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G-protein receptor kinases 2, 5 and 6 redundantly modulate Smoothened-GATA transcriptional crosstalk in fetal mouse hearts

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Abstract

G-protein receptor kinases (GRKs) regulate adult hearts by modulating inotropic, chronotropic and hypertrophic signaling of 7-transmembrane spanning neurohormone receptors. GRK-mediated desensitization and downregulation of β-adrenergic receptors has been implicated in adult heart failure; GRKs are therefore a promising therapeutic target. However, germ-line (but not cardiomyocyte-specific) GRK2 deletion provoked lethal fetal heart defects, suggesting an unexplained role for GRKs in heart development. Here we undertook to better understand the consequences of GRK deficiency on fetal heart development by creating mice and cultured murine embryonic fibroblasts (MEFs) having floxed GRK2 and GRK 5 alleles on the GRK6 null background; simultaneous conditional deletion of these 3 GRK genes was achieved using *Nkx*2-5 Cre or adenoviral Cre, respectively. Phenotypes were related to GRK-modulated gene expression using whole-transcriptome RNA sequencing, RT-qPCR, and luciferase reporter assays. In cultured MEFs the atypical 7-transmembrane spanning protein and GRK2 substrate Smoothened (Smo) stimulated Gli-mediated transcriptional activity, which was interrupted by deleting GRK2/5/6. Mice with *Nkx*2-5 Cre mediated GRK2/5/6 ablation died between E15.5 and El 6.5, whereas mice expressing any one of these 3 GRKs (i.e. GRK2/5, GRK2/6 or GRK5/6 deleted) were developmentally normal. GRK2/5/6 triple null mice at E14.5 exhibited left and right heart blood intermixing through single atrioventricular valves or large membranous ventricular septal defects. Hedgehog and GATA pathway gene expression promoted by Smo/Gli was suppressed in GRK2/5/6 deficient fetal hearts and MEFs. These data indicate that GRK2, GRK5 and GRK6 redundantly modulate Smo-GATA crosstalk in fetal mouse hearts, orchestrating transcriptional pathways previously linked to clinical and experimental atrioventricular canal defects. GRK

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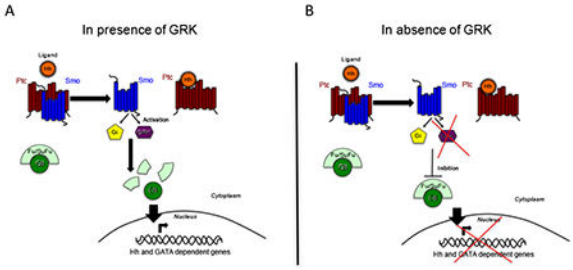
modulation of Smo reflects convergence of conventional neurohormonal signaling and transcriptional regulation pathways, comprising an unanticipated mechanism for spatiotemporal orchestration of developmental gene expression in the heart.

Graphical abstract



Schematic depiction showing how GRK regulates Hh Smo-GATA transcriptional crosstalk. **(A)** In the presence of GRK, Hedgehog family members (Hh; orange) bind to Ptc (Patched: rust), releasing and activing Smo (blue) that, via G_i- and GRK-transduced signals, relieves Fused (Fu) and Suppressor of Fused (SuFu) proteins (light green) that sequester the transcription factor Gli (forsest green) in the cytoplasm, thus permitting it to translocate to the nucleus where it stimulates Hh- and GATA-dependent gene expression. **(B)** In absence of GRK. Hh bind to Ptc thus activing Smo, but the absence of GRK suppressed Smo-stimulation of the transcription factor Gli, that is now not able to migrate into the nucleus to promote GATA- and Hh-dependent transcriptions.

Keywords

G-protein coupled receptor kinase; Smoothened; GATA; atrio-ventricular septal defect; heart development

Introduction

G-protein coupled receptor (GPCR) kinases (GRKs) bi-directionally modulate GPCR signaling. Their recruitment to, and phosphorylation of, ligand-occupied receptors terminates conventional signaling transduced by heterotrimeric G-proteins while simultaneously initiating alternate signaling events transduced by β-arrestins[1]. Over 1,000 mammalian GPCRs are modulated by just 7 GRKs, 2 of which (GRK1 and GRK7) are exclusive to the retina; another (GRK4) is limited to the testes. Thus, GRKs 2, 3, 5, and 6 regulate almost all GPCR signaling. A 20-year-old unsolved mystery of cardiac G-protein signaling is why germ-line murine *Grk2* ablation provokes embryonic lethality (E12.5-E15.5 embryonic stage), myocardial hypoplasia and ventricular septal defects [2], whereas cardiomyocyte-directed deletion of *Grk2* in early embryonic mouse hearts (E9.5-E10.5) produces viable mice with normal hearts [3]. Together, these data suggested the possibility that extra-cardiac *Grk2* was involved in one or more vital processes at a later stage of development. On the other hand, role for other GRKs in heart or other critical aspects of fetal development was not supported by germ-line ablation of GRKs 3, 5 or 6, which evoked only limited and well-tolerated phenotypes not involving the heart [4–6].

Embryonic development is orchestrated by transcriptional control mechanisms reflecting maturational stage and tissue type. GRK2 and β-arrestin 2 have been implicated in zebrafish development through their regulation of the GPCR-like protein Smoothened (Smo) [7, 8]. During vertebrate development Smo functions as the on-off switch for Hedgehog (Hh) activation of Gli-dependent gene transcription [9]. In zebrafish, Smo-mediated transcriptional regulation requires GRK2 [8, 10], and studies in *Drosophila* [11, 12] or mouse embryo cells [8, 13] support evolutionary conservation of GRK-Smo signaling. However, it an *in vivo* role for GRK-Smo signaling, if any, has not been detected in mammals.

We posited that cardiac GRK regulation of Hh/Smo signaling in mice would be obscured in conventional GRK knockout models if GRKs exhibited functional redundancy during fetal development. Here, we tested this notion using combinatorial genetic ablation of GRKs in cultured mouse embryonic fibroblasts and the *in vivo* developing mouse heart. Our results demonstrate that GRKs 2, 5 and 6 redundantly promote Hh/Smo signaling, thereby modulating cross-talk between Hh and GATA gene dependent expression pathways, interruption of which perturbs atrioventricular septal development.

Results

GRK2, GRK5 and GRK6 collectively promote Smo signaling in cultured MEFs.

To assess GRK-Smo interactions we needed an *in vitro* mammalian cell system in which Smo signaling could be experimentally perturbed and the consequences of GRK presence or absence then examined. Thus, we first measured Hh/Smo-dependent gene expression in cultured murine embryonic fibroblasts (MEFs) before and after forced expression of Smo. Our readout was activity of the transcription factor Gli, which is specifically activated and transcriptionally upregulated by Hh/Smo signaling [14]. Adenoviral expression of human Smo provoked a 4-fold increase in activity of a dual luciferase Gli reporter (Figure 1A), a 6-fold increase in *GUI* mRNA levels (Figure 1B), and 12-20 fold increased transcript levels of two other Hh/Smo-responsive genes, Wnt inhibitor factor 1 (*Wif1*) and hedgehog interacting protein (*Hhip*) (Figure 1B**)**. Thus, Smo-Gli signaling in MEFs modulates expression of Hh pathway genes.

GRKs have been observed to modulate Smo signaling in *Drosophila* and zebrafish [12, 15]. We interrogated GRK modulation of Smo-mediated Gli activation in MEFs prepared from combined *Grk2fl/fl*, *Grk5fl/fl*, *Grk6−/−* mice (Figure 1C) after infection with adeno-Cre, thereby creating GRK2/5/6 triple deficiency. As in WT MEFs, Gli reporter activity in *Grk2fl/fl*, *Grk5fl/fl*, *Grk6−/−* MEFs without adeno-Cre (control) was low at baseline and increased ~5-fold after forced Smo expression (Figure 1D). Combined Cre-mediated deletion of *Grk2, Grk5*, and *Grk6* markedly suppressed Smo-stimulated Gli reporter activity (Figure 1D). Because Smo has the potential to activate Gli via G-dependent [16] or GRK/p-arrestin-dependent [7, 8] pathways, we also assayed Gli reporter activity after inhibiting G_i with pertussis toxin (PTX). PTX incompletely suppressed Smo-stimulated Gli reporter activity (Figure 1D), whereas simultaneously deleting GRKs and inhibiting G_i abrogated Smo-stimulated Gli activity (Figure 1D).

GRK2, GRK5 and GRK6 are functionally redundant for heart development.

Gli is transcription factor for Hh-dependent genes, some of which are thought to be important for mouse heart development[17].To delineate the developmental consequences of cardiac GRK isoform expression we interbred mice carrying different GRK isoform knockout or foxed alleles. Although the mouse *Grk2* and *Grk5* genes are on the same arm of chromosome 19, the floxed alleles of both were recombined through intensive breeding to achieve a cross-over event. *Grk6* knockout alleles (Ch 13) were then crossed onto *Grk2/Grk5* double floxed mice. In the absence of Cre the resulting mice (*Grk2fl/fl*, *Grk5fl/fl*, *Grk6−/−*) lack GRK6, but express all other GRKs; like conventional GRK6 knockout mice [6] these mice were viable and phenotypically unremarkable.

The *Nkx*2-5 Cre knock-in allele induces loxP recombination before E7.5 in cardiac myocytes and pharyngeal arch ectoderm[18]. We selected it to delete GRK2 or GRK5 from cardiac myocytes so that the current results could be directly compared with our previously reported *Nkx*2-5 Cre GRK2-deficient mouse [3]. *Nkx2-5* Cre driven GRK2/GRK5, GRK2/GRK6 and GRK5/GRK6 double deficient mice were viable with no overt cardiac phenotype (not shown). Moreover, *Grk2f1/WT*, *Grk5fl/fl*, *Grk6−/−*, *Nkx2-5* Cre knock-in mice were also viable **(**Figures 2A, 2B; designated as “GRK2 het”), not only demonstrating that a single cardiac *Grk2* allele is sufficient for mouse viability, but providing a means, by breeding with *Grk2fl/fl*, *Grk5fl/fl*, *Grk6−/−* mice, to achieve the triple GRK cardiac knocout. By comparison, *Nkx2-5* Cre GRK2/GRK5/GRK6 triple knockout mice (*Grk2fl/fl*, *Grk5fl/fl*, *Grk6−/−*, *Nkx2-5* Cre knock-in; *Nkx*2-5 Cre GRK TKO) died between E14.5 and E16.5 (Figures 2A, 2B). Embryonic lethality in cardiac GRK TKO mice, but not any of the double GRK-deficient mice, revealed GRK isoform redundancy during development that contrasts with their individual roles described in adult hearts[19].(Supplemental figure 1)

Cardiac GRK and GPCR expression change after birth.

GRK isoform expression has been defined in adult, but not fetal, mouse hearts. We measured mRNA levels of GRKs 1-6 (mice do not have GRK7[20]) in mouse hearts at different ages. GRK2 was highly expressed in fetal and adult hearts, whereas GRK6 was equal in abundance to GRK2 in fetal hearts, but declined postnatally (Figure 3A). GRK3 and GRK5 were comparatively minor isoforms (Figure 3A).

To understand GRK functioning it was necessary to also define GRK substrate (i.e. GPCR) expression. Smo was abundant in fetal hearts (Figure 3B, down arrow) whereas the major cardiac GPCRs in adult hearts, such as α- and β-adrenergjc (AR) or ATI angiotensin **II** receptors, were scarce (Figure 3B, up arrows). Co-expression of multiple GRK isoforms with their putative substrate, Smo, in fetal hearts supported the likelihood of a meaningful functional interaction.

Loss of cardiac GRKs induces atrioventricular septal defects.

To identify the cause of fetal mortality in cardiac GRK TKO mice we employed intrauterine echocardiography at E14.5. Normal hearts at this developmental stage have septated right and left atria and ventricles separated by right (tricuspid) and left (mitral) atrioventricular (A-V) valves. Accordingly, the right and left ventricular Doppler inflow patterns are

individually distinct (Figure 3C **left** and Supplemental Movies S1–S4). By comparison, GRK TKO hearts at E14.5 had either a single large diastolic ventricular inflow jet (Figure 3C **middle** and Supplemental Movies S5 and S6) or perpendicular systolic flow between the left and right ventricles (Figure 3C **right** and Supplemental Movies S7–S10), reflecting structural defects within the atrioventricular canal in transverse and coronal sections **(**Supplemental Figure-2**)**. Left ventricular chamber size (end diastolic volume; EDV) and contractile performance (ejection fraction; EF) were normal (Figure 3-D**)**. Thus, embryonic cardiac GRK deficiency causes anatomic heart abnormalities, but not impaired pump function defects.

Consistent with the echo/Doppler findings, postmortem examination of cardiac GRK TKO hearts typically revealed a single A-V valve with large membranous ventricular septal defects (VSD) and low atrial septal defects (ASD) (Figure 3-E). Orthogonal sectioning (analogous to an echocardiographic short axis view) showed that the malformed A-V valve, VSD and ASD were manifestations of a general abnormality of A-V canal development (Figure 3-E), clinically referred to in human patients as complete atrioventricular septal defect (AVSD) [21]. Restoration of one *Grk2* allele ("GRK2 hets" in Figure 2) rescued embryonic lethality and moderated the phenotype (Supplemental Figure 3 A-B), supporting a dose-dependent relationship between GRK gene ablation and A-V canal abnormalities.

GRK deficiency does not impair formation of A-V endocardial cushions

The endocardial cushions are formed after endocardial cells invade the myocardium and undergo an epithelial-meseynchymal transition. We considered that atrioventricular canal defects might result from degeneration of endocardial cushion elements destined to form the A-V valves. Atrioventricular canal defects can result either from impaired genesis of the endocardial cushions or from disorganization of different cushion elements collectively destined to form the A-V valves and adjoining chamber septae. The general abundance and localization of endocardial cushion tissue appeared normal in cardiac GRK TKO hearts **(**Figure 4-A**)**. Moreover, there was no evidence for accelerated death **(**Figure 4-B**)** or impaired proliferation of endocardial cushion cells **(**Figure 4-C**)**. While these results cannot identify the precise cellular pathology underlying A-V canal defects caused by multiple GRK deficiency, they suggest a defect in the arrangement, rather than the genesis, of atrioventricular endocardial cushion tissue **(**Figure 4-D**)**.

GRK deficiency impairs Hh/Smo-mediated gene expression in fetal hearts.

Fetal heart development is orchestrated by conserved transcriptional control mechanisms. Because proper modulation and integration of gene expression is essential to normal development, dysregulation of these pathways can cause developmental disease[22]. Similarities between the cardiac GRK TKO mouse cardiac phenotype and human AVSD prompted us to measure expression of human AVSD culprit genes in our mouse model, which were unaltered (Figure 5-A). Myocardial expression of Smo was likewise unaltered by GRK deficiency (Figure 5-B). Accordingly, we performed genome-wide RNA sequencing. 207 RNA transcripts were dysregulated (changed at least 2-fold, $p<0.01$) in cardiac GRK TKO mouse hearts (Supplemental Dataset 1). This transcriptional signature discriminated between cardiac GRK TKO and control hearts (Figure 5-C). Regulated

mRNAs segregated into three distinct clades: clade 1 having no unifying gene-ontology theme (likely representing compensatory expression of functionally diverse genes), clade 2 enriched in developmental genes, and clade 3 enriched in stimulus-response pathways (Figure 5-C; Supplemental Dataset 2).

Dysregulated Hh/Smo pathway genes clustered in clade 2 (Supplemental Dataset 2), and the Hh/Smo pathway transcript signature nicely segregated control from GRK-deficient hearts, largely determined by decreased levels of normally abundant Hh/Smo genes including GRK-Smo regulated Wnt-inhibitory factor 1 (Wif1) (Figures 1B and 5D). Wnt signaling in the developing heart is integrated by GATA transcription factors[23], mutations of which can cause human and experimental AVSD[24–27]. Indeed, the GATA mRNA profile also segregated cardiac GRK TKO from normal fetal hearts (Supplemental Figures 4A-C); GRK haploinsufficient ("hef") hearts had GATA and Hh transcriptional signatures that tended to be transitional between control and cardiac GRK TKO hearts (Supplemental Figure 4). GATA factors were not, however, themselves dysregulated by GRK deficiency (Figure 5A; Supplemental Dataset 2).

GRK2/GRK5/GRK6 abrogation impacts Hh/Smo gene expression in developing mouse hearts and cultured MEFs.

The above results pointed to modulation of Hh/Smo signaling that in turn impacts GATA pathways required for normal A-V canal formation, as the link between cardiac GRKs and the AVSD-like phenotype. To validate this paradigm if GRK TKO hearts supported interruption of GRK-transduced Hh/Smo signaling, we interrogated GRK regulation of Hh/GATA pathway cross talk using RT-qPCR of Gli1 and other Hh/Smo pathway genes dysregulated in cardiac GRK TKO hearts **(**Figure 6A**)** with parallel studies in GRK TKO MEFs **(**Figure 6B**)**. In the presence of GRKs (i.e. in the absence of Cre), forced Smo expression in MEFs increased abundance of Wif1, Gli1, Hhip, and Hdac5 mRNAs (Foxa2 mRNA was not detectible in MEFs) (Figure 6B). By contrast, deletion of GRK2, GRK5, and GRK6 reversed Smo-mediated increases in Wif1, Gli1 and Hdac5 (increased Hhip was unaffected by GRK ablation, consistent with GRK-independent/Gi-dependent regulation of this gene). As in hearts, MEF GATA6 mRNA levels were not altered either by Smo expression or GRK deletion **(**Figure 6A**)**.

Discussion/Conclusions

GRK isoforms are important for viability of the heart and cardiac development. While germline (systemic) *Grk2* deletion provoked embryonic lethality at E12.5-E15.5, including cardiac defects, cardiomyocyte-specific *Grk2* deletion at E9.5-E10.5 did not affect embryonic development of the heart, nor did it provoke lethality. Thus, we hypothesized that functional redundancy between GRKs required the combined absence of multiple functionally convergent isoforms to uncover true GRK impact on cardiac development. For this reason, we generated a series of *Nkx*2-5 Cre-directed GRK2, GRK5, GRK6 deficient mice in all combinations. Remarkably, only the cardiac GRK TKO mice recapitulated some of the original cardiac findings described with germ-line GRK2 ablation [2]. Moreover, our

studies of cardiac GRK TKO mice provide the first direct evidence that enabling Hh/Smo signaling is a critical GRK function during mammalian heart development.

GRK2 regulation of Hh signaling via Smo phosphorylation has been described during zebrafish development, but its relevance in mammals has been unclear [8, 10] likely because functional redundancy between mammalian GRKs obscured the developmental significance of the GRK/Hh/Smo signaling interactome [3, 5, 6]. The presence of ventricular septal defects in both systemic GRK2 −/− [2] and *Nkx*2-5 Cre-directed GRK2/GRK5/GRK6 deficient mice suggests that A-V cushion abnormalities are one of the consequences of generalized GRK deficiency. It is notable that a single functioning *Grk2* allele, in the absence of GRK5 and GRK6, moderated the phenotype from completed AVSD to small VSD and prevented fetal death.

It has been recognized for some time that mutations of GATA4 and GATA6 cause clinical AVSD [24, 25]. Loss of GRK function is an unanticipated mechanism provoking and interrupting fetal heart GATA signaling to produce AVSD-like endocardial cushion abnormalities. The current results support adding GRKs to GATAs and other candidate genes when screening for AVSD-associated human DNA sequence variation. These results also emphasize the roles that GRKs may play in biology, aside from their canonical functioning as GPCR “off” switches. Here, we show how GRKs can be transcriptional regulators. A recent study in adult mice [28] revealed that GRK5 competes with calcineurin for access to Ca2+-calmodulin, thus modulating GATA-dependent cardiac hypertrophy signaling by regulating dephosphorylation of NFAT . Our results in MEFs and the general importance of Hh/Smo signaling in mammalian embryonic development and cancer [29–31] suggest that non-canonical GRK functioning will likely also have importance outside the heart.

It is worth noting several limitations of this study. Ours was an unbiased discovery study designed to define the role(s) of GRK signaling in mouse heart development. Prior results in flies and zebrafish suggested that GRK-Smo signaling might be activated in the embryonic heart, but we had no notion at the outset that we would find atrio-ventricular septal defects or that Hedgehog and GATA transcriptional pathways would be regulated in parallel by the GRK-Smo pathway. Therefore, our study design is not necessarily optimal for an *a priori* investigation of murine AVSD or of GATA transcriptional control mechanisms. For example, whereas GRK2 and GRK5 were ablated conditionally, the GRK6 knockout was germ-line, leaving open the possibility for systemic GRK6 deletion effects to impact the cardiac phenotype. We think this unlikely based on the minimal phenotype of the GRK6 knockout mice [6], but it remains a formal possibility. Likewise, we used *Nkx2-5* Cre to conditionally ablate GRK2 and GRK5 in the GRK6 knockout background because the previous comparator study of selective vs germ-line GRK2 deletion used this Cre [3], and it was necessary to delete GRKs at exactly the same stage of cardiac development in order to rigorously compare the prior and current work in early stage embryonic development. We recognized (and noted; *vide supra*) that *Nkx2-5* Cre is not purely cardiac myocyte-specific, and so again there is the formal possibility that cardiomyocyte non-autonomous effects are influencing AVSD in GRK triple null mouse hearts. The potential for extra-cardiac influences does not negate any of our conclusions about GRK-Smo signaling, the AVSD

phenotype, or Smo-GATA crosstalk regulation by GRKs. A further limitation of this study involves the regulation of the transcription factor Gli. We infer that Gli is activated via GRK-Smo signaling on the basis of previous studies, but it is beyond the scope of this work to define exact Smo phosphorylation events that affect Gli 1-regulated transcription. More details of how the Hh/GRK/Smo/Gli and GATA pathways interact will be the subject of future investigations.

Materials and Methods

Animal Use:

Grk2 floxed allele (#012458), *Grk5* floxed allele (#010960), and *Grk6* null mice (#010961) are available from The Jackson Laboratory. The *Nkx2-5* Cre knock-in mouse (*9*) was a gift from Dr. Robert Schwartz (Baylor Medical School, Houston Texas). All experimental procedures were approved by the Animal Studies Committee at Washington University School of Medicine. PCR genotyping used the following primers:

GRK2 floxed

5′-tgaggctcagggatacctgtc at-3′

5′-gttagctcaggccaacaagcc-3′

5′-caggcattcctgctggactag-3′

GRK5 floxed

5′-ctatccattcacctccatgctccc-3′

5′-gcaactctggtacagacaggatctc-3′

GRK6 KO

5′-gctcatgagctttgcacctga-3′

5′-aggaactttctagactagtaa-3′

GRK6 WT

5′-gctcatgagctttgcacctga-3′

5′-gtggcagtcattatctcttcagaaccc-3′

NKX Cre

5′-ccttcagagctgtgcgcgctgcag-3′

5′-tagttacccccaggctaagt-3′

GRK TKO mice are *Grk2*fl/fl, *Grk5*fl/fl, *Grk6*−/−, *nkx2-5* Cre +; littermate controls are Cre negative. GRK haploinsufficient mice are *Grk2*fl/WT, *Grk5*fl/fl, Grk6−/−, *Nkx2-5* Cre +. Littermate controls (i.e. *Grk6* KO mice) have normal hearts and normal cardiac gene expression compared to C57/Bl6 mice of the same developmental stage.

Mouse embryos were harvested from timed pregnancies and genotyped by PCR.

Echocardiography:

Transuterine embryonic imaging was conducted in pregnant mice after surgical exteriorization of the uterus. In isoflurane-anesthetized pregnant mice (1.5% gaseous Isoflurane administered through customized nose cone), one uterine horn (containing 5–7 embryos) was exposed through a 1 cm low abdominal midline incision. The uterine horn was laid out over the maternal abdomen on a sterile gauze pad presoaked in PBS at 37°C. Care was taken to avoid any tension or pressure on the uterine vessels. Maternal body temperature as well as the temperature of the surgical field was maintained at 36–38°C. Embryos were imaged individually using a VisualSonics Vevo 2100 ultrasound system (VisualSonics, Toronto, Canada) equipped with a 55 MHz ultrasound transducer. Standardized imaging planes (apical 4-chamber and parasternal long-axis) were obtained with careful attention paid to the position of the embryo within the uterus. EKG monitoring was of the dame. Approximately 8 to 10 embryos were studied in sessions limited to 1.5 h. At the end of the study, dames were euthanized by cervical dislocation while still anesthetized. Embryos were decapitated and collected for histopathological studies.

Histology:

Mouse embryos were harvested from timed pregnancies, frozen or fixed in 4% paraformaldehyde, and genotyped by PCR. Fluorescence of anti-phospho histone 3 (pHH3; Anti-Histone H3 (phospho S10) antibody; Abeam ab47297) and nuclear DAPI was performed on a Nikon Ti Confocal microscope using a 20× objective. TUNEL staining used the DeadEnd™ Fluorometric TUNEL System (Promega G3250).

RNA Isolation:

RNA sequencing was performed using a modification of our previously described methods (27). Briefly, total RNA extracted from mouse embryo hearts was treated with RNase-free DNase I, Amp Grade (Invitrogen) and subjected to ribosomal RNA depletion using the RiboZeroGold reagent system (Illumina). RNA libraries were sequenced (single end 50 nt reads) on a Hi Seq 2500 instrument. Sequencing reads were aligned and mapped to mouse transcriptome (UCSC mm10) using Bowtie2 and Tophat 2.0.10. The mean number of transcriptome-aligned reads per embryonic heart was 7.6 million. Detectable RNAs were assessed as those present at or above 1 read per million in at least 6 of 8 hearts from any genotype group, resulting in ~13,900 “cardiac-expressed” mRNAs for further analyses. Statistical analyses for differentially expressed RNAs used the R/Bioconductor package edgeR. Partek Genomics Suite v6.6 (Partek, St. Louis, MO) was used for heat map construction: normalized read counts from underwent unsupervised hierarchical clustering with Euclidean distance and average linkage; map colors represent the extent of deviation from the row average. Gene expression levels in column graphs are rendered as FPKM (Fragments Per Kilobase of exon per Million reads mapped to the transcriptome).

Studies in vitro:

Effects of GRKs on Smo-mediated Gli transcriptional activity were determined in primary MEFs cultured from E13.5 Grk2fl/fl, Grk5fl/fl, Grk6 −/− embryos. MEFs were plated in Dulbecco’s MEM (Invitrogen) supplemented with 10% fetal bovine serum and 2 mM L-

Glutamine, 100U/mL Penicillin and 10 μg/ml Streptomycin. MEFs were transfected with CRE adenovirus (Ad-CMV-iCre; Vector Biolabs #1045) at multiplicity of infection (m.o.i.) of 50 to delete GRK2 and GRK5, 72h prior to assays to delete GRK2 and GRK5; MEFs not given adeno-Cre served as controls. 48 h prior to assay cells were transduced with lentiviral firefly luciferase coupled to a Gli-responsive promoter together with Renilla luciferase coupled to a constitutive CMV promoter (40:1; Cignal GLI Reporter kit: Qiagen CLS-3030L) at m.o.i. of 20, with or without a custom adenoviral human Smo (created by Vector Biosystems) at m.o.i of 50, together with 8 μg/ml hexadimethrine bromide (Polybrene: Sigma) to improve lentiviral efficiency. In some experiments pertussis toxin (PTX; Sigma P2980) was added at 100ng/mL. Renilla (control) and firefly luciferase activates were measured after lysing cells in 1× Glo Lysis Buffer (Fisher Scientific/Promega PR-E2661) and assaying lysate aliquots with the Promega DualGlo Luciferase Assay System (Fisher Scientific/PR-E2920) in a Promega GloMax Luminometer.

RT-qPCR:

RT-qPCR used Invitrogen Superscript III reverse transcriptase and TaqMan assays from ThermoFisher Scientific: Foxa2 (Mm00839704), Gata6 (Mm00802636), Gli1 (Mm00494654), Hhip (Mm00469580), Wif1 (Mm01156569), Wnt5a (Mm01220918), Hdac5 (Mm01246080), GAPDH(Mm99999915). qPCR of deleted exons for GRK2, GRK5, and GRK6 used SYBR Green and the following primer pairs:

GRK2 exons 3-6

5′-gagacagaggaggaggaacgtgt-3′

5′-gaaaagggatgygagcaggc-3′

GRK5 exons 7,8

5′-ttgaccgttttctgcagtgg-3′

5′-aggcagacctctccaaag-3′

GRK6 exons 3-9

5′-cctggatggggtgtctgaat-3′

5′-ccgtggctcagaaagttc-3′

GAPDH

5′-atggtgaaggtcggtgtga-3′

5′-gaatttgccgtgagtggagt-3′

Statistical analysis:

Results are expressed as mean ± s.e.m. Except for RNA sequence analysis statistical comparisons (two-sided) used unpaired Student t-test. $P<0.05$ was considered statistically significant except for genome-wide RNA-sequencing analyses, in which $P<0.01$ was the threshold for significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors declare no conflicts. Unprocessed and transcriptome-aligned RNA sequencing reads were deposited in the NCBI Gene Expression Omnibus with accession number GSE87321. Supported by NIH HL087871 (GWD). GWD and AF conceived of the research, performed and analyzed studies, and wrote the manuscript. AF, LZ, SJM, and AK performed and analyzed studies. AF, SJM and GWD prepared the revised manuscript.

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Highlights

- GRK(s) are fundamental in Hh/Smo signaling in heart mammalian development
- Atrio-Ventricular(A-V) cushion abnormalities are enclosed to GRK(s) deficient mouse
- GRK loss function induces produces AVDS in GATA signaling

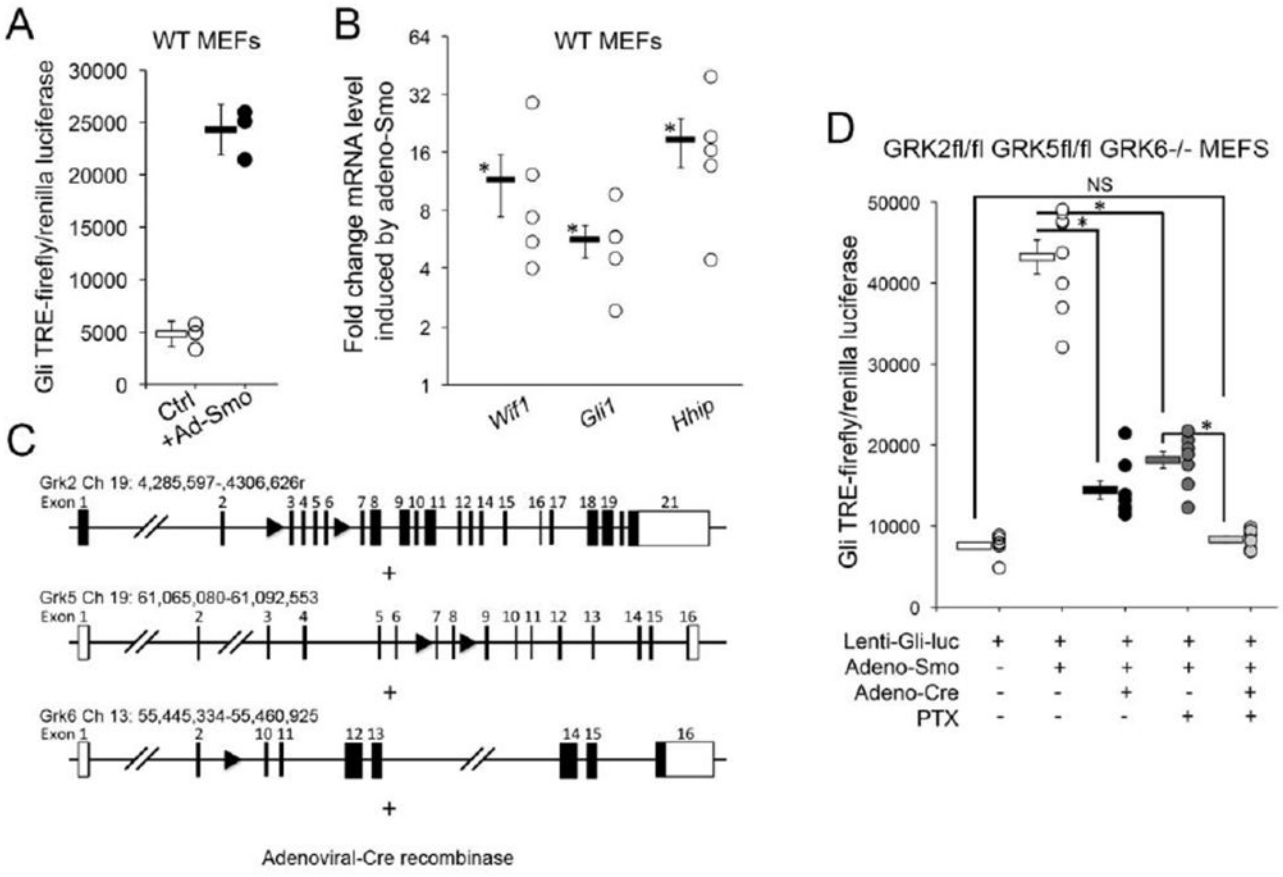

Figure 1. GRKs regulate Hh/Smo transcriptional activity in cultured MEFs.

(A) Gli transcription reporter activity in MEFs without and after ad-hSmo transduction (n=3). **(B)** Hedgehog/Smoothened pathway gene expression assayed by RT-qPCR in cultured MEFs as a function of adenoviral-hSmo transduction (fold-changeover no Smo; $\log_2$ scale). Each point is an independent experiment; bars are means±SEM. N=5/group; * is $p<0.05$ vs no Smo Ctrl MEFs (ANOVA). **(C)** Conditional multiple *Grk* gene ablation strategy. **(D)** Regulation of Smo-stimulated Gli transcriptional activity by GRKs and PTX-sensitive Gi signaling in MEFs. n=7 or 8/group; * is $p<0.05$; NS is non-significant (ANOVA).

A

Grk2 fl/fl, Grk5 fl/fl, Grk6 KO × *Grk2* fl/WT, *Grk5* fl/fl, *Grk6* KO + *nkx2.5*-Cre				
Genotype E13.5 (n=87)	**#Embryos**	**Predicted#**	**%Embryos**	**Predicted%**
GRK2,5,6 TKO	24	21.75	27.5	25
GRK2 het; GRK5,6 DKO	20	21.75	23	25
Cre negative	43	43.5	49.5	50
Genotype E14.5 (n=153)	**#Embryos**	**Predicted#**	**%Embryos**	**Predicted%**
GRK2,5,6 TKO	39	38.25	25.5	25
GRK2 het; GRK5,6 DKO	30	38.25	20	25
Cre negative	84	76.5	55	50
Genotype P7 (n=116)	**# Pups**	**Predicted#**	**% Pups**	**Predicted%**
GRK2,5,6 TKO	4	29	3	25
GRK2 het; GRK5,6 DKO	44	29	38	25
Cre negative	68	58	59	50

B

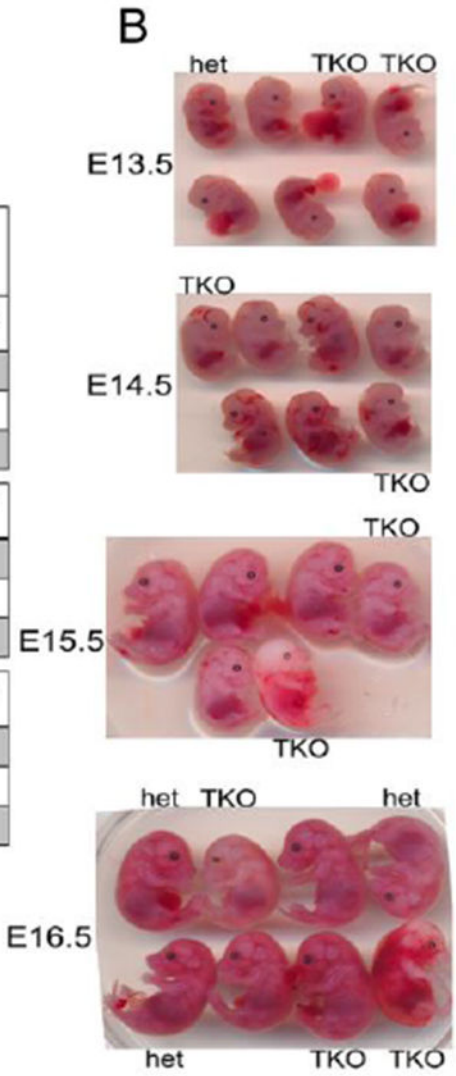

Figure 2. Fetal mortality in Nkx2-5 GRK TKO mice.

(A) Results of timed pregnancies and pups survived. (B) Representative litters at embryonic days E13.5-E16.5. "TKO" is *Grk2* fl/fl, *Grk5* fl/fl, *Grk6* −/−, *Nkx2-5* Cre +; "het" is *Grk2* fl/WT, *Grk5* fl/fl, *Grk6* −/−, *Nkx2-5* Cre +; unlabeled are Cre negative controls. *Nkx2-5* GRK TKO embryos are indistinguishable from littermates at E13.5 and E14.5, but show evidence of non-viability (pale or translucent) at E15.5 and E16.5.

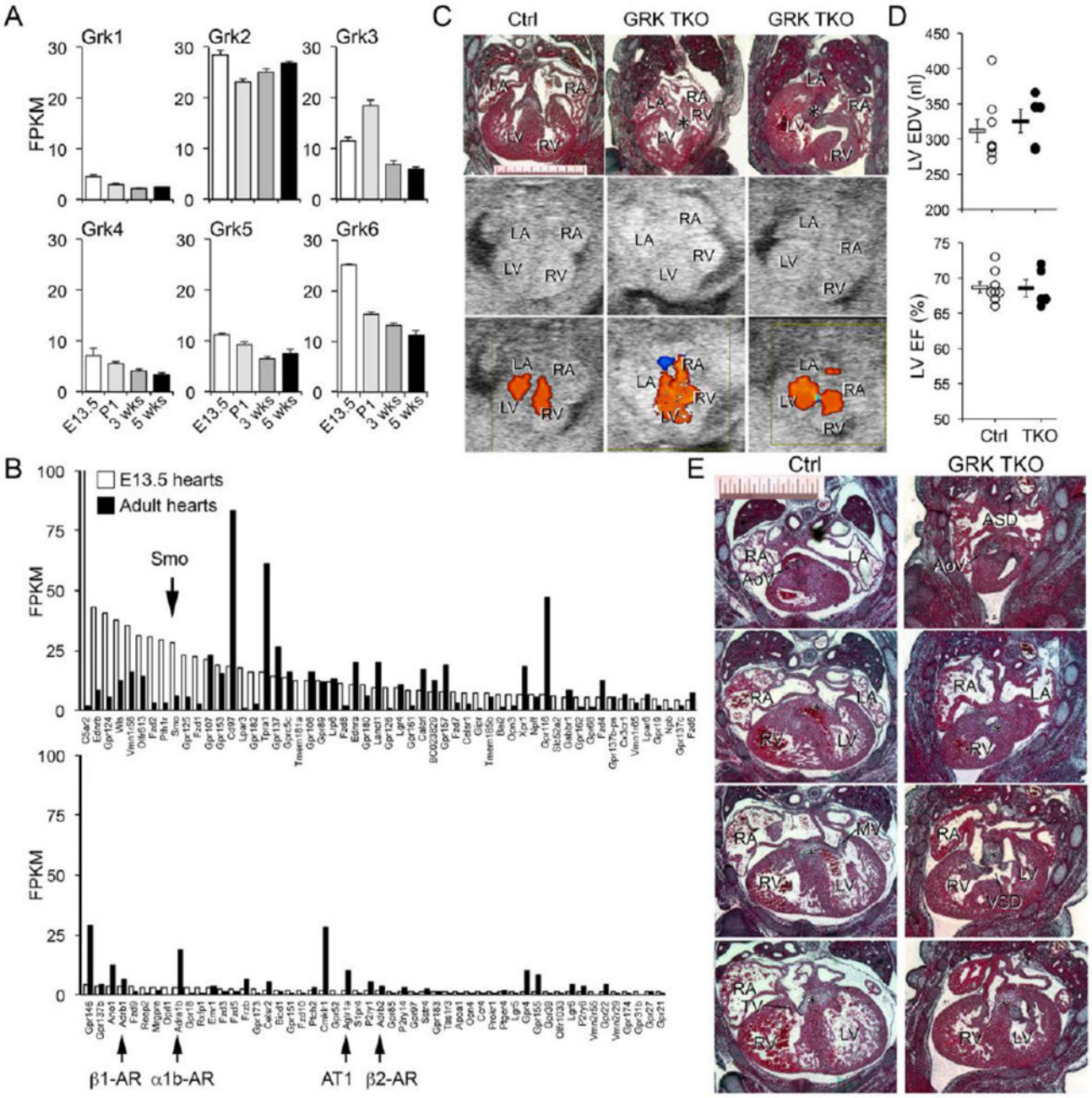

Figure 3. Effects of combined Nkx2-5 mediated GRK2 GRK5 GRK6 ablation on the developing mouse heart.

(A) Mouse *Grk* gene expression in hearts of embryonic (E13.5), immediate perinatal (P1), weanling (3 wks), and juvenile (5 wks) mice. n=3-6/group. **(B)** GPCR mRNA abundance ranked by expression in E13.5 fetal hearts; corresponding adult levels are in black. AR is adrenergic receptor; AT1 is angiotensin type 1 receptor, Smo is Smoothened. **(C)** Interventricular flow abnormalities in GRK TKO mice. *Top*, transverse cardiac sections. *Middle*, 2-dimensional echocardiographic images. *Bottom*, corresponding color flow

Doppler studies (flipped vertically for clarity). **(D)** Quantitative intra-uterine echocardiogram data for left ventricular end diastolic volume (EDV) and ejection fraction (EF). N = 8 Ctrl and 5 TKO; no significant differences. **(E)** Representative serial H&E-stained sections of E14.5 hearts. **(C and E)** RA – right atrium; LA – left atrium; ASD – atrial septal defect; AoV – aortic valve; MV – mitral valve; TV – tricuspid valve; RV – right ventricle; LV – left ventricle; VSD – ventricular septal defect; * indicates A-V endocardial cushion. Scale bar is 1mm (0.01 mm increments).

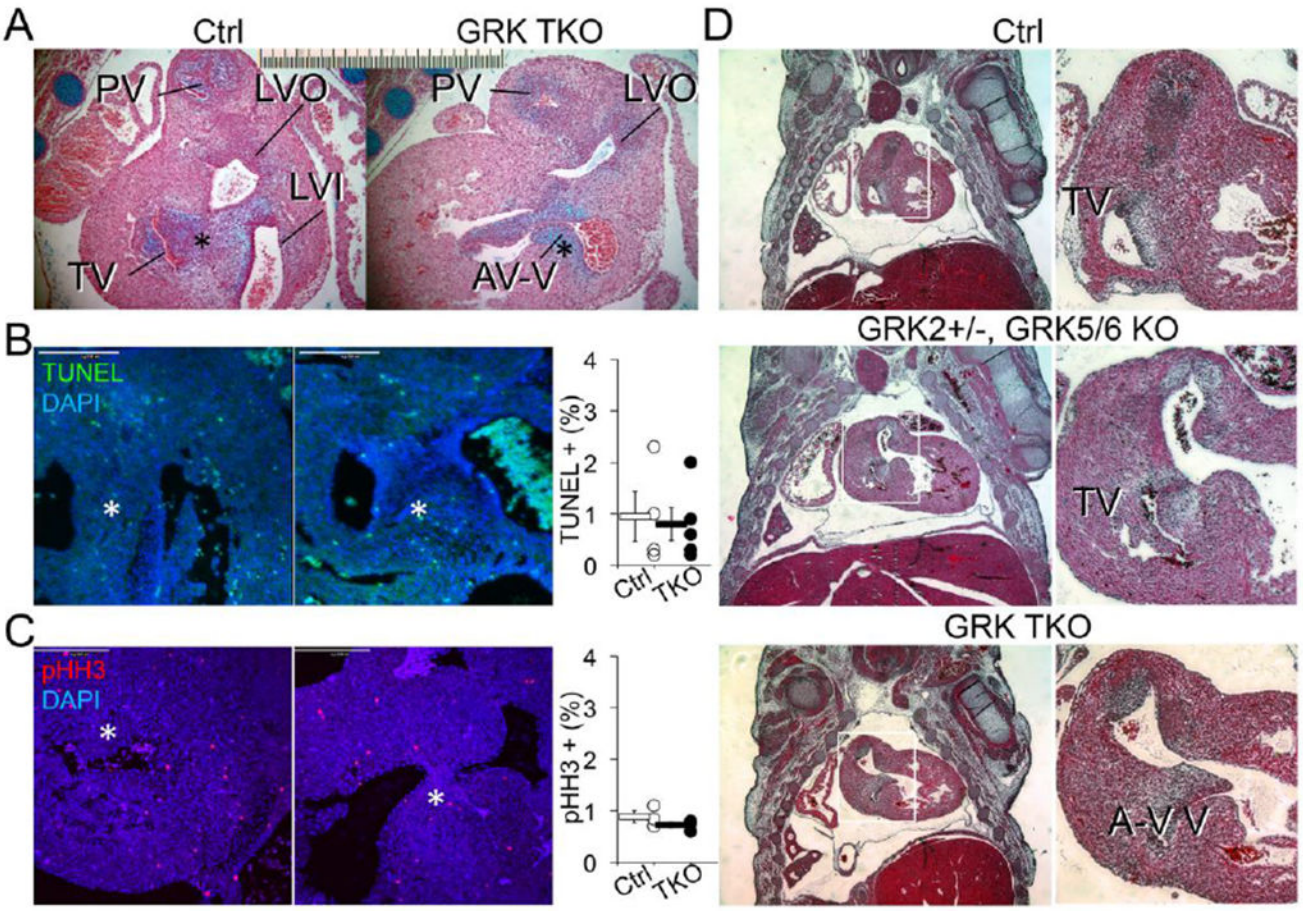

Figure 4. A-V endocardial cushions of Nkx2-5 GRK TKO mice.

(A) Alcian blue staining of A-V endocardial cushions (1mm scale bar). (B) TUNEL staining (200 micron scale bar). (C) Anti-phospho histone 3 (red). * denotes endocardial cushion tissue. (D) Coronal sections through A-V endocardial cushions of Ctrl (top), GRK haploinsufficient (*Grk2* fl/WT, *Grk5* fl/fl, Grk6 −/−, *Nkx2-5* Cre +; middle) and Nkx2-5 GRK TKO (bottom) E14.5 hearts. Exploded views are from areas framed in white.

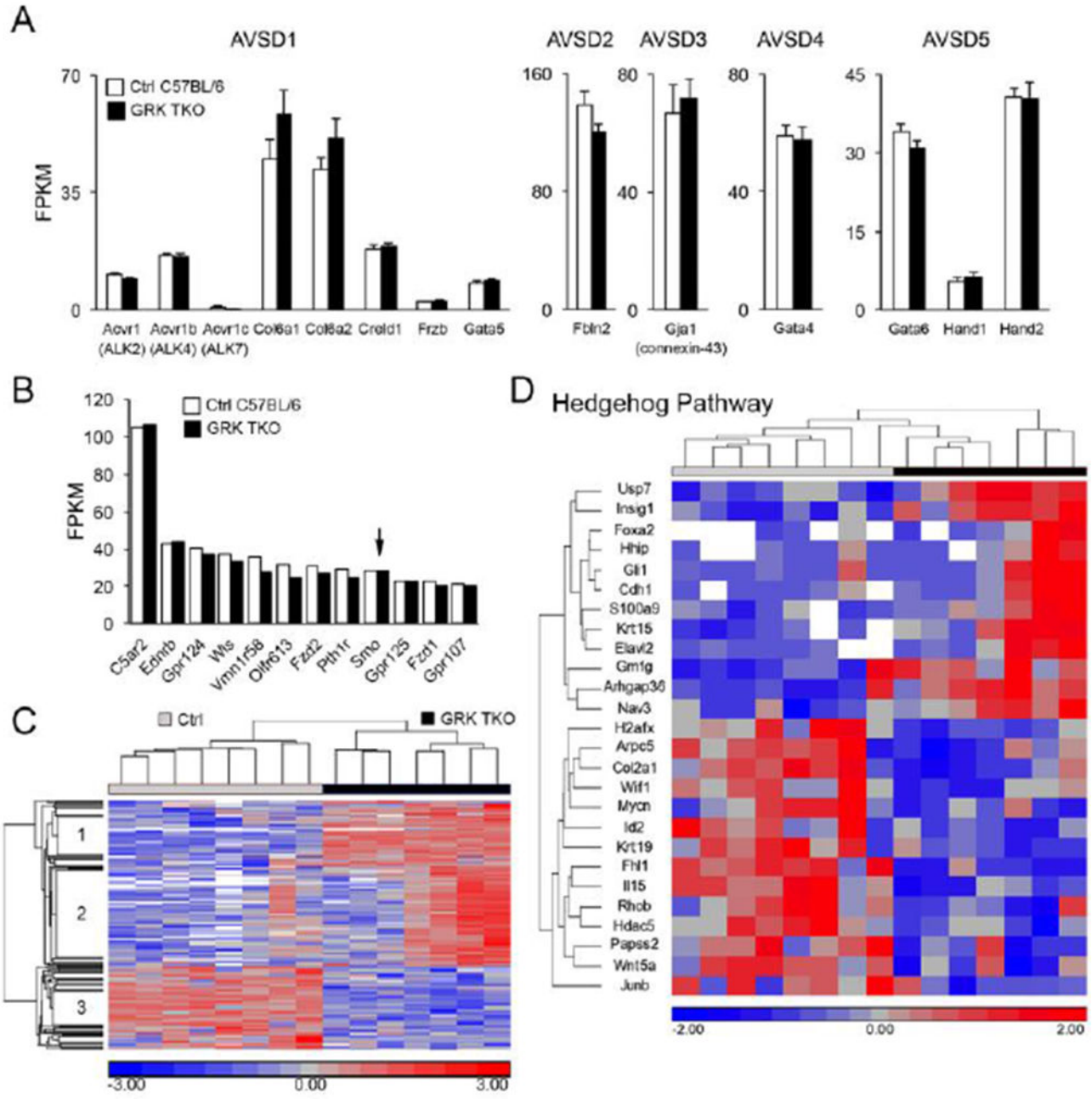

Figure 5. Gene expression in Nkx2-5 GRK2/5/6-deficient fetal hearts.

(A) Expression of the mouse orthologs for human AVSD1-5 genes listed by OMIM for "endocardial cushion defect" in E13.5 mouse hearts. N=8 Ctrl (white); N=7 GRK TKO (black); there are no differences, including for GATA4 and GATA6 that are human AVSD genes. **(B)** Twelve most abundant GPCR family members in E13.5 hearts; arrow indicates Smo. **(C)** Unsupervised hierarchical clustering of 207 dysregulated cardiac RNA transcripts in E13.5 GRK TKO mouse hearts. Each column is the result from a different mouse. **(D)** Heatmap of regulated Hh pathway genes in E13.5 GRK TKO hearts.

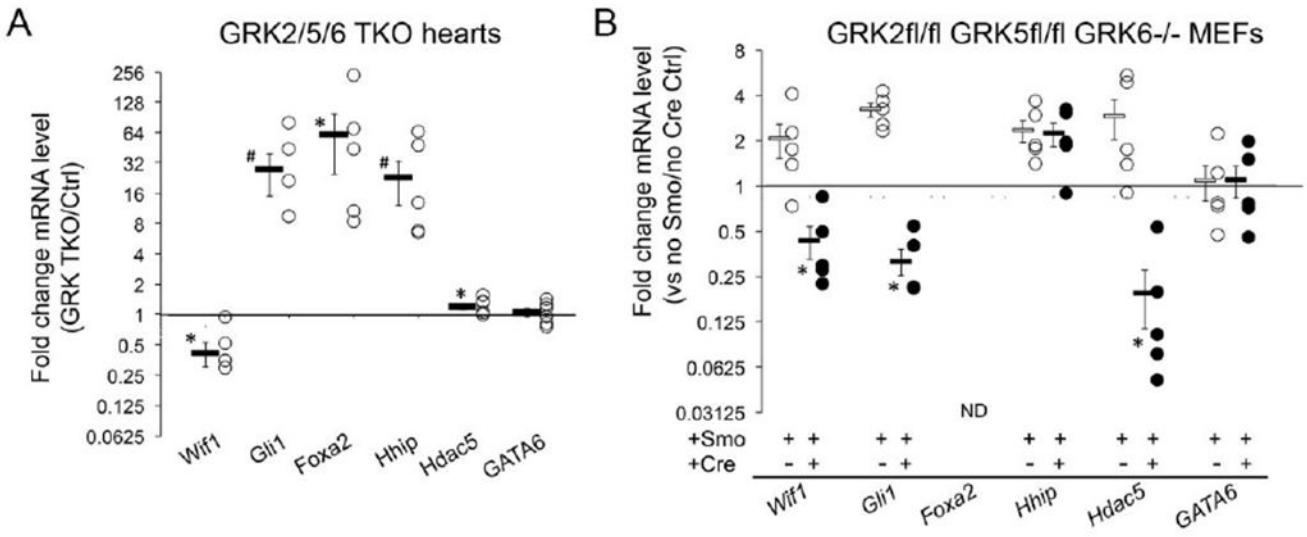

Figure 6. GRK deficiency suppresses Smoothened-dependent gene expression in developing mouse hearts and cultured MEFs.

(A) RT-qPCR of selected genes in E13.5 *Nkx2-5* GRK TKO hearts compared to Ctrl hearts. N=4-6; * is $p<0.05$ vs Ctrl; # is $p<0.07$ vs Ctrl. **(B)** RT-qPCR of genes from panel (A) in GRK gene conditional KO MEFs, and their regulation by Smo in the presence (white, no Cre) and absence (black, after Cre) of GRKs. N=5; * is $p<0.05$ vs no Cre (t-test).