 73]. Further, under conditions of chronic β -AR stimulation, GRK2 overexpression has been shown to increase mitochondrial ROS generation via up-regulation of NADPH oxidase-4 (NOX-4) leading to myocyte cell death [73]. Increased mitochondrial ROS induced by GRK2 also appears to negatively affect mitochondrial metabolism [69]. Heightened GRK2 levels in myocytes such as those observed in HF, significantly depress fatty acid oxidation, a primary source of energy in the adult heart [69]. Conversely, β ARKct expression can greatly enhance basal respiration rate and ATP production in myocytes [69]. These findings have been reaffirmed in a study wherein GRK2 levels were shown to be upregulated resulting in reduced fatty-acid oxidation in an in vivo model of perturbed energy substrate utilization and HF [74]. Further, GRK2 inhibition by GRKInh, a novel peptide inhibitor of GRK2, prevented the dysfunctional cardiac energetics present in this model [74]. Taken together, it is evident that GRK2 is a critical regulator of mitochondrial bioenergetics, and up-regulation of GRK2 within this cellular compartment leads to an altered redox state that appears to make the myocardium more susceptible to injury.

2.2 GRK2 Inhibition in the SNS aids in HF Rescue

One constant of the mouse studies with GRK2 KO or β ARKct expression in HF or post-I/R is that these interventions normalized the cardiac β -AR system demonstrating a resensitization of adrenergic signaling, which was hypothesized to restore the fight-or-flight response. Interestingly, this underlying hypothesis has been supported not only by cardiac studies in mice and β ARKct gene therapy studies described in detail below, but also in studies investigating GRK2 activity within the SNS, specifically at the level of the adrenal gland and catecholamine secretion in HF. Data has revealed that analogous to the β -AR signaling dysregulation observed in the failing heart when SNS activity is high, the α 2-AR signaling system in the adrenal gland is dysregulated with receptors being overly desensitized and downregulated due to a significant upregulation of GRK2 expression [75]. Since the α 2-ARs represent the only negative-feedback mechanism for catecholamine secretion in this system, elevated GRK2 expression in this organ induces heightened catecholamine release contributing to the aforementioned vicious cycle. First using β ARKct gene delivery to the adrenal gland [75, 76] and subsequently, sympathetic-targeted GRK2 KO [77], it was found that lowering GRK2 activity could reset α 2-ARs and reduce catecholamine release. Thus, this sympatholytic effect of GRK2 inhibition could in itself lead to positive outcomes in HF. Interestingly, exercise in HF was also found to not only improve GRK2 levels and β -AR signaling in the heart, but also lead to reduction of GRK2 levels in the adrenal gland supporting the notion that these sympatholytic effects can improve cardiac responses [78]. β -blockers also appear to have the capacity to lower GRK2 levels in both the heart and the adrenal gland, which appears critical to any sympatholytic effect of this HF therapy [79].

2.3 Translational HF Studies with GRK2 Inhibition

The results of GRK2 inhibition in transgenic mouse models had proven promising; however, translational studies were needed to determine whether this is a viable option for HF treatment. Indeed, studies have been done to support successful translation. Studies have been ongoing for several years with myocardial, viral-mediated β ARKct gene delivery [80]. Early studies using an adenoviral- β ARKct vector showed that within the confines of short-term myocardial β ARKct expression in rabbits with cardiac injury and HF, there was pronounced improvement of cardiac function and normalization of the β -AR system [48, 81–83]. The latter results appear to be consistent with resensitization of β -AR signaling as well as the sympatholytic effects of adrenal targeting of β ARKct. Importantly, indirect sympatholysis with cardiac β ARKct expression in failing myocardium was proven in a chronic rat HF model using adeno-associated virus serotype 6 (AAV6) delivery of β ARKct where not only was GRK2 inhibited and β -AR reset in the heart, but this led to significant decreases in circulating catecholamines demonstrating that this is an added benefit of GRK2 inhibition in failing myocardium [33]. Moreover, AAV6- β ARKct delivery chronically led to enhanced cardiac function and reverse remodeling of the LV [33].

Recent large animal studies with AAV6- β ARKct have taken this HF gene therapy approach to the brink of human translation. Studies in sheep and post-MI HF pigs have demonstrated that GRK2 inhibition with β ARKct is a viable gene therapy strategy [84, 85]. Delivery of AAV6- β ARKct during cardiac arrest and coronary pump-perfusion in an ovine HF model resulted in preserved cardiac function and significant reduction in myocyte cross-sectional area [84]. Moreover, in a porcine model of HF, AAV6-mediated delivery of β ARKct post-MI, using a coronary sinus retrograde perfusion method, led to significantly increased cardiac function, normalization of the neurohormonal axis, and inhibition of adverse remodeling and induction of the fetal gene program [85]. Taken together, these studies suggest that GRK2 inhibition is a promising therapy for the treatment of HF. To this end, the next logical progression of this work, a Phase I clinical trial utilizing AAV6- β ARKct is actively being pursued through the United States Food and Drug Administration.

While β ARKct gene therapy translation is being pursued, the development of a novel small molecular inhibitor of GRK2 may provide a safer, less costly approach to treating HF. As a result, the Tesmer group identified the selective serotonin reuptake inhibitor (SSRI) paroxetine as a small molecular inhibitor of GRK2 utilizing high-throughput screening [86]. This study demonstrated that paroxetine, as an off-target (μ M range of affinity), could bind to and inhibit the catalytic activity of GRK2 ultimately leading to in vitro enhancement of myocyte contractility. Utilizing paroxetine as a model for GRK2 inhibition, this work has progressed leading to the development of a more specific GRK2 inhibitor which has 20-fold lower inhibition of serotonin reuptake [87]. Further, GRK2 inhibitor screens have been performed on known compounds resulting in identification of several new inhibitors and renewed interest in identifying novel small molecule GRK inhibitors [88].

Following promising in vitro data demonstrating GRK2 inhibition by paroxetine led to increased myocyte contraction, our laboratory has applied this therapy to a mouse model of MI. Two weeks post-injury, treatment was initiated with either GRK2 inhibitor paroxetine or another SSRI, fluoxetine, which lacks GRK2 inhibitory effects. Following four weeks of

paroxetine treatment, mice demonstrated improved LV structure and function as well as decreased activation of the fetal gene program [31]. Fluoxetine treatment displayed none of these effects demonstrating that the effects of paroxetine were due to GRK2 inhibition [31]. This study provided a proof-of-concept for the use of small molecule inhibitors of GRK2 in the treatment of HF and provides a basis for designing more selective GRK2 inhibitors which could ultimately reverse the progression of HF.

Another potential GRK2 inhibitory small molecule target, analogous to β ARKct, is $G\beta\gamma$. Interestingly, inhibition of $G\beta\gamma$ by the compound M119 prevented HF in an acute mouse model utilizing infusion of β -agonist isoproterenol [89]. Further, gallein, a highly related M119 analog, prevented the progression of chronic HF in the aforementioned CSQ transgenic mouse [89]. More recently, gallein was utilized in a model of trans-aortic constriction (TAC)-induced HF, wherein gallein improved cardiac function, prevented adverse remodeling and most importantly promoted cell survival [90]. Of note, the authors attributed these benefits not only to the role of $G\beta\gamma$ inhibition in the myocardium, but in the adrenal glands as well consistent with the above-described adrenal studies targeting GRK2 with β ARKct. Thus, systemic inhibition of GRK2 by a small molecule may have additional benefits over myocardial targeted gene therapy.

3. GRK5 in Pathology

As recently reviewed, GRK5, another GRK highly expressed in the myocardium, has also been shown to be upregulated in HF [91]. Studies involving mice with cardiac-specific GRK5 overexpression (TgGRK5) have revealed GRK5's role within the myocardium. TgGRK5 mice displayed increased β -AR desensitization, but unlike GRK2 overexpressing mice were incapable of attenuating angiotensin II induced contractile effects suggesting GRKs possess in vivo receptor specificity [92]. Perhaps more importantly, TgGRK5 mice have been used implicate GRK5 as a key regulator of the pathological cardiac hypertrophy with this role being independent of any GRK activity on receptors (Figure 5). Specifically, TgGRK5 mice present with exaggerated hypertrophy and early onset HF after TAC injury [93]. Mechanistically, in a $G\alpha_q$ dependent process, GRK5 exhibits its non-canonical activity by translocating to the nucleus of cardiomyocytes via a functional nuclear localization sequence (NLS) post-TAC [93]. Accordingly in mice where a mutant GRK5 lacking a functional NLS is overexpressed (GRK5-NLS), translocation of GRK5 to the nucleus is prevented leading to preservation of cardiac function and the absence of severe pathology present in TgGRK5 mice [93]. The role of GRK5 in the nucleus was first found to be as a Class II histone deacetylase (HDAC) kinase such that when GRK5 is elevated in the nucleus it phosphorylates HDAC5 resulting in the de-repression of myocyte enhancer factor 2 (MEF2), the hypertrophic transcription factor [93].

This landmark study prompted intense investigation into this non-canonical activity of GRK5. It was discovered that upon $G\alpha_q$ stimulation, intracellular Ca^{2+} levels rise activating calmodulin (CaM), and this results in activated CaM binding to the N-terminus of GRK5 leading to GRK5's nuclear accumulation [94]. Interestingly, receptor specificity has been shown to play a role in GRK5's non-canonical activity. Activation of α_1 -AR and angiotensin II receptors, both of which GRK5 does not desensitize, leads to GRK5 nuclear

accumulation, while stimulation of the endothelin receptor, a receptor desensitized by GRK5, does not [94]. In addition, aldosterone also has been shown to promote GRK5 nuclear translocation [72]. Thus, it is likely that GRK5 is prone to translocate to the nucleus when it is not actively engaged with an activated GPCR. However, Gq-mediated activity is a key event in nuclear GRK5 actions and in pathological hypertrophy subsequent to elevated GRK5 in HF [95].

While in the nucleus, GRK5 clearly exhibits class II HDAC kinase activity phosphorylating HDAC5, leading to its nuclear export and de-repression of MEF2 [93, 94, 96]. This de-repression leads to increased transcription of hypertrophic genes and subsequently pathological hypertrophy. These findings in post-TAC TgGRK5 mice have been reaffirmed in the spontaneous hypertensive HF rat model such that GRK5 has been shown to accumulate in myocyte nuclei [97, 98]. In a subsequent study, it was found that in addition to GRK5's HDAC kinase activity, nuclear GRK5 can act as a facilitator, downstream of hypertrophic stimuli (TAC, phenylephrine and Angiotensin II) of NFAT, another key hypertrophic transcription factor in the heart [99]. In a cardiac-specific NFAT luciferase reporter transgenic mouse, GRK5 overexpression led to increased NFAT activity, while ablation of GRK5 reduced it [99]. In addition, mice with cardiac-specific GRK5 overexpression and NFATc3 KO were protected from the early onset HF observed with increased cardiac levels of GRK5. Mechanistically, GRK5 was shown to act in a kinase-independent manner thereby facilitating NFAT activity as part of a DNA binding complex [99]. Thus, both kinase-dependent and kinase independent activity of GRK5 appears responsible for its pathological role in maladaptive hypertrophy and HF development.

GRK5 has also been shown to interact with the NF- κ B pathway, which may also participate in cardiac hypertrophy after stress. In fact, both GRK 2 and 5 have been shown to regulate p105, the NF- κ B subunit [100, 101]. Further, both of these GRKs are capable of phosphorylating I- κ B α leading to increased inflammation, and the association of GRK5 and NF- κ B has been shown to be evolutionarily conserved [102]. However, the interaction of GRK5 and NF- κ B in the heart has proven somewhat controversial. It has been reported that GRK5 activates NF- κ B through upregulation of two subunits, p50 and p65 [103], which directly opposes a study in which GRK5 was found to inhibit NF- κ B signaling [104]. Thus, it is evident that GRK5 plays a role in regulation of NF- κ B signaling, however further studies are needed to delineate how this interaction affects the cardiovascular regulation imparted by GRK5.

The above studies have clearly delineated that GRK5 overexpression has detrimental effects in cardiomyocytes following several hypertrophic stimuli due to its nuclear activity; however, it was not known if this was indeed physiologically relevant. The absolute requirement of GRK5 in the myocardium has been elucidated utilizing both global and cardiomyocyte-specific GRK5 KO mice [96]. These transgenic mice were subjected to chronic G α_q stimulation via either TAC or osmotic pumping with phenylephrine. Importantly in both models of GRK5 KO, mice exhibited prevention of maladaptive hypertrophy, preserved cardiac function, reduced HDAC5 phosphorylation, and inhibition of fetal gene program activation [96]. This demonstrated that GRK5 is a physiologically relevant HDAC5 kinase and a potential therapeutic target for human HF.

3.1 GRK5 in Physiological Hypertrophy

Physiological hypertrophy, another form of hypertrophy, naturally occurs in the heart during pregnancy and exercise [105]. Physiological hypertrophy is characterized by more uniform myocyte growth with respect to proportional increases in cell width and length resulting in favorable cardiac adaptations (i.e. stimulation of cell survival) [106–107]. In pathology, myocyte length increases in the absence of a proportional increase in cell width leading to reduced chamber size and septal thickening [106]. Recently, it has been reported that GRK5 is solely a regulator of pathological hypertrophy, but not physiological hypertrophy since exercise leads to normal adaptive growth in TgGRK5 mice, and does not induce GRK5 nuclear accumulation [108]. This finding corresponds with a study in which NFAT, a pathological molecule regulated by GRK5, was not shown to regulate physiological hypertrophy [109]. In both forms of cardiac hypertrophy, intracellular Ca^{2+} levels rise. However, it is possible activation of specific Ca^{2+} pools is required for the induction of the pathological events observed in HF (i.e. GRK5 nuclear accumulation, HDAC kinase activity, DNA binding and increased NFAT activity), thus explaining why these events are absent in exercise. Interestingly, exercise has been shown to ameliorate cardiac dysfunction, but not cardiac growth in Post-TAC TgGRK5 mice [108]. This observation presents an interesting avenue by which the detrimental effects of GRK5 can be slowed, however further studies are needed to clearly understand the mechanism by which this occurs.

3.2 Potential Translation of GRK5 Targeting in the Heart

In humans, unlike GRK2, GRK5 has non-synonymous polymorphisms that may have translational significance. Four single nucleotide polymorphisms of GRK5 are known. The GRK5-Leu41 polymorphism, in which at position 41 a leucine is substituted for glutamine, appears common in the African-American population [110]. Interestingly, this point mutation was found to protect against death or cardiac transplantation in a prospective trial following African American HF patients [110–111]. Subsequently, it has been postulated that GRK5-Leu41 provides a genetic β -blockade that leads to increased survival in the African American population [110].

Similar to what has been proposed for GRK2, recent work has suggested small molecule inhibitors of GRK5 as potential cardiac therapeutics. Several compounds have been identified in high throughput drug screens with the most promising of these being amlexanox, an FDA approved anti-inflammatory medication [112]. Importantly, amlexanox was shown to inhibit GRK5 activity in vivo utilizing a MEF2 luciferase reporter assay [112]. Unfortunately, kinase inhibitors tend to lack specificity as they are able to inhibit the catalytic domain of structurally similar kinases. Accordingly, amlexanox has been shown to bind and inhibit I κ B kinase ϵ and presumably other kinases, potentially limiting its use as a therapeutic [113]. The Tesmer lab, while seeking to develop a more specific small molecular inhibitor of GRK2 based on the compound GSK180736A, developed a novel compound which is capable of both GRK2 and GRK5 inhibition with IC_{50} values of 150 and 380 nM, respectively [114]. While this compound was not developed for GRK5 inhibition, it was found to be a potent inhibitor. Interestingly, this compound was used to stabilize GRK5 augmenting its crystallization providing for structure determination via single crystal X-ray diffraction [114]. Publishing the structure of GRK5 in complex with this inhibitor provides

valuable insight into the structure of GRK5 and will aid in the development of more specific small molecule inhibitors in the near future.

Similar to GRK2, peptide inhibitors have also been suggested as a method of GRK5 inhibition. Recently, studies have been performed utilizing peptides that mimic the domains of GPCRs and GRKs which demonstrated inhibition of GRK5. One such protein which was successful in inhibiting rhodopsin phosphorylation by GRK5 was helix 9 of the GRK5 RH domain [115]. Furthermore, alanine substitution at the 169th amino acid of this peptide was able to augment its inhibitory capacity. However, this peptide lacked specificity as it was also able to inhibit GRK2, 6 and 7 [115]. While a promising result, further work must be undertaken to design a peptide inhibitor which exhibits a high level of specificity for GRK5.

Another area of increasing interest is the role of biased agonists in activation of distinct GPCR signaling pathways and the role of GRKs in this process. GPCRs were originally thought to reside in either an inactive or active conformation. In essence, activated receptors would signal through both their heterotrimeric G protein as well as through β -arrestin-mediated pathways. Recently, it was demonstrated that ligands exist that can bias receptor signaling through either G protein or through β -arrestin-mediated pathways [116]. GRKs are central to this biased agonism paradigm since they initiate GPCR phosphorylation resulting in β -arrestin-mediated signaling. Further, It has been hypothesized that GRKs alter GPCR signaling due to their preference for distinct GPCR phosphorylation sites, and each GRKs respective phosphorylation pattern exists as a molecular “barcode” [117]. Importantly, the role of GRKs in biased signaling has been confirmed in the myocardium. It has been reported that mechanical stretch in cardiomyocytes leads to conformational change in the angiotensin receptor (AT1R), activating β -arrestin-mediated cell survival signaling in the absence of G protein signaling [118]. Interestingly, β -arrestin-mediated signaling was not detected in GRK5 KO or GRK6 KO hearts, demonstrating a necessary role for these GRKs in this process. Moving forward, the implications of GRKs vital role in biased agonism suggests that inhibition of specific GRKs is a very intriguing therapeutic target.

When considering translation of a GRK5 inhibitor it may be most beneficial to directly target its nuclear activity, but the targeting of GRK5 should also be considered since studies have revealed non-kinase dependent effects at the level of DNA binding [99]. This is even more important since GRK5 has been shown to play a role in transactivation of β 1-AR and epidermal growth factor receptors (EGFRs) conferring cardioprotection [119]. It is possible that GRK5 may be protective while present at the myocyte membrane, only to become detrimental to cell survival upon pathological hypertrophic stimuli due to nuclear targeting. Thus, regulation of GRK5 compartmentalization may be an important issue to review if GR5 inhibition is to be utilized therapeutically. Accordingly, an effective strategy may be to develop a therapy that is capable of inhibiting the nuclear accumulation of GRK5 allowing this enzyme to exert known cardioprotective effects at the sarcolemma through transactivation of the β -AR receptor and EGFR while preventing its activation of MEF2 and NFAT in the nucleus [119].

4. Potential MicroRNA Regulation of GRK expression in the Heart

While dysregulation of GRKs, especially the up-regulation of GRK2 and GRK5 in the injured heart has been well-established in disease, the underlying reasons for alterations in GRK expression are less clear. Non-coding RNAs are potent post-transcriptional regulators of gene expression, and have garnered interest recently for their relevance in disease. For example, microRNAs are short, ~22 nucleotide transcripts that canonically bind to the 3' untranslated region (3' UTR) of mRNA transcripts and promote repression of mRNA expression through decapping and deadenylation or inhibition of translation [120]. Importantly, the expression of microRNAs is dynamic, and may be a potential mechanism by which gene expression changes in disease. Recently, the expression of several miRNAs has been shown to be altered in both murine and human HF [120–123]. Further, data involving β -AR alterations potentially implicate GRK2 and GRK5. MiRNA-133a is known to be downregulated in HF, and has been implicated in regulation of cardiac hypertrophy as well as in β -AR function [124–125]. MiRNA-133a overexpression in a transgenic mouse model conferred significant cardioprotection in pressure-overload induced HF leading to diminished cell death and preserved cardiac function due to fine-tuning of the β 1-AR pathway [121–125]. Additionally, viral delivery of miRNA-133a protected against hypertrophy in mice, suggesting miRNAs as a potential gene therapy target [125]. Thus, influencing gene expression at the post-transcriptional level using gene therapy technology is an attractive option, due to the specificity of miRNAs for their gene targets and could help eliminate off-targets effects [126].

Recently, a Kaposi's sarcoma-associated herpesvirus microRNA has been shown to directly target GRK2 resulting in migration and invasion of endothelial cells [127]; however, post-transcriptional regulation of GRK genes in cardiovascular disease have yet to be explored as a potential therapy. As we know, in the context of HF, GRK2 and GRK5 expression is increased [128–131], but the mechanism governing the GRK transcriptome is currently unknown. Analysis of the 3' UTR of both GRKs has revealed several miRNAs that may regulate either expression or activity of GRK2 and GRK5. In theory, this potential therapeutic approach would employ a candidate miRNA that is decreased in HF but also displays GRK specificity. Subsequently, downregulation of this candidate miRNA would constitute a mechanism to enhance GRK translation. Adenoviral-mediated delivery to an area of injury could allow the miRNA to normalize GRK expression to pre-HF levels, in effect slowing or halting the progression of HF by preventing the pathogenic mechanisms of GRKs. Studies are presently underway to determine the viability of miRNA targeting of GRKs in disease. Altogether, the therapeutic potential of gene therapy with miRNAs in the context of GRK dysregulation could represent a novel form of intervention that could have far reaching implications for treatment of cardiovascular diseases.

5. Conclusion

It is clear that GRK2 and GRK5 can be pathological when up-regulated in the failing heart. The mechanism behind the detrimental effects are multiple and diverse encompassing both canonical and non-canonical activities of these molecules. Indeed, a complex and ever-expanding “GRK interactome” is emerging, and it is believed to be a rich environment for

potential development for novel therapies to combat pathological hypertrophy and HF. Due to the growing aged population and HF, new and innovative treatments are desperately needed since existing therapies can be underwhelming. As a result of nearly two decades of research, it is our belief that targeted therapies against GRK2 and GRK5 can be developed successfully, and this will lead to a novel drug class that will be effective in cardiovascular diseases.

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Nonstandard Abbreviations and Acronyms

3'	UTR 3' untranslated region
AAV6	Adeno-associated virus serotype 6
Ad	Adenoviral
AT1R	Angiotension receptor
AR	Adrenergic receptor
AngII	Angiotensin II
C	Carboxyl
Ca²⁺	Calcium
CaM	Calmodulin
CSQ	Calsequestrin
DCM	Dilated cardiomyopathy
EGFR	Epidermal growth factor
eNOS	Endothelial Nitric Oxide Synthase
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GRK2-C340S	Mice wherein GRK2 cannot be S-nitrosylated
GRK5-NLS	Mice wherein GRK5 cannot be targeted to the nucleus
HDAC	Histone deacetylase
Hsp90	Heat Shock Protein 90
HF	Heart failure

I/R	Ischemia-Reperfusion
KO	Knockout
LV	Left ventricular
MEF2	Myocyte enhancer factor 2
MI	Myocardial Infarction
MLP	Muscle Lim Protein
NLS	Nuclear localization sequence
NO	Nitric Oxide
NOX-4	NADPH oxidase-4
RGS	Regulator of G protein signaling
RH	Region of Homology
ROS	Reactive Oxygen Species
SNS	Sympathetic nervous system
SSRI	Serotonin reuptake inhibitor
TAC	Transverse aortic constriction
TgGRK5	Transgenic GRK5

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Figure 1. Classical GPCR signaling

Upon ligand binding (blue sphere), GPCRs undergo a conformational change allowing exchange of GDP for GTP on the G protein α subunit ($G\alpha$) which in turn activates $G\alpha$ subunit. $G\alpha$ and $G\beta\gamma$ subunits dissociate leading to downstream signaling. The agonist occupied receptor is then phosphorylated by a GRK which leads to a conformational change in the receptor and in turn provides a binding site for β -arrestin. β -arrestin binding initiates the recruitment of clathrin and AP-2 which are responsible for catalyzing the endocytosis of the receptor. Following endocytosis, the receptor either undergoes proteasomal degradation or is recycled and trafficked back to the cell membrane. Meanwhile, GTP is hydrolyzed to GDP terminating $G\alpha$ signaling.

Figure 2. Topology of GRK2, β ARKct and GRK5

Classic tri-domain structure of GRK2 and GRK5 contains similar N-terminal regions which interact with activated GPCRs. N-terminal region includes calmodulin (CaM) binding domain (orange) and regulator of G protein signaling (RGS) homology (RH) domain (pink). Interrupting the RH domain is the centrally located kinase domain (blue) with catalytic lysine residue (red). GRK5 differs from GRK2 in that it contains both a nuclear export sequence (NES, tan) and nuclear localization sequence (NLS, green). C-terminus of GRK2 and GRK5 differ in that GRK2 contains a pleckstrin homology (PH) domain (purple) which binds G β γ . GRK5 lacks this domain; however, contains a second CaM binding domain and amphipathic helix (brown) which allows for membrane association. Also shown is β ARKct which is identical to the 194 residue C-terminus of GRK2.

Figure 3. Downregulation of GPCR signaling by GRK2 and restoration of signaling following GRK2 inhibition with β ARKct

(A) Upregulation of GRK2, as seen in human heart failure, results in decreased GPCR density and signaling due to desensitization and downregulation of GPCRs at the cell membrane. (B) GRK2 inhibition by β ARKct increases GPCR density at the cell membrane and restores receptor signaling responsiveness.

Figure 4. Negative effects of GRK2 upregulation

Upregulation of GRK2, which occurs in human heart failure, results in adrenergic desensitization. (A) β_1 adrenergic receptors are downregulated in the heart leading to decreased sympathetic responsiveness and consequently decreased inotropic reserve. (B) Within the vascular smooth muscle, β_2 adrenergic receptors are downregulated by GRK2 leading to increased vascular tone. This in turn increases afterload on the heart. (C) Within the adrenal medulla, chromaffin cell α_2 adrenergic receptors are downregulated by GRK2 leading to altered feedback inhibition of the sympathetic nervous system and ultimately increased adrenal catecholamine release. This augmented catecholamine release perpetuates the cycle of GRK2 upregulation and impaired β adrenergic receptor responsiveness. Additionally, the upregulated sympathetic activity results in increased vascular tone as well as cardiomyocytes apoptosis. (D) Following ischemia reperfusion injury (I/R), GRK2 phosphorylates eNOS leading to decreased catalytic activity which results in increased cardiomyocyte apoptosis. (E) Additionally, I/R stimulates GRK2 translocation to the mitochondria of cardiomyocytes resulting in altered metabolism and increased ROS production. These detrimental effects within the mitochondria augment cardiomyocytes apoptosis.

Figure 5. Schematic Depicting the Facilitation of Hypertrophic Transcription by GRK5
Nuclear translocation of GRK5 occurs via stimulation of the Gαq pathway following pathologic stimulus such as transaortic constriction (TAC) or phenylephrine (PE) stimulation. This occurs following activation of calmodulin (CaM) by calcium (Ca^{2+}) and subsequent binding to the N-terminus of GRK5. CaM binding causes GRK5 to dissociate from the plasma membrane and translocate to the nucleus. 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. Adapted from Hullmann et. al. “GRK5-Mediated Exacerbation of Pathological Cardiac Hypertrophy Involves Facilitation of Nuclear NFAT Activity”, *Circ Res* (2014) [99].