Molecular Biology of the Cell
Vol. 19, 5478–5489, December 2008

Smoothened Signaling in Vertebrates Is Facilitated by a G Protein-coupled Receptor Kinase

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Submitted May 2, 2008; Revised August 29, 2008; Accepted September 17, 2008
Monitoring Editor: Marianne Bronner-Fraser

Smoothened, a heptahelical membrane protein, functions as the transducer of Hedgehog signaling. The kinases that modulate Smoothened have been thoroughly analyzed in flies. However, little is known about how phosphorylation affects Smoothened in vertebrates, mainly, because the residues, where Smoothened is phosphorylated are not conserved from *Drosophila* to vertebrates. Given its molecular architecture, Smoothened signaling is likely to be regulated in a manner analogous to G protein–coupled receptors (GPCRs). Previously, it has been shown, that arrestins and GPCR kinases, (GRKs) not only desensitize G protein–dependent receptor signaling but also function as triggers for GPCR trafficking and formation of signaling complexes. Here we describe that a GRK contributes to Smoothened-mediated signaling in vertebrates. Knockdown of the zebrafish homolog of mammalian GRK2/3 results in lowered Hedgehog transcriptional responses, impaired muscle development, and neural patterning. Results obtained in zebrafish are corroborated both in cell culture, where zGRK2/3 phosphorylates Smoothened and promotes Smoothened signal transduction and in mice where deletion of GRK2 interferes with neural tube patterning. Together, these data suggest that a GRK functions as a vertebrate kinase for Smoothened, promoting Hedgehog signal transduction during early development.

INTRODUCTION

The seven-transmembrane–spanning receptor (7TMR, also known as G protein–coupled receptor [GPCR]) family represents the largest class of cell surface receptors, comprising several hundred genes in humans. These receptors enable cells to respond to a wide variety of structurally unrelated, extracellular cues. Their physiological importance is highlighted by the fact that nearly 60% of currently prescribed pharmaceuticals target pathways controlled by 7TMRs. Most signaling from 7TMRs has been studied classically from the standpoint of heterotrimeric G-protein activation by the ligand-occupied receptor, which results in the intracellular up- and down-regulation of a variety of second messengers and cellular response (Lefkowitz, 2007).

In spite of the number and diversity of these proteins, 7TMR signaling is regulated by a relatively small number of proteins. These proteins include the kinases that phosphorylate activated receptor, so called G protein–coupled receptor kinases (GRKs), and the multiadaptor proteins, arrestins, which bind to the phosphorylated receptor (Claing *et al.*, 2002). Binding of arrestin to the receptor acts not only to uncouple the receptor from G-protein activation, but also to function as scaffolds to facilitate the internalization of the receptor and to promote signaling through alternative, non-G protein–dependent cascades (Lefkowitz and Shenoy, 2005). Excluding the visual system, mammals have five GRKs (GRK2–6) and two arrestins, (also known as β-arrestin 1 and 2 [barr1 and 2]). The GRKs are divided by sequence homology into two subfamilies: GRK2 and 3 (previously called βARK1 and 2 for their ability to phosphorylate the β-adrenergic receptor) and GRK4–6. These genes display an apparent high functional redundancy in mammals, thwarting the analysis of GRK and barr function during development, as only the GRK2 knockout (KO) mice die during embryonic development (Jaber *et al.*, 1996). More recently, experiments in our laboratory in the vertebrate *Danio rerio* have uncovered a role for barr2 in promoting Hedgehog (Hh) signaling in vivo (Wilbanks *et al.*, 2004). Knockdown of barr2 resulted in a phenotype with multiple defects, including faulty somite patterning and craniofacial development, aspects of zebrafish development regulated by Hh. These findings were consistent with the simultaneous report of the activity-dependent interaction of barr2 with the Hh pathway component Smoothened (Smo) in a cellular system (Chen *et al.*, 2004).

The Hh pathway regulates multiple aspects of embryonic development in both vertebrates and invertebrates. Reduced Hh signaling leads to the human developmental disorders of polydactyly and holoprosencephaly, as well as severe craniofacial and skeletal malformations (McMahon *et al.*,

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08–05–0448) on September 24, 2008.

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Abbreviations used: barr, β-arrestin; GPCR, G protein–coupled receptor; GRK, G protein–coupled receptor kinase; Hh, Hedgehog; Ptc-1, Patched-1; Smo, Smoothened; 7TMR, 7-transmembrane spanning receptor.

2003). Constitutive pathway activity is implicated in the formation of basal cell carcinoma and medulloblastoma and in the incidence of an increasing number of cancers (Beachy *et al.*, 2004). The core components of the pathway include the ligand Hh; its receptor, the 12TM membrane protein, Patched (Ptc); the 7TM protein Smo; and the Gli transcription factors (called Cubitus interruptus, Ci in flies). Ptc catalytically inhibits the activity of Smo in the absence of Hh (Taipale *et al.*, 2002). When Hh binds to Ptc, Smo is released from tonic repression and undergoes rapid changes in its subcellular localization (Denef *et al.*, 2000; Corbit *et al.*, 2005). Recent studies in mice and in mammalian cell culture implicate Smo localization to the primary cilia as critical to Hh signal transduction (Huangfu *et al.*, 2003; Corbit *et al.*, 2005; Huangfu and Anderson, 2005; Rohatgi *et al.*, 2007), an organelle not found in most Hh sensing cells in flies (Huangfu and Anderson, 2006). Also, phosphorylation, as demonstrated in flies, represents one important event regulating Smo signaling. The kinases, PKA and CK1, phosphorylate Smo at multiple sites promoting full pathway activity (Jia *et al.*, 2004; Zhang *et al.*, 2004; Apionishev *et al.*, 2005), but the phosphorylation sites are not conserved from *Drosophila* to vertebrates. Recently, it has been reported, that Smo activity in the wing disk anterior–posterior compartment boundary requires and enhances the expression of Gprk2 (Molnar *et al.*, 2007), which represents the *Drosophila* homolog of mammalian GRK5 (Fan and Schneider, 2003).

Regulation of Smo signaling by direct phosphorylation has not been described in vertebrates; indeed, this is critical as the intracellular mechanisms controlling pathway activity appear to have diverged considerably between vertebrates and invertebrates (Varjosalo *et al.*, 2006). In heterologous cell systems it has been demonstrated, that Smoothened-mediated signal transduction can be regulated by GRK2 (Chen *et al.*, 2004). However, it has remained unanswered, whether this regulation can be applied to Hedgehog signaling in the whole organism. To determine if GRK2 functions in the facilitation of Smo signal transduction in vertebrates, we chose a trifold approach using mammalian cells, zebrafish and KO mice.

MATERIALS AND METHODS

Zebrafish Strains and Husbandry

A single outcross of ekwill and AB inbred lines (EK/AB) produced adult fish that were used for egg production for all of the studies. Zebrafish were maintained according to standard procedures in accordance with Duke University approved animal use IACUC protocols. The islet-GFP zebrafish line has been described (Higashijima *et al.*, 2000).

Cloning of Zebrafish GRK2/3 and Mutagenesis

A sequence with significant homology to the mammalian G protein–coupled receptor kinases GRK2 and GRK3 was identified by BLAST search of the Sanger zebrafish genome database (http://www.ensembl.org/Danio_rerio/index.html). A partial clone (amino acids 88–688 of final sequence) was obtained by PCR with *pfu* polymerase (Stratagene, La Jolla, CA) from cDNA (Protoscript, New England Biolabs, Ipswich, MA) generated from 24 hpf (hours after fertilization) embryos using the following primers: zGRK2/3 forward 5′-ATTAAAGAGTACGAGAAGTTGGACTCA; zGRK2/3 reverse 5′-TCACAGGCCGTTGCTGTTGCGGTG. 5′RACE PCR was performed (first choice RLM RACE, Ambion, Austin, TX) to obtain the full-length coding sequence and 5′-untranslated region (UTR). Full-length cDNA was cloned into the pCS2+ vector for expression in HEK293T cells and generation of capped mRNA for rescue experiments. Alignment was compiled using T-coffee (Notredame *et al.*, 2000) and illustrated using the Boxshade software. Mutagenesis of the full-length clone was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Morpholino Design and Microinjections

All zGRK2/3 morpholino antisense oligos (MOs) were designed and synthesized by Gene Tools (Philomath, OR), based on submitted sequences. The Shh MO has been published before (Nasevicius and Ekker, 2000). The zGRK2/3 ATG MO with the sequence, AGGTCCGCCATCTTCGCCCTCTGGG, was designed to the region encompassing the 5′-UTR and sequence downstream of the start codon. A five-base mismatch control MO (5_mis CT MO) was designed with the following sequence: AGCTCCCCCATCTTCCCCGTCTCGG. The genomic sequence spanning from exon 5 to exon 9 was amplified, cloned, and sequenced. Splice-blocking MOs were created to the exon/intron junctions of exon 6 and 7 (Supplemental Figure S3). Effective dose concentrations of 0.05 mM for the ATG and 5_mis MOs were determined by dose dilution experiments where the 5_mis control MO exhibited no morphological effects to 120 hpf. One nanoliter of 0.05 mM ATG and 5_mis MOs were injected into the yolk of 1–2-cell embryos using a Femtojet microinjector (Eppendorf, Fremont, CA), and pulled needles were calibrated with a micrometer under magnification. Capped mRNAs for injections were generated using the T7 and SP6 message machine kit (Ambion) using linearized and purified cDNA as the template.

In Situ Hybridization and Immunohistochemistry

In situ hybridization was performed following standard protocols. DIG-labeled probes were generated for the following zebrafish genes as described for *ptc-1*(Concordet *et al.*, 1996), *shh* (Krauss *et al.*, 1993), *nkx2.2* (Barth and Wilson, 1995), *smo* (Chen *et al.*, 2001), and *myoD* (Weinberg *et al.*, 1996), as well as for mouse patched (Adolphe *et al.*, 2004). A 1002-base pair fragment amplified from NM_130952.1 was used to detect zebrafish dmrt2. The probe for wnt11 was ordered from ZIRC (clone ID cb748). Pictures of stained zebrafish embryos were obtained with a Zeiss Axiovert compound microscope under Nomarski optics (Thornwood, NY) or alternatively with a Nikon stereoscope (Melville, NY). Immunostainings of whole zebrafish embryos and mouse cryosections (20 μm) were performed following standard protocols. The 4d9 antibody used 1:100, which recognizes the *engrailed* protein in the nuclei of muscle pioneer cells, was kindly provided by Nipam Patel (University of California, Berkeley, CA; Patel *et al.*, 1989). The Prox1 antibody (R&D Systems, Minneapolis, MN), which labels the nuclei of slow muscle fibers in zebrafish (Ochi *et al.*, 2006) was used at 1:500 dilution. Fluorescent secondary antibodies (Molecular Probes, Eugene, OR) were used for detection. Stained embryos were photographed on a Zeiss Axiovert compound microscope utilizing the Apotome or using a Zeiss LSM510 confocal microscopy system. Antibodies used on cryosections of E11.5 mouse embryos were obtained from the following sources: HB9 (1:2000, Abcam, Cambridge, MA), Pax7 (1:100, Developmental Hybridoma Bank, University of Iowa, Iowa City, IA).

Quantification of Slow Muscle Defects

Twenty-seven hpf embryos were fixed and stained using Prox1 and 4d9 antibodies. Numbers of slow-twitch muscle nuclei and muscle pioneer cells were counted in five somites over the yolk extension per embryo. Statistical analysis was carried out using GraphPad software (San Diego, CA).

Luciferase Assay

C3H10T1/2 cells were transfected using TransIT-LT1 reagent (Mirus, Madison, WI) at a density of 10^5 cells/well in six-well dishes with indicated plasmids along with a Gli-luciferase reporter and CMV–β-galactosidase (β-gal) as transfection control (Meloni *et al.*, 2006). Cells were harvested 72 h after transfection in 1× reporter buffer (Promega, Madison, WI). The Luciferase assay system (Promega) was used to measure the raw Gli-promoted signaling, which was normalized to the β-gal activity.

Smo Phosphorylation by zGRK2/3

HEK293T cells were transfected with human myc-tagged Smo and bovine GRK2 or zGRK2/3. After 48 h, the cells were washed in phosphate-free medium and labeled with ^{32}P-orthophosphate (0.1 mCi/ml) for 1 h. Cells were washed with cold PBS and lysed with buffer A (20 mM HEPES, 0.5% Nonidet P-40, 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 100 μM sodium orthovanadate, 50 mM sodium fluoride, and 1 μM microcystin). Smo was immunopurified from clarified supernatant with the use of anti-Myc affinity gel (Covance, Princeton, NJ). Immunocomplexes were washed with buffer A and analyzed on 10% SDS-PAGE gels and detected by autoradiography.

Western Blotting

Fifteen to 20 zebrafish embryos (24 hpf) were deyolked (Link *et al.*, 2006), and homogenized in 200 μl of lysis buffer (50 mM HEPES, 250 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 0.5% NP-40) containing proteinase inhibitors (Complete Mini, Roche, Alameda, CA). The lysates were cleared by short centrifugation. Samples were loaded with an equal amount of protein as measured using Bradford reagent (Bio-Rad, Richmond, CA) on SDS 10% polyacrylamide gel, resolved by electrophoresis (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. zGRK2/3 was probed using the C-14 antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) and detected by chemiluminescence. Recombinant zGRK2/3 was expressed in HEK293T cells by standard transfection using

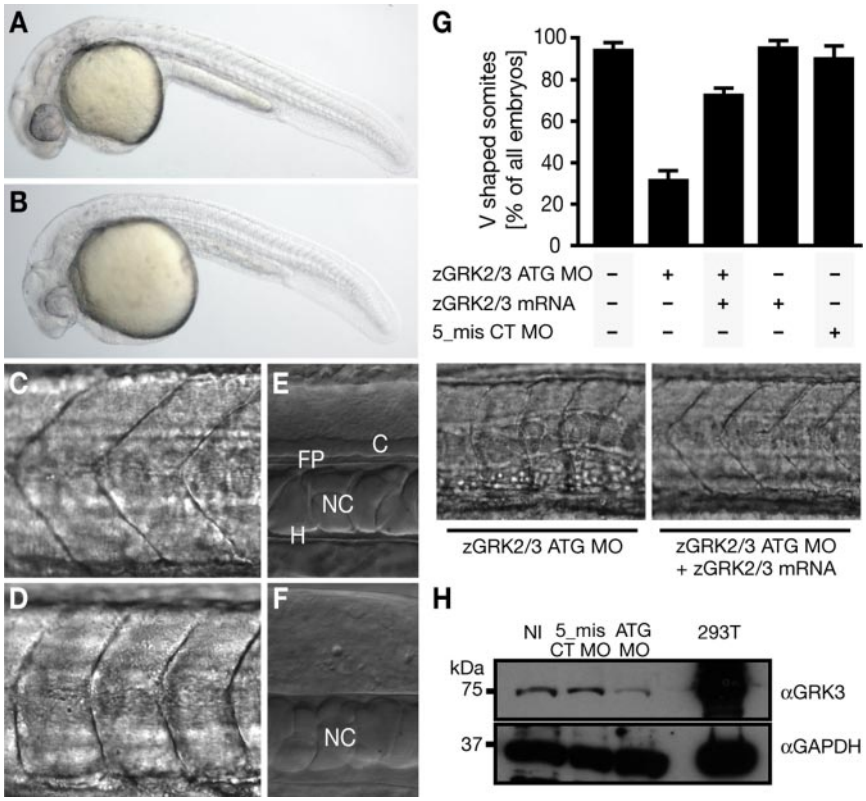

Figure 1. Role of zGRK2/3 in somite and neural development. (A, C, and E) Embryos injected with 5_mis CT MO; (B, D, and F) zGRK2/3 ATG MO-injected embryos. (A and B) Morphological defects in zGRK2/3 morphants, showing a slightly shortened body axis, impaired brain development, and malformed somites compared with the 5_mis CT MO-injected embryos at 30 hpf. (C–F) Nomarski view of somites and neural tube, illustrating the malformed somites and absence of cavitation in neural tube of morphant embryos at 24 hpf. (G) Rescue of somite development by simultaneous injection of zGRK2/3 mRNA at nontoxic doses and zGRK2/3 ATG MO. Bars represent the mean number of embryos out of three individual experiments; error bars, SEM. (H) MO-induced knockdown of zGRK2/3 expression in zebrafish embryos at 24 hpf. Western blots were probed with an antibody against mammalian GRK3. Loading control blot probed with an anti-GAPDH antibody. Figure shows representative blot out of three independent experiments. ATG MO, zGRK2/3 ATG MO; 5_mis CT MO, control MO; NI, not injected, 293T, lysate of HEK293 T-cells transfected with zGRK2/3 expression plasmid. NC, notochord; C, canal; FP, floor plate; H, hypochord.

calcium phosphate and extracted for subsequent gels by lysing cells in lysis buffer. Anti-GAPDH antibody (1:500, Abcam) was used as loading control.

RESULTS

Cloning of zGRK2/3

To provide an initial analysis for a possible in vivo role(s) of GRKs in vertebrate development, we chose the zebrafish because of the possibility to use a rapid reverse genetic approach in this model. Using the zebrafish genome at Sanger to mine for GRK homologues, we discovered a partial sequence bearing significant homology to the mammalian GRK2/3 cDNAs, the βARK kinase subfamily. A strong PCR product was produced from cDNA generated from 24-hpf zebrafish embryos, and upon RACE extension and sequencing a complete sequence for the zebrafish cDNA was obtained (GenBank accession number EU924796). The cDNA sequence shows the highest significance to the GRK2 cDNAs from mouse, rat, bovine, and human sources. However, multiple sequence alignments of the translated protein sequences reveal a slightly higher homology to the mammalian GRK3 (Supplemental Figure S1).

GRKs are defined by a highly conserved kinase domain. The kinases are modular proteins with domains both C- and N-terminal to the common kinase domain that control their localization and activity (Penela *et al.*, 2003; Supplemental Figure S1). Searching all available sequences, we did not identify another GRK with significant homology to the βARK subfamily of GRKs, suggesting that the zebrafish may have only one βARK-like kinase. Therefore, we have called this kinase the *zebrafish* GRK2/3 (zGRK2/3). Given the high homology in all regions between the human and fish proteins, zGRK2/3 is likely to possess the biochemical activities of the mammalian proteins.

Knockdown of zGRK2/3 in Zebrafish

To study the function of zGRK2/3 in early development, we utilize MOs directed at the ATG of the first codon and sequences in the 5′-UTR to knock down zGRK2/3 expression. To control for nonspecific effects, another MO with five base changes (5_mis CT MO) is included in all of the experiments. Furthermore, we characterize two additional splice-blocking MOs (Supplemental Figure S3). Western blot analysis using antibodies raised against the mammalian GRK3 protein reveals a single protein of the correct predicted molecular weight in 24 hpf whole embryo lysates of the noninjected and 5_mis CT MO-injected embryos (control embryos), but the signal is markedly reduced in zGRK2/3 ATG MO-injected embryos, demonstrating effective knockdown of zGRK2/3 expression (Figure 1H). Embryos with knocked down levels of zGRK2/3 develop a reproducible, distinct phenotype that is lethal between 72 and 96 hpf. In comparison, the control embryos survive and develop no apparent phenotype. The salient morphological features of the zGRK2/3 ATG MO-injected embryos include a shortened anterior–posterior axis, a smaller head, and malformed somites (Figure 1, A and B). Nomarski optics of 24 hpf embryos reveals U-shaped somites in the morphant embryos in comparison to control embryos, which display the typical "chevron" shape (Figure 1, C and D).

This phenotype is reversed by coinjection of zGRK2/3 mRNA, bearing silent mutations in the MO-binding site (103/142 embryos total displaying V-shaped somites in three injections; Figure 1G). Embryos injected with zGRK2/3 mRNA alone are indistinguishable (125/131 embryos in three injections) from uninjected fish (190/202 embryos) and from fish injected with a 5_mis CT MO (109/121 embryos in three injections), whereas injection with the zGRK2/3 ATG MO reproducibly decreases the number of

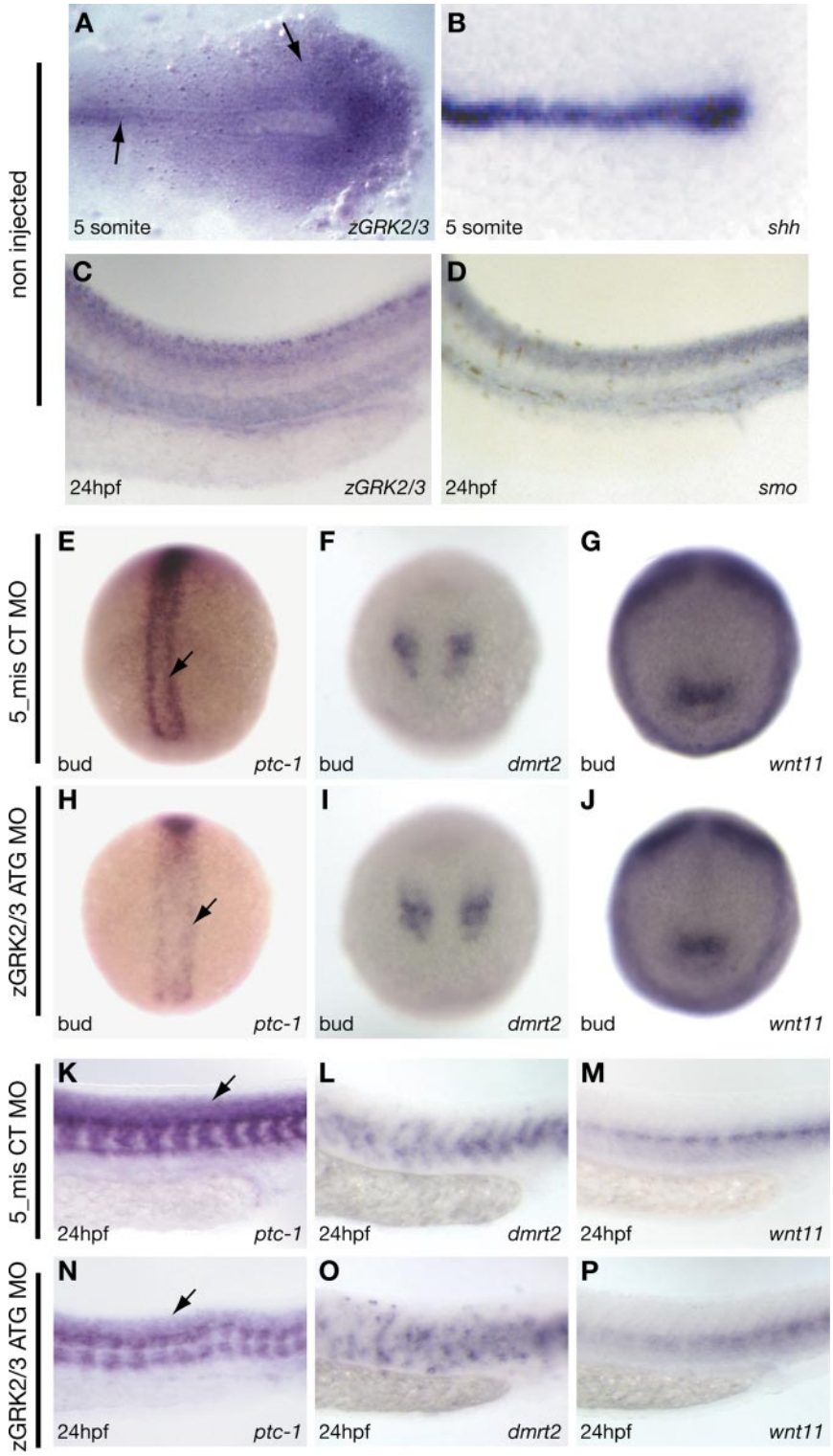

Figure 2. Implication of zGRK2/3 in the Hh pathway. (A) Flat-mounted embryos viewed dorsally reveal enriched GRK2/3 message in the tail bud caudally and in the notochord more rostrally (five-somite stage). (B) The expression of *shh* at the five-somite stage is restricted primarily to the notochord. (C) At 24 hpf, zGRK2/3 is expressed broadly in the embryo but is excluded from the notochord. (D) Expression pattern of *smo* at 24 hpf is complementary to the zGRK2/3 pattern. (E–P) In situ hybridization of *ptc-1* (E, H, K, and N) and the Hh-independent control genes *dmrt2* (F, I, L, and O) and *wnt11* (G, J, M, and P) in embryos injected with either the 5_mis CT MO (E–G at bud stage, K–M at 24 hpf) or the zGRK2/3 ATG MO (H–J at bud stage, N–P at 24 hpf).

fish with normal V-shaped somites (36/115 in three injections). Optical sectioning indicates the absence of cavitation in the spinal cord of the zGRK2/3 ATG MO embryos (Figure 1, E and F), suggesting that there may also be deficiencies in patterning of the neural tube. The absence of cavitation obscures the presence of the floor plate, which is present as revealed in subsequent experiments (see Figure 7, C and D, *shh* panel). The expression pattern of zGRK2/3 during early development, as determined by in situ hybridization, is consistent with zGRK2/3 playing a role in the patterning of these systems (Figure 2, A and C, and Supplemental Figure S2), because mRNA is detected in Hh-responsive areas and at later stages overlaps with Smo expression (Figure 2D).

To determine if the zGRK2/3 morphant embryos are deficient in Hh signaling, we analyzed the expression of *patched-1* (*ptc-1*) during early development. Ptc-1 is a direct

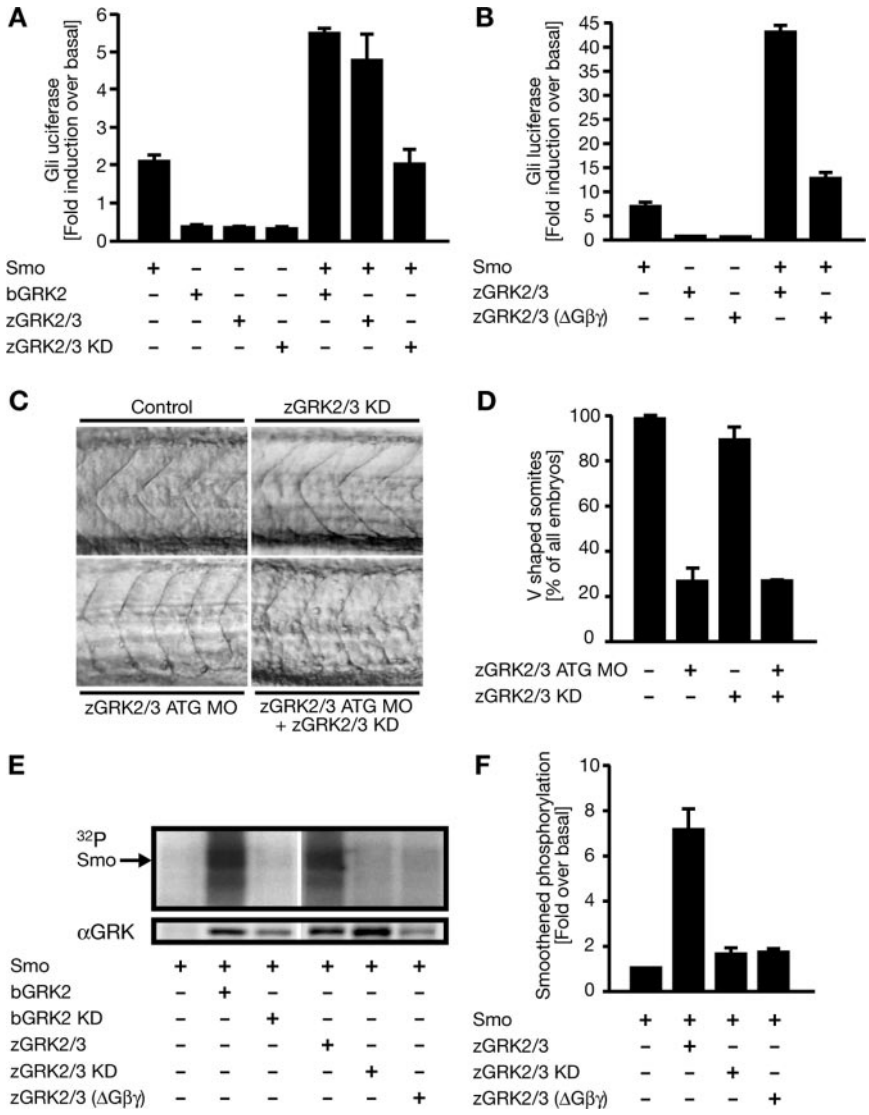

Figure 3. zGRK2/3 stimulates Hh signaling through Smo and directly phosphorylates Smo. (A) Smo (human) signaling in C3H10T1/2 cells promoted by zGRK2/3 and the kinase-dead zGRK2/3 KD (K220R) mutant is compared with the bovine protein (bGRK2). (B) Recruitment by G proteins is indispensable for the effect of zGRK2/3 on Smo-mediated signaling. zGRK2/3 ($\Delta G\beta\gamma$) is characterized by a point mutation (R587Q) described as necessary for interaction with the $\beta\gamma$-subunit of a G protein. (C) Endogenous Smo signaling depends on the kinase function of zGRK2/3 in fish, as rescue experiments using zGRK2/3 KD mRNA failed. Embryos injected with either zGRK2/3 ATG MO alone or in combination with zGRK2/3 KD mRNA still developed U-shaped somites (n = three sets of injections). (D) Graph showing percentages of embryos developing V-shaped somites under different treatments, depicted as mean ± SEM (n = 2 experiments). (E) Representative gel showing the phosphorylation of Smo by GRKs in HEK293T cells, which were transfected either with Smo alone or in combination with bovine bGRK2, bGRK2 kinase-dead (KD), zGRK2/3, or zGRK2/3 KD. White line indicates deletion of blank lanes in the gel. (F) Quantification of phosphorylation of Smo by zGRK2/3. Error bars, SD of two experiments.

transcriptional target of the Hh pathway, providing a mechanism for feedback inhibition (Alexandre *et al.*, 1996; Concordet *et al.*, 1996). During early somitogenesis, we detect a down-regulation of *ptc-1* transcripts in the hypoblast adjacent to the axial mesoderm in embryos injected with MOs against zGRK2/3 (18/27 embryos; Figure 2, E and H), whereas two Hh-independent genes (Ochi *et al.*, 2006), doublesex-related (dmrt2; Figure 2, F and I), and wnt11 (Figure 2, G and J) were unaffected. This effect persists at later stages, where *ptc-1* levels in the somites and the neural tube remain decreased compared with control embryos (34/50 embryos; Figure 2, K and N). Similarly to the earlier stage analyzed, dmrt2 levels are not altered in zGRK2/3 morphants (Figure 2, L and O). However, wnt11 appears to be down-regulated in the medial somite, where muscle pioneer cells are located (Figure 2, M and P). These results suggest a specific reduction in Hh signaling in embryos with reduced zGRK2/3 expression.

zGRK2/3 Directly Phosphorylates Smo and Promotes Gli-directed Transcription

Given the morphology of the zGRK2/3 morphants and the down-regulation of *ptc-1* transcript levels, we analyzed the effect of zGRK2/3 and two mutated versions on Hh signaling in a cell-based model. The assay in C3H10T1/2 cells utilizes a luciferase reporter under the control of the Gli1 promoter, providing a sensitive readout of Smo signaling (Figure 3A; Meloni *et al.*, 2006). zGRK2/3 alone, like bovine GRK2 (bGRK2), does not activate Gli-promoted transcription in these cells. However, when cotransfected with Smo, zGRK2/3, and bGRK2 enhance Smo-mediated activity of Gli-promoted transcription to a high level, indicating a synergistic effect. This synergy is dependent on the kinase activity of zGRK2/3, as the kinase dead mutant (K220R) is unable to increase Smo-induced pathway activity (Kong *et al.*, 1994). We also tested a previously characterized point mutant (R587Q) that was described to maintain full kinase activity but was unable to interact with $G\beta\gamma$ subunits (Carman *et al.*, 2000). GRKs of the βARK-like kinase subfamily have the ability to interact with $\beta\gamma$ subunits of G proteins through a binding motif in the C-terminus. This interaction has been shown to be critical for targeting of the kinase to the plasma membrane during receptor activation. Indeed, this mutant displayed a substantially reduced ability to synergize with Smo to promote Gli signaling, suggesting that

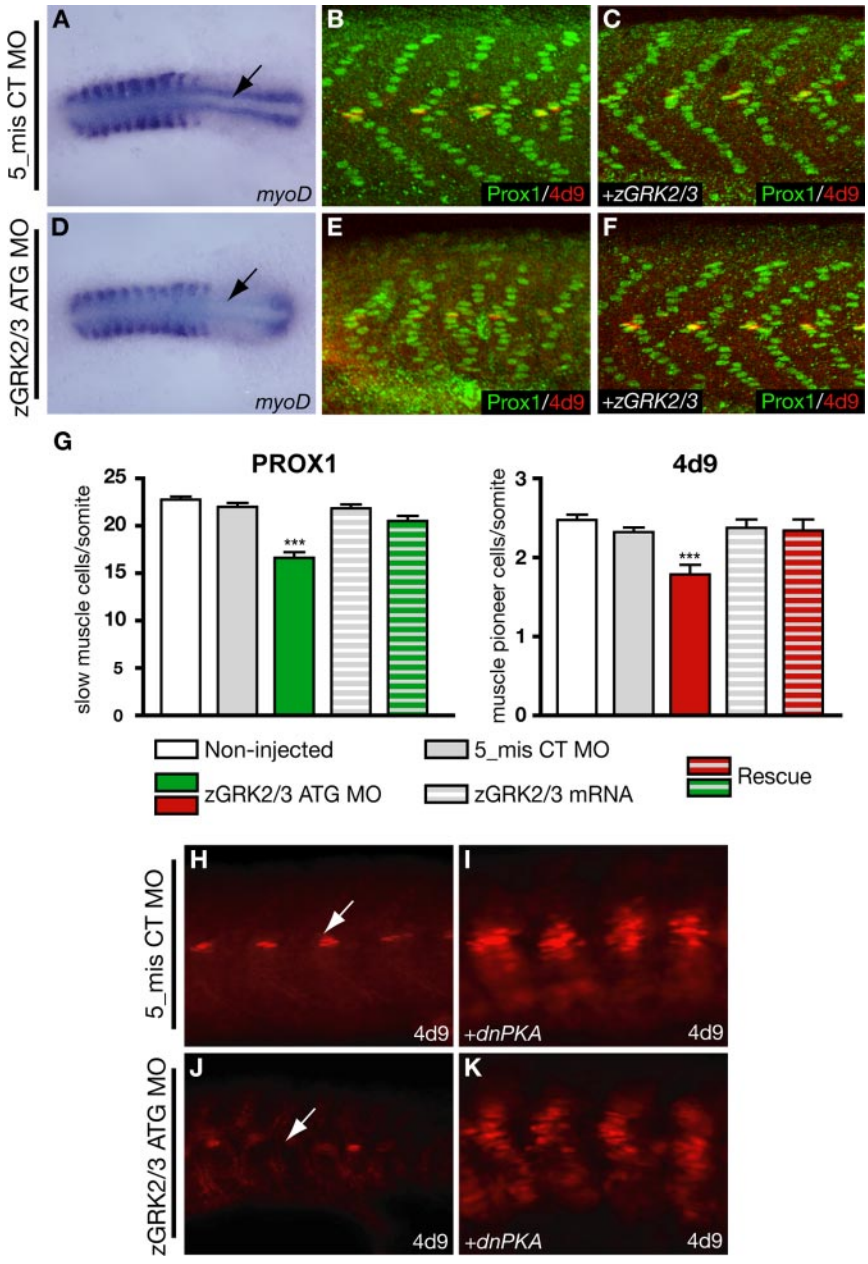

Figure 4. zGRK2/3 is important for proper slow muscle development. (A and D) Analysis of *myoD* expression during somitogenesis in 15 hpf embryos injected with the zGRK2/3 ATG MO or 5_mis CT MO. Embryos are flat mounted and viewed dorsally. Arrows indicate the adaxial staining, which is reduced in the knock-down embryos. (B, C, E, and F) Analysis of slow muscle development by whole-mount immunostaining of 27 hpf embryos with antibodies labeling slow muscle nuclei (Prox1, green) and engrailed+ muscle pioneer cells (4d9, red). Lateral view of embryos injected with 5_mis CT MO (B) or zGRK2/3 ATG MO (E) as well as embryos co-injected with low doses of MO resistant zGRK2/3 mRNA (C and F). (G) Graphical representation of numbers of slow muscle nuclei and engrailed+ nuclei indicating muscle pioneer cells. Both, slow muscle fiber and muscle pioneer development could be fully rescued by zGRK2/3 mRNA bearing silent mutations for the MO-binding site. Data represent average ± SEM $< p <$ 0.0001. (H and J) Whole-mount immunohistochemistry using the 4d9 antibody of 27 hpf embryos injected with either 5_mis CT MO (H) or zGRK2/3 ATG MO (J). White arrows in 4d9 panel indicate reduction of engrailed staining in the knock-down embryos compared to controls. (I and K) Increase of 4d9-expressing cells in somites of embryos injected with indicated MO and mRNA encoding for dominant negative PKA (dnPKA).

zGRK2/3 may function in Hh signaling in a manner analogous to other 7TM receptor systems (Figure 3B).

To validate these results in vivo, we used again the zebrafish system. We hypothesized that kinase dead zGRK2/3 would be unable to rescue the zGRK2/3 morphant zebrafish. Indeed, coinjection of kinase dead zGRK2/3 mRNA with the zGRK2/3 ATG MO does not reverse the effects of the zGRK2/3 ATG MO on somite development (25/93 embryos total in two injections vs. 53/54 in control embryos, 21/77 in zGRK2/3 ATG MO-treated embryos and 72/81 embryos injected with zGRK2/3 K220R mRNA; Figure 3, C and D).

Given that zGRK2/3 can synergize with Smo to enhance Hh signaling in cells, we analyzed the ability of zGRK2/3 to directly phosphorylate Smo (Figure 3, E and F). Human Smo overexpressed in HEK293T cells is constitutively phosphorylated at low levels. However, phosphorylation of Smo is stimulated about sevenfold by cotransfection of zGRK2/3. This activity is similar to the observed activity for bGRK2 (Chen *et al.*, 2004). In accordance with the signaling data in cells and rescue data in zebrafish embryos, the kinase dead mutant of zGRK2/3 is unable to phosphorylate Smo. Taken together, the signaling and phosphorylation data imply that zGRK2/3 stimulates Hh signaling in cells and that this activity likely relates to its ability to enhance Smo activity by directly phosphorylating Smo.

zGRK2/3 in Slow Muscle Development

Because the kinase activity of zGRK2/3 promotes Smo phosphorylation and Smo-directed Hh signaling in cellular assays and zebrafish, we sought to understand the role of this kinase in Hh signaling in vivo. Studies in the zebrafish have revealed that the Hh pathway mediates many aspects of the development of the somites and skeletal musculature (Lewis *et al.*, 1999; Barresi *et al.*, 2000; Wolff *et al.*, 2003; Ingham and Kim, 2005). Mutations identified in forward genetic screens

have demonstrated that a reduction of Hh signaling during development hinders the development of the two slow-muscle cell types, the superficial slow muscles and the slow muscle pioneer cells. In brief, Hh released from the notochord specifies the identity of these slow muscle precursors emerging from the surrounding presomitic mesoderm (Blagden *et al.*, 1997). During somitogenesis, adaxial cells adjacent to the notochord as well as cells in the forming somites express the myogenic transcription factor *myoD* in response to Hh (Barresi *et al.*, 2000).

To assess the role of zGRK2/3 in the development of slow muscle, we analyzed the affect of zGRK2/3 knock-down on the expression of slow muscle markers. Accordingly, the zGRK2/3 morphant embryos display a marked reduction in *myoD* expression in the adaxial cells as well as a slight reduction in the somites compared with control embryos (53/79 embryos in three injections; Figure 4, A and D). Moreover, differentiation of slow muscle cells is perturbed in the morphant embryos. Both, superficial slow fibers as visualized by Prox1 staining of their nuclei (Figure 4, B and E; green; 22–28 embryos per condition in two separate injections) and *engrailed*+ muscle pioneers were significantly reduced in numbers in zGRK2/3 ATG MO-injected embryos (Figure 4, B and E, as well as H and J; 4d9, red; 67/79 embryos from three injections). This phenotype could be successfully rescued by coinjection of zGRK2/3 mRNA-bearing silent mutations for the MO-binding site (Figure 4, C and F; n = 24–25 embryos per condition in two separate injections). The incomplete penetrance of the slow muscle phenotype may be a result of a functionally redundant kinase partially compensating for the loss of zGRK2/3 or alternatively, that low levels of zGRK2/3 activity persist in these tissues. Indeed, similar varying levels of slow muscle developmental defects have been described in the different Hh pathway genetic mutants (Wolff *et al.*, 2004), potentially indicating compensatory effects by other genes or pathways in the development of slow muscle. Nevertheless, these data demonstrate that embryos with reduced zGRK2/3 expression are unable to respond effectively to Hh signals during early segmentation, resulting in significant impairment in the development of slow muscle.

To assess whether these embryos are still competent to signal through the Hh pathway and also at what level in the pathway zGRK2/3 is active, we coinjected mRNA for a dominant negative version of PKA (dnPKA). This construct when overexpressed stimulates ectopic formation of muscle pioneers through constitutive activation of Hh signaling at the level of Gli (Hammerschmidt *et al.*, 1996; Schauerte *et al.*, 1998; Barresi *et al.*, 2000), as visualized by an increase in the number of *engrailed*+ muscle pioneer cells (Figure 4, I compared with H). In the absence of normal levels of zGRK2/3, the expansion of the number of muscle pioneer cells still occurs, suggesting that zGRK2/3 acts upon the pathway upstream of Gli (12/12 embryos; Figure 4, K compared with J). To further investigate the point at which zGRK2/3 exerts its activity on the Smo signaling cascade, we examined the expression of the Hh target gene *nkx2.2* in the developing brain (Barth and Wilson, 1995). As shown in Figure 5, A and B, zGRK2/3 ATG MO causes a notable reduction of *nkx2.2* in the brain. As previously reported, overexpression of Shh leads to the expansion of ventral cell fates in the nervous system as can be seen by the ectopic induction of *nkx2.2* (27/34 embryos; Figure 5C; Wilbanks *et al.*, 2004). Consistent with the notion that zGRK2/3 functions downstream of Smo, coinjection of the zGRK2/3 ATG MO with Shh mRNA normalizes the ectopic expression of *nkx2.2* in the brain toward control levels (16/24 embryos; Figure 5D, compare with 5A).

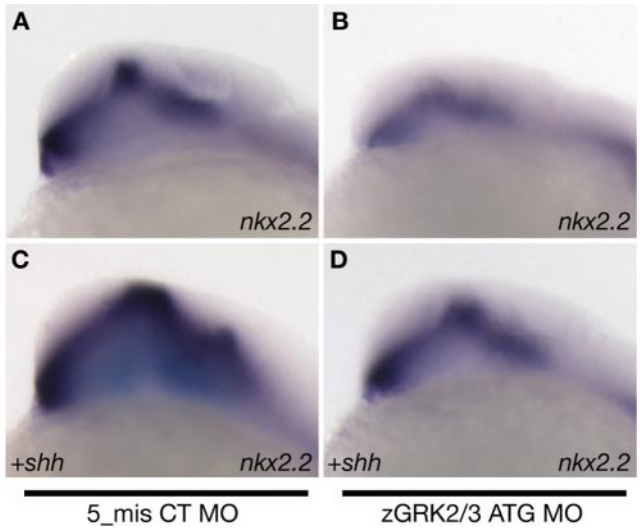

Figure 5. Amelioration of the effects of Shh overexpression by zGRK2/3 knockdown. (A and B) Level of *nkx2.2* expression in control embryos and in zGRK2/3 morphants. (C) Embryos injected with capped *shh* mRNA. (D) Knockdown of zGRK2/3 is able to normalize unrestrained Hh signaling to control levels. All pictures: 24 hpf, anterior to the left, dorsal to the top.

Low Levels of Hh Augment the zGRK2/3 Phenotype

GPRK2, the closest homolog of nonvisual GRKs in vertebrates, is expressed in areas of high Hh levels and activity in the imaginal wing disk of *Drosophila*. Loss of GPRK2 by RNAi resulted only in a phenotype resembling a moderate loss of Hh signaling. However, complete abolishment of Hh signaling can be achieved by simultanous reducing Hh. Thus, it was suggested, that GPRK2 might be necessary for the signal transduction of high Hh levels (Molnar *et al.*, 2007).

In our studies we see a similar, moderate decrease of Hh signaling if zGRK2/3 levels are reduced. Therefore we tested for synergistic effects with zGRK2/3 by titrating Shh levels. Ptc-1 has been reported to be a transcriptional target associated with high Hh-signaling levels in flies and fish (Sekimizu *et al.*, 2004; Molnar *et al.*, 2007). As demonstrated earlier in here, knockdown of zGRK2/3 decreases *ptc-1* (Figure 6, A and B). Likewise, embryos injected with a low dose of Shh MO express lower levels of *ptc-1* (Figure 6C). Simultaneous injection of zGRK2/3 ATG MO and Shh MO synergistically abrogated Smo-mediated Hh signal transduction, as *ptc-1* was barely detectable (Figure 6D; n = 35–63 embryos per condition).

In fish, the development of muscle pioneer cells depends on highest levels of Hh signaling (Wolff *et al.*, 2004), whereas slow muscle fibers require moderate to low Hh. Accordingly, zGRK2/3 morphants display a stronger defect in muscle pioneer development, although slow muscle fiber formation is affected, too. Gradual depletion of Shh by injection of increasing doses of MO reduces more strongly the number of muscle pioneer cells than those of slow muscle fibers (Figure 6, G and I). Consistently with our gene expression result above, coinjection of Shh MO and zGRK2/ATG MO synergistically decreased both muscle subtypes (Figure 6, H and I; n = 18–35 embryos per condition in two separate

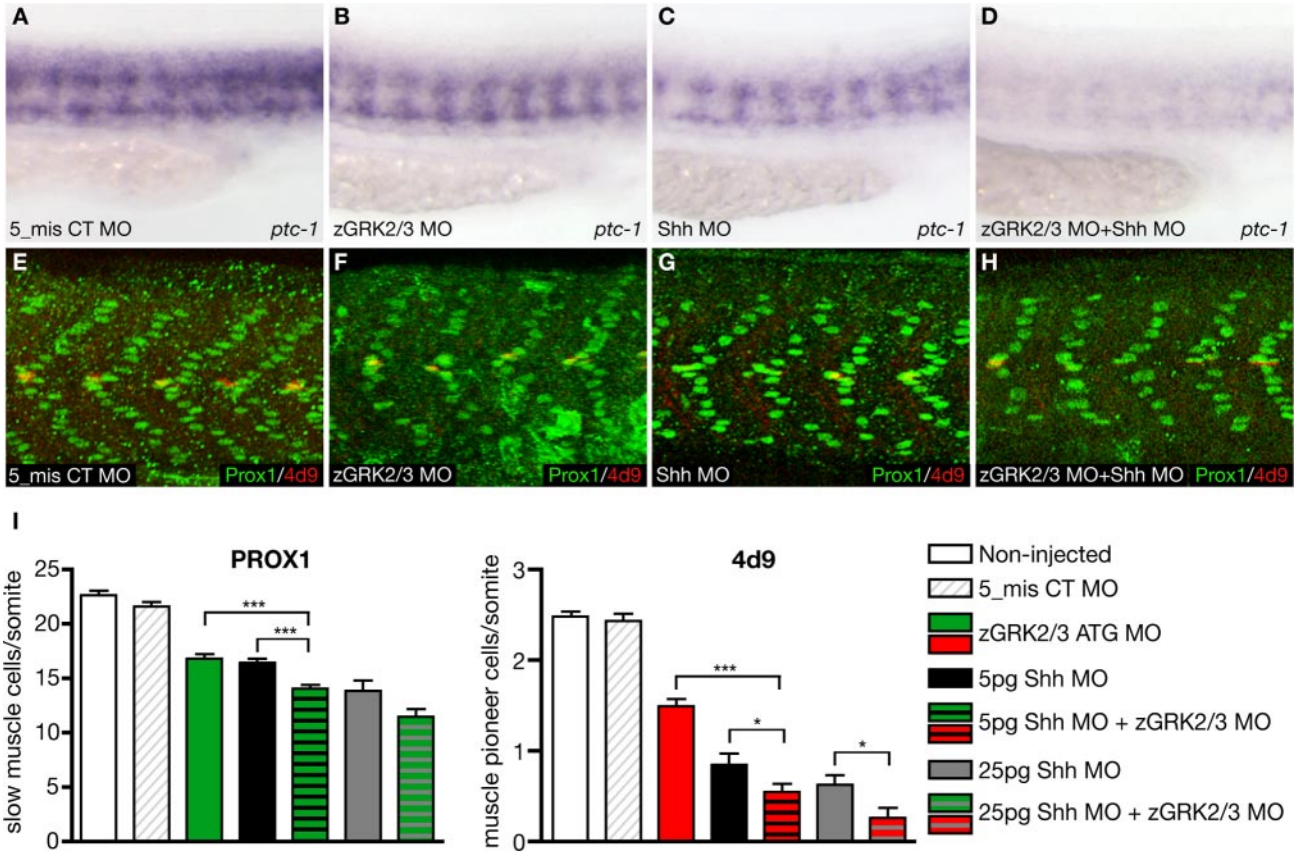

Figure 6. Shh reduction potentiates the effects of the zGRK2/3 knockdown. (A–D) Coinjection of zGRK2/3 ATG MO and an intermediate dose of Shh MO synergistically decreases the expression of *ptc-1* (D) when compared with embryos injected with 5_mis CT MO (A), zGRK2/3 ATG MO (B), or Shh MO (C). (E–H) Confocal images (z-stacks) of injected fish colabeled for slow muscle fiber nuclei (Prox1, green) and muscle pioneer cells (4d9, red) in control (E), zGRK2/3 morphants (F), Shh MO-injected (G) and coinjected embryos (H). The Shh MO dose for the picture shown was 5 pg per egg. (I) Numbers of Prox1+ and *engrailed*+ (4d9) cells, respectively. Data represent average ± SEM; $p < 0.0001$ for zGRK2/3 ATG MO versus 5pg Shh MO + zGRK2/3 ATG MO (Prox1 and 4d9) and 5 pg Shh MO versus 5 pg Shh MO + zGRK2/3 ATG MO (Prox1); $p < 0.05$ for 5 pg Shh MO + 5 pg Shh MO + zGRK2/3 ATG MO (4d9); $p < 0.03$ for 25 pg Shh MO + 25 pg Shh MO + zGRK2/3 ATG MO (4d9). All photographs: Lateral view of 27-hpf embryos, anterior is to the left.

injections). Thus, similarly to Gprk2 in flies, low levels of Hh can augment the phenotype of zGRK2/3 in zebrafish.

zGRK2/3 Is Involved in Patterning of the Neural Tube

Developmental studies in the fish, mouse, and chick have identified Hh as a key morphogen in the patterning of the ventral neural tube (Ruiz i Altaba *et al.*, 2003). To assess the contribution of zGRK2/3 to Hh mediated signaling in the neural tube, we analyzed again the expression of *nkx2.2*. In noninjected and control injected embryos, *nkx2.2* is expressed in the middiencephalic boundary regions of the brain and in lateral floor plate of the neural tube at 24 hpf (Figures 5A and 7A). In the zGRK2/3 morphant embryos, tissues express significantly lower levels of *nkx2.2*, consistent with the idea that zGRK2/3 is important in transducing the Hh signal (36/45 embryos in two injections; Figure 7B). These data indicate a requirement of zGRK2/3 in the developing neural tube for the appropriate cellular response to the Hh signals from the floor plate and notochord. Indeed, in the morphant embryos, the floor plate and notochord produce the *shh* transcript at normal levels, suggesting that the defect is in the response to Hh not in its production (Figure 7, C and D). Moreover, *smo* transcript levels are comparables in the control and zGRK2/3 ATG MO-treated embryos suggesting that the Hh signaling defects are not a result of reduced levels of Smo (Figure 7, E and F).

Hh signaling is important for the differentiation of all motoneurons in the zebrafish, and embryos with reduced levels of Hh signaling show reductions in the number of islet1-positive motoneurons (Chen *et al.*, 2001; Lewis and Eisen, 2001). Accordingly injection of islet1-GFP transgenic embryos with zGRK2/3 ATG MO causes a marked reduction in both the number of isletl1-positive cells in the neural tube and in motor axon outgrowth (Figure 7, G and H, arrows and arrowhead; 23/31 embryos observed from two injections).

Similarity of GRK2-deficient Mice with Hh Mutants

In mice, deletion of GRK2 causes embryonic lethality, which has been attributed to a defect in heart development (Jaber *et al.*, 1996). However, the embryonic lethality of the constitutive KO presumably cannot solely be explained by a heart defect (Matkovich *et al.*, 2006). Apart from the apparent failure of cardiac development, GRK2 KO embryos display multiple aberrations from normal embryonic development. Approximately half of the dissected GRK2 KO embryos at embryonic day (E)11.5 are dramatically retarded in growth and embryonic development stalls around E9.5. They usu-

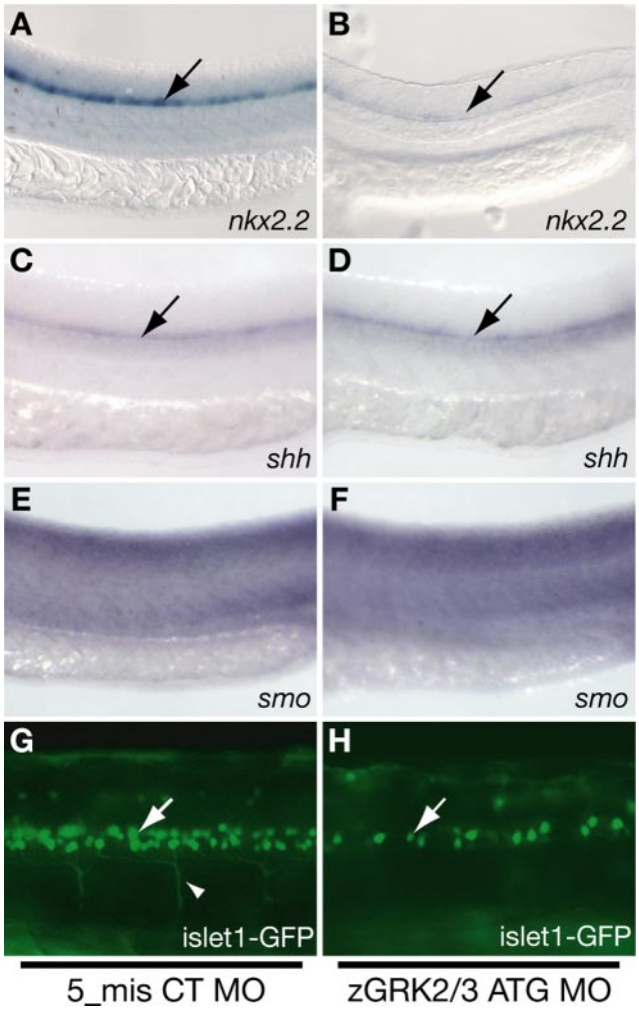

Figure 7. Patterning defects in the neural tube of zGRK2/3 morphant embryos. (A and B) Expression of *nkx2.2* in injected embryos at 27 hpf in the neural tube (black arrow) between somites 5 and 10. (C–F) Expression of *shh* (C and D) and *smo* (E and F) in the trunk of injected embryos at 27 hpf. (G and H) Lateral views between somites 5 and 10 of 48-hpf injected *islet1*-GFP transgenic embryos. GFP labeling indicates significantly fewer motoneurons (white arrow) in the spinal cord and motor axon outgrowth defects of zGRK2/3 morphant embryos (white arrowhead). All pictures: anterior to the left, dorsal to the top.

ally lack visible external eye structures and limbs (Figure 8, A and B). Similar phenotypes have been described in mouse models of impaired Hh signaling (Chiang *et al.*, 1996; Zhang *et al.*, 2001; Kawakami *et al.*, 2002). Consistent with the hypothesis that zGRK2/3 is required for Hh signal transduction, we observe a reduction in the number of motoneurons in the lumbosacral region of the spinal cords of the GRK2 KO animals, when compared with sections of wild-type (wt) littermates (GRK2 KO: n = 4, wt: n = 4). Also, the remaining motoneurons are not concentrated in the ventrolateral region of the spinal cord; instead they appear scattered along the dorsal–ventral axis of the neural tube (Figure 8, C and D).

Previously, it has been demonstrated, that impaired Hh signaling causes disruption of normal spinal cord patterning. Although KOs or mutants for negative regulators of Hh signaling such as FKBP8 or Rab23 have been characterized by the expansion of ventral cell fates as motor neurons and

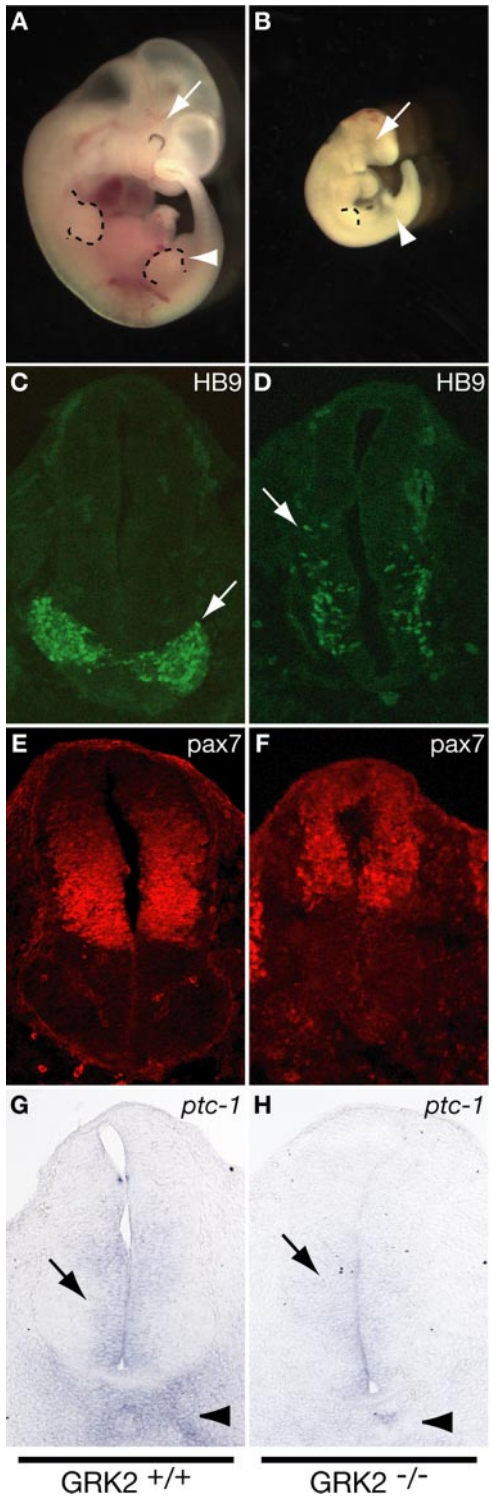

Figure 8. Mouse GRK2 KO embryos display defects characteristic of impaired hedgehog signaling. External and spinal cord morphology of wt (A, C, E, and G) and GRK2 KO embryo (B, D, F, and H) at E11.5. (A and B) GRK2 KO embryos are severely retarded in growth and lack externally visible eye structures (arrow). In addition, limb development is impaired (arrowhead). Images are taken at same magnification. Dashed lines indicate the contour of limbs. (C–F) Expression of HB9 (C and D) and Pax7 (E and F) in the caudal neural tube at E11.5 as assessed by immunohistochemistry. (G and H) *Ptc1* mRNA is reduced in spinal cords of GRK2 KO embryos.

adjacent cell types (Bulgakov *et al.,* 2004; Eggenschwiler *et al.,* 2006), it has been shown, that loss of Smo or Shh itself caused the complete absence of ventral neural cell types such as motoneurons (Liu *et al.,* 2005; Eggenschwiler *et al.,* 2006). In contrast to Shh KOs, more dorsal populations of interneurons appear less affected in GRK2 KO embryos, as Pax7-positive cells are still present although potentially less abundant in the mutant mice (Figure 8, E and F). Similar findings have been reported in mice bearing a hypomorphic mutation for IFT88, a protein involved in ciliary function, which has been shown to act as an important modulator of Smo function (Liu *et al.,* 2005).

In our zebrafish studies, we find, that zGRK2/3 is necessary for Smo signaling. By in situ hybridization for *ptc-1* we reproducibly detect a decrease in ptc-1 in commissural neurons and motoneurons of spinal cords of GRK2 KO embryos (KO: n = 5; wt: n = 4), consistent with a reduction in Hh signaling (Figure 8, G and H). Together, these data reveal a significantly blunted Hh transcriptional response in the neural tube with defects in the differentiation of motor neurons of teleost and mammalian embryos.

DISCUSSION

In the biology of conventional GPCRs, phosphorylation of the activated receptor by GRKs desensitizes the ability of the receptor to signal through heterotrimeric G proteins. Instead it promotes the interaction with arrestin family proteins. However, these interactions trigger two other important events. First, they act as signals for receptor trafficking toward the endocytic machinery and subsequent internalization. Second, through the ability of β-arrestin to scaffold signaling complexes, they promote G protein–independent signaling. Therefore either of these mechanisms could play a role in the Hh-signaling pathway. Smo activity is closely tied to its localization in cells in both vertebrates and invertebrates. In flies, Smo accumulates at the cell surface in response to activation and phosphorylation (Denef *et al.,* 2000; Jia *et al.,* 2004; Zhang *et al.,* 2004; Apionishev *et al.,* 2005). However, in vertebrates, strong evidence suggests that a prerequisite of Smo activity is its translocation to the primary cilia (Corbit *et al.,* 2005). In fact, Kovacs *et al.* (2008) recently demonstrated, that in NIH3T3 cells Smo is driven into cilia by an interaction between β-arrestin and the ciliary motor protein Kif3a. Phosphorylation of Smo by GRK2 may be the prerequisite for a stable Smo/β-arrestin interaction, in turn regulating the trafficking of Smo to and from the cilia. Interestingly, flies without cilia develop morphologically normally, most probably because most cells in flies do not form ciliary structures with the exception of sperm and sensory neurons (Basto *et al.,* 2006).

Another possible mode for GRK regulation of Hh signaling is through its promotion of a signaling complex. The scaffolding activity of β-arrestin 2 in the signal transduction of other 7TMR systems has been described extensively in cell culture and more recently in mice (DeFea *et al.,* 2000; McDonald *et al.,* 2000; Luttrell *et al.,* 2001; Beaulieu *et al.,* 2005; Lefkowitz and Shenoy, 2005). Hh signaling in *Drosophila*, involves the kinesin-like protein, Costal-2, which regulates signaling by orchestrating a series of interactions between Ci, Fused, Suppressor of Fused, and Smo (Lum *et al.,* 2003). Morpholino studies in zebrafish have suggested that the Costal-2 homolog can regulate Hh signaling similarly to its role in flies (Tay *et al.,* 2005). So far, Kif7/Kif27, the mammalian costal-2 homologues have not been genetically ablated in mice. However, the studies in zebrafish suggest that deletion of Kif7/Kif27 in mice may result in ectopic activation of the Hh pathway. These studies and others intimate that there may be significant differences in the spatial regulation of the fundamental Hh pathway components between vertebrates and invertebrates, but that there may also be variation in the fundamental players of the pathway between vertebrates (i.e., fish and mice).

Indeed, it appears, that a single GRK may be sufficient to facilitate Smo signaling in some tissues in mice, as the deletion of GRK2 only, already causes embryonic lethality. GRK2 KO embryos display many of the defects that have been reported in mice with impaired Hh signaling (Chiang *et al.,* 1996; Zhang *et al.,* 2001; Kawakami *et al.,* 2002; Liu *et al.,* 2005; Eggenschwiler *et al.,* 2006). Conversely, the GRK3 KO mice develop normally. However, these KO mice express detectable levels of GRK3 mRNA, which may explain why developmental abnormalities are not observed (Peppel *et al.,* 1997). β-arrestin 1 and 2 KO mice, on the other hand, are viable as single KOs and are anatomically indistinguishable from their wt littermates (Conner *et al.,* 1997; Bohn *et al.,* 1999; Gainetdinov *et al.,* 2004). However, a double deletion of barr1 and 2 is embryonic lethal and possibly mimics mice defective in Hh signaling (DeWire *et al.,* 2007). GRK2 KO embryos display a mild Hh phenotype, more similar to mutants of intraflagellar transport (IFT) proteins than to mice lacking Smo. As with the GRK2 KOs, motoneurons in IFT mutant mice are scattered throughout the spinal cord, whereas dorsal neuronal populations are less affected (Liu *et al.,* 2005). Consistent with the diminished expression of *ptc-1* in GRK2 KO embryos, the embryonic lethal phenotype of GRK2 KO may indeed be due to a pleiotropic, extracardiac function of GRK2 and not simply be borne from insufficiencies in heart function (Matkovich *et al.,* 2006).

At present, we cannot exclude the importance of any other GRKs for Hh signal transduction. The zebrafish genome contains three more nonvisual GRKs with significant homology to mammalian GRKs 4–6. It remains to be uncovered, if any of those may correlate to *Drosophila* Gprk2 in function. However, considering, that deletion of any of the GRK4–6 family kinases does not interfere with embryonic development, strongly suggests that GRK2 is the vertebrate G protein–coupled receptor kinase enabling Smo signaling.

In summary, herein we provide evidence for a potential physiological role for a component of the GPCR desensitization machinery to facilitate the signaling of a GPCR-like protein. We demonstrate in zebrafish embryos that a βARK-like GRK is critical to Hh-mediated patterning. Our data indicate that by direct phosphorylation of Smo zGRK2/3 acts as a permissive factor in high level Hh signaling. As in the zebrafish, interfering with the function of this kinase in mice leads to embryonic phenotypes that resemble loss-of-function mutants of the Hh-signaling pathway. Although further studies will be necessary to fully elucidate the mechanism(s) of GRK2 action in Hh signaling, our observations in multiple experimental systems, strongly suggest a model where this kinase actively participates in positively mediating cellular signaling and tissue patterning through the direct regulation of Smo in vertebrates.

ACKNOWLEDGMENTS

We thank Philip Dilorio (University of Massachusetts, Worcester, MA), Nipam Patel (University of California, Berkeley, CA), Kenneth Poss (Duke University, Durham, NC), Ian Woods (Harvard University, Cambridge, MA) and Zeng-Jie Yang (Duke University, Durham, NC) for reagents, fish, and technical advice. G.B.F. thanks the instructors and organizers of the zebrafish course hosted at the Marine Biological Laboratories, Woods Hole, MA. We thank Tama Evron, Jeff Kovacs, Kenneth Poss, Erik Myers, and Robert J. Lefkowitz for critically reading the manuscript. The authors also thank Kelly

Nembhard for excellent laboratory assistance, Alexandra Lepilina for assistance with the zebrafish facility maintenance, and Wendy Roberts for mouse care. This work was supported by National Institutes of Health Grants GM069086 (G.B.F), GM74349 (A.R.M.), HL61635 (L.S.B.), CA113656 (W.C.), and NS19576 (M.G.C.). M.P. received support from the Novartis Foundation, Human Frontier Science Progam, and the Marie Curie Outgoing International Fellowship of the European Union and A.R.M from the Raychem/Rogers/Morris Postdoctoral Fellowship.

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