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The transcriptional repressor REST/NRSF modulates hedgehog signaling

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ABSTRACT

The spatial and temporal control of gene expression is key to generation of specific cellular fates during development. Studies of the transcriptional repressor REST/NRSF (RE1 Silencing Transcription Factor or Neural Restrictive Silencing Factor) have provided important insight into the role that epigenetic modifications play in differential gene expression. However, the precise function of REST during embryonic development is not well understood. We have discovered a novel interaction between zebrafish Rest and the Hedgehog (Hh) signaling pathway. We observed that Rest knockdown enhances or represses Hh signaling in a context-dependant manner. In wild-type embryos and embryos with elevated Hh signaling, Rest knockdown augments transcription of Hh target genes. Conversely, in contexts where Hh signaling is diminished, Rest knockdown has the opposite effect and Hh target gene expression is further attenuated. Epistatic analysis revealed that Rest interacts with the Hh pathway at a step downstream of Smo. Furthermore, we present evidence implicating the bifunctional, Hh signaling component Gli2a as key to the Rest modulation of the Hh response. The role of Rest as a regulator of Hh signaling has broad implications for many developmental contexts where REST and Hh signaling act.

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Introduction

The emergence of the diverse cell types that comprise the vertebrate nervous system is dependent on a carefully orchestrated program of gene expression. The transcriptional repressor REST (also known as NRSF) has been implicated as a major regulator of neural gene expression (Ballas et al., 2005; Lunyak et al., 2002; Westbrook et al., 2008). *Rest* is expressed in neuronal precursors and *in vitro* studies demonstrate that the degradation of REST protein is a key step in differentiation of neural progenitors in culture (Ballas et al., 2005; Westbrook et al., 2008). Recent studies have identified hundreds of REST target sites that potentially regulate an immense set of transcripts, including miRNAs (Ballas et al., 2005; Bruce et al., 2004; Conaco et al., 2006; Johnson et al., 2007; Mortazavi et al., 2006; Otto et al., 2007; Singh et al., 2008; Su et al., 2004; Watanabe et al., 2004).

However, only a fraction of putative RE1-regulated transcripts are upregulated when REST is degraded (Johnson et al., 2008). Many studies highlight the importance of developmental context on the activity of REST (Bergsland et al., 2006; Chen et al., 1998; Johnson et al., 2008; Jorgensen et al., 2009). For instance, while overexpression of REST blocks terminal differentiation *in vitro* (Ballas et al., 2001), mosaic expression of REST in chick did not prevent overt differentiation of neural precursors (Paquette et al., 2000). However, upregulation of terminal differentiation genes was observed upon inhibition of REST function in chick spinal cord (Chen et al., 1998). This upregulation was shown to require the presence of upstream activators of those genes (Bergsland et al., 2006). Most importantly, premature neurogenesis was not observed in the mouse knockout of *Rest*, and only one of several RE1 containing genes tested was inappropriately expressed (Chen et al., 1998). *Rest*$^{-/-}$ embryos are retarded in growth and do not survive past E11.5, precluding in-depth studies of REST function during maturation of the nervous system. However, early induction and patterning of the nervous system appeared normal in *Rest* knockouts. In contrast, interference with REST function in Xenopus embryos resulted in downregulation of some target genes, possibly due to early patterning defects attributed to involvement of REST in the BMP pathway (Olguin et al., 2006). The embryonic lethality of the mouse knockout demonstrates the necessity of REST, but a broader understanding of the requirement for Rest in regulation of specific developmental processes is lacking.

In this study, we demonstrate a novel role for zebrafish Rest in modulation of the Hedgehog (Hh) pathway. Hh signaling is involved in many aspects of development including regulation of cell type specification, neurogenesis, cell survival and proliferation (Briscoe and Novitch, 2008; Cayuso et al., 2006). In vertebrates, Sonic Hedgehog (Shh) has perhaps been best characterized as a morphogen that establishes dorsal–ventral patterning of the neural tube. Shh secreted from the ventral midline of the neural tube induces ventral cell fates in a dose dependent manner, generating distinct neural

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subtypes. The transcription factors expressed in response to the Hh gradient are categorized as class I genes (e.g. *pax3*, *pax7*, and *dbx1a*) or class II genes (e.g. *nkx2.2a* and *nkx6.1*), which are repressed or induced, respectively, in response to Hh signaling (Briscoe and Novitch, 2008).

Members of the Gli family of transcription factors are key effectors of Hh signaling (Huangfu and Anderson, 2006; Jacob and Briscoe, 2003; Ruiz i Altaba, 1999; Stamataki et al., 2005). Like Drosophila Ci, vertebrate Gli2a and Gli3 are bifunctional and act as both activator and repressors of Hh target genes. In the absence of Hh signaling, protein kinase A (PKA)-dependent proteolytic cleavage produces a repressor protein (Gli^R), while activation of the Hh pathway allows full-length or near full-length Gli protein to function as an activator (Gli^A). Gli1, in contrast, lacks a repressor domain and is thought to function only as an activator (Dai et al., 1999; Ruiz i Altaba, 1999). In zebrafish, *gli1* is transcriptionally regulated by Gli2a and Gli3, and is thought to amplify Hh signaling after the initial activation of Gli2a and Gli3 (Karlstrom et al., 2003; Tyurina et al., 2005). Although both Gli2a and Gli3 have early activator roles in zebrafish, they act chiefly as repressors during later stages as their expression becomes limited to cells outside the zones of strong Hh signaling. This downregulation of *gli2a* and *gli3* is in part mediated by Hh signaling (Karlstrom et al., 2003; Tyurina et al., 2005). Recently, a second zebrafish Gli2 orthologue, Gli2b, which also functions in the nervous system, was identified (Ke et al., 2005; Ke et al., 2008).

Our *in vivo* studies demonstrate that Rest influences Hh signaling through regulation of Gli2a activity. We observed that when Rest levels are decreased, Hh signaling is enhanced and the response to ectopic Hh is elevated. Conversely, when Hh signaling is diminished, reduction of Rest levels leads to diminished expression of Hh target genes. Several lines of evidence support the hypothesis that this phenotype results from excess Gli2a activity. These include observations that *gli2a* expression is expanded in *rest* morphants and that disruption of Gli2a alters the consequences of Rest knockdown on Hh signaling. Regulation of *gli2a* transcription by Rest may be a wide-ranging mechanism to modulate the Hh response. These results reveal a novel requirement for Rest during zebrafish embryogenesis.

Materials and methods

Zebrafish stocks and embryo maintenance

Adult zebrafish stocks were maintained at 28.5 °C. Embryos were produced by natural matings, collected and stored at 28.5 °C in embryo medium until desired stage according to Kimmel et al. (1995). *Tg[islet:efp]* are described by (Higashijima et al., 2000). The following mutant alleles were used in this study: *gli1/dtr*^{te370} , *gli2a/yot*^{ty17}.

Quantitative real time PCR

Embryos (10/tube for control, 15/tube for rest morphants) were collected at the appropriate stage and placed in TRIzol reagent (Invitrogen) for RNA extraction. cDNA was synthesized from .5–1 µg mRNA with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Real time PCR was performed and analyzed as previously described (Londin et al., 2005).

Zebrafish rest cDNA isolation

Full-length rest cDNA was isolated from 12 hpf cDNA using the following primers: Forward TTTCAGTGGTCCAGCATGTC and Reverse ACATCTGACCCAGTTCGGTT. The PCR product was cloned into a pCS2+ vector, using the BD infusion method (BD Biosciences).

CLUSTAL W (Thompson et al., 1994) and BOXSHADE version 3.3.1 were used for protein sequence alignment.

Whole mount in situ hybridization and photography

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4 °C then stored in 100% methanol for storage at −20 °C. In situ hybridizations were done as previously described (Thisse et al., 1995). Constructs used to synthesize the following probes have been described previously: *nkx2.2a* (Barth and Wilson, 1995), *axial/foxA2* (Macdonald et al., 1997; Odenthal and Nusslein-Volhard, 1998), *nkx6.1* and *olig2* (Guner and Karlstrom, 2007), *pax6a* (Krauss et al., 1991) *pax3a* and *pax7* (Seo et al., 1998), *ptc1*(Concordet et al., 1996), *gli1* (Karlstrom et al., 2003), *gli2a* (Karlstrom et al., 1999), *gli3* (Tyurina et al., 2005), *phox2a* (Guo et al., 1999), *shh* (Krauss et al., 1993). After in situ hybridization, embryos were mounted in 75% glycerol and photographed using a Zeiss Axiocam mounted on a Zeiss Axioplan microscope.

Histology

After whole mount RNA *in situ* hybridization, embryos were dehydrated in ethanol, infiltrated and embedded in JB-4 resin (Ted Pella) 10 µM sections were obtained using a ultramicrotome (LKB 8800 ultratome III; Bromma).

mRNAs and morpholino microinjections

Capped *rest*, *shh* (Krauss et al, 1993) and dnPKA (Ungar and Moon, 1996) mRNA was made using the mMESSAGE mMACHINE RNA synthesis kit (Ambion). One- to two-cell embryos were injected with 50–100 pg (*dnPKA*, *shh*) or 500 pg (*rest*) of mRNA diluted in 0.2 M KCl and phenol red. A splice inhibiting morpholino (MO) against the intron–exon boundary of zebrafish *rest* exon 3
(5′-GGCCTTTCACCTGTAAAATACAGAA-3′) and a translation blocking MO
(5′-AAACACCGGCTGAGACATGCTGGAC-3′) were synthesized by Gene Tools (Philomath, OR). Unless otherwise noted, the splice blocking MO was used for all described experiments. Prior to microinjections, embryos were dechorionated in 1 mg/ml pronase (Sigma-Aldrich). Morpholinos were diluted in .2 M KCl and phenol red from a 34 mg/ml stock to 8–10 mg/ml. MO was injected at the one cell stage, using 4 ng for the shh mRNA combination experiments and 5 ng for all other experiments. Equivalent amounts of the standard control morpholinos provided by Gene Tools (5′-CCTCTTACCTCAGTTACAATTTATA-3′) were used in all experiments. For mRNA and MO combination injections, embryos were first injected with rest MO or control MO, then mRNA was injected into each of these. At the appropriate stage, embryos were fixed in 4% paraformaldehyde for *in situ* hybridization or placed in TRIzol reagent (Invitrogen) for RNA extraction.

Cyclopamine treatments

Cyclopamine (CyA) (Calbiotech) was diluted in EM from a 10 mM stock dissolved in DMSO. Embryos were incubated in the desired concentration of CyA media from 4 hpf (Fig. 5) or at shield stage (Fig. 4) on, with control embryos in the equivalent concentration of DMSO in EM.

Results

Identification and characterization of zebrafish rest

To examine the role of Rest in neurogenesis, we isolated a zebrafish *rest* cDNA using primers derived from the zebrafish genome sequence. The predicted zebrafish Rest protein (855 residues) is 39% identical and 54% similar to the human Rest protein (1097 residues). Like the mammalian REST proteins, the zebrafish protein is predicted

to encode 9 zinc fingers (8 of which comprise the DNA binding domain) (Supplemental Fig. 1). In zinc fingers domains, the human and zebrafish proteins have a higher degree of similarity (89%) and identity (81%). This suggests the Rest homologues interact with similar DNA elements. Computer algorithms predict over 1000 putative RE1 sites in the zebrafish genome (Mortazavi et al., 2006) and data not shown), which is comparable to the numbers of RE1 sites present in other vertebrate genomes (Bruce et al., 2004; Mortazavi et al., 2006). Zebrafish Rest is also highly similar to human REST within the domains that are key to interactions with the Sin3 and CoREST co-repressor complexes (Nomura et al., 2005; Tapia-Ramirez et al., 1997). Phosphodegron motifs, which are required for post-translational regulation of REST levels, are also conserved (Guardavaccaro et al., 2008; Westbrook et al., 2008).

We have analyzed *rest* expression in zebrafish by whole mount RNA *in situ* hybridization. *rest* is present as a maternal transcript (data not shown), and is expressed ubiquitously until mid-somitigenesis (Figs. 1A, B). *rest* expression then becomes increasingly confined to non-neural tissue and proliferative zones within the nervous system. For example, *rest* is expressed at low levels throughout the extent of the hindbrain at 16 hpf (Fig. 1D), a stage when cells are largely proliferating and few neurons have differentiated. Around 20 hpf, (1E) *rest* transcript is downregulated in the ventrolateral domains where neuronal differentiation is occurring (arrowheads E–G).

During the accelerated period of neurogenesis from 24 hpf to 48 hpf (Lyons et al., 2003), *rest* persists in the undifferentiated dorsal rhombic lip (Figs. 1G, K), and in proliferative midline (ventricular zone, vz) cells extending to the floorplate. Dorso-ventral differences in expression likely reflect the pattern of differentiation in the neural tube, where maturation occurs ventrally before dorsally. This is most clearly demonstrated in the midbrain region during late day one development (Fig. 1J). *rest* is expressed throughout the undifferentiated dorsal midbrain, (optic tectum, OT). Whereas the ventral midbrain (tegmentum, tg) has undergone extensive neurogenesis and *rest* transcript is largely restricted to the vz. *rest* is expressed in domains outside the neural tube, but is excluded from the mature somites (Figs. 1K, L). In addition, *rest* is excluded from differentiated sensory cranial ganglia adjacent to the neural tube (Figs. 1E, F, G, K asterisks).

The expression pattern of *rest* is largely coincident with proliferative and nonneural tissue, as seen in mouse (Chong et al., 1995)) and chick (Chen et al., 1998). However, the significance of *rest* transcript must be interpreted with caution. Rest levels are regulated post-translationally (Guardavaccaro et al., 2008; Westbrook et al., 2008; Ballas et al., 2005). Therefore, the sites of *rest* expression may not equate with the sites of Rest activity. It is also likely that the availability of individual components of the co-repressor complexes will affect the repression mediated by Rest. Conversely, because the

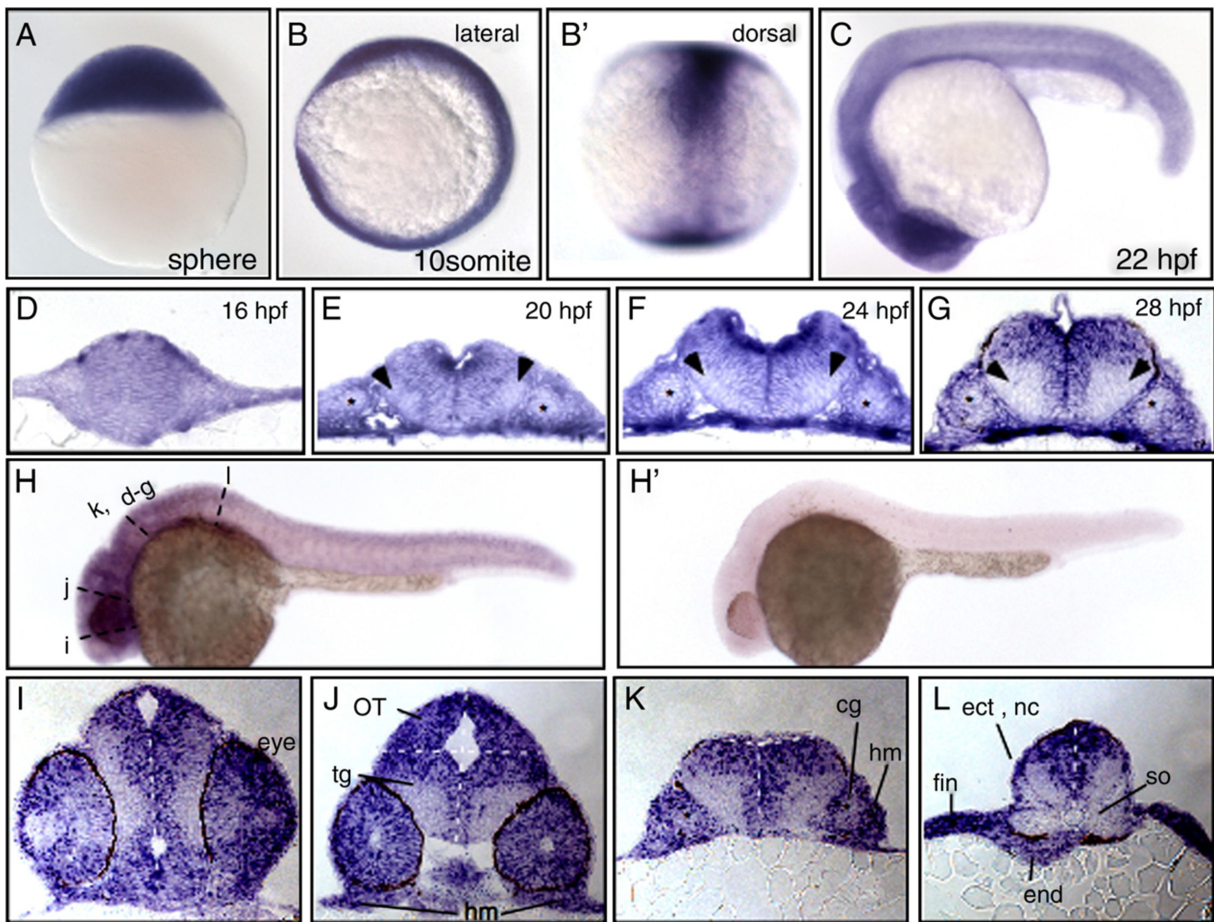

Fig. 1. Expression of *rest* during early zebrafish development. mRNA in situ hybridization with antisense *rest* probe, except H' where *rest* sense strand was used. Wholemounts (A–C, H) and transverse sections (D–G, I–L) of wild-type embryos during the first 42 h of development. (A, B, D) Early expression is ubiquitous. (C–G) Expression remains widespread at 22 hpf (C) and 25 hpf (H) but transverse sections of hindbrain reveal progressive downregulation as neurogenesis takes place in ventrolateral domains (arrowheads, E–G). (I–L) Sections of 42 hpf embryo, taken at levels indicated in (H). The pattern of *rest* expression is similar along the anterior–posterior axis. *rest* is expressed in mitotic cells of the ventricular zone, (marked by white dashed line) and undifferentiated neural structures such as the eyes (I, J), and optic tectum (OT, J). (J–L) Most tissue outside the neural tube still expresses *rest* at this stage, such as the head mesenchyme (hm J, K), developing fins (L) endodermal tissue (end, L) neural crest (nc, L) and ectoderm surrounding the somites (ect, L) but not the the already differentiated somites (L, so) and sensory cranial ganglia (cg, K). cg, cranial ganglia; ect, ectoderm; end, endoderm; hm, head mesenchyme; OT, optic tectum; tg, tegmentum.

mechanism of Rest repression involves epigenetic changes to the chromatin environment of target genes, the effects of Rest activity may outlast the presence of Rest protein.

Rest limits expression of Hh target genes within the neural tube

To investigate the role of Rest during development, we generated two independent *rest* morpholinos (MOs). The first targets the translation start site, while the second targets an intron–exon boundary to block mRNA splicing. While injection of both MOs led to similar defects, the splice blocking MO was more potent and was used for the following studies. The splice blocking morpholino binds to the intron–exon boundary of the third exon, and this is predicted to produce a truncated protein due to inclusion of the second intron in the mature mRNA (Supplemental Fig. 2A). The predicted protein produced from the mis-spliced transcript lacks zinc fingers 6–8 of the DNA binding domain and the C-terminal CoREST interaction domain. Quantitative real time PCR was used to assay the amount of wild-type

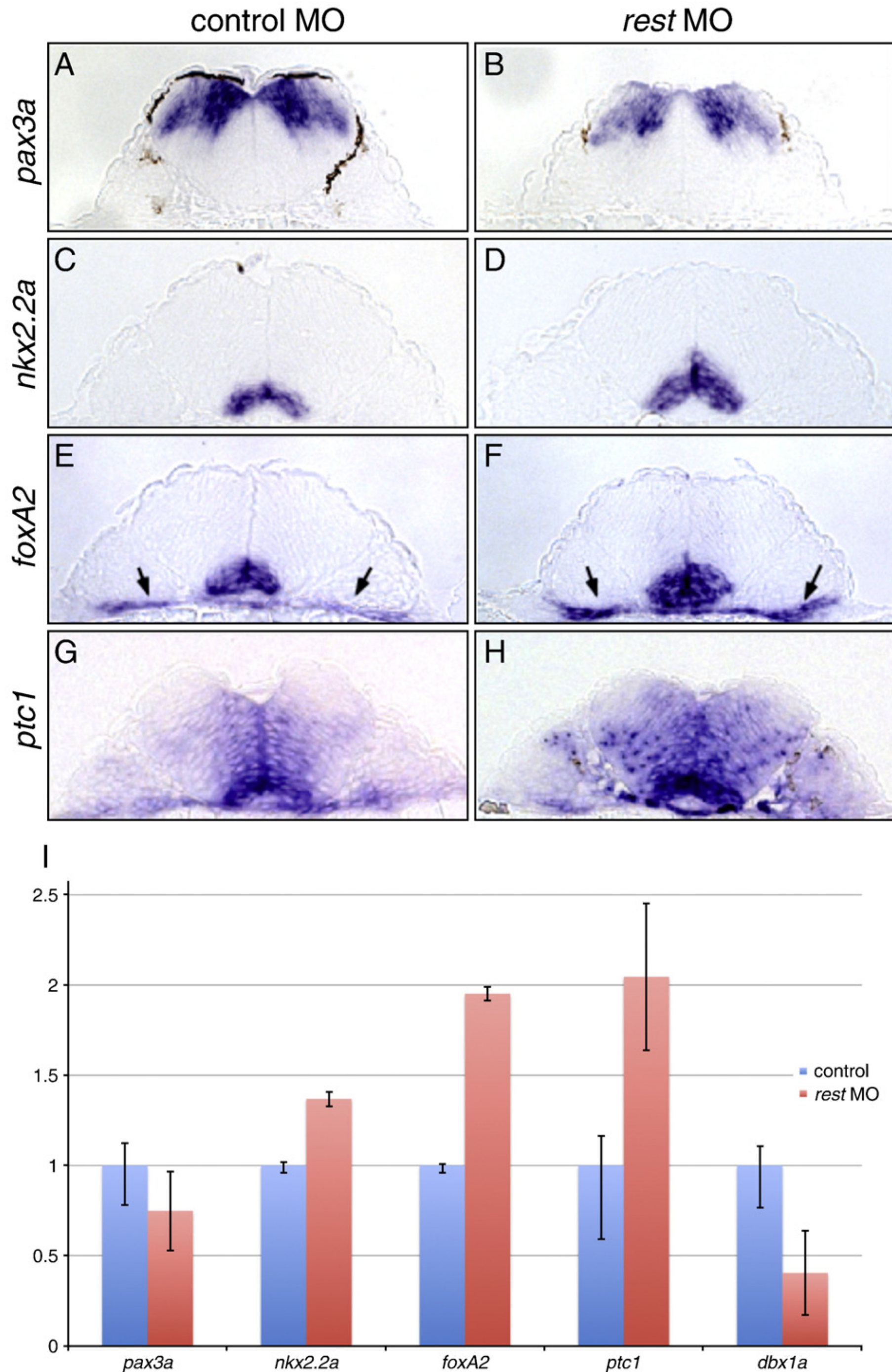

Fig. 2. The neural tube is ventralized in Rest knockdown embryos. Transverse sections of 29hpf (A, B) or 26hpf (C–H) wild-type embryos processed for RNA *in situ* hybridization and sectioned at the level of the hindbrain at anterior rhombomere 4. Control (A, C, E, G) and rest MO injected (B, D, F, H) embryos stained with antisense probes for Hh response genes *pax3a*, *nkx2.2a*, *foxA2* and *ptc1*. *pax3a* expression (A, B) is reduced in rest morphants, while expression of *nkx2.2a* (C, D), *foxA2* (E, F) and *ptc1* (G, H) is expanded compared to stage matched control embryos. This suggests that Rest represses Hh signaling. Arrows (E, F) mark pharyngeal endoderm. (I) qPCR analysis on 29hpf control (red bars) and stage matched rest morphant (blue bars) cDNA for markers shown in A–H, plus class I gene *dbx1a*. Overall levels of class II Hh target genes *nkx2.2a*, *foxA2*, and *ptc1* are increased while class I genes *pax3a* and *dbx1a* levels are reduced.

rest mRNA present following *rest* mo treatment (Supplemental Fig. 2B). By 6 hpf, only about 50% the amount of wild-type *rest* mRNA is present in *rest* morphants compared to controls. The amount of wild-type *rest* mRNA in *rest* mo treated embryos drops to less than 10% the control amount by 12 hpf. This demonstrates that the morpholino is effective in reducing the levels of wild-type *rest* transcript. However, the experiment also reveals that the morpholino does not entirely eliminate wild-type *rest* mRNA. In addition, the morpholino is less effective at disrupting mature mRNA production at early stages. This is presumably due to maternal *rest* mRNA, which is impervious to the splice blocking morpholino.

A second set of primers that detect the mis-spliced product containing intron sequences reveals that this product is significantly enriched in the *rest* morphant cDNA (Supplemental Fig. 2C). This provides additional proof that the predicted mis-splicing event occurred. Interestingly, wild-type cDNA also contains low levels of this product. This may result from trace amounts of immature, partially spliced mRNA in the wild-type sample. Alternatively, zebrafish may have a Rest splice variant akin to the Rest4 form that has been observed in mammals (Magin et al., 2002; Palm et al., 1998; Shimojo et al., 1999). However, we have not detected any activity of the truncated Rest form in overexpression assays (data not shown).

By gross morphological examination, *rest* morphants appear wild-type through 3 dpf, except that they tend to be smaller, and progress more slowly than control-injected embryos. Examination of neural tube markers by RNA *in situ* hybridization revealed that the neural tube of *rest* morphants is mildly ventralized (Fig. 2). Expression of the ventral marker, *nkx2.2a*, was expanded in *rest* morphants (Fig. 2D), while expression of the dorsal marker *pax3a* was reduced in hindbrain sections (Fig. 2B). *nxk2.2a* expression is induced by Hh signaling and *pax3a* expression is repressed by Hh (Guner et al., 2008; Liem et al., 1995). The polarity of the vertebrate neural tube is established by a gradient of Shh (Briscoe and Ericson, 1999), and we wondered if Rest knockdown enhanced expression of other Hh target genes within the developing neural tube. Therefore, we determined the expression of two direct Hh target genes *ptc1* and *foxA2* (Vokes et al., 2007) in Rest compromised embryos. As with *nkx2.2a*, the expression of *foxA2* and *ptc1* was stronger within their normal domain and expanded 1–2 cell widths laterally and dorsally in *rest* morphants (Figs. 2F, H).

To quantitatively examine the alterations in Hh target gene expression in these embryos, qPCR was performed on 29 hpf control and stage-matched *rest* MO injected embryos. The levels of *nkx2.2*, *foxA2* and *ptc1* transcripts are enhanced in *rest* morphants (Fig. 2I). Conversely, the levels of *pax3a* transcript were slightly reduced, while a second class I gene, *dbx1a*/*hlx1* (Fjose et al., 1994), which is expressed ventral to *pax3a* (Guner and Karlstrom, 2007; Hauptmann et al., 2002) was also reduced (Fig. 2I).

Regulation of Hh signaling by Rest is not limited to the neural tube, as Rest knockdown also enhanced expression of *nkx2.2a* in the developing pancreas (Supplemental Fig. 3) and *foxA2* in the pharyngeal endoderm (Fig. 2H, arrows.) These results indicate the Rest function is involved not only in the proper dorso-ventral patterning of the neural tube, but in Hh signaling in general.

Rest represses the Hh response

Enhancement of Hh target gene expression in the neural tube was strongest near the floor plate, which is a source of Hh. This suggested

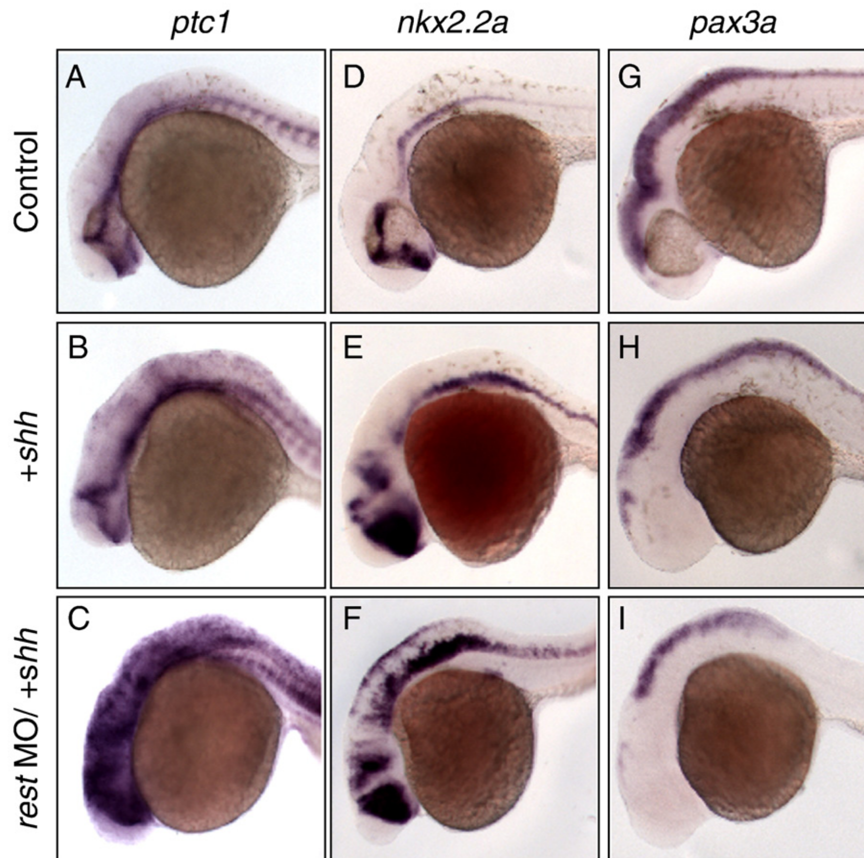

Fig. 3. Rest knockdown sensitizes embryos to exogenous *shh*. Lateral, head and hindbrain views of 28 hpf control (A, D, G), *shh* mRNA (B, E, H) and *shh* mRNA/REST mo (C, F, I) microinjected embryos stained with antisense probes for Hh response genes. Injection of *shh* mRNA results in enhancement of *ptc1* (B) and *nkx2.2a* (E), while *pax3a* (H) expression is reduced. *rest* morphants treated with the same amount of *shh* mRNA have increased expression of Hh target genes compared to *shh* mRNA treated embryos. These embryos have enhanced expression of *ptc1* (C) and *nkx2.2a* (F), while *pax3a* is further reduced (I). This demonstrates that Rest knockdown enhances the response to high levels of Hh.

Table 1
Rest knockdown enhances induction of *nkx2.2a* by *shh* mRNA treatment.

IA. *rest* splice MO					
Hindbrain *nkx2.2a* expression at 1 dpf					
Treatment	*N*	Severe	Moderate	Mild	Wild-type
shh mRNA	154	9 (6%)	26 (17%)	65 (42%)	54 (35%)
rest MO/*shh* mRNA	148	74 (50%)	38 (26%)	21 (14%)	15 (10%)
$p = 8.1407E-120$					
(Total from 7 experiments)					

IB. *rest* ATG MO				
Hindbrain *nkx2.2a* expression at 1 dpf				
Treatment	*N*	Moderate	Mild	Wild-type
shh mRNA	73	6 (8%)	23 (31%)	44 (60%)
rest ATG MO/ *shh* mRNA	68	19 (28%)	33 (48%)	16 (23%)
$p = .03538E-119$				
(Total from 3 experiments)				

Severe = strongly expanded and strong ectopic dorsal (as in Fig. 2F).
Moderate = ventral domain expanded and weak ectopic dorsal expression (as in Supplemental Fig. 4D).
Mild = ventral domain expanded or weak ectopic dorsal expression but not both (as in Fig. 2E and Supplemental Fig. 4C).
Wild-type = expression within the range observed in control embryos.

that *rest* morphants show increased sensitivity to high levels of Hedgehog. To determine whether Rest knockdown augmented the response to Hh, we examined the expression of markers in wild-type embryos and *rest* morphants injected with *shh* mRNA. Ectopic activation of Hh signaling causes a dorsal expansion of Hedgehog targets *ptc1* and *nkx2.2a*, while the domain of the dorsal class 1 marker, *pax3a* is reduced (Dessaud et al., 2008; Guner and Karlstrom, 2007) and Fig. 3). Co-injection of *rest* MO and *shh* mRNA enhanced the ventralizing effects of Shh on the neural tube in a synergistic manner (Fig. 3, Table 1A). The expression domains of *ptc1* and *nkx2.2a* were expanded compared to *shh* treatment alone, while *pax3a* expression was further suppressed. Other patterning genes in the neural tube that showed a synergistic response to Rest knockdown in conjunction with Shh activation include *shh*, *foxA2*, *phox2a*, *nkx6.1*, *olig2*, *pax6a* and *pax7* (Supplemental Fig. 4). The expanded domains of patterning genes such as *nkx6.1* and *phox2a* are the likely cause of excess production of *islet-eGFP* positive cranial motor neurons seen in later staged embryos (Compare Figs. S4W and S4X). We conclude that Rest is required to limit Hh signaling and that Rest likely modulates a fundamental aspect of the Hh pathway.

Importantly, a similar enhancement of the effects of *shh* mRNA overexpression was observed with the second *rest* morpholino which blocks translation (*rest* ATG) (Supplemental Figs. 5 A–D and Table 1B). To confirm that the effects of the *rest* morpholino treatment result from Rest knockdown, we assayed the ability of *rest* mRNA to rescue the effects on Shh signaling produced by the splice morpholino. *rest* mRNA was co-injected into *rest* morphants treated with *shh* mRNA and RNA *in situ* hybridization was used to assay *pax3a* expression. *rest* mRNA microinjection largely restored the *pax3a* expression (Supplemental Fig. 5H) compared to *rest* MO/*shh* injected embryos (Supplemental Fig. 5G, and Table 2). These experiments demonstrate that the effects of the morpholino treatment are produced by knockdown of Rest.

Table 2
Rest mRNA injection rescues Rest mo mediated enhancement of Shh treatment.

pax3 expression at 1 dpf					
Treatment	*N*	Severe	Moderate	Mild	Wild-type
shh mRNA	61	7 (11%)	12 (19%)	26 (43%)	16 (27%)
rest MO/*shh* mRNA	98	46 (47%)	17 (17%)	25 (26%)	10 (10%)
rest MO/*shh* mRNA *rest* mRNA	63	7 (11%)	18 (28%)	17 (27%)	21 (34%)

Rest knockdown enhances the effects of cyclopamine

Our initial findings suggest that Rest is a negative regulator of the Hh pathway. We therefore treated wild-type embryos with low doses of the Hh antagonist cyclopamine (CyA) (Chen et al., 2002; Hirsinger et al., 2004; Wolff et al., 2003) to determine whether blockage of Hh signaling could be alleviated by Rest knockdown. Wild-type embryos were microinjected with control or *rest* morpholino and incubated in a low concentration of CyA (1.5 μM) or control media from shield stage (6 hpf) onward. Treated embryos were fixed at 24 hpf and stained for the class II gene *nkx2.2a* (A–D) As expected, *rest* MO treatment led to an increase in *nkx2.2a* expression (Fig. 4B). Surprisingly, in the presence of CyA, Rest knockdown caused further reduction in Hh signaling as revealed by diminished *nkx2.2a* expression (Fig. 4D). At this concentration of CyA, most control embryos showed a mild (33%, $n = 75$) or moderate (47%, as in Fig. 4C) reduction of *nkx2.2a* expression in hindbrain and spinal cord, while retaining *nkx2.2a* expression in the basal forebrain and diencephalon. The remaining embryos (20%) had a severe reduction overall in *nxk2.2a* expression. *rest* morphants were more severely affected compared to control embryos treated with the same dose of CyA. Most morphants displayed a total absence of hindbrain expression and a strong loss in the head region (74% $n = 78$, as in Fig. 4D, compared to 20% of control embryos). The remaining *rest* morphants showed a moderate reduction of *nkx2.2a* expression.

Since cyclopamine treatment also results in the upregulation of genes negatively regulated by Hh signaling (Hammond et al., 2007; Martin et al., 2007), embryos were also assayed for ventral expansion of class I gene *pax3a*. This concentration of CyA resulted in a mild ventral expansion of *pax3a* in control embryos (6/16 as in 4G, 10/16 comparable to wild-type) and downregulation of *nkx2.2a* (compare sibling control embryos, in 4C, G). In contrast, *rest* morphants often had significant expansion of *pax3a* (8/18 as in 4H, the remainder were similar to control in 4G) Thus, Rest knockdown increases response to high levels of Hh signaling (Fig. 3), but further attenuated Hh signaling in the context of low levels of Hh signaling (Fig. 4). We conclude that regulation by Rest positively or negatively influences the Hh response depending on the state of Hh signaling.

Rest interacts with the Hedgehog pathway downstream of Smo

RE1 sites are associated with hundreds of genes and many direct or indirect interactions with the Hh pathway can be postulated to account for the bimodal phenotype observed. To determine whether Rest interacts with the Hh pathway by modulating an intracellular signaling step in the cascade, we activated the Hh pathway cytoplasmically by overexpressing a dominant-negative form of PKA (dnPKA) (Ungar and Moon, 1996). PKA has multiple roles, and in context of the Hh pathway, PKA activity is required to generate the repressor forms of Gli2a and Gli3. Overexpression of *dnPKA* strongly activates Hh target gene expression, presumably because Gli activator forms predominate in that condition (Hammerschmidt et al., 1996).

As expected, injection of *dnPKA* mRNA resulted in a dorsal expansion of *nkx2.2a* expression (Fig. 5C). As with *shh* overexpression, upregulation of *nkx2.2a* by dnPKA was further enhanced by simultaneous co-injection of *rest* MO (Fig. 5D). While this result suggests that Rest knockdown acts intracellularly on the Hh pathway, Hh signaling also results in transcription of Hh ligands and amplification of the signal (Blader et al., 1997; Neumann and Nuesslein-Volhard, 2000) Supplemental Figs. 4B, C). It is therefore possible that some of the effects on Hh signaling of Rest knockdown is a result of heightened extracellular Hh ligand activity due to ligand production, processing or diffusion. To address this possibility we injected *dnPKA* mRNA to activate

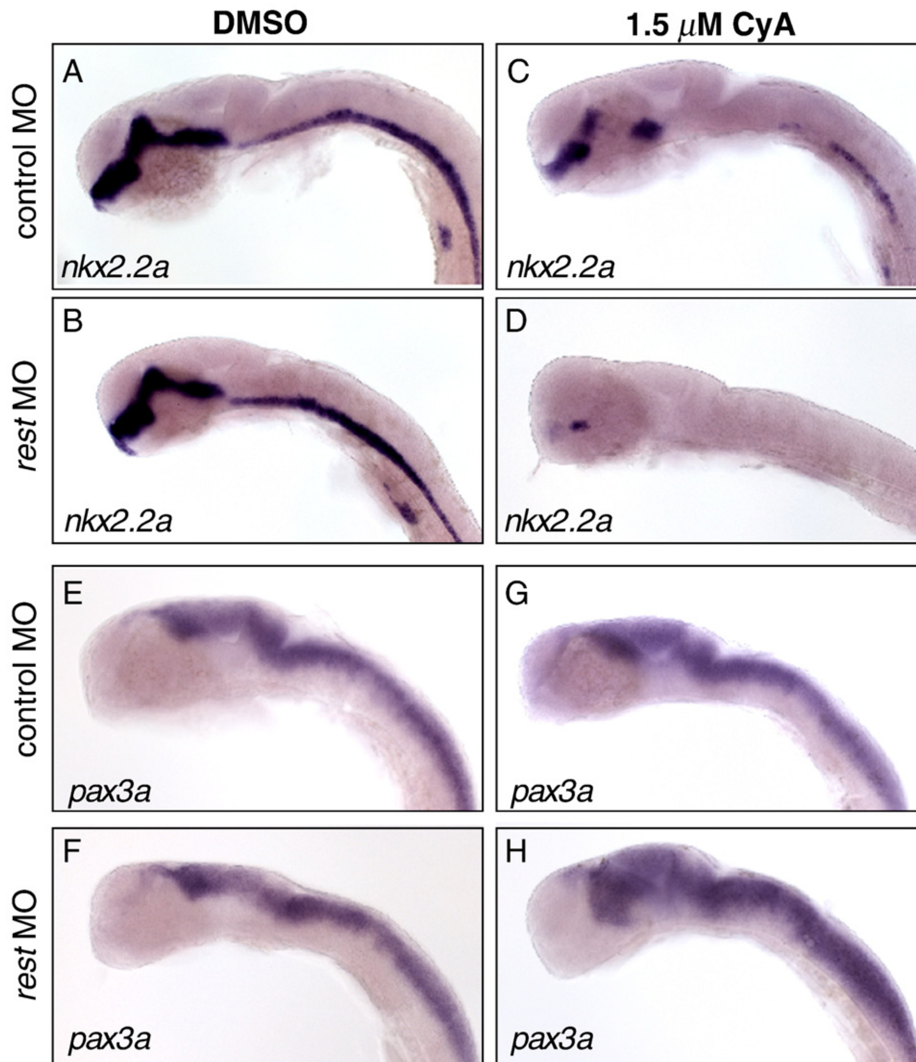

Fig. 4. CyA mediated attenuation of Hh signaling is enhanced by Rest knockdown. Lateral views of 26 hpf control (A, C, E, G) and *rest* morphants (B, D, F, H) embryos stained for *nkx2.2a (A–D)* or *pax3a* (E–H). Embryos were incubated in control media (A, B, E, F) or 1.5 μM cyclopamine (CyA) media from 6 hpf on (C, D, G, H). *rest* morphants incubated in control media show a modest increase in *nkx2.2a* expression (B, compare to control, A) and a modest decrease in *pax3a* (F, compare to control, E) grown under the same conditions. 1.5 μM CyA decreases *nkx2.2a* expression (C) and modestly expands *pax3a* (G) in control embryos. Rest knockdown produces a greater reduction in *nkx2.2a* expression, and a greater expansion of *pax3a*, then CyA treatment alone.

the pathway intracellularly, but subsequently blocked extracellular Hh signaling at the level of the transmembrane receptor Smo with a high dose of CyA (50 μM). If Rest regulates Hh signaling components downstream of Smo, then CyA treatment will not alter the enhanced dnPKA overexpression effects resulting from Rest knockdown (as in Fig. 5D). CyA treatment largely eliminated *nkx2.2a* expression in embryos injected with control MO (Fig. 5B) or *rest* MO (Fig. 5D). Importantly, Rest knockdown enhanced the effects of *dnPKA* mRNA treatment on *nkx2.2a* expression in the presence of 50 μM CyA (compare Fig. 5H with 5D). Thus, blocking extracellular signaling events did not significantly alter the enhanced effects of dnPKA on Hh signaling produced by Rest knockdown. We conclude that Rest interacts with the Hh pathway at an intracellular step downstream of Smo.

Rest is required for dynamic regulation of gli2a expression

Intracellular pathway components include the mediators of Hh signaling, the Gli family of transcription factors. To determine whether Rest regulates *gli* transcription, we examined expression of the four zebrafish *gli* genes (*gli1*, *gli2a*, *gli2b* and *gli3*) in *rest* morphants. *gli1* is expressed in regions where Hh signaling is active and expression is enhanced in *rest* morphants (Fig. 6B). The stronger expression of *gli1* in *rest* morphants is consistent with enhanced Hh signaling because *gli1*, like *ptc1*, is a Hh response gene. Gli1 is not thought to have repressor activity (Dai et al., 1999; Ruiz i Altaba, 1999), so upregulation of *gli1* is unlikely to produce the negative effects we observed on Hh signaling in CyA treated embryos (Fig. 4D, H).

Both *gli2a* and *gli3* are initially expressed in ventral regions of the CNS and act as weak activators of the initial Hh response. As development proceeds, *gli2a* and *gli3* are repressed by Hh signaling. Expression of both transcripts becomes restricted to regions distant from the ventral source of Hh (Karlstrom et al., 2003; Tyurina et al., 2005; Vanderlaan et al., 2005, and Fig. 5). At these stages, both proteins function as repressors of the Hh response. We did not detect significant alterations of *gli3* mRNA in *rest* morphants from 18–24 hpf (Fig. 6D). *gli2b* expression was also unaltered by Rest knockdown (data not shown).

In contrast, *gli2a* expression was enhanced in *rest* morphants. In the hindbrain of wild-type embryos, *gli2a* is initially expressed across the dorso-ventral extent of the neural tube before 15 hpf (Vanderlaan et al., 2005) and then is progressively reduced in the ventral and midline regions as development proceeds (Figs. 6E, G). In *rest*

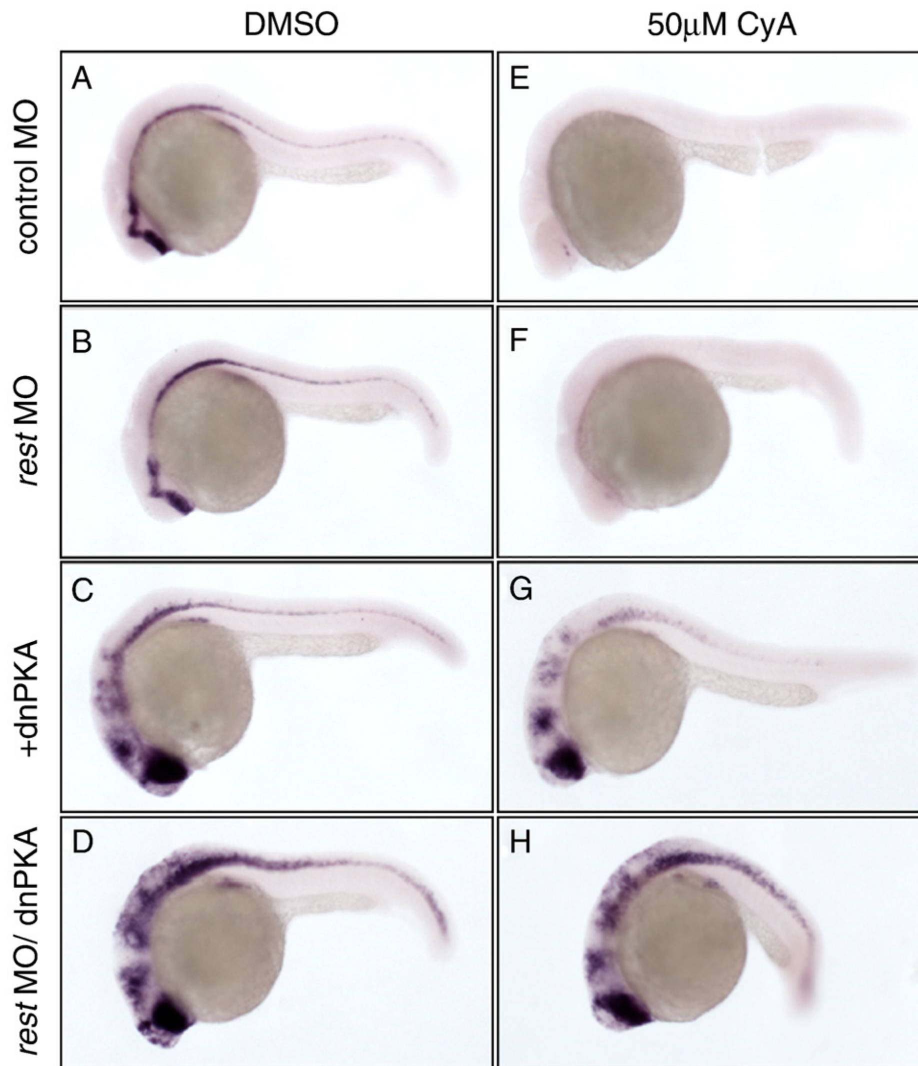

Fig. 5. Rest acts downstream of Smo in the Hh pathway. Lateral views of 24 hpf embryos injected with control MO (A, E) *rest* MO (B, F), dominant-negative PKA mRNA (dnPKA, C, G), or both (D, H) and stained for *nkx2.2a*. Embryos were placed in control media (A–D) or media containing 50 μM cyclopamine (E–H). Injection of *dnPKA* mRNA expands *nkx2.2a* expression (C). This expansion is augmented by co-injection with *rest* MO (D). CyA treatment partially attenuates the effects of dnPKA mRNA injection on *nkx2.2a* expression (G). *dnPKA* mRNA/*rest* mo injected embryos (F) are more resistant to CyA treatment then dnPKA treated embryos.

morphants, *gli2a* fails to be properly downregulated at 18 hpf, and remained in the ventral CNS at 24 hpf (Figs. 6F, H). Thus, Rest knockdown results in inappropriate expression of *gli2a* in ventral domains. In these regions, cells are exposed to higher levels of Hh ligand and Gli2a^{A} is presumably generated. *gli2a* expression is repressed by Hh (Supplemental Fig. 6C), so the expansion of the *gli2a* domain is unusual among the Hh targets we have observed in *rest* morphants. In the experiments described earlier (Fig. 3 and Supplemental Fig. 6D'), Rest knockdown enhanced Hh signaling when ectopic *shh* mRNA was applied. However, double injection of *rest* MO with *shh* mRNA did not enhance the Hh-mediated repression of *gli2a* expression (Supplemental Fig. 6D). We conclude that Rest plays a role in repressing *gli2a* expression, and that this repression is independent of effects resulting as a consequence of enhanced Hh signaling.

These experiments do not establish whether regulation of *gli2a* by Rest is direct or indirect. Using an algorithm optimized to identify mammalian RE1 sites (Otto et al., 2007), we did not detect any canonical RE1 sites in the zebrafish *gli2a* locus, which is large and encompasses over 100 kb. Recently, two groups have identified RE1 site variants termed split-RE1 sites (Johnson et al., 2007; Otto et al., 2007). The error rate for predicting split-RE1 sites is higher. We identified three split RE1-like sequences in the *gli2a* locus. One site is positioned at ~15 kb upstream and two at about 80 kb from the transcription start site. Functional data suggests that optimal repression from RE1s occurs when the site is within 2–3 kb of the transcriptional start site (Johnson et al., 2008). Therefore, these sites are not strong candidates to mediate repression of *gli2a* by Rest. Rest may indirectly bind DNA and in addition, Rest appears to interact with sites that lack characterized RE1 motifs (Johnson et al., 2008).

Rest knockdown diminishes Hh target expression in yot/gli2a mutants

The failure to downregulate *gli2a* transcription (Figs. 6F, H) combined with the context dependent response of *rest* morphants to manipulations of Hh signaling (Figs. 3 and 4) led us to hypothesize that Gli2a activity is central to the interaction between Rest and the Hh pathway. To further investigate the role of Gli2a in the Rest–Hh interaction, we assayed the consequences of blocking Rest activity in *yot/gli2a* mutants. This mutation is a lesion in *gli2a* that results in the production of a truncated protein and acts as a dominant repressor of Hh signaling (Gli2aDR), most likely through interference of Gli activator function (Karlstrom et al., 1999; Karlstrom et al., 2003). While most *nkx2.2a* expression is lost in *yot* mutants, domains remain in the ventral forebrain and hindbrain. If enhanced Hh signaling in *rest*

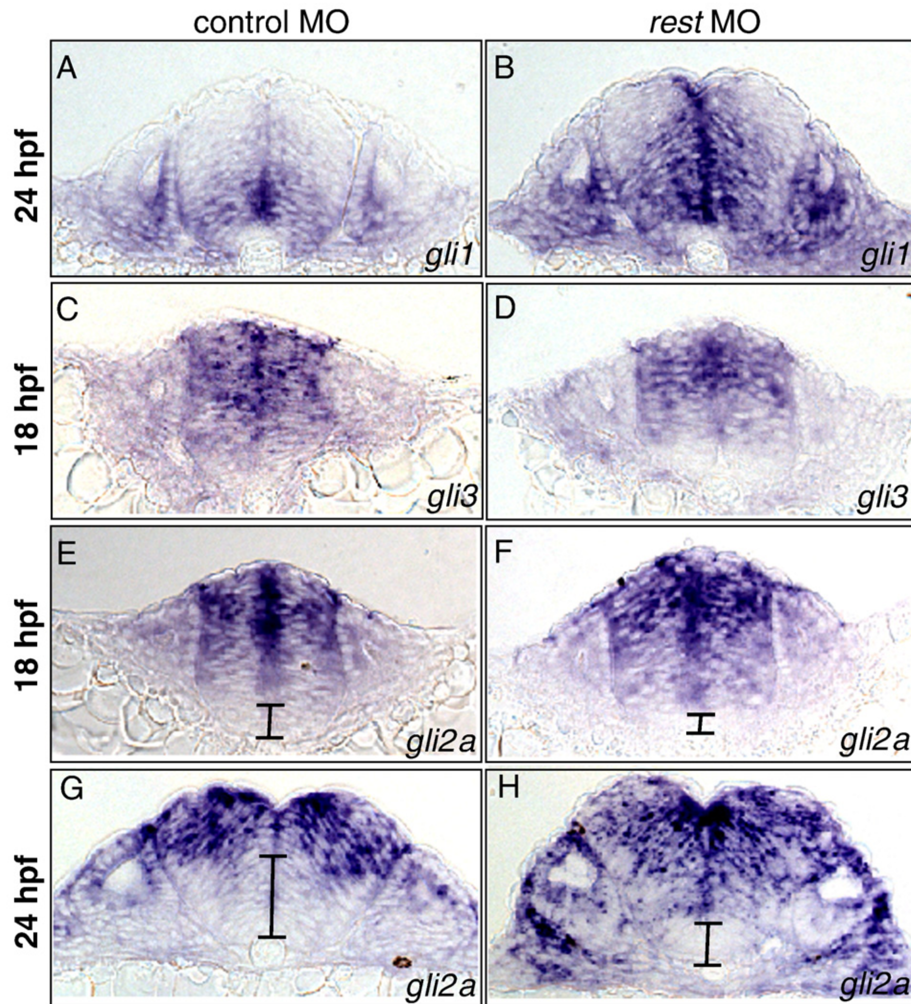

Fig. 6. Rest represses *gli2a* expression. Transverse sections of the hindbrain of control (A, C, E, G) and stage-matched *rest* morphants (B, D, F, H). RNA *in situ* hybridization to monitor *gli1* (A, B) *gli3* (C, D) and *gli2a* (E–H) expression. (A, B) *gli1* expression is enhanced by Rest knockdown. (C, D) *gli3* expression is unaltered by Rest knockdown. (E, G) *gli2a* expression is downregulated ventrally and in the midline ventricular zone (vz) as development proceeds in control embryos. (F, H) gli2a expression is maintained in the vz and is expressed more ventrally in *rest* morphants.

morphants is primarily dependent on $Gli2a^A$, then restoration of *nkx2.2a* expression will not occur in *yot* mutants treated with *rest* mo. Alternatively, if the *rest* morphant phenotype was due to excess activity by Glis other than Gli2a, Rest knockdown in *yot* mutants might have either no effect, or restore Hh signaling by compensation or competition.

As expected, loss of Rest function led to an increase in *ptc1* and *nkx2.2a* expression in wild-type siblings (Figs. 7B, F). However, in *yot* mutants, which are readily identified by characteristic loss of Hh target gene expression, Rest knockdown did not alleviate, but instead further reduced expression of *ptc1* and *nkx2.2a* in the midbrain region (Figs. 7D, H arrows). This result suggests that Rest knockdown increases Gli^R activity in *yot* embryos, which is opposite to the effect of Rest knockdown in wild-type embryos. Because *yot* mutants only generate Gli2aDR and not $Gli2a^A$, this finding is consistent with the model that misregulation of Gli2a accounts for the effects of Rest knockdown on the Hh pathway.

Enhancement of Hh signaling by Rest knockdown requires Gli2a

The *yot*/Gli2DR results above led us to hypothesize that excess Gli2a activity produced the observed phenotypes. We therefore sought to test the ability of Rest knockdown to alleviate defective Hh signaling due to loss of Gli1 function. *Detour (dtr)* mutants lack functional Gli1 and exhibit a loss of Hh target expression, including a complete loss of *nkx2.2a* in the hindbrain (Karlstrom et al., 2003). While Gli1 is the main activator of Hh target genes in zebrafish, Gli2a is a weak activator of Hh targets, and is partially redundant with Gli1 (Karlstrom et al., 2003; Park et al., 2000). Embryos from a *dtr*+/− intercross were injected with *rest* MO and assayed for *nkx2.2a* expression at 24 hpf. *rest* MO injected *dtr* mutants resemble *dtr* mutants (reduced or absent *nkx2.2a* expression) throughout most of the embryo except, strikingly, in the hindbrain where some *nxk2.2a* expression is restored (23/33 in *dtr*−/− embryos, Fig. 8B). *nkx2.2a* expression was not detected in 26/26 in control-injected *dtr*−/− embryos. Restoration of *nkx2.2a* expression in *rest* morphants indicates that while Gli1 function is required for the expansion of *nkx2.2a* in most tissues in *rest* morphants, there is partial compensation for loss of Gli1 in the hindbrain. This is significant because *dtr* mutants are largely refractory to exogenous *shh* mRNA (Fig. 8C, Karlstrom et al, 2003). However, injection of *shh* mRNA and *rest* MO into *dtr* mutants resulted in ectopic *nkx2.2a* in the dorsal hindbrain (6/16 *dtr*−/− embryos as in Fig. 8D) or partial restoration of ventral *nkx2.2a* expression (14/16 dtr−/− embryos) in the hindbrain. These results demonstrate that while the enhancement of Hh signaling in *rest* morphants largely depends on Gli1 function (possibly in response to $Gli2a^A$), Rest also regulates Gli1-independent activities.

This is consistent with the hypothesis that derepression of *gli2a* accounts for the biphasic alterations of Hh signaling in *rest* morphants. In that case, simultaneous knockdown of Gli2a would negate the

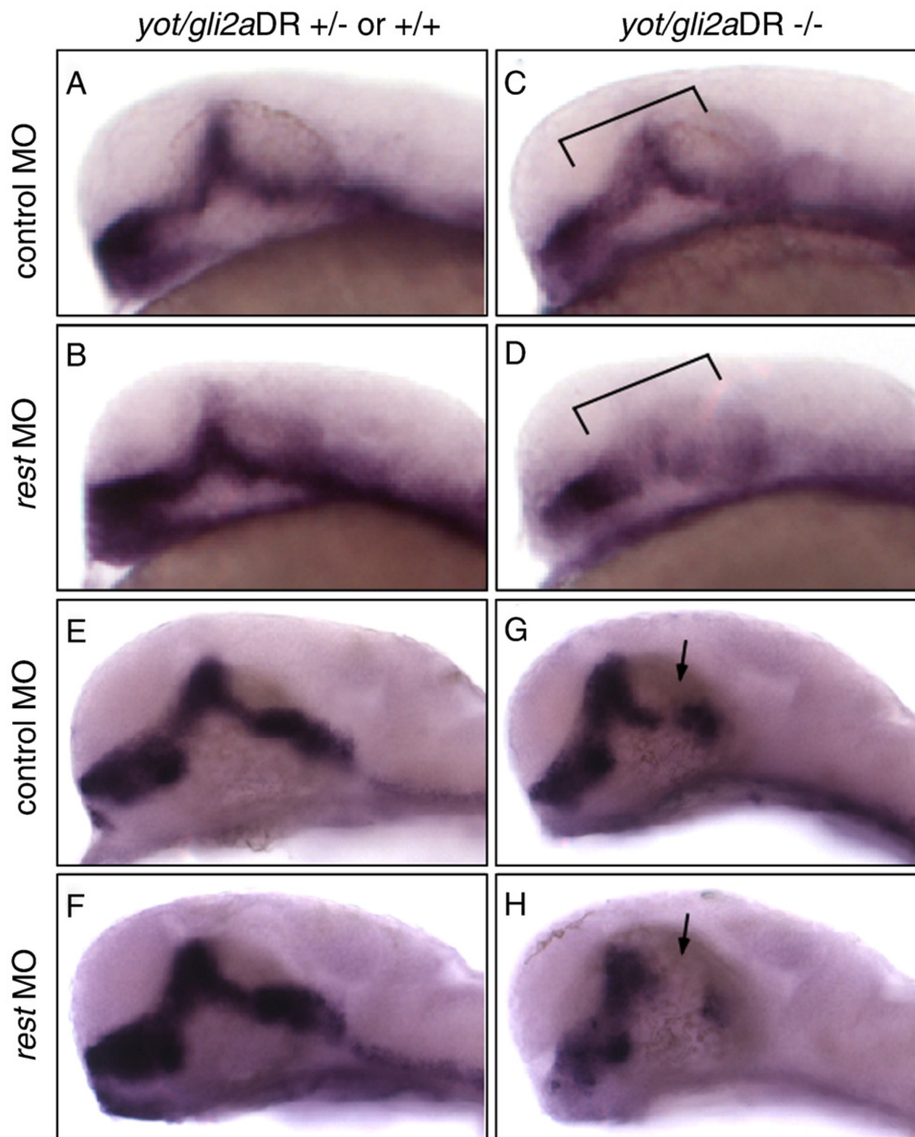

Fig. 7. Rest knockdown in *yot*/*gli2a* mutants represses Hh target gene expression. Lateral view, 30 hpf embryos stained for *ptc1* (A–D) or *nkx2.2* (E–H) mRNA. *rest* knockdown in wild-type embryos (B, F) results in modest enhancement of *ptc1* and *nkx2.2a* expression (compare B with A, and F with E). *yot*/*gli2a* mutants show characteristic loss of *ptc1* (C) and *nkx2.2a* (G). In *yot* mutants with compromised Rest function (D, H), the loss of *ptc1* (D) and *nkx2.2a* (H) expression is more pronounced in the midbrain/diencephanlon region (indicated by brackets, D and arrows, H) than in control-injected mutants (C, G).

observed effects of Rest knockdown in *dtr*/*gli1* mutants. To determine if the restoration of *nkx2.2a* in the hindbrain of *dtr* mutants treated with *rest* morpholinos was due to excess Gli2a^A activity, *rest* MO and *gli2a* MO were injected into a *dtr* intercross. While *dtr* mutants injected with rest MO displayed *nkx2.2a* expression in the hindbrain (9/11 in *dtr*−/− embryos, as in Fig. 7B), this expression was suppressed by Gli2a knockdown (10/15 absent, ($p = .000001$) 5/15 reduced in *dtr*−/− embryos, Fig. 7F). Therefore, we conclude that restoration of Hh target gene expression in *dtr* by Rest knockdown requires Gli2a.

Discussion

Rest plays a central role in regulation of gene expression required for cell proliferation and differentiation in a variety of contexts. Recent analysis of Rest function has led to proposals of a number of novel and seemingly contradictory roles for Rest. How the myriad of potential interactions mediated by Rest is translated into biologically relevant outcomes is poorly understood. We provide *in vivo* genetic evidence for an essential function for zebrafish Rest in regulation of the Hh pathway.

Rest has been best characterized as a transcriptional repressor, and we hypothesized that Rest directly or indirectly represses one or more Hh signaling components. Several lines of evidence implicate the Gli transcription factors, particularly Gli2a, as the key Rest target. First, Rest knockdown enhanced Hh target gene expression in multiple tissues implying that Rest regulates a fundamental aspect of the Hh pathway. Second, the effects of both positive and negative alterations in Hh signaling were enhanced with Rest loss of function. This is consistent with excess Gli activator function in contexts of strong Hh signaling, and excess Gli repressor activity when Hh signaling is reduced. Thus, excess Gli activity could account for the exaggerated response to both high and low levels of Hh signaling. In addition, our epistatic experiments reveal that Rest interacts with the Hh pathway downstream of Smo and upstream of Gli1 with the exception of the hindbrain, where Gli1-independent activities are present. Gli2a and Gli3 transduce Hh signaling chiefly through transcriptional regulation of *gli1*, but are also weak activators of other Hh targets (Fig. 9). We

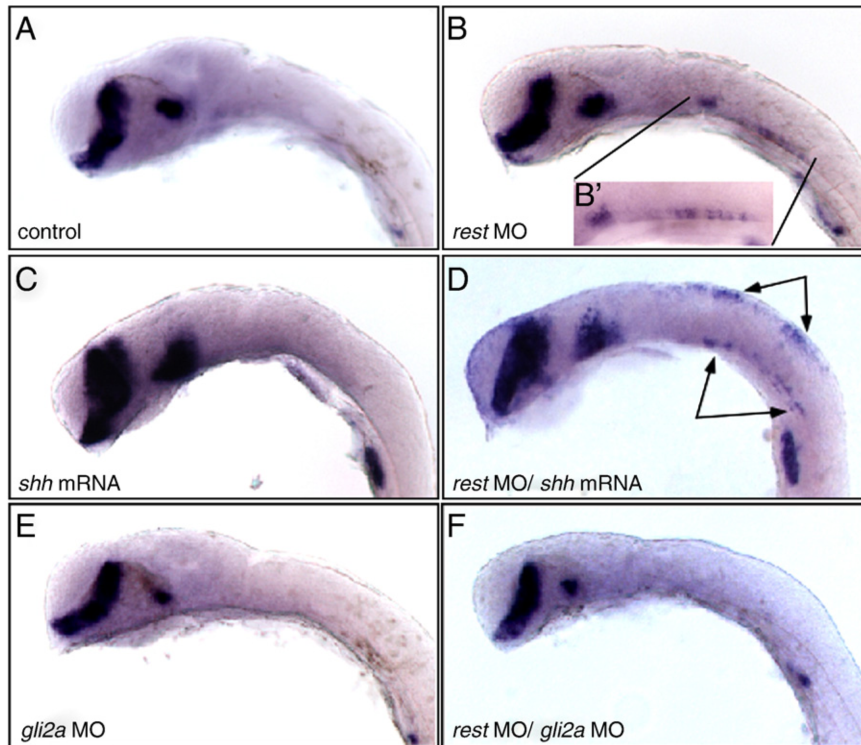

Fig. 8. Rest mediated repression of Hh target gene expression in *gli* mutants requires Gli2a. *dtr*/*gli1* mutants, lateral views, head and hindbrain, stained for *nkx2.2a* by whole mount RNA *in situ* hybridization. Embryos were injected with control MO (A), *rest* MO (B), *shh* mRNA (C), *rest* mo/*shh* mRNA (D), *gli2a* MO (E) or *rest* MO/*gli2a* MO (F). In *dtr*/*gli1* mutants, *nkx2.2a* expression is absent in control-injected embryos (A). However, hindbrain expression is partially restored in *dtr*/*gli1* mutants treated with *rest* morpholino (arrows, B). *dtr* mutants are largely refractory to exogenous *shh* mRNA (C) *dtr*/*gli1* mutants treated with *rest* morpholino have a qualitatively different response to *shh* mRNA treatment (D). Gli2a knockdown results in decreased midbrain *nkx2.2a* expression (E) The restoration of hindbrain *nkx2.2a* expression in *dtr* mutants produced by Rest knockdown (arrows, inset in B) is eliminated by simultaneous knockdown of Gli2a (compare F with B). This reveals that the enhancement of Hh signaling produced by Rest knockdown requires Gli2a and Gli1.

cannot rule out the possibility that Gli1 and/or Gli3 activity is also increased, independently of excess Gli2a activity.

The expansion of the *gli2a* domain into regions of active Hh signaling in *rest* morphants provides a mechanism to account for the effects on Hh signaling by Rest. The expression pattern of *gli2a* in *rest* morphants runs counter to alterations in other Hh pathway genes tested, including *gli3* and *gli2b*, because enhanced Hh signaling normally results in decreased *gli2a* expression.

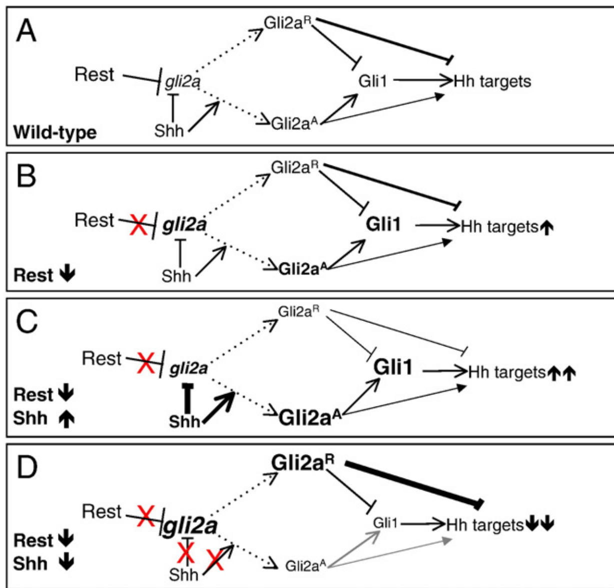

Fig. 9. Model for Rest interaction with the Hh pathway. *gli2a* transcription is directly or indirectly repressed by Rest. When Rest activity is attenuated, more Gli2a protein is produced. Depending on the level of Shh, Gli2a is processed to activator or repressor forms. An alternate model where Rest regulates nuclear transport of GliA and GliR is also consistent with our data.

In addition, derepression of *gli2a* also accounts for enhanced repression of Hh targets by Rest knockdown in *yot* mutants, in which only the dominant repressor form of Gli2a is produced. Finally, restoration of Hh signaling in the hindbrain of *dtr*/*gli1* mutants with compromised Rest function is blocked by Gli2a knockdown. This demonstrates a requirement for Gli2a^A in Rest mediated enhancement of Hh signaling. Together, our results support a model in which Gli2a is the principal point of Rest interaction within the Hh pathway (Fig. 9). Following ectopic activation of the Hh pathway (*shh* or *dnPKA* mRNA treatment), conversion of Gli2a to Gli2a^A predominates resulting in synergistic enhancement of Hh target gene expression. In CyA treated *rest* morphants, the excess Gli2a would be converted to Gli2a^R, which would repress Hh target gene expression.

Ectopic *gli2a* expression is most evident in the midline and ventral domains in *rest* morphants (Fig. 6H), and we were not able to discern any consistent differences from control embryos in the dorsolateral domains. In a wild-type embryo, this repression may serve to dampen the response to high levels of signaling. While Rest function in the developing embryo tempers the cellular response to Hh, the modest alteration in target gene expression seen in *rest* morphants may be due to redundant regulation of Hh signaling (Dessaud et al., 2007; Jeong and McMahon, 2005). Subtle phenotypes in zebrafish arise from mutations in negative regulators of the pathway including *ptc2*, *sufu* and *hip* (Koudijs et al., 2008; Koudijs et al., 2005).

Although the absence of strong RE1 sites near or within the *gli2a* locus does not rule out direct regulation by Rest, the transcriptional upregulation of *gli2a* in response to reduced levels of Rest may indeed be indirect. In addition to Hh, other signaling pathways including the FGF, Notch and Wnt pathways also regulate Glis (Alvarez-Medina

et al., 2008; Brewster et al., 2000; Ke et al., 2005). A model in which Rest knockdown activates one of these pathways, which in turn enhances transcription of one or more *gli* genes is consistent with our observations.

Rest function during development

Here, we show that reduced levels of Rest during zebrafish development leads to alterations in the progenitor domains responsible for generation of distinct neural subtypes. In addition to a role for REST in repressing neural genes in non-neural cells, REST has been implicated in the control of neurogenesis at multiple steps (Ballas et al., 2005; Bergsland et al., 2006; Otto et al., 2007; Su et al., 2004). Outside the developing nervous system, Rest has been placed upstream of the network controlling pancreatic islet development (Johnson et al., 2007; Kemp et al., 2003). This is an interesting finding as the Hh pathway regulates both neural and pancreatic development. We also find that Rest knockdown enhances expression of *nkx2.2a* in the developing zebrafish pancreas (Fig. 7, Supplemental Fig. 3). The wide range of potential activities proposed for REST underscores the importance of considering the unique cellular environment in which REST is acting.

REST mutant mice undergo widespread apoptosis beginning at day E 9.0 and die by day E11.5 (Chen et al., 1998). In contrast, we observed that the *rest* MO treated zebrafish present with a much subtler phenotype, ventralization of the neural tube. The expansion of ventral cell types in *rest* morphants, is unlikely to be produced by increased apoptosis. However, there are key differences in the mouse and fish experiments. Primarily, our treatments produce a knockdown, not a knockout of Rest. In addition, zebrafish *rest* is supplied as a maternal transcript, which may allow for adequate Rest activity during early stages.

REST has been identified as both a tumor suppressor (Coulson et al., 2000; Westbrook et al., 2008; Westbrook et al., 2005) and an oncogene (Lawinger et al., 2000; Su et al., 2006). It will be important to determine whether REST regulates Shh signaling in transformed cells. It is perplexing that REST downregulation results in differentiation in some cell populations and proliferation in others. For example, ß-TRCP dependent degradation of REST allows differentiation in neural stem cell culture, but proliferation in human mammary epithelial cell culture (Westbrook et al., 2008). How loss of REST allows activation of such different pathways is not well understood, but it is clear that differential target regulation depends on cellular context. For example, in neural progenitors, cell cycle progression relies on degradation of REST during the G2 phase for optimal expression of *mad2*, a direct REST target (Guardavaccaro et al., 2008). Needless to say, the full repertoire of genes under the control of REST is not activated during the G2 phase of mitotic cells. Differential target regulation may depend on many factors, including preexisting epigenetic modifications and the unique combinations of co-repressors and/or transcriptional co-regulators present. Unique combinations of such factors determine and are determined by the unique cellular context, allowing a small number of signaling pathways to affect a wide array of transcriptional networks and produce diverse outcomes.

Our studies reveal a novel and unexpected interaction between Rest and the Hh pathway. We find that Rest acts as a modulator of the Hh signal by regulation of *gli2a*. Rest likely fine-tunes the response of cells to Hh signaling by controlling transcription levels of *gli2a* and possibly additional factors. Transcriptional repression by Rest may thus be an additional limiting factor for Hh signal transduction, independent of antagonistic pathway components such as Ptc and Hip. Hh signaling plays a key role in regulation of progenitor cell proliferation and differentiation in many places within the developing nervous system and other tissues. Regulation of Hh signaling by Rest may be critical in many of these domains. These findings have broad implications for regulation of signaling in the many places where Hh acts and provide an avenue for future studies into Hh-mediated cell fate decisions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.01.029.

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