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A Role for the Transcriptional Repressor REST in Patterning the Zebrafish Neural Tube

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Abstract of the dissertation

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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/Neural Restrictive Silencing Factor) have provided important insight into the role that epigenetic modifications play in differential gene expression. Functional studies place REST within multiple developmental pathways and transcriptional networks. However, findings between different groups are often incongruent, and little progress has been made on understanding the embryonic lethality of the Rest knockout mice. What emerges from the controversies surrounding REST function is that the cellular context of REST is paramount.

Here, zebrafish embryos are used to study REST function within the broader context of a developing organism. The approach was to assay changes in gene

expression following Rest knockdown in various backgrounds. This method revealed a novel interaction between zebrafish Rest and the Hedgehog (Hh) signaling pathway. It was observed that Rest knockdown enhances or represses Hh signaling in a context-dependent 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, the findings demonstrate that the bifunctional transcription factor Gli2a is key to 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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Chapter 1. Background

1.1 Historical significance

The emergence of the diverse cell types that comprise the vertebrate nervous system is dependent on a carefully orchestrated program of gene expression. The generation of specific neural subtypes and the development of proper circuitry ultimately rely on the battery of genes that not only must be activated, but also repressed, at the right time and place. Studies of the transcriptional repressor REST (also known as NRSF) have provided important insight into the role that epigenetic modifications play in this differential gene expression.

REST was discovered as the transcription factor that binds to the Restrictive Element 1 (RE1), a ~23 bp regulatory element known to confer negative regulation of several neural specific genes (Ishiguro et al., 1993; Kraner et al., 1992; Li et al., 1993; Mori et al., 1992; Wuenschell et al., 1990). The discovery of REST by two independent groups (Chong et al., 1995; Schoenherr and Anderson, 1995) was of great interest because REST was proposed to be a key repressor in the neural default model, which proposes ectodermal cells

become neurons unless specifically prevented from doing so. While this view of REST has proved to be wrong, REST function has been shown to be necessary for embryonic survival (Chen et al., 1998), and has been implicated as a major regulator of neurogenesis (Ballas et al., 2005; Conaco et al., 2006; Lunyak et al., 2002; Roopra et al., 2000; Westbrook et al., 2008). REST has remained an enigma in terms of its exact role played in cell differentiation and identity. However, details on the mechanisms of gene repression by REST and its co-repressors have been elegantly demonstrated in biochemical studies.

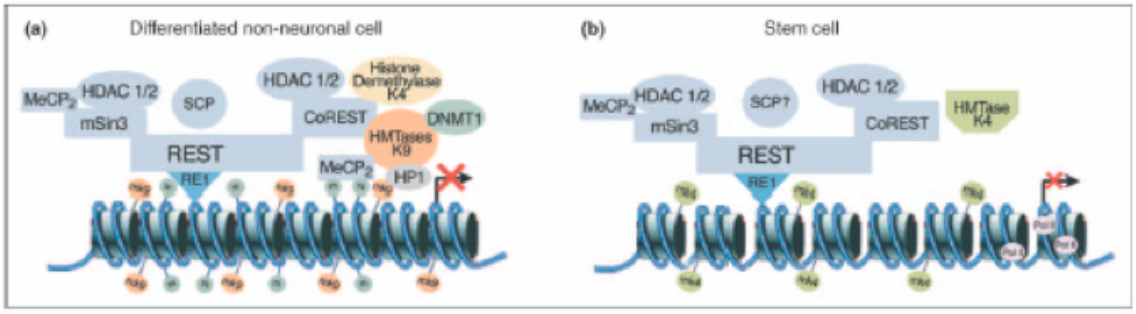


Figure 1. 1. Mechanism of REST repression. REST mediated repression in differentiated non-neural cells, left, and in stem cells, right. REST binds to an RE1 site within the regulatory region of the target gene and recruits a host of histone and DNA modifying enzymes. C- and N-terminal domains mediate repression independently by direct interaction with CoREST and Sin3, respectively. These corepressors in turn interact with more general chromatin modifying complexes. Epigenetic modifications of target gene chromatin in stem cells (right) leave the chromatin relatively loose compared to target genes in non-neural cells (left). Transcriptional activation in stem cells is therefore repressed but "poised for action". Adapted from Ballas and Mandel, 2005.

1.2. Mechanisms of repression

The discovery and study of REST has coincided with, and has greatly contributed to, the expanding field of epigenetic research. Understanding the influence of chromatin modifications is a prerequisite for understanding differential gene expression. Repression by REST involves the recruitment of co-repressors to the REST-DNA complex, leading to modification of chromatin structure around target genes (Fig. 1.1). REST functional domains consist of an 8 zinc finger DNA binding domain, and C- and N-terminal protein interaction domains, both sufficient by themselves for repressor activity (Ballas et al., 2001). The amino terminal interacts with a Sin3a/HDAC complex, which has been proposed to be involved with more dynamic, temporary repression (Grimes et al., 2000; Roopra et al., 2000; Silverstein and Ekwall, 2005). CoREST interacts directly with REST through a C-terminal zinc finger and recruits multiple DNA and histone-modifying proteins (Battaglioli et al., 2002; Lunyak et al., 2002). The REST/CoREST complex in embryonic stem cells and progenitors differs from that found in differentiated non-neural cells (Figure 1.1, Ballas et al., 2005; Ballas and Mandel, 2005). Target genes in non-neural cells are thought to be silenced by tighter compaction of chromatin and subsequent inaccessibility to transcriptional activators and basal transcription machinery. In contrast, the chromatin of repressed target genes in embryonic stem cells ESCs and progenitors is relatively less compacted and gene expression may be allowed in response to developmental signals. This difference in chromatin modifications is thought to center on the methylation status of the DNA and histones. Methylation of discrete histone residues and DNA not only affects transcription factor accessibility but recruitment of other enzymes and subsequent further modifications (Ballas and Mandel, 2005). Thus, while REST binding to the RE1 sequence confers specificity, preexisting epigenetic modifications and the

particular corepressor complex assembled will determine the nature of repression.

1.3. The RE-1 binding site.

DNA *cis*-regulatory sequences provide the instructions for proper spatiotemporal gene expression by recruiting the transcription factors that aid or hinder the basal transcription machinery. The typical transcription factor binding site (TFBS) is around 5-8 bp, which makes the 23 bp RE1 unusually long. This makes the search for putative targets feasible, and the RE1 site favorable for those using bioinformatics to study transcriptional networks through TFBS analysis. Consequently, using various computational models to detect RE1s, several thousand putative targets have been found (Bruce et al., 2004; Johnson et al., 2007; Johnson et al., 2008; Mortazavi et al., 2006; Otto et al., 2007). Additionally, REST has been implicated in multiple transcriptional networks. However, the presence of an RE1 within the regulatory region of a gene does not necessarily confer regulation by REST. To this end several methods have been used to determine which RE1s are occupied, and which RE1s are actually functional. Various methods combining ChIP and sequencing of DNA bound by REST have confirmed some of the bioinformatically predicted targets and have also identified non-canonical RE1s and sequences having no resemblance to RE1s. Functional assays following confirmed occupancy of RE1s consist of assaying target or reporter upregulation upon interference with REST activity. While the identification of non-canonical RE1 sites has greatly expanded the list of potential targets, consensus sequences are associated with much higher occupancy and greater functional significance (Johnson et al., 2008; Mortazavi et al., 2006; Otto et al., 2007).

Whether a given RE1 is occupied and/or functional has been found to be context dependent, with significantly different outcomes from loss of function

occurring based on the particular cell line and conditions. For example, Otto et al., 2007, using kidney cell lines found that of the REST occupied RE1 sites tested, around half were functional. In contrast, an extensive microarray assay found a much smaller percentage of functional RE1s in embryonic stem (ES) cells. (Johnson et al., 2008) The same study found significant functional differences between REST activity in ES cells compared to neural stem (NS) cells and fibroblasts.

Different levels of Rest transcript and protein in a given cell type likely accounted for some of the differences seen due to differential target regulation. Differential binding of REST on a given gene may depend on its relative affinity for the genes RE-1 site and surrounding sequence. For example, in the human glioma cell line U373 a majority of genes tested for REST occupancy were not bound at endogenous levels but could bind following overexpression of REST (Bruce et al., 2004). Two genes that did have REST bound at low levels, *SNAP25* and L1CAM, contained adjacent, closely spaced consensus RE-1 sequences. The authors speculate that the tandem RE-1s in those genes could account for the differential occupancy of REST on different promoters. Differential recruitment of REST may also be influenced by the presence of degenerate RE-1's in the surrounding sequence (Qiang et al., 2005; Zhang et al., 2006). Although REST has only low affinity for these sites, they attract REST and keep it in the vicinity of the promoter as natural dissociation occurs from the DNA. Functionally, the location of the RE1 sequence has been found to be an important factor. Target upregulation following interference with REST as assayed by ChIP-chip finds optimal distance of functional RE1s to be within 2-3kb of the transcriptional start site (Johnson et al., 2008). This proximity bias stands in contrast to proposed RE1 sites that are far distances away from the promoter (Lunyak et al., 2002). Thus, differences in the location, the surrounding sequence, and the degree of consensus of an RE1 are all factors determining the extent of regulation conferred by REST.

1.4. Functional studies*, in vitro*

In it's initial discovery as the factor that binds to the RE1, *Rest* was found expressed in the embryonic ventricular zone of the neural tube and so early on was posited to play a role in repressing neural genes in precursors. Subsequent functional studies carried out in cell cultures provided support for the model of REST as a potent determinant in maintaining non-neural characteristics. Loss of function studies were typically accomplished by introduction of a dominant-negative Rest (D/N REST), consisting of the DNA binding domain only (Ballas et al., 2001). Alternatively, the RE1 was removed or mutated to abolish binding activity. These types of manipulations result in inappropriate expression of endogenous target or reporter genes (Chong et al., 1995; Lunyak et al., 2002; Tapia-Ramirez et al., 1997). Likewise, gain of function experiments suggest potent repressor functions for REST. PC12 cells, which do not express REST, differentiate upon exposure to NGF. Conditional expression of REST, however, prevented this acquisition of a neural phenotype (Ballas et al., 2001).

The scope of influence of REST has also been tested by fusing a powerful activator (VP16) domain to the DNA binding domain of REST (RESTVP16). Expressed as a constitutive activator, REST is able to induce neurogenesis in otherwise non neurogenic cells. RESTVP16 has been reported to drive C17.2 NS cells to differentiate (Su et al., 2004), and even convert myoblasts into mature cells with neuronal like properties (Watanabe et al., 2004). These studies suggest the entire battery of genes necessary for neurogenesis is under the control of REST. This idea of REST as a master regulator has also been fueled by bioinformatic centered studies. Hundreds, if not thousands of putative REST binding sites that potentially regulate an immense set of transcripts have been discovered (Watanabe et al., 2004) (Bruce et al., 2004; Johnson et al., 2009; Johnson et al., 2008; Mortazavi et al., 2006; Mu et al., 2005; Otto et al., 2007; Wu and Xie, 2006). These studies implicate REST at multiple hierarchal levels in various transcriptional networks. Besides controlling neuronal differentiation,

REST may be similarly involved in cell specification in the pancreas (Johnson et al., 2007; Kemp et al., 2003). Putative regulation of miRNAs greatly expands the scope of possible influence by REST (Conaco et al., 2006; Otto et al., 2007; Singh et al., 2008; Wu and Xie, 2006; Yoo et al., 2009). For example Singh et al. have reported that REST maintains the undifferentiated state in ESCs by repression of the microRNA miR-21, which antagonizes major pluripotency factors Sox2, Nanog, Oct4 and c-Myc.

However, the extent of regulation by REST and its actual role in cell differentiation is hotly contested. Other groups report REST is not involved in stem cell maintenance or regulation of proneural genes. With respect to the pluripotency factors Sox2, Oct4 and Nanog, REST may function downstream, and/or share common targets (Johnson et al., 2008; Kim et al., 2008). However, stem cell characteristics were not affected in LOF assays. Loss of REST had no effect on those pluripotency factors, or miR-21, or proneural genes such as *Mash1a* in these studies (Buckley et al., 2009; Jorgensen et al., 2009a; Jorgensen et al., 2009b; Sun et al., 2008).

In fact, *in vitro* and *in vivo* manipulations of REST ultimately affect only a relatively small subset of target genes (Chen et al., 1998; Johnson et al., 2008). Recent functional studies show that despite the presence of an RE1 in the vicinity of a gene, or even despite occupancy of the RE1 by REST, interference does not cause derepression in vast majority of identified targets. Further, that derepression differs depending on cell type. (Chen et al., 1998; Johnson et al., 2008). Discrepancies between findings from different groups have been attributed to the use of different cell lines and different culture conditions.

These issues will need to be resolved, but what has become clear is the crucial role that context has on REST function. The elucidation of the variable and complex mechanisms that REST and its corepressors carry out their functions may indeed require the isolation available *in vitro*. However, a more complete understanding of REST/CoREST function will require *in vivo* studies within the context of a full range of developmental signaling. Animal studies to

date have confirmed the basic repressor function, but have not established REST's full role during embryonic nervous system development.

1.5. Functional studies *in vivo*

in vivo functional studies, while confirming a basic function of repressing neural genes, have not found REST a master regulator of neural phenotype. The definitive functional study *in vivo* uses the mouse knockout, a homozygous null mutant that dies by E10 (Chen et al., 1998). While this demonstrates the necessity of REST during early development, the cause of lethality has not been discovered. Embryos are stunted in growth by E8.5, and massive cell is apparent. Mutants showed cellular disorganization in the head mesenchyme and myotomes, and ectopic blood cells were also evident. However, germ layer formation, neural induction and early CNS patterning appeared largely normal. Therefore, in contrast to *in vitro* reports by Singh et al, embryonic stem cells lacking REST function in a developing embryo can progress normally for some time without lineage being affected and without overt premature neurogenesis occurring. Of several targets tested in the mouse, only neuronal TUBB3 (Neuron-specific class III beta-tubulin) was upregulated. This primarily occurred in the head mesenchyme, in the ectoderm overlying the CNS and in the myotome. Upregulation of TUBB3 was also observed following electroporation of a D/N REST into chick spinal cord. (ibid) However, this upregulation required the presence of upstream activators of TUBB3 (Bergsland et al., 2006), demonstrating that derepression by itself was insufficient for gene expression. This phenotype has been described as very complicated and the mouse knockout has not been further explored. Still, the eventual widespread cell death and lethality indicate a crucial deregulation of genes and cellular processes.

These *in vivo* studies suggest REST function is most important downstream of neural induction, during the stages of differentiation. However,

functional studies done in Xenopus reveal an earlier role. Interference with REST activity in frog embryos using an inducible D/N REST resulted in decreased REST target gene expression (Armisen et al., 2002; Olguin et al., 2006). This was thought to be due to early ectodermal patterning defects, as there was an expansion of the neural plate at the expense of epidermal and neural crest. This loss of function mimics a BMP decrease, and was shown to counteract BMP misexpression. However, it is not clear how the expansion of neural plate is related to the later loss of markers. Whether this proposed earlier function is species specific or reflects experimental differences has not been established.

The mouse and frog studies result in embryonic lethality and prepatterning defects, respectively, and so preclude *in vivo* study of REST's role in neurogenesis. The results of the Xenopus study, while intriguing, are difficult to interpret. Unfortunately, despite the multitude of *in vitro* functional studies conducted to date, little light has been shed on the possible events that lead to embryonic lethality. Lethality in the null mutant occurs at a time when positive activators of neural genes are increasing. This leaves open the possibility of a disorderly activation of the neuronal program, which could hypothetically lead to the widespread apoptosis that was observed. Alternatively, lethality may be due to REST function that is not directly related to neuronal differentiation. For example, REST null ES cells have decreased proliferation and undergo cell death, a phenotype attributed to dysregulation of several extracellular matrix molecules (Sun et al., 2008). Other roles for REST, such as the smooth progression of the cell cycle via regulation of mad2 (Guardavaccaro et al., 2008), also remain plausible avenues for investigation.

Chapter 2. Zebrafish as a model organism for vertebrate development

2.1. Introduction

The rapid, well-characterized CNS development of the zebrafish and the availability of transgenic and mutant stocks can help elucidate the role REST during embryonic development. One strength of zebrafish is the large number of offspring produced and the ease at which genetic pathways can be manipulated in these embryos. Two or more genes or pathways can simultaneously be perturbed with relative ease on hundreds of embryos in one sitting. This allows elucidation of epistatic relationships between factors, and the large sample size gives confidence in results. Homologs of genes of interest can be found in the nearly completed and assembled zebrafish genome database. (www.ensembl.org/Danio_rerio). One issue frequently encountered is that zebrafish often have more than one homolog of mammalian genes. This is believed to be due to an entire genome duplication in ancestors of zebrafish that occurred after the lineage split from the fish that would give rise to land vertebrates (Meyer and Schartl, 1999; Taylor et al., 2001), and see Fig 2.3A for example). The resultant redundancy can make some loss of function studies more complicated. However, the presence of a second allele may also prevent embryonic lethality in those cases, leading to a milder phenotype in which function may still be uncovered.

2.2.1 Formation of the zebrafish nervous system

The fundamental processes of nervous system development and neurogenesis are well conserved throughout vertebrate evolution. Zebrafish primary neurulation occurs fundamentally the same as in other vertebrates, with minor differences; the neural plate folds into a 'neural keel' first and cavitation to form the lumen is secondary (Lowery and Sive, 2004). The proliferative expansion of the single cell layer of the epithelial neural keel begins from around 10 hpf (hours post fertilization) and peaks around 30 hpf (Figure 2.1A, (Lyons et al., 2003) As proliferation proceeds, early born cells migrate or are pushed out laterally toward the pial surface by later born cells growing at the ventricle surface. Those early born and most lateral cells are generally the first to differentiate. During this major period of neural tube expansion, a small fraction of the cells exit the cell cycle to become primary neurons. These early born neurons make up a simple scaffold of bilateral nerve clusters, commissures and tracts by 24 hpf (Chitnis and Kuwada, 1990; Kimmel et al., 1995; Strahle and Blader, 1994; Wilson et al., 1990).

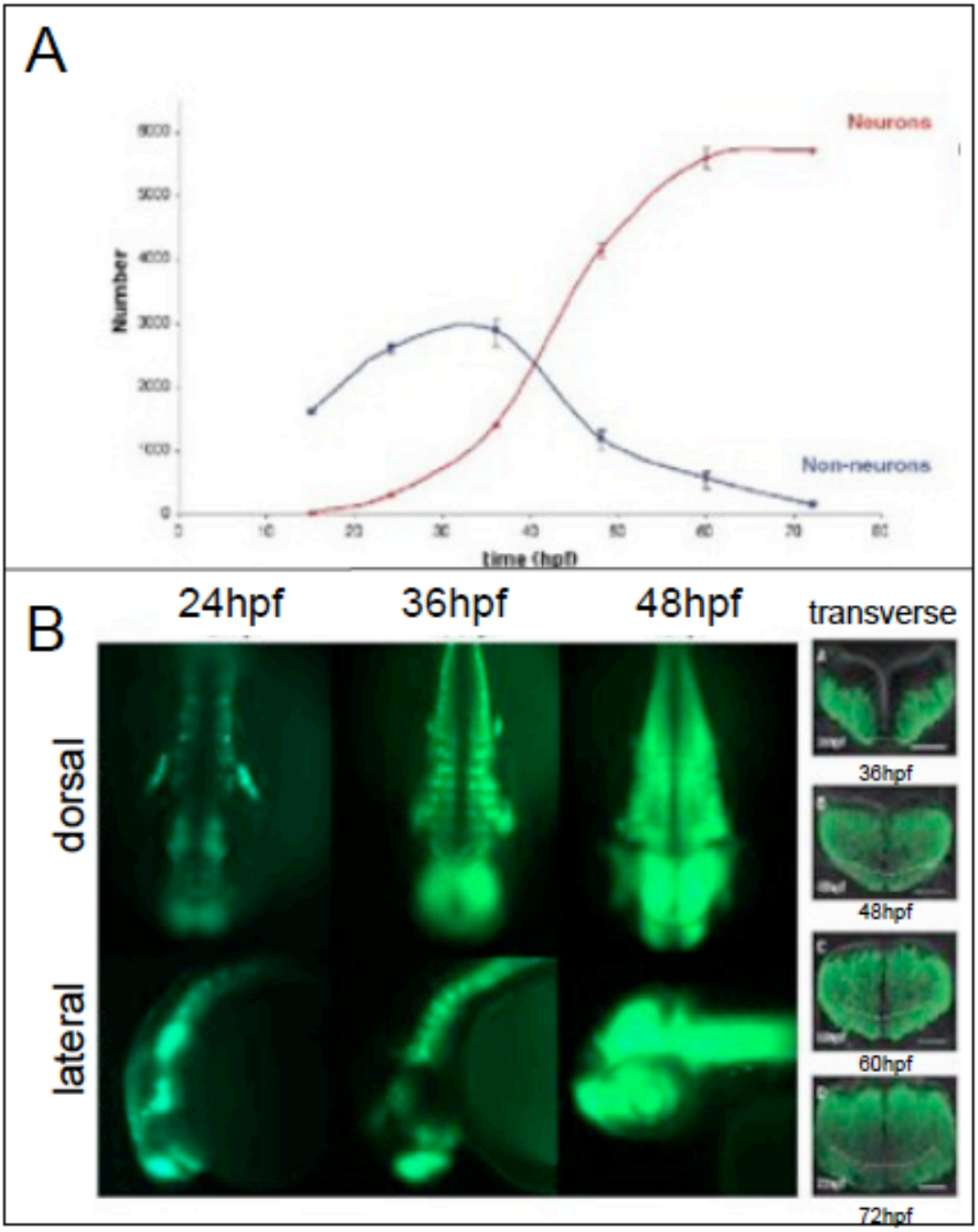


Figure 2.1. Formation of embryonic nervous system in zebrafish. (A) Graph summarizing total number of progenitors and neurons in a section of the embryonic zebrafish hindbrain during first 3 days of development. (B) tg[*elavl3*-eGFP] embryos demonstrating dynamics of neurogenesis. elavl3 is a marker of post mitotic neurons. Graph in (A) and transverse sections in (B) adapted from Lyons et al., 2003

This is referred to as ‘primary neurogenesis’, and the neurons generated at this stage amount to a small percentage of the total neurons that will be generated by 72 hpf. The major neurogenic period, where the fraction of cells leaving the cell cycle to become neurons is greatest, occurs roughly between 36 hpf and 60 hpf. Primary neurogenesis does not occur in the eyes or optic tectum, and the first post-mitotic cells are seen after 36hpf in those structures. In the rest of the embryo, this secondary neurogenesis generally expands upon and adds new clusters to the primary scaffold established in the first.

The pan-neural marker *elavl3* is one of the first neural markers to appear in committed progenitors within these fields (Kim et al., 1996). The bulk of zebrafish CNS development takes place over a 72-hour period and follows a stereotypical pattern of well defined events. The use of GFP expressing transgenic zebrafish lines allow one to follow this course of development and quickly assay experimentally induced changes. For example, the *elavl3*-GFP line (Park et al., 2000a) is a convenient way to follow neurogenic events in live embryos (Figure 2.1B). The overall maturity of the CNS as widespread neurogenesis is occurring can be visualized by the decreasing proliferative ventricular zone and the increasing number of *elavl3*-GFP positive cells, as shown in the developing hindbrain (Fig. 2.1C). Other cell type specific promoter-reporter lines are also available (See Figures 4.2, 4.3, 5.5).

2.2.2 Zebrafish proneural genes

The brief period of neurogenic events in the zebrafish are well characterized at the level of the whole organism. This allows a broad overview of gene expression patterns in which general functions may be inferred. Neurogenesis takes place within proneural fields marked by expression conserved bHLH genes. These proneural transcription factors drive differentiation and also help determine cell identity.

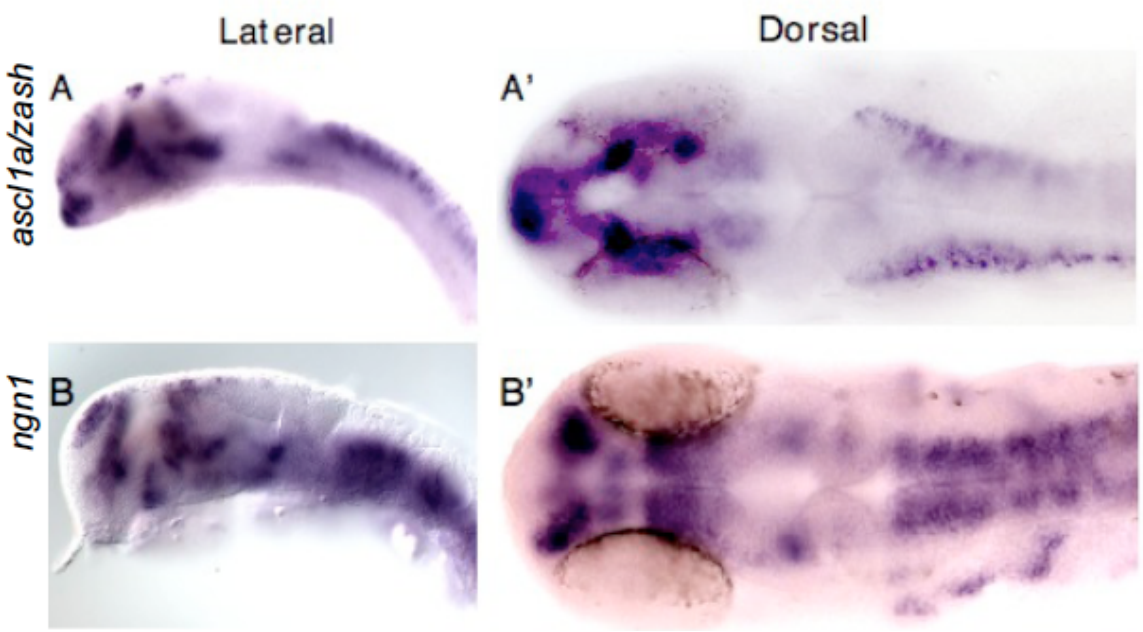


Figure 2.2. Proneural gene expression. RNA in situ hybridization, zebrafish embryos fixed around 24hpf. Lateral (A-C) and dorsal (A'-C') views of head and hindbrain. (A-B') Proneural genes acheate-scute complex- like 1a (*ascl1a*, homolog of mammalian *MASH* gene) and *neurogenin1* (*ngn1*) form complex neural progenitor domains that are largely complementary.

Zebrafish proneural genes *ngn* and *zash1A/asc1a* are expressed in fields that are mostly complementary to each other, and together largely make up the proneural field (Ma et al., 1996), and Fig 2.2.).

2.2.3 Zebrafish neural tube patterning genes

The proneural factors are expressed within progenitor domains created by prepatterning factors. These transcription factors responsible for patterning the neural tube are conserved throughout evolution. Retinoic Acid, FGF and Wnt ligands secreted from the surrounding mesodermal tissue and signaling centers within the neural tube combine to give rise to *otx2, pax2*, the *hox* genes, and other transcription factor domains that define anterior-posterior regions (Figure 2.3A). Signaling centers such as the anterior border cells (Houart et al., 1998),

the zona limitans intrathalamica (ZLI) (Scholpp et al., 2006), the midbrain/hindbrain boundary (Wurst and Bally-Cuif, 2001) and rhombomere 4 (Maves et al., 2002), play similar roles in zebrafish and other vertebrates in defining specific regions along the axis of the embryo. The stereotypical development of the cranial nerves from the segments of embryonic hindbrain is a good example of these conserved processes (Figure 2.3B)

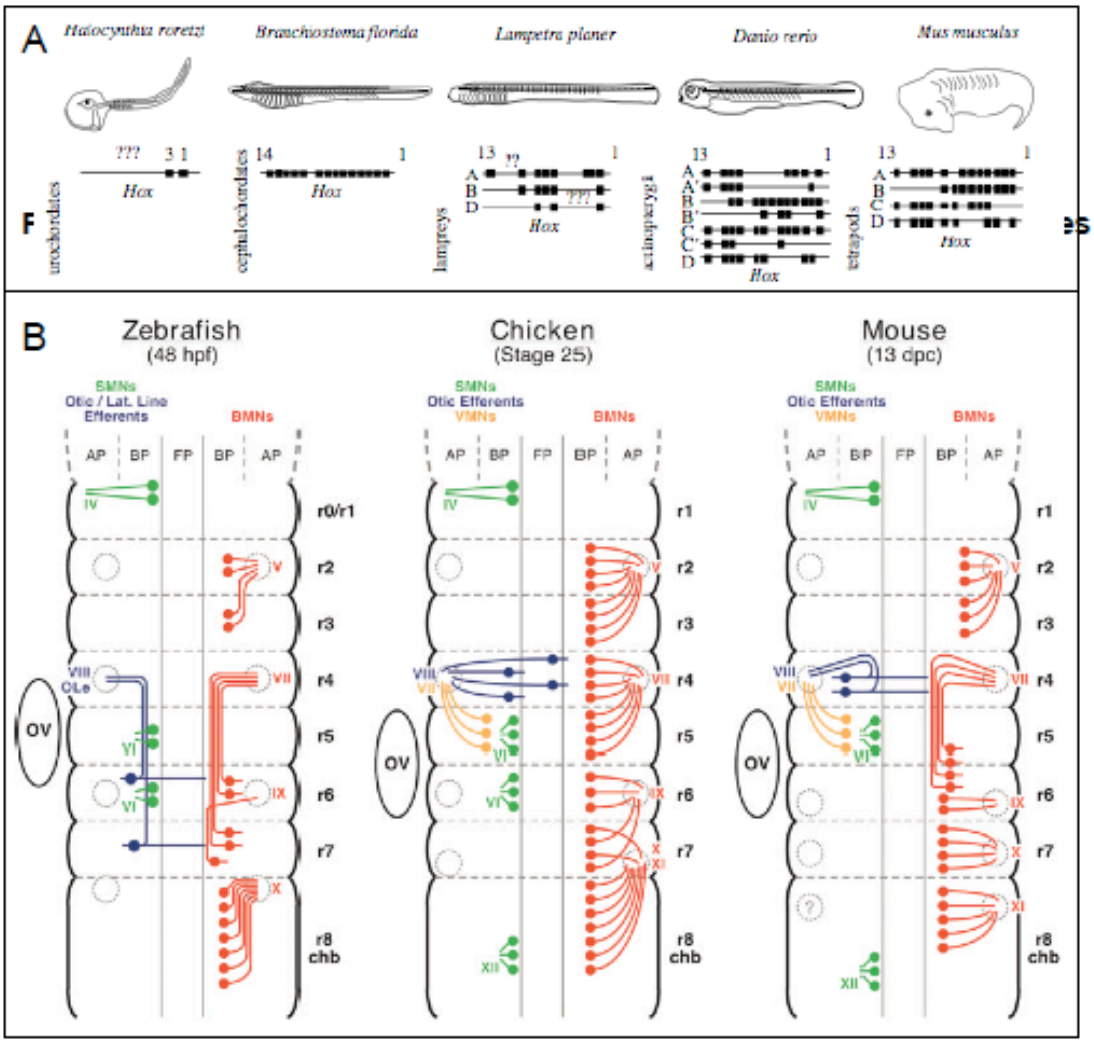


Figure 2.3. Conservation of anterior posterior (AP) patterning in vertebrates. (A) Hox gene locus throughout evolution. Hox genes expressed in different hindbrain segments give cells their AP identity. Note gene copy number in zebrafish compared to mouse. Adapted from Schilling and Knight, 2001.
(B) Cranial nerves labeled in embryonic hindbrains. Specific cranial nerves arise out of the same hindbrain segments (rhombomeres, r) in vertebrates. Adapted from Chandasekhar et al. 2004

Cells of the neural tube that compose the roof plate (Chizhikov and Millen, 2004) and floor plate (Strahle and Blader, 1994) secrete the molecules that establish dorsoventral patterning of the nervous system. The exact role of BMP signaling in dorsal cell type identity is unclear in zebarfish, and Wnt signaling from the neural tube roofplate may play a larger role in this. However, the pathway to ventral specification is well conserved (Huangfu and Anderson, 2006). Sonic Hedgehog secreted from the ventral midline of the neural tube induces ventral cell fates in a dose dependent manner, generating distinct neural subtypes (Lee and Jessell 1999; Guner and Karlstrom 2007, Figure 2.4). In addition to the conserved *shh* gene *shha*, zebrafish have a closely related gene, *shhb*/*tiggywinkle hedgehog* (Ingham and McMahon, 2001). *shhb* has both unique and redundant functions with *shha* in patterning the ventral neural tube, and is able to compensate for loss of *shha* (Lewis and Eisen, 2001). The effectors of Hh signaling, the Gli transcription factors, while largely conserved, show some functional divergence. For example, while the main activator of Hh targets in mammals appears to be Gli2, that function is fulfilled by Gli1 in zebrafish. (Figure. 2.5, (Bai and Joyner, 2001; Karlstrom et al., 2003) The use of zebrafish Hh pathway mutants and transgenic lines have contributed to a better understanding of the many roles and mechanisms of Hh signaling.

While some functional divergence in the details of these conserved pathways is apparent between fish and mammals, the basic processes and pathway components are conserved. Zebrafish have proved to be useful models in which to study the development of vertebrate nervous system, from the early patterning events throughout the course of neurogenesis.

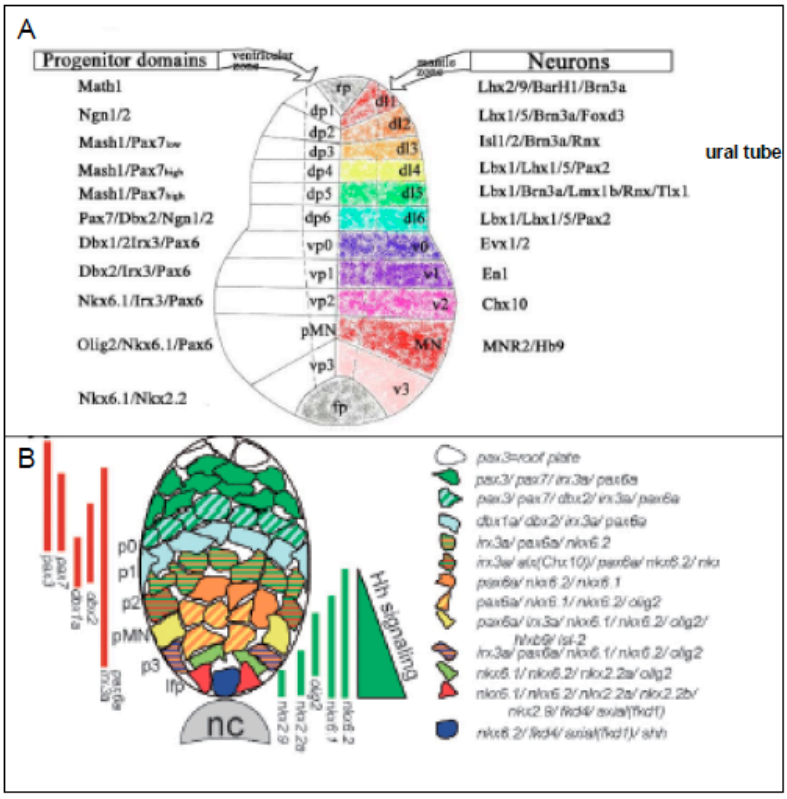


Figure 2.4. Conservation of dorsoventral patterning of the vertebrate neural tube. Neural progenitor domains defined by expression homeodomain transcription factors in mouse (A) and zebrafish (B) spinal cords. A gradient of Hh signaling from the floorplate (fp) creates the domains of transcription factors. (A) adapted from Wilson and Maden, 2005. (B) adapted from Guner et al., 2007.

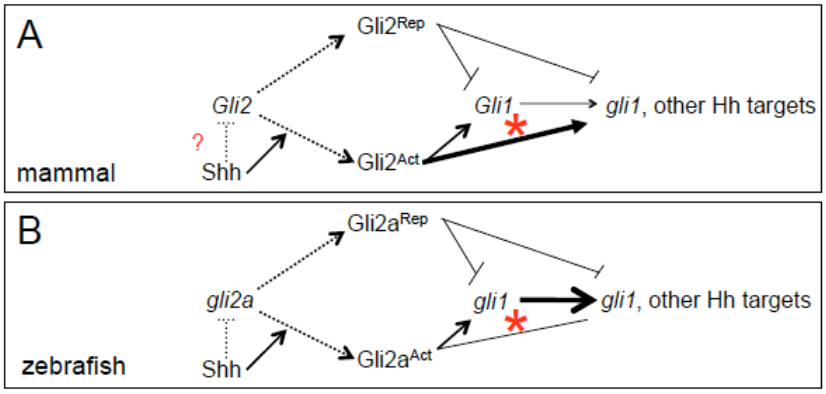


Figure 2.5. Gli family of TFs mediate Hh signaling. Human (A) and zebrafish (B) Gli activity models. Gli3 is omitted for clarity, but has important Repressor roles and weak Activator ones in both species. Gli2 functions of an activator or repressor depending on the presence of active Hh signaling. The Gli2 activator form transduces Hh signaling by direct transcriptional activation of Hh targets, including gli1. Gli1 is always an activator and plays a larger role in zebrafish than it does in mammals. Gli2 is the main activator in mammals, whereas Gli2a is only a weak activator in zebrafish. Strong or prolonged Hh signaling results in transcriptional downregulation of gli2a in zebrafish.

Chapter 3. Conservation and expression of Zebrafish *rest*

3.1 Introduction

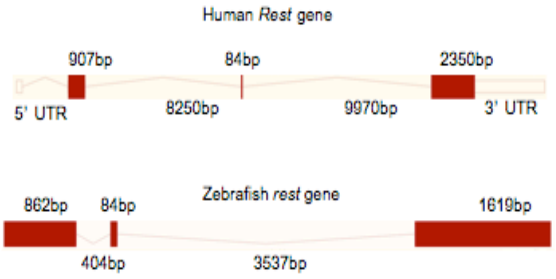
Rest expression has been studied in mouse, chick and frog. Although not assayed extensively, expression patterns in these three appear similar, and consistent with maintaining non-neuronal properties. Expression in both frog and mouse embryos has been described as being widespread early on, then becoming more restricted as development proceeds. The expression in frog is ubiquitous until neurola stage (Armisen et al., 2002), and found in the neural tube at stage 35, although the study did not differentiate between medial (proliferative) verses lateral expression (post mitotic). In mouse, expression has been described as ubiquitous up until E9.5 (Chen et al., 1998) and present in nonneural and ventricular cells at E14 (Chong et al., 1995). Likewise, *Rest* transcript is found in nonneural tissue and in the ventricular zone at E5 in chick (Chen et al., 1998).

3.2 Results

3.2.1 Conservation of zebrafish *rest* gene

To begin to examine the role of Rest in zebrafish, zebrafish *rest* was cloned to determine conservation of the protein and of expression patterns, and to create antisense probes for in situ hybridization. Like Human Rest, the zebrafish *rest* gene contains three coding exons (Fig 3.1A). While these exons are approximately (exons 1 and 3) or exactly (exon 2) the same size, expansion of the introns make the human *Rest* locus around eight times larger overall.

A.



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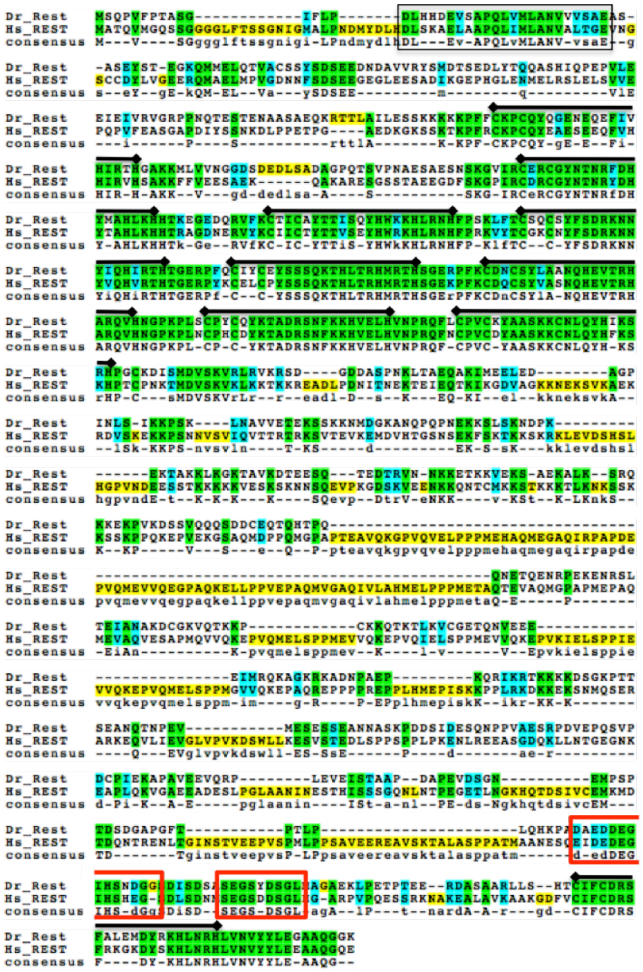

Figure 3.1. Conservation of zebrafish Rest. (A) Genomic structures of human *Res*t (on top) and zebrafish *rest.* While exon sizes are roughly the same, the human Rest locus has expanded non-coding regions (B) Alignment of Human REST and predicted zebrafish Rest proteins. Sin3a interaction domain in black box. Overlying bars indicate zinc fingers of DNA binding domain, and C-terminal zinc finger CoREST interaction site). Phosphodegron motifs in red boxes. Adapted from Gates et al., 2010

Additionally, a 5' UTR found in humans and other mammals has not been found in zebrafish. This expansion of noncoding DNA is typical during evolution, and may allow for additional regulatory control.

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, zebrafish Rest is predicted to encode 9 zinc fingers (8 of which comprise the DNA binding domain) (Fig. 3.1B). 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 to identify RE1 sites 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 corepressor 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).

3.2.3 *rest* expression during development

rest expression was analyzed 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 (Fig 3.2A, 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.3.2D), a stage when cells are largely proliferating and few neurons have differentiated. Around 20 hpf, (FIG. 3.2E) *rest* transcript is downregulated in the ventrolateral domains where neuronal differentiation is occurring (arrowheads 3.2E-G).

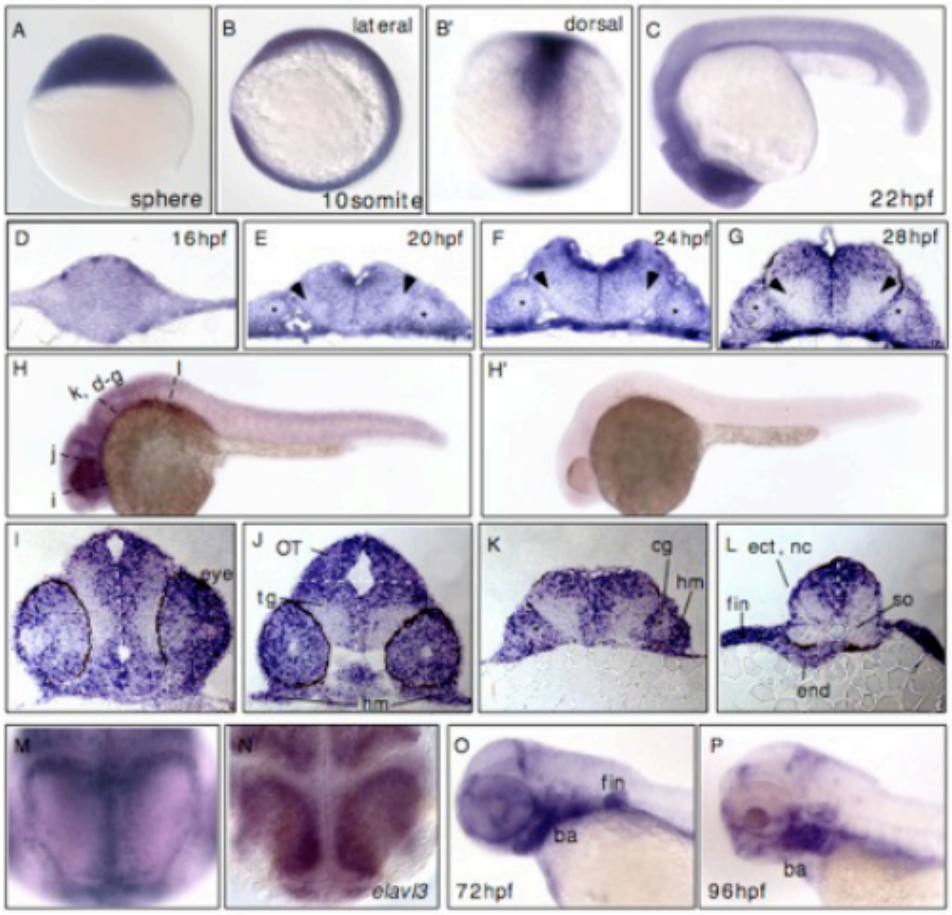

Figure 3.2. Expression of *rest* during early zebrafish development. mRNA *in situ* hybridization with antisense *rest* probe, except H' where *res*t sense strand was used, and M, *elavl3*. Wholemounts (A-C, H, M-P) and transverse sections (D-G, I-L) of wild-type embryos during the first days of development. (A, B, D) Early expression is ubiquitous. (C-G) Expression remains widespread at 22hpf (C) and 25hpf (H) but transverse sections of hindbrain reveal progressive downregulation as neurogenesis takes place in ventrolateral domains (arrowheads, E-G). (I-L) Sections of 42hpf 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) 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 already differentiated somites (L, so) and sensory cranial ganglia (cg, K). (M-N) dorsal view of 48hr optic tectum, anterior down. (M) the peripheral borders of the optic tectum lobes remain proliferative and expresses rest. (N) Panneural marker *elavl3* expressed in a complementary pattern in the postmitotic center of each lobe. (O, P). *rest* remains expressed at later stages in the developing fins and branchial arches,ba. cg, cranial ganglia; ect, ectoderm; end, endoderm; hm, head mesenchyme; OT, optic tectum; tg, tegmentum. From Gates et al., 2010

During the accelerated period of neurogenesis from 24 hpf to 48 hpf (Lyons et al., 2003), *rest* persists in the undifferentiated dorsal rhombic lip (Fig. 3.2G, K), and in proliferative midline (ventricular zone, vz) cells extending to the floorplate. Dorsoventral 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. 3.2J). *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 (Fig 3.2K, L). In addition, *rest* is excluded from differentiated sensory cranial ganglia adjacent to the neural tube (Fig 3.2E, F, G, K asterisks).

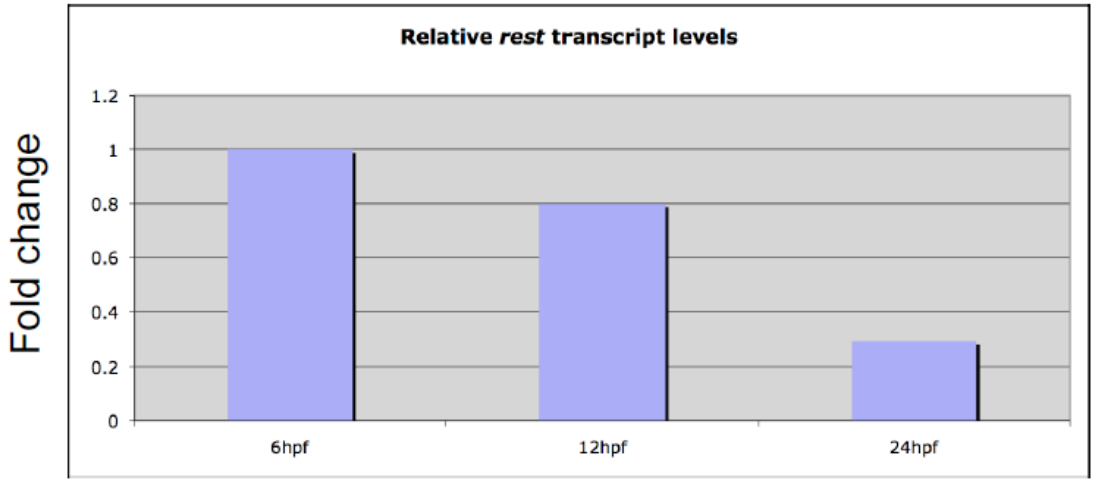

Figure 3.3. Relative rest transcript levels during early development. qPCR of rest transcript levels in cDNA derived from whole embryos. The fold change (Y axis) of rest at 12 and 24hpf are graphed relative to levels from 6hpf embryos. Overall *rest* levels are downregulated dramatically as development proceeds from early gastrulation periods (6 hpf) to late somitigenesis (24 hpf)

As neurogenesis rates increase during the second day of development, *rest* levels decrease correspondingly. Expression remains in the narrowing ventricular zone, in the eyes and the periphery of the optic tectum lobes (Fig 3.2. M), which are still proliferative. There, *rest* is complementary to that of pan-neural marker *elavl3* in the post-mitotic center (Fig. 3.2, N). Post-embryonically, *rest* is expressed at low levels in the vz but still present in fins (Fig. 3.1, O), the branchial arches (Fig. 3.2, O, P), and head mesenchyme.

Overall, levels of rest transcript decreases as the embryo matures, qPCR reveals that relative levels drop up to 75% from 6 hpf to 24 hpf (Fig. 3.3). Presumably, this reflects a greater proportion of differentiated cells at this period.

3.3.4 *rest* is expressed in proliferating cell populations

While details of the posttranslational degradation of REST have been reported, (Ballas et al., 2005; Guardavaccaro et al., 2008; Westbrook et al., 2008) its transcriptional regulation remains poorly understood. Some evidence has been provided for negative and positive regulation by the Retinoic Acid and Wnt pathways, respectively (Ballas et al., 2005; Nishihara et al., 2003). The signaling pathways responsible for regionalizing the embryos will also influence cell cycle exit and differentiation (Alvarez-Medina et al., 2009; Bally-Cuif and Hammerschmidt, 2003). However, *rest* expression appears to coincide with mitotic markers in general, with no domain specific differences. This pattern suggests that downregulation of *rest* is a cell intrinsic response to differentiation rather than a result of unique extracellular cues. The Notch pathway is a major regulator of neurogenesis, controlling the rate of neurogenesis within proneural fields throughout the extent of the embryo. The zebrafish mutant *mindbomb* has defective notch signaling, and these embryos exhibit uncontrolled neurogenesis and a depletion of progenitors by around 24 hpf (Bingham et al., 2003; Itoh et al.,

2003). *rest* expression in *mind bomb* was assayed to determine if *rest* mRNA levels decrease correspondingly in the context of premature and accelerated neurogenesis. While Rest protein is likely degraded rapidly in this context, transcript levels might be less affected if downregulation was independent of the proneural activity driving neurogenesis. Expression in mutants was compared in wildtype siblings and with *elavl3* expression. While *rest* expression is not sharply delimited, it is broadly complementary to post mitotic neural marker *elavl3* (Fig. 3.4 A ,C and see Fig. M, N). In *mindbomb* mutants, *elavl3* is dramatically expanded in the hindbrain, and *rest* is correspondingly downregulated there (Fig. 3.4 compare B with D and A). *rest* levels remained wildtype in areas that were *elavl3* negative, such as in tissue adjacent to the neural tube, the eyes and the midbrain. The significant downregulation in neurogenic regions indicates the *rest* gene may be one target of proneural activity downstream of notch signaling.

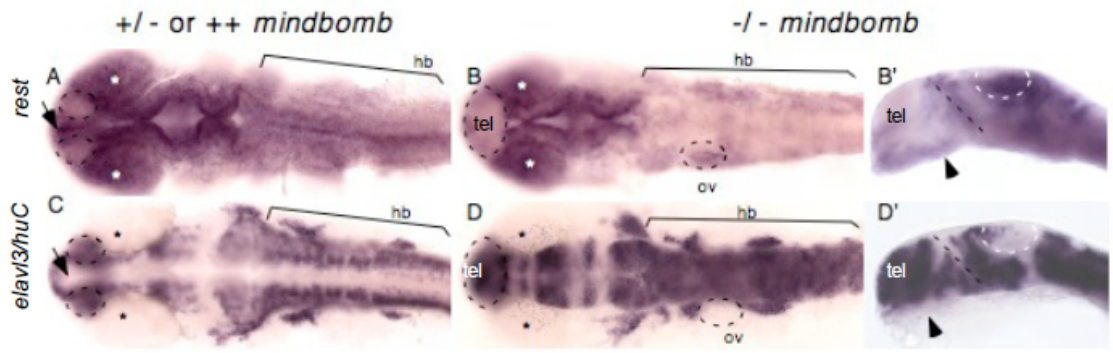

Figure 3.4. *rest* is downregulated as a consequence of neurogenesis. Dorsal views of Head and hindbrain (A-D) or lateral head (B', D") . 24hpf wildtype siblings (A,C) or neurogenic mindbomb mutants (B B',D, D') Fixed embryos stained for rest (A- B') or elavl3/huC (C- D'). (A, C) Rest expression is ubiquitous in wildtype 24hr but strongest in domains that are *elavl3* negative such as the eyes (asterisks A, C) and forebrain ventricular zone (arrows, A,C).(B- D') In *mindbomb* mutants, CNS hindbrain cells (hb, brackets, B, D) and telencephalic neurons (tel, D,D') express the post mitotic pan-neural marker *elavl3*. (D) *rest* transcript has been correspondingly downregulated in those cells (B, B'). *rest l*evels remains at wildtype levels in *elavl3* negative structures, such as the otic vesicle (ov, dashed oval line B, D) and optic tectum (dashed circle B', D').

3.4 Discussion

The general wildtype pattern and the depletion in *mindbomb* indicate downregulation of *rest* is a natural consequence of differentiation events. The expression pattern of zebrafish *rest* is largely coincident with proliferative and nonneural tissue, as seen in mouse and chick. However, the significance of *rest* transcript must be interpreted with caution. Rest levels are regulated posttranslationally (Ballas et al., 2005; Guardavaccaro et al., 2008; Westbrook et al., 2008). 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 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.

3.5 Materials and Methods

3.5.1. 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. *mindbomb*^{ta52b} mutants, described in (Bingham et al., 2003) from heterozygote in-crosses can be readily identified by the prominent midbrain structure and overall curve of the trunk and tail.

3.5.2. Zebrafish *rest* cDNA isolation and alignment

Full-length rest cDNA was isolated from 12hpf 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.

3.5.3. Quantitative Real Time PCR

Embryos 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 according to AB1 Prism User Bulletin # 2, relative quantification of gene expression. Beta-actin levels were used to standardize sample amounts.

3.4.4. 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., 1993). After *in situ* hybridization, embryos were mounted in 75% glycerol and photographed using a Zeiss Axiocam mounted on a Zeiss Axioplan microscope.

3.4.5. Microtome sections

After whole mount RNA *in situ* hybridization, embryos were dehydrated in ethanol, infiltrated and embedded in JB-4 resin (Ted Pella). For orientation of the embryos, embryos were embedded twice, as follows: For the first embedding, the infiltrated embryos were placed in a standard 2mL microcentrifuge tube with .750mL of embedding media. The tubes were closed and placed upside down so

that the embryo lay flat on the bottom of the well located on the inside of the tube lid. This was allowed to harden overnight at 4°C. These samples were cut out and re-embedding in a BEEM embedding capsule (Ted Pella), and oriented so that the sample faced forward toward the tip of the tube. 10μM sections were obtained using an ultramicrotome (LKB 8800 ultratome III; Bromma).

Chapter 4: Rest as a modulator of the Hedgehog pathway

(The following is modified from Gates et al., 2010).

4.1 Introduction

The *Rest* -/- mouse null phenotype has been described as complicated and difficult to interpret (Nurit Balls, personal communication). Further, lethality occurs at an early stage, impeding analysis on its role in nervous system development. Overall, the mouse knockout study left many unanswered questions about REST function during embryonic development, so functional studies within the context of a living organism were still desirable. My approach to studying Rest function in zebrafish embryos was to assay changes in gene expression after morpholino induced knockdown in various genetic backgrounds. A reduction in Rest levels, rather than total loss of function, was desired to avoid embryonic lethality and to allow grossly normal development of the nervous system.

Given the current model of REST function, my initial experiments focused on the role of Rest in zebrafish neurogenesis. Preliminary results did not indicate that Rest function was necessary for proper spatiotemporal expression of proneural or neural genes (see chapter 5). However, I proposed that Rest might play a modulatory role that would be revealed if other factors were simultaneously perturbed. For this, misexpression of *sonic hedgehog* (*shh*) mRNA was used due to its demonstrated ability to induce neurogenesis in some populations of precursors in zebrafish (Blader et al., 1997; Chandrasekhar et al., 1999; Strahle et al., 1997).

Surprisingly, Rest knockdown had its effect on neural patterning rather than on neurogenesis. This study reveals 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, 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 subtypes. The transcription factors expressed in response to the Hh gradient are categorized as class I genes (e.g. *pax3. pax7, dbx1a*) or class II genes (e.g. *nkx2.2a, 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^{Rep}), while activation of the Hh pathway allows full-length or near full-length Gli protein to function as an activator (Gli^{Act}). 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).

This *in vivo* study demonstrates that Rest influences Hh signaling through regulation of Gli2a activity. 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.

4.2 Results

4.2.1 Morpholinos

To investigate the role of Rest during development, two independent *rest* morpholinos (MOs) were generated. 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 thus 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 a stop codon within the inclusion of the second intron in the mature mRNA (Fig. 4.1A). The predicted protein produced from the misspliced transcript lacks zinc fingers 6-8 of the DNA binding domain and the C-terminal CoREST/HDAC interaction domain.

4.2.2 PCR confirmation of knockdown

Quantitative Real Time PCR was used to assay the amount of wild-type rest mRNA present following *rest* mo treatment (Fig. 4.1B). By 6 hpf, only about 50% the amount of wild-type *rest* mRNA is present in rest morphants compared to controls. By 12 hpf, levels are reduced to less then 10% of the control amount. 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 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 (Fig. 4.1C). 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)

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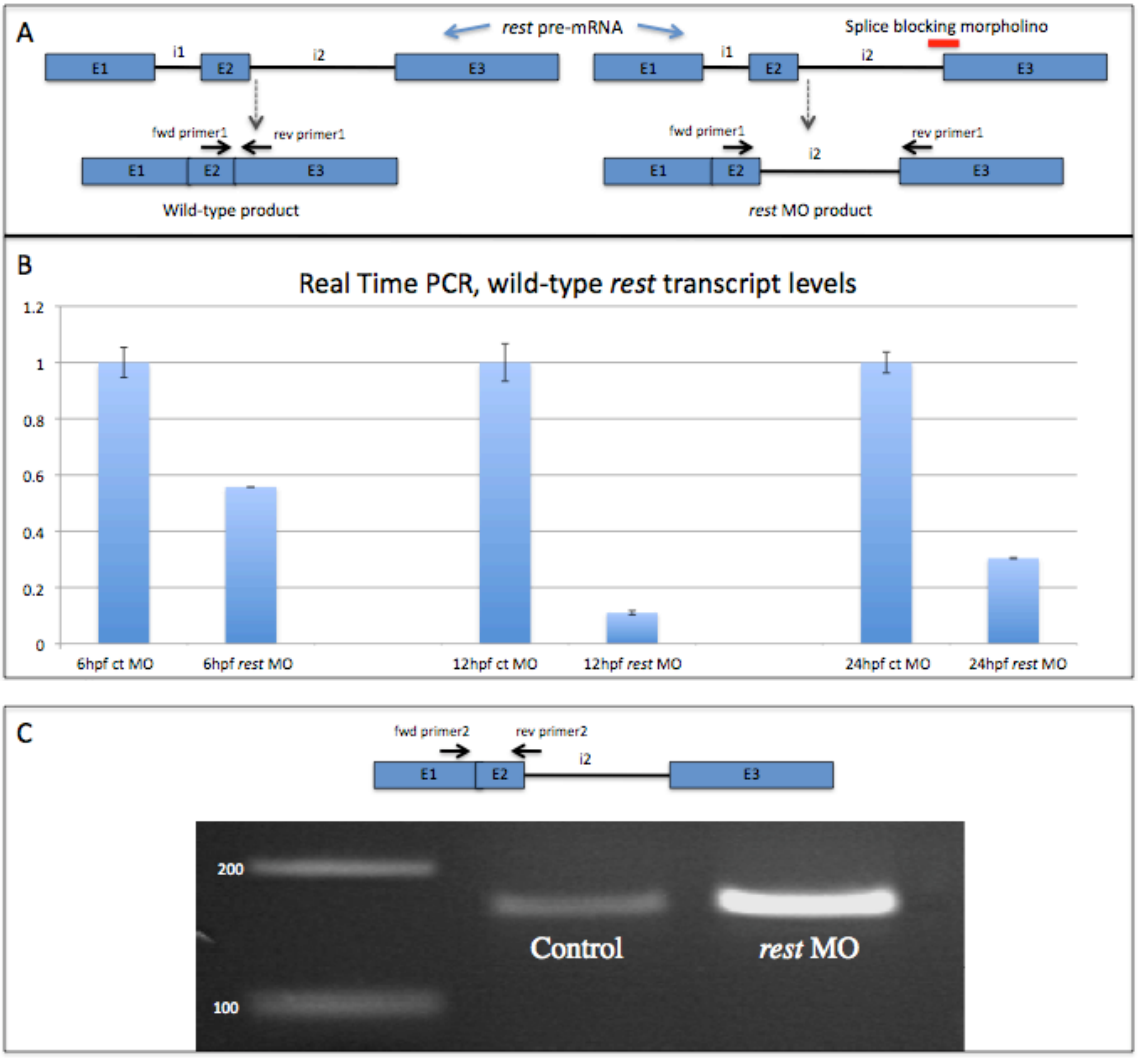

Figure 4.1. *rest* MO effectively reduces wild-type *rest* transcript levels. (A) Diagram of the *rest* pre-mRNA showing the oligonucleotides and morpholino used for these experiments. *rest* splice blocking morpholino (MO) binds the intron 2-exon3 boundary of zebrafish *rest* and is predicted to result in the inclusion of intron 2. Primers (arrows) that amplify a 130 bp region spanning exon 2 and exon 3 in the wild-type mRNA are separated by 3,674 bp after morpholino treatment if intron 2 is not removed. This product is not amplified under the PCR conditions used. (B) Real Time PCR analysis of *rest* transcript levels at 6 hpf, 12 hpf and 24 hpf after micro-injection of control or *rest* mo. Wild-type *rest* mRNA is reduced following treatment with *rest* morpholino (C) RT-PCR of 24hpf control or *rest* morphant cDNA using primers designed to amplify a 173 bp mRNA product containing intron 2. The PCR product predicted to be produced as a result of the morpholino treatment is enriched in *rest* morphant cDNA. Note: a similar product is present at low levels in control and may represent a splice variant (Rest4) that is present in other species.

In these embryos, maternally supplied *rest* mRNA is available during early gastrulation periods, then declines dramatically around 12 hpf, the beginning of primary neurogenesis. Between 12 hpf and 24hpf *rest* levels then return 50% of normal levels. Additionally, the misspliced transcript created may act like natural splice variant Rest4. Various functions, sometimes opposing, have been reported for Rest4. However, we have not detected any activity of the truncated Rest form in preliminary overexpression assays (data not shown). This may be due to any number of procedural reasons, but wild-type *rest* levels may also need to be reduced in conjunction with the overexpression of the splice product construct.

4.2.3 *Shh* induced expansion of cranial motor neurons is enhanced by Rest knockdown

Progenitor domains determined by the Hh gradient give rise to specific subpopulations of neurons, and ectopic activation of the Hh pathway in zebrafish produces supernumerary Islet-1 positive branchiomotor neurons (Chandrasekhar et al., 1998; Vanderlaan et al., 2005). If Rest function played a role in countering positive regulators of neurogenesis, then Rest knockdown might sensitize the embryo to exogenous expression of such activators.

As a first measure of the response, Tg[*islet-egfp*] embryos (Higashijima et al., 2000) were used, both to observe immediate results and to follow developing embryos over time. *rest* MO, s*hh* mRNA or both were microinjected into outcrossed Tg[*islet-egfp*] embryos. Injection of *rest* MO by itself produced no change in Islet motor neuron development when compared to stage matched control embryos (See Chapter 5, Fig 5. 6). As expected, *shh* mRNA treatment resulted in an enhancement of *islet:*eGFP+ motor neurons at 22hpf that was still evident at 32 hpf (Fig 4.2C,D). In the *rest* morphants treated with *shh* mRNA, a dorsal expansion of similar magnitude was observed at 22 hpf (figure 4.2C). However, the expansion of *islet1*:GFP+ motor neurons in those embryos became increasingly more severe as development proceeded (fig 4.2F), dynamics not observed in the embryos injected with *shh* alone. Early born hindbrain clusters of

cranial nerve five (cnV, trigeminal) and cnVII (facial) motor neurons were the most severely affected, and cnX showed significant enhancement as well.

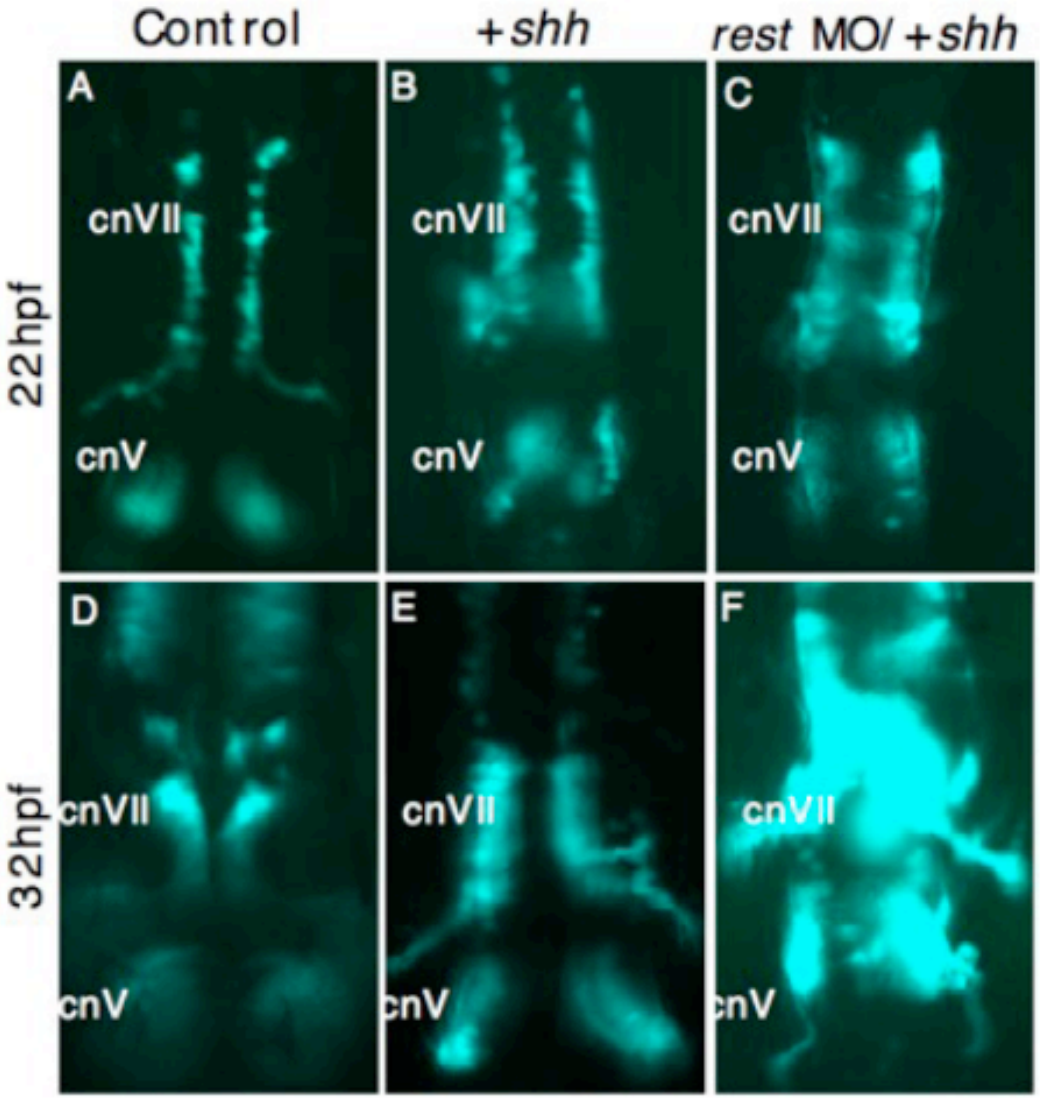

Figure 4.2. *Shh* induced expansion of cranial motor neurons is enhanced by *rest* knockdown Dorsal views of the hindbrains of live transgenic Islet1-eGFP embryos. Anterior is down. Embryos were injected with control morpholino (A, D) *shh* mRNA (B, E) or REST morpholino (REST MO) and *shh* mRNA (C, F). At 22hpf, modest expansion of Islet-GFP positive motor neurons is observed in embryos injected with *shh* mRNA (B) or *shh* mRNA and *rest* MO (C). At 32 hpf (C'), *shh* treated r*est* morphants show dramatic increase in the number of cranial motor neurons (cnV, cnVII) compared to *shh* treatment alone (B')

4.2.4 Hh induced neural defects in rest morphants are domain specific.

Given its proposed role in regulating neurogenesis, it would be tempting to suggest Rest knockdown led to a more permissive state for Shh induced neurogenesis. The late onset of the enhancement suggested otherwise, because the expansion of branchiomotor neurons in that case should have occurred earlier The examination of other neural and proneural markers suggested the effect was more likely related to the role of Shh in neural patterning. For example, the proneural marker *zash/asc1a* is expressed in discrete clusters of progenitors located in both dorsal and ventral domains. Injection of *shh* results expansion of *zash/asc1a* positive progenitors close to Hh sources (Fig. 4.3B,zli, tg), while dorsally located progenitors in the hindbrain are reduced (Fig. 4.3B, bars). Co-injection of *rest* MO results in an exaggeration of this shh patterning defect, resulting in more expanded (Fig. 4.3C tg, zli) or more reduced (Fig. 4.2C, bars) *asc1a* expression. As seen in embryos probed for pan-neural marker *elavl3*, Rest knockdown affected overall neurogenesis in a domain specific manner. In hindbrain, the overall enhancement of ectopic Hedgehog neurons was largely confined to neurons arising from rhombomeres 2 and 4 (Fig. 4.2E, F arrowheads), corresponding to the segments that give rise to cnV and cnVII, (see Figure 4.1) Early born Islet-eGFP+ motor neurons were the only population of post-mitotic neurons known to be enhanced. Further, other neuronal subtypes were relatively reduced after Rest knockdown. For example, *gata2*-GFP labels specific neural populations, including diencephalic (asterisks) and tegmental (arrows) clusters, which are expanded and shifted dorsally by *shh* injection (Fig. 4.2H). This phenotype was not enhanced by simultaneous Rest knockdown (Fig. 4. 2I), in contrast to the enhancement of hindbrain *islet*-GFP+ motor neurons in embryos from the same injection (Fig. 4.2I').

Thus, Rest knockdown only enhanced the Shh misexpression phenotype for a subset of neurons, indicating the effect stemmed from patterning, not neurogenesis defects.

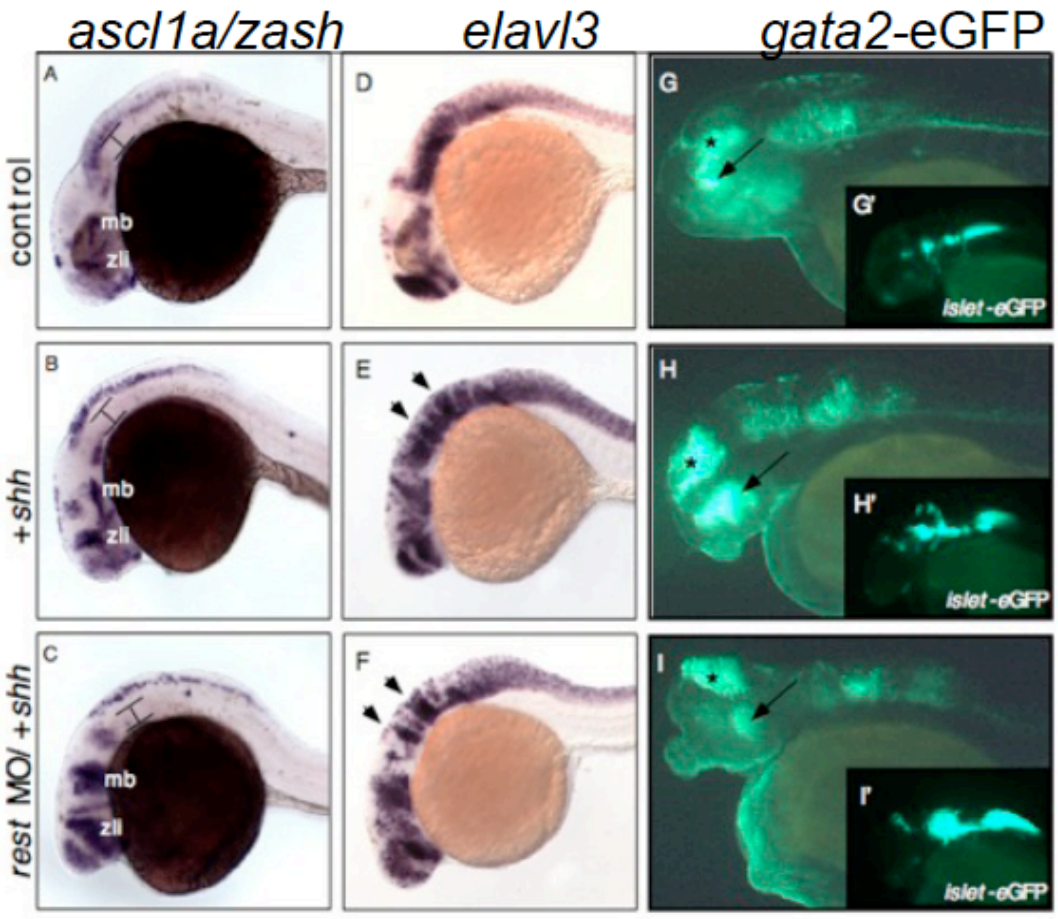

Figure 4.3. Domain specific neural defects upon shh misexpression in rest morphants. Lateral views of 24hpf fixed and stained with antisense probes (A-F) or 48hpf transgenic GFP live (G-I') embryos. (B) injection of *shh* mRNA alone expands proneural marker *ascl1a* in anterior regions (B, zli and midbrain, mb) and reduces expression in hindbrain (B, brackets, compare to A).(C) Co-injection of *rest* MO produces an exaggeration of this phenotype (compare C to B). (D-F) Embryos stained for panneural marker elavl3. Rest morphants injected with *shh* show a general downregulation of *elavl3* in hindbrain (compare F to E), except in rhombomeres 2 and 4 (arrows, F). (G-I) tg[*gata2*-eGFP] embryos. Shh overexpression causes expansion of diencephalic (arrows) and tegmental (asterisks) neural clusters. These neural populations are not expanded with co-injection of *rest* MO. (compare I to H) (G'-I') tg[*islet*-eGFP] from same injection is shown for comparison.

4.2.4 Progenitor domains of cranial motor neurons are expanded

I predicted that decreased Rest levels would ease constraints on the ability of exogenous Shh to drive neurogenesis. Instead, the effects I observed appeared more related to earlier role of Shh in patterning the neural tube. The excess Islet branchiomotor neurons seen at later stages in that case should be due to expansion of the ventral progenitor domains that give rise to them. *phox2a* and *nkx6.1* are expressed in the progenitors of these cranial motor neurons prior to expression of *islet* (Cheesman et al., 2004; Guo et al., 1999), so expression patterns of these were examined to see if they were enhanced by Rest knockdown. Wild-type embryos and *rest* morphants injected with *shh* mRNA were fixed between 24-28hpf and stained for *phox2a* and *nkx6.1*. As with *islet*-GFP expression, there was no noticeable differences when injecting *rest* MO at this concentration (not shown). Injection of *shh* mRNA resulted in ectopic dorsal expression of *phox2a* (Fig.4.4B), and a general expansion of *nkx6.1* (Fig.4.4E). Co-injection of *rest* MO produced a more severe phenotype in both markers (Fig. 4.4C, F) as seen with *islet*-GFP at later stages. With *islet*-GFP neurons, significant differences were not evident until around 30hpf, whereas these progenitor populations were expanded at 24hpf. Thus, excess production of Islet:GFP positive cranial motor neurons seen in later staged embryos (compare *phox2a, nkx6* with islet 22h), appear due to the increased domains of patterning genes such as *nkx6.1* and *phox2a* at earlier stages.

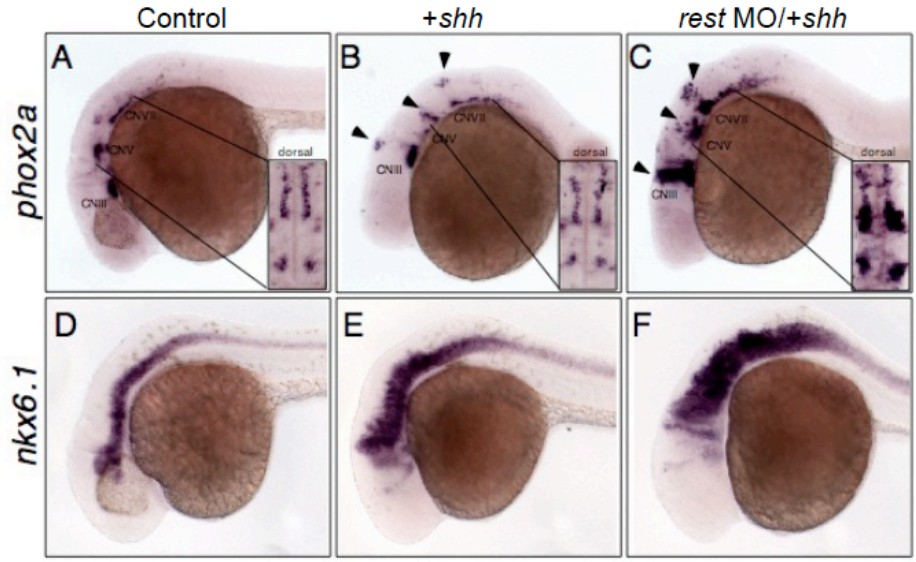

Figure 4.4. Progenitors of branchiomotor neurons are increased. Lateral (A-F) or dorsal (A-C, inserts) views of 24-27hpf embryos stained for *phox2a* (A-C) or *nkx6.1* (D-F). (A-C) *phox2a* is expressed specifically in the cranial motor neuron progenitors that will express *islet* after cell cycle exit. Ectopic dorsal expression of *phox2a* after injection of *shh* mRNA (arrows, B), is greatly enhanced by co-injection of *rest* MO (C). (D-F) nkx6.1 is expressed in the more general ventral progenitor domain that gives rise to the cranial motor neurons. This domain is expanded with Shh injection (E), and is moreso when done in conjunction with rest MO (F).

4.2.5 Rest knockdown augments the Hh response

The induction of cell-type specific transcription factors such as *phox2a* and *islet* depends on sharply defined progenitor domains marked by Hh dependent expression of homeobox transcription factors. Those genes expressed in response to the Hh gradient are categorized as class I genes (e.g. *pax3*) or class II genes (e.g. *nkx2.2a, nkx6.1*), which are repressed or expressed, respectively, by high levels of Hh signaling (Guner and Karlstrom, 2007; Liem et al., 1995). *nkx6.1* is expressed at high or medium levels of Hh signaling, but is not thought to be a direct Gli target (Vokes et al., 2007). To determine whether Rest knockdown augmented the response to Hh in general, I examined the expression of representative markers *nkx2.2a*, *ptc1*, and *pax3a*. *nkx2.2a* is a direct Hh targets, and expression depends on high levels of Hh. The expression domain of

nkx2.2a is limited by class I transcription factors, so expression of the Hh receptor *ptc1* transcript was also assayed. *ptc1* is also a direct response gene, and its expression can be viewed as a more direct readout of Hh signaling. *pax3a* expression was assayed to determine how dorsal class I markers respond compared to ventral markers. *pax3a*, like *nkx2.2a* and *nkx6.1*, labels progenitor cells and it was important to determine if Rest knockdown caused greater proliferation in dorsal populations as well.

Embryos were treated as described above and fixed between 24-28hpf. As expected, 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, *pax3* is reduced (Fig. 4.5E, H). Co-injection of *rest* MO and *shh* mRNA enhanced the ventralizing effects of Shh on the neural tube in a synergistic manner (Fig. 4.5 and Table 1A). The expression domains of *ptc1* and *nkx2.2a* were expanded compared to *shh* treatment alone, while *pax3* expression was further suppressed. Quantification by qPCR of changes in *nkx2.2a* expression is shown in Fig 4.5J. Other patterning genes in the neural tube that showed a synergistic response to Rest knockdown in conjunction with Shh activation include *shh*, *foxA2, olig2*, *pax6a* and *pax7* (Fig. 4.6). Rest knockdown also sensitized embryos to Hh signaling in non-neural tissues. Shh specifies slow muscle fiber identity in the embryonic mesoderm (Barresi et al., 2000; Wolff et al., 2003) Overexpression of *shh* mRNA results in increased expression of the adaxial cell marker *myoD*. As in the neural tube, the severity and penetrance of the *shh* overexpression phenotype was increased in *rest* morphants (Fig. 4.6S compare with Fig. 4.6R). These embryos have a greater expansion of *myoD* expression in the tailbud compared to wild-type embryos treated with *shh* mRNA. In addition, we also observed that the Hh dependant expression of *nkx2.2a* in the developing pancreas was also expanded in *rest* morphants treated with *shh* mRNA compared to wild-type embryos exposed to *shh* mRNA (see de-yolked embryo in Figure 4.9C, for example). It was concluded from these experiments

that Rest is required to limit Hh signaling and that Rest likely modulates a fundamental aspect of the Hh pathway.

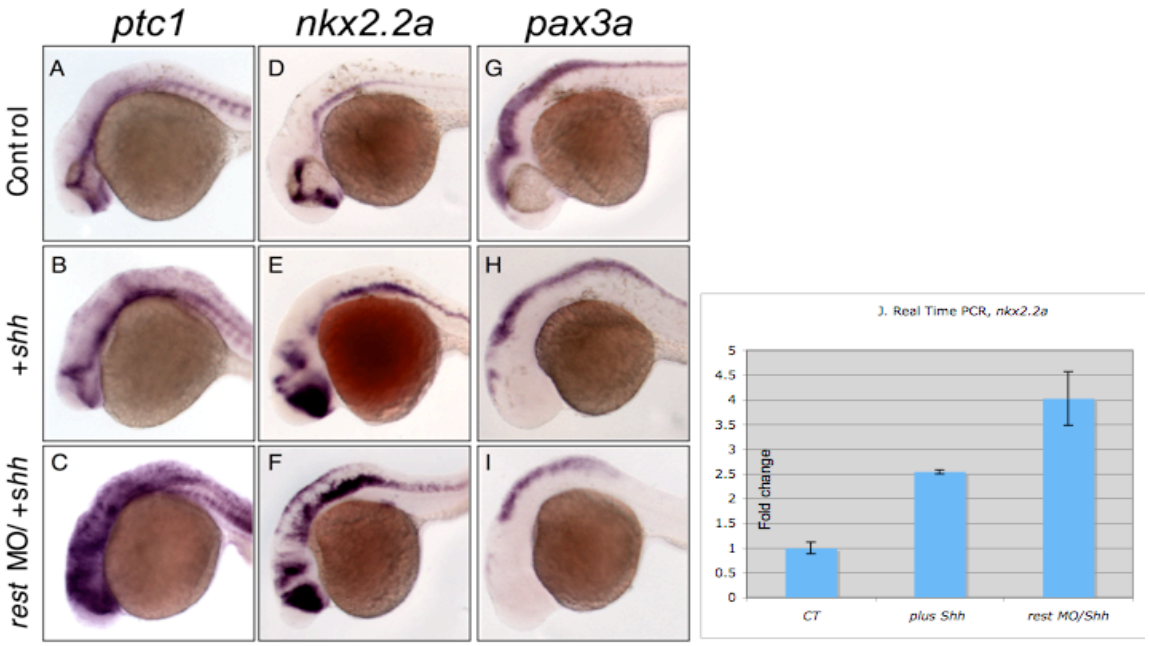

Figure 4.5. Rest knockdown sensitizes embryos to exogenous *shh*.
Lateral, head and hindbrain views of 28hpf control (A, D, G), *shh* mRNA (B, E, H) and *shh* mRNA/REST mo (C, F, I) microinjected embryos stained with antisense probes for Hh response genes. Injection of *shh* mRNA results in enhancement 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). (J) Quantification by qPCR of overall levels of *nkx2.2a* levels. cDNA sample size for each condition is an average of 80 embryos. This demonstrates that Rest knockdown enhances the response to high levels of Hh.

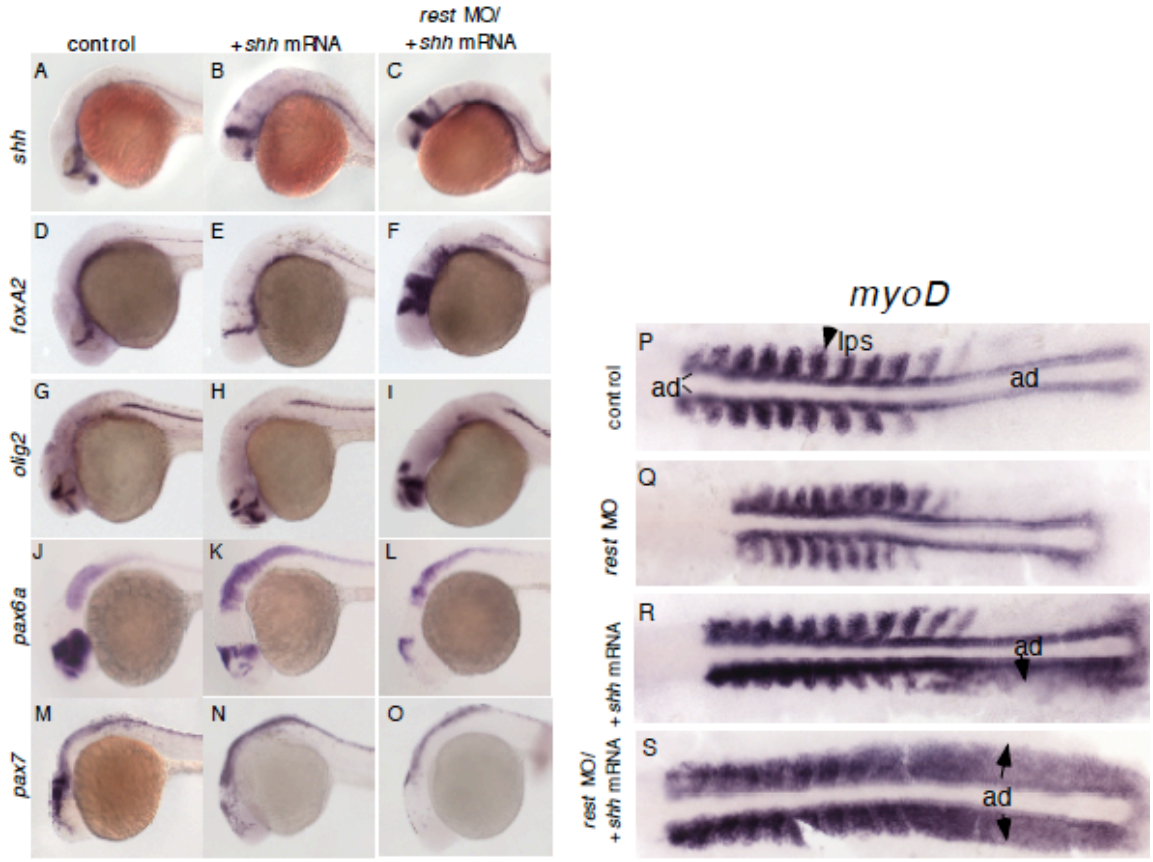

Figure 4.6. Rest knockdown enhances the effects of ectopic Hh signaling in general. (A-O) Lateral views of embryos injected with control MO (Left column), *shh* mRNA (middle column) or *shh* mRNA in conjunction with *rest* MO (right column). Embryos fixed around 24-28hpf and stained for Hh response genes. (Middle column) Injection of *shh* mRNA results in dorsal expansion (B, E, H) or reduction (K, N) in displayed markers. (Right column) Co-injection of *rest* MO alongside *shh* mRNA causes greater dorsal expansion (C, F, I) and a greater reduction in genes negatively regulated by Hh signaling (L, U). (P-S) Dorsal view of 14hpf flatmounted embryos stained for paraxial mesodermal marker myoD. Rest knockdown enhances the Hh induced expansion (arrows R, S) of adaxial (ad) cells into lateral presomitic mesoderm (lps) domain (Compare S to R)

4.2.6 Specificity of Rest knockdown effects

Importantly, a similar enhancement of the effects of *shh* mRNA overexpression was observed with the second *rest* morpholino which blocks translation (*rest* ATG) (Fig. 4.7 A-D and Table 1B). To confirm that the effects of the *rest* morpholino treatment result from Rest knockdown, I 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 *pax3* expression. *rest* mRNA microinjection largely restored the *pax3* expression (Fig. 4.7H) compared to *rest* MO/shh injected embryos (Fig. 4.7G, and Table 2). These experiments demonstrate that the effects of the morpholino treatment are produced by knockdown of Rest.

Table 4. I Rest knockdown enhances induction of *nkx2.2a* by *shh* mRNA treatment.

4. 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 value from Chi square test, 8.1407E-120
(Total from 7 experiments)

4. 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 value from Chi square test, 9.03538E-11
(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).
Wildtype= expression within the range observed in control embryos

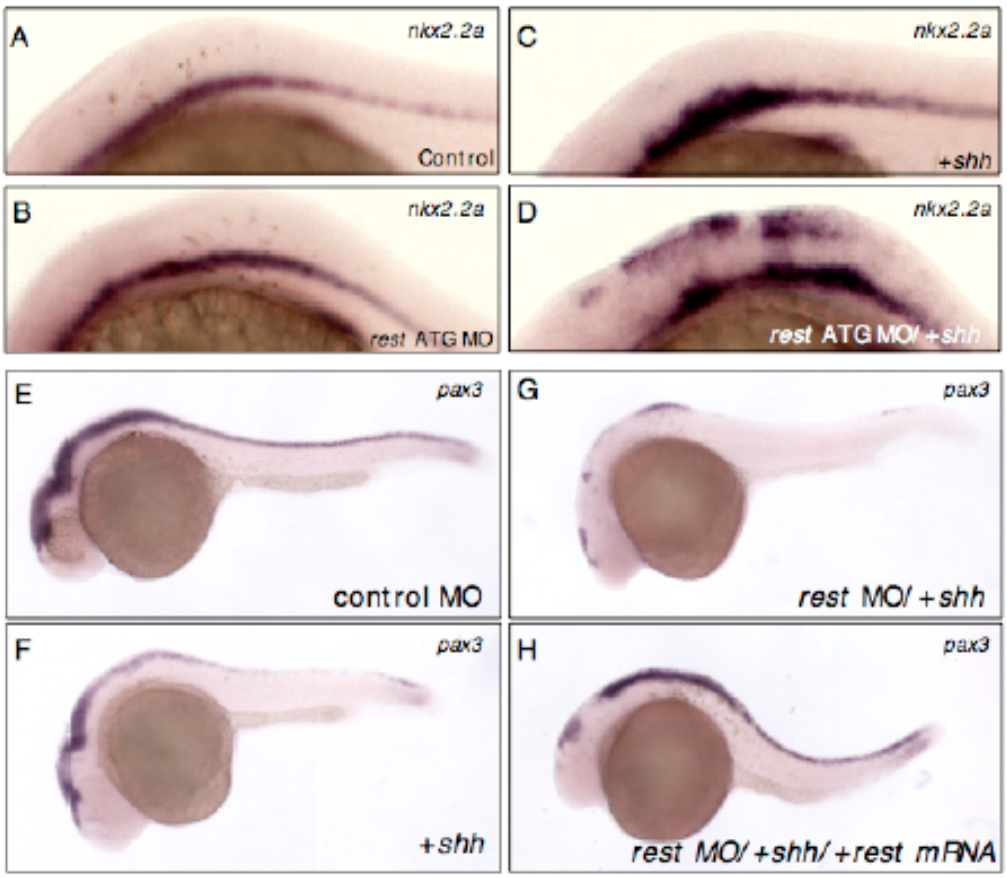

Figure 4.7. *rest* morphant phenotypes are specific to Rest knockdown. Lateral views, 1dpf embryos stained with *nkx2.2a* (A-D) or *pax3* antisense riboprobes (E-H). Hindbrain, anterior trunk views of embryos treated with control MO (A, C) or translation blocking *rest* ATG MO (B, D) and co-injected with *shh* mRNA (C, D) *rest* ATG MO sensitizes embryos to ectopic Hh signaling, (compare D to C). This phenocopies the effects of the *rest* splice blocking MO. (E-H) Injection of *shh* mRNA represses pax3 expression (F, compare with E). The heightened effect of exogenous Shh in *rest* morphants (G, compare with F) is reversed by *rest* mRNA (H, compare with G).

Table 4.II. *rest* mRNA injection rescues Rest mo mediated enhancement of Shh treatment.

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%)

pax3 expression at 1 dpf

4.2.7 A failure to dampen the Hh response

Rest knockdown in context of Shh misexpression reveals that proper levels of Rest help determine the response to Hh signaling. Additionally, these experiments provide clues as to a mechanism. Hh pathway relies on several negative feedback mechanisms that dampen signaling. The best characterized are the Hh ligand antagonists that are transcriptionally upregulated in response to pathway activation (Jeong and McMahon, 2005). The Hh receptor Ptc1 is one such antagonist, but transcription of *ptc1* is enhanced in the *rest* morphants (Fig. 4.5C). Still, the differential dynamics of the Hh response produced by Rest knockdown are indicative of an absence of such negative feedbacks. To demonstrate this effect, embryos from the same injection batch were collected at different stages and assayed for relative differences in *nkx2.2a* expression. At 14hpf, the expansion of *nkx2.2a* in *shh* injected control and *rest* morphants is comparable. By 24hpf the difference between the two conditions has increased dramatically. (Fig 4.8, compare D to C, then compare H to G) Over the next 12 hours, while control embryos exhibit progressively milder phenotypes, *rest* morphants exhibit both an increase in severity and penetrance (Fig. 4.9). These dynamics indicate that one or more negative feedback mechanisms employed by control embryos had failed to engage in *rest* morphants.

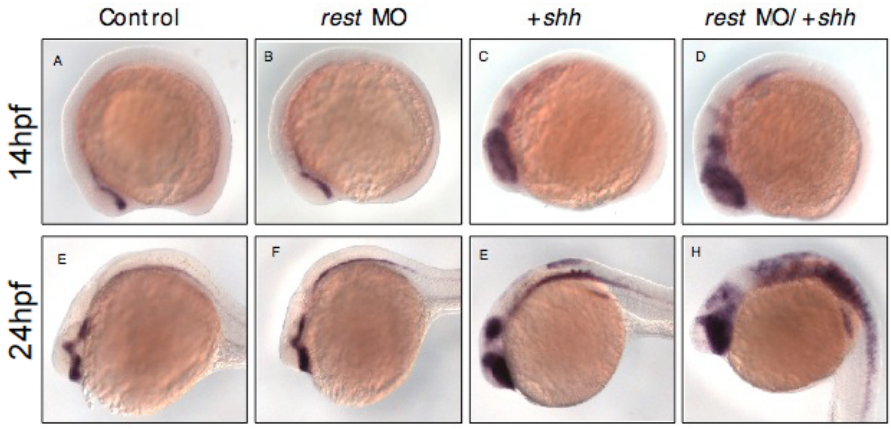

Figure 4.8. Enhancement of shh induced phenotype is increases over time in *rest* morphants. Lateral views, 14pf embryos (A-D) or 24hpf siblings from the same experiment (E-H) stained with *nkx2.2a*. (A-D) Embryos injected with *rest* MO (B, F), *shh* mRNA (C, G) or both (D, H). (A-D) Shh induced expansion of *nkx2.2a* is not significantly enhanced by co-injection of *rest* MO at this early stage. In contrast, expansion by 24hpf is much greater in *rest* morphants injected with *shh* (compare H with G).

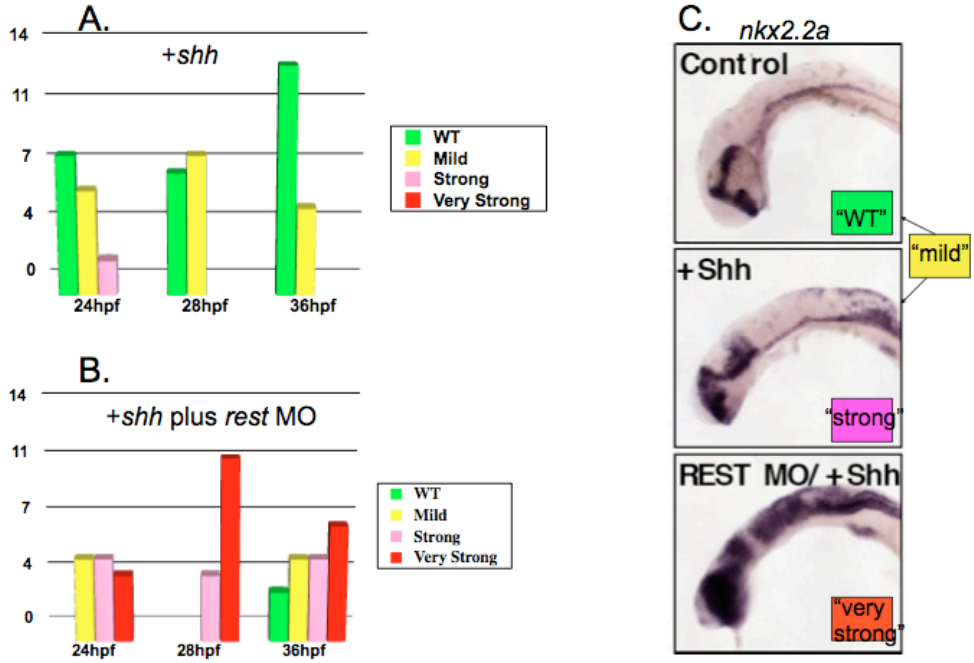

Figure 4.9. Differential dynamics of Shh overexpression in rest morphants. (A, B) graphs representing numbers of embryos with a particular phenotype of *nkx2.2a* expression. (C) representative embryos of graph categories . "Mild", not shown, was intermediate between WT (wild-type) and "strong". Categorization was based only on enhancement of *nkx2.2a*. (A) *nkx2.2a* expansion due to shh overexpression reverts to wildtype levels over time in a majority of embryos. (B) The same amount of shh injected alongside rest MO induces a more severe and more penetrant phenotype, which increases from 24-28hpf). Only A small number of rest morphants revert to wildtype levels of nkx2.2a by 36hpf.

4.2.8. Reduced Rest levels cause a mild ventralization

Loss of function experiments in context of ectopic high signaling revealed a role for zebrafish Rest in determining the response to Hh signaling. We reasoned that *rest* morphants in a wildtype context should also exhibit greater sensitivity in endogenous zones of high signaling. The amount of *rest* MO (4ng) that sensitized embryos to ectopic Hh did not initially appear to produce consistent effects in a wildtype context. However, a slight increase in *nkx2.2a* was repeatedly observed in a small number of embryos within each experiment. To explore the effect of reduced levels of Rest during development more thoroughly, higher doses of *rest* MO (5ng) were used in the remainder of the experiments described below.

Examination of representative neural tube markers by RNA *in situ* hybridization and microtome sectioning revealed that the neural tube of *rest* morphants is mildly ventralized (Fig. 4.10). Expression of direct Hh targets *nkx2.2a, ptc1* and *foxA2* were expanded in *rest* morphants (Fig. 4.10D, F), while expression of the dorsal marker *pax3* was reduced (Fig. 9B). The expression of *nkx2.2a* and *foxA2* was stronger within their normal domain and expanded 1-2 cell widths laterally and dorsally in *rest* morphants. *ptc1* expression (Fig. 9G,H) is more strongly upregulated than the others, perhaps because its expression is not limited by cross-repressive interactions as are class I and II transcription factors.

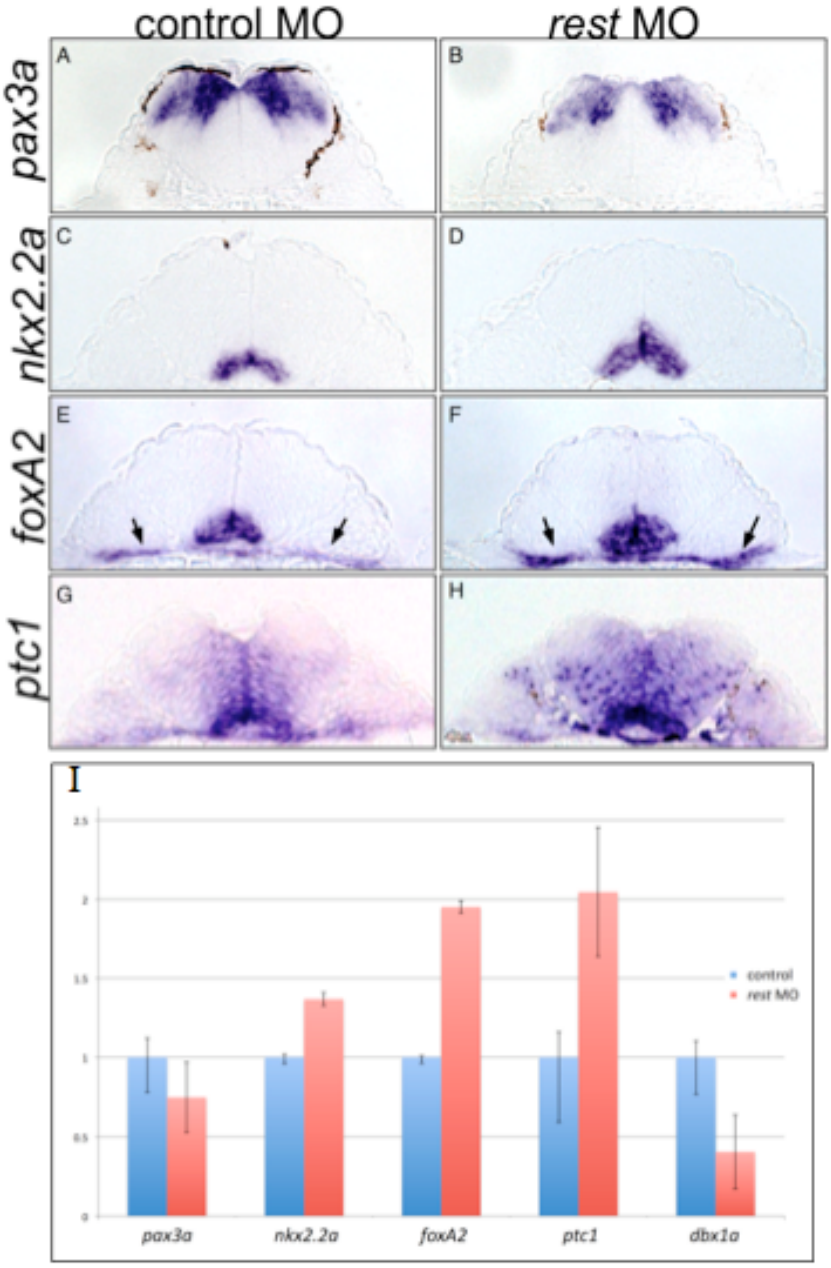

Figure 4.10. The neural tube is ventralized in *rest* knockdown embryos. Transverse sections of 29hpf (A, B) or 26hpf (C-H) wild-type embryos processed for RNA *in situ* hybridization and sectioned at the level of the hindbrain at anterior rhombomere 4. Control (A,C, E, G) and *rest* MO injected (B, 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.

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. cDNA from this later stage was selected because at earlier stages, the effect of Rest knockdown on reduction of *pax3* is not apparent. The levels of *nkx2.2a, foxA2* and *ptc1* transcripts are enhanced in *rest* morphants (Fig. 4.10I). 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* (Hauptmann et al., 2002) was also reduced.

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 (Fig. 4.11) and *foxA2* in the pharyngeal endoderm (Fig. 4.10H, 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.

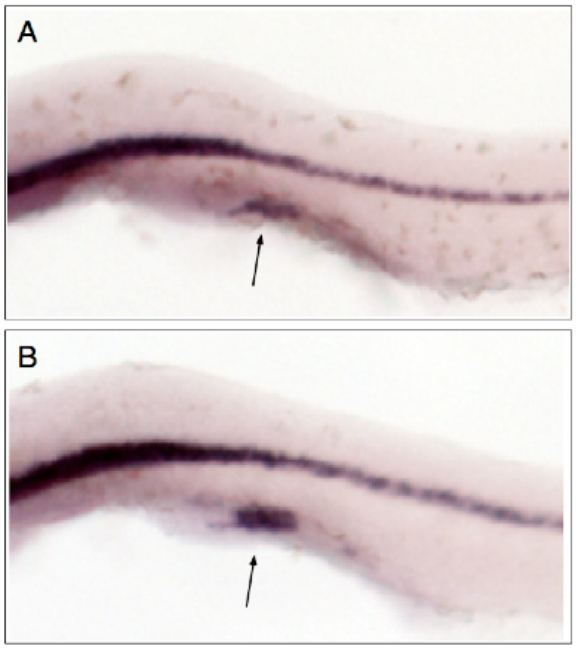

Figure 4.11. Rest represses *nkx2.2a* expression in pancreas. Lateral view at anterior trunk level of de-yolked embryos stained with antisense probe for *nkx2.2a*. Pancreas (arrows) is more prominent in stage matched *rest* morphants (B) than in embryos injected with control mo (A)

4.2.9 Rest knockdown enhances the effects of cyclopamine.

Initial findings suggested that Rest is a negative regulator of the Hh pathway. In that case, *rest* morphants should be less sensitive to ectopically induced blockage of Hh signaling. To determine whether blockage of Hh signaling could be alleviated by Rest knockdown, wild-type embryos were treated with low doses of the Hh antagonist cyclopamine (CyA) (Chen et al., 2002; Hirsinger et al., 2004; Wolff et al., 2003). Wild-type embryos were microinjected with control or *rest* morpholino and incubated in a low concentration of CyA (1.5μM) 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* (4.12A-D). As expected, *rest* MO treatment led to an increase in *nkx2.2a* expression (Fig. 12B) Surprisingly, in the presence of CyA, Rest knockdown caused further a reduction in Hh signaling as revealed by diminished *nkx2.2a* expression (Fig. 12D). 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. 4.12D, compared to 20% of control embryos). The remaining *rest* morphants showed a moderate reduction of *nkx2.2a* expression.

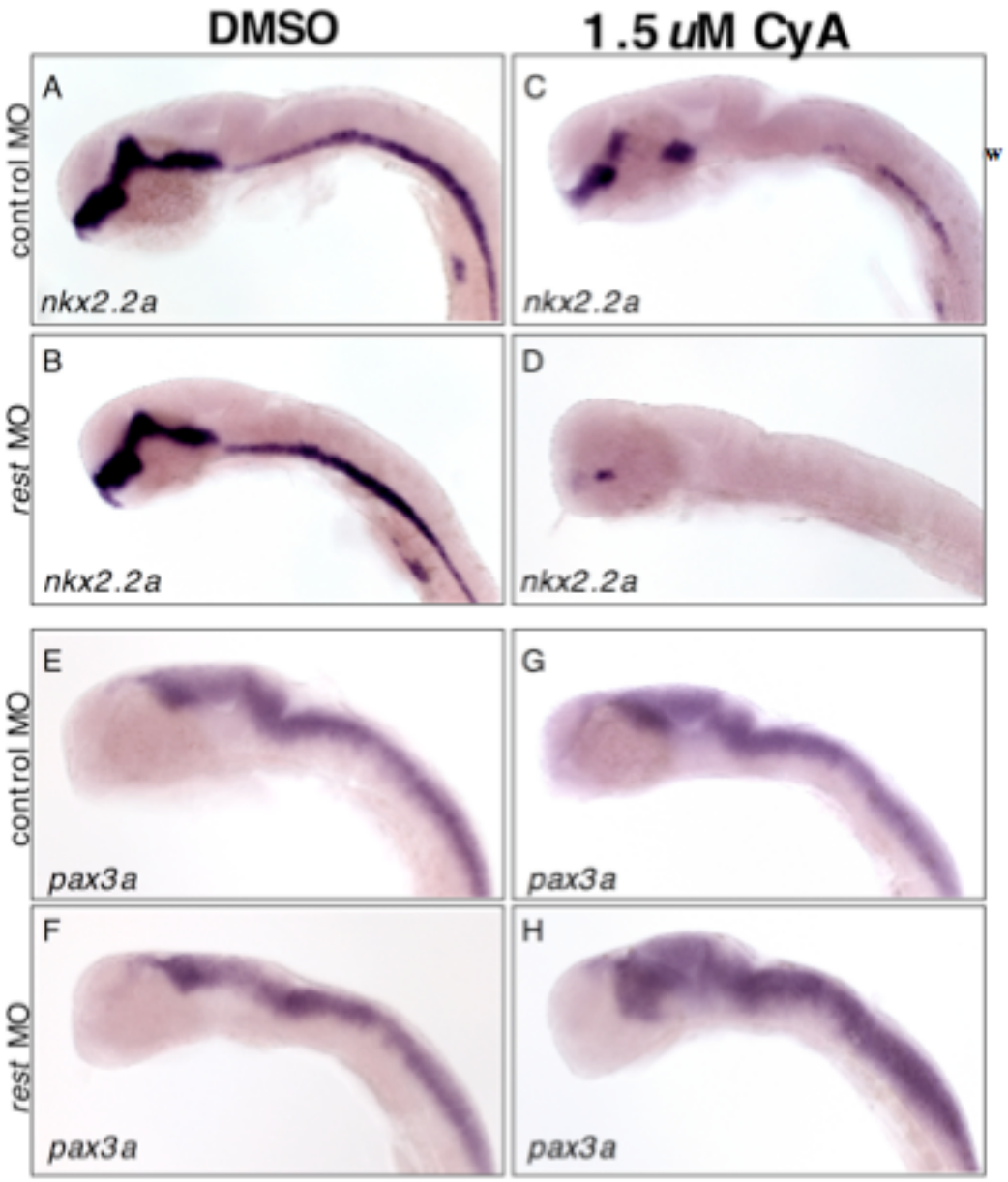

Figure 4. 12. CyA mediated attenuation of Hh signaling is enhanced by *rest* knockdown. Lateral views of 26 hpf control (A, C, E, G) and *rest* morphants (B, D, F, H) embryos stained for *nkx2.2a (A-D)* or *pax3a* (E-H). Embryos were incubated in control media (A, B, E, F) or 1.5µM cyclopamine (CyA) 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. 1.5µM CyA decreases *nkx2.2a* expression (C) and modestly expands *pax3a* (G) in control embryos. Rest knockdown produces a greater reduction

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 4.12G, 10/16 comparable to wild-type) despite downregulation of *nkx2.2a* (compare sibling control embryos, in 4.12C, G). In contrast, *rest* morphants often had significant expansion of *pax3a* (8/18 as in 4.12H, while the remainder were similar to control in 4.12G) Thus, Rest knockdown increases response to high levels of Hh signaling (as in Fig. 4.5, 4.6), but further attenuated Hh signaling in the context of low levels of Hh signaling (Fig. 4.12). We conclude that regulation by Rest positively or negatively influences the Hh response depending on the state of Hh signaling.

4.2.10 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. 4.13C). As with *shh* overexpression, upregulation of *nkx2.2a* by dnPKA was further enhanced by simultaneous co-injection of rest MO (Fig. 13D). While this result suggests that

Rest knockdown heightens activity intracellularly, excess activity of extracellular components could not be ruled out, because activation of the Hh signaling pathway induces Hh transcription and amplification of the signal (Blader et al., 1997; Neumann and Nuesslein-Volhard, 2000). It is therefore possible that some of the enhancement of dnPKA and Shh overexpression seen in *rest* morphants (Fig 4.13D) was a result of heightened extracellular Hh ligand activity due to ligand production, processing or diffusion. To address this possibility we repeated the dnPKA injections to activate 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). In this experiment, if Rest regulates components downstream of Smo, then CyA treatment will not affect the enhancement of Hh target gene expression observed with Rest knockdown, as in Fig. 4.13D.

CyA treatment largely eliminated *nkx2.2a* expression in embryos injected with control MO (Fig. 4.13E) or *rest* MO (Fig. 4.13F) only. Importantly, Rest knockdown enhanced the effects of *dnPKA* mRNA treatment on *nkx2.2a* expression in the presence of 50 μM CyA (compare Fig. 4.13H with 4.13D). Thus, so long as the pathway is activated, extracellular ligand is not required. This demonstrates that Rest interacts with the Hh pathway at an intracellular step downstream of Smo.

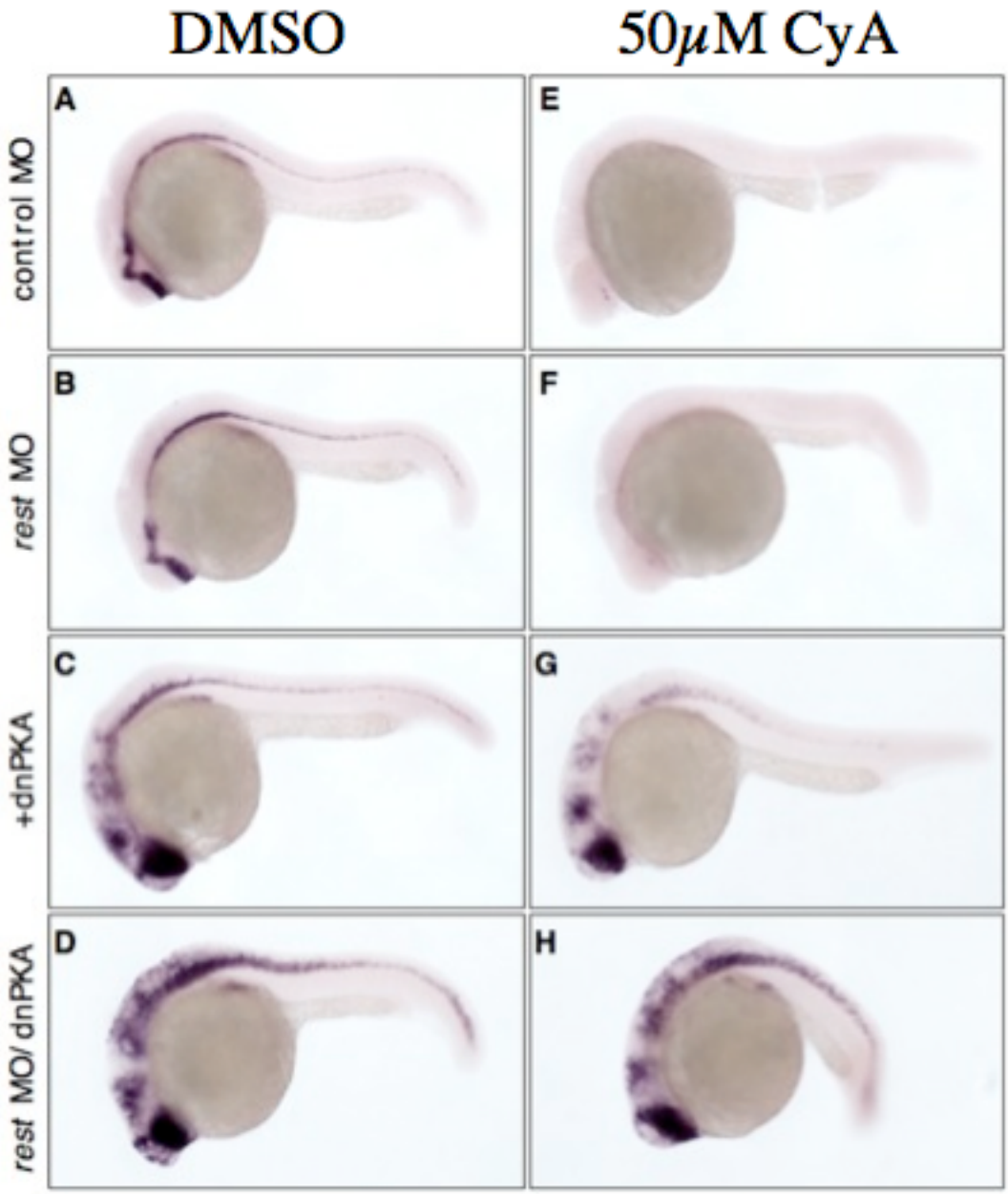

Figure 4.13. Rest acts downstream of Smo in the Hh pathway. Lateral views of 24 hpf embryos injected with control MO (A, E) *rest* MO (B, F), dominant-negative PKA (dnPKA, C, G), or both (D, H) and stained for *nkx2.2a*. Embryos were placed in control media (A-D) or media containing 50μM cyclopamine (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.

4.2.11 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. 4.14B). 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, so upregulation of *gli1* is unlikely to produce the negative effects we observed on Hh target gene expression in CyA treated embryos (Fig 4.12D, 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 become restricted to regions distant from the ventral source of Hh (Karlstrom et al., 2003; Tyurina et al., 2005; Vanderlaan et al., 2005) and Fig. 4.14E, G). 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. 4.14D). *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 (Fig. 4.14E, G). In *rest* morphants, *gli2a* fails to be properly downregulated at 18 hpf, and remained in the ventral CNS at 24 hpf (Fig. 4.14F, 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, 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. 4.5), 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 (Fig. 4.15D). We conclude that Rest plays a role in repressing of *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. With the assistance of Sean McCorkle of Brookhaven National Lab, we used 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 100kb. 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 ~15kb upstream and two at about 80kb from the transcription start site.

Functional data suggests that optimal repression from RE1s occurs when the site is within 2-3kb 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).

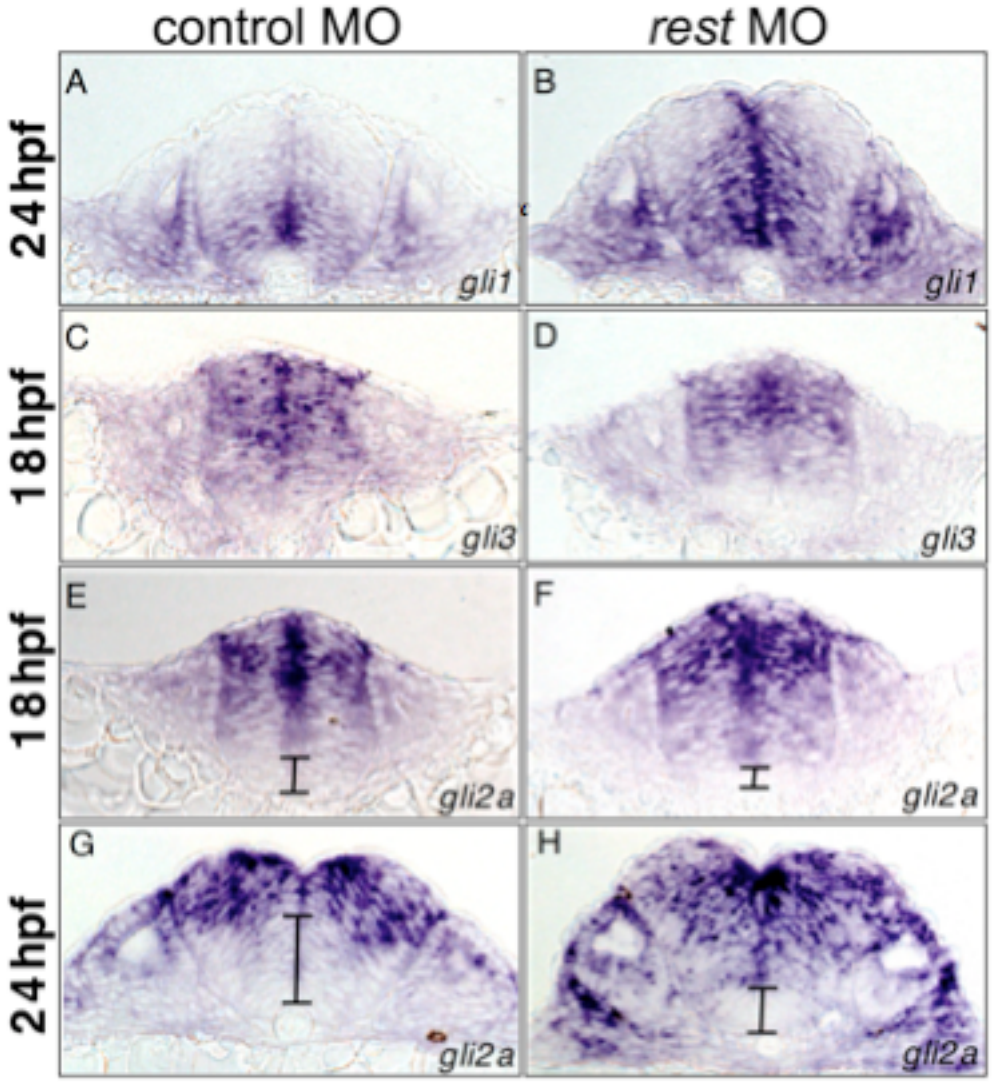

Figure 4.14. Rest represses *gli2a* expression. Transverse sections of the hindbrain of control (A,C, E, G) and stage-matched *rest* morphants (B, D, F, H). RNA *in situ* hybridization to monitor *gli1* (A, B) *gli3* (C, D) and *gli2a* (E-H) expression. (A, B) *gli1* expression is enhanced by Rest knockdown. (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.

While it would be ideal to test for Rest occupancy of these sites by ChIP, there are no antibodies for zebrafish Rest yet. An available alternative was the transgenic line with inducible, myc tagged Rest gene (tg[*hsp70-rest-myc*]) (Sirotkin, unpublished). While control ChIP attempts were successful, experimental ChIP using this line were not. I was unable to determine occupancy of the *gli2*a sites by Rest-myc, nor of several higher consensus RE1s from promoters of *snap25a, elavl3, neuroD* and other known REST targets. This could have been due to several reasons. Among the many possibilities is the stability of the transgene product, the inability of Rest-myc to displace endogenous Rest, or interference of DNA binding by the Myc tag.

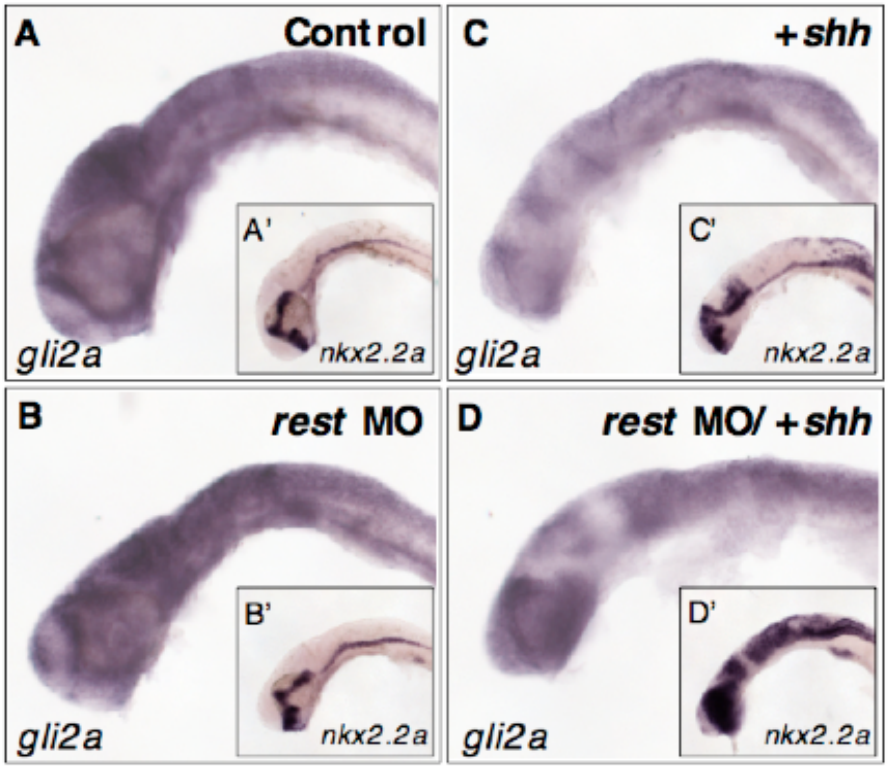

Figure 4. 15. **Hh-induced downregulation of *gli2a* is not enhanced by co-injection of *rest* MO**. Lateral view, head and hindbrain of embryos probed for either *gli2a* (A-D) or *nkx2.2a* (inserts A'-D') for comparison. (A, B) *gli2a* expression in control and age matched *rest* morphants. C) Overexpression of *shh* results in decreased levels of *gli2a* and increased levels of *nkx2.2a* (C'). (D) Embryos injected with both *rest* mo and *shh* mRNA have dramatically enhanced *nkx2.2a* expression (D'), co-injection does not correspondingly downregulate *gli2a* expression (D).

4.2.12 Rest knockdown diminishes *Hh target* expression in *yot/gli2a* mutants.

The failure to downregulate *gli2a* transcription (Fig. 4.14F, H) combined with the context dependent response of *rest* morphants to manipulations of Hh signaling (Fig. 4.5, Fig. 4.12) led us to hypothesize that Gli2a activity was central to the interaction between Rest and the Hh pathway. To further investigate the role of Gli2a to 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). While most *nkx2.2a* expression is lost in *yot* mutants, domains remain in the ventral forebrain and hindbrain. If enhanced Hh signaling in *rest* 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. Indeed, Rest knockdown alleviated loss of *nkx2.2a* expression in embryos co-injected with a Xenopus Gli2 dominant repressor form (XGli2DR) (Ruiz i Altaba, 1999), a form similar to that produced in the *yot* mutant (Fig. 4.16).

As expected, loss of Rest function led to an increase in *ptc1* and *nkx2.2a* expression in wild-type siblings (Fig. 4.17B, F). However, in *yot* mutants, Rest knockdown did not alleviate, but instead further reduced expression of *ptc1* and *nkx2.2a* in the midbrain region (Fig. 4.17D, 7H 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. Unlike *yot* mutants, XGli2DR-injected embryos can produce wildtype Gli2a. This suggested that excess Gli2a activity played a role in counteracting the interference of the XGli2DR form. Because *yot* mutants only generate Gli2DR 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.

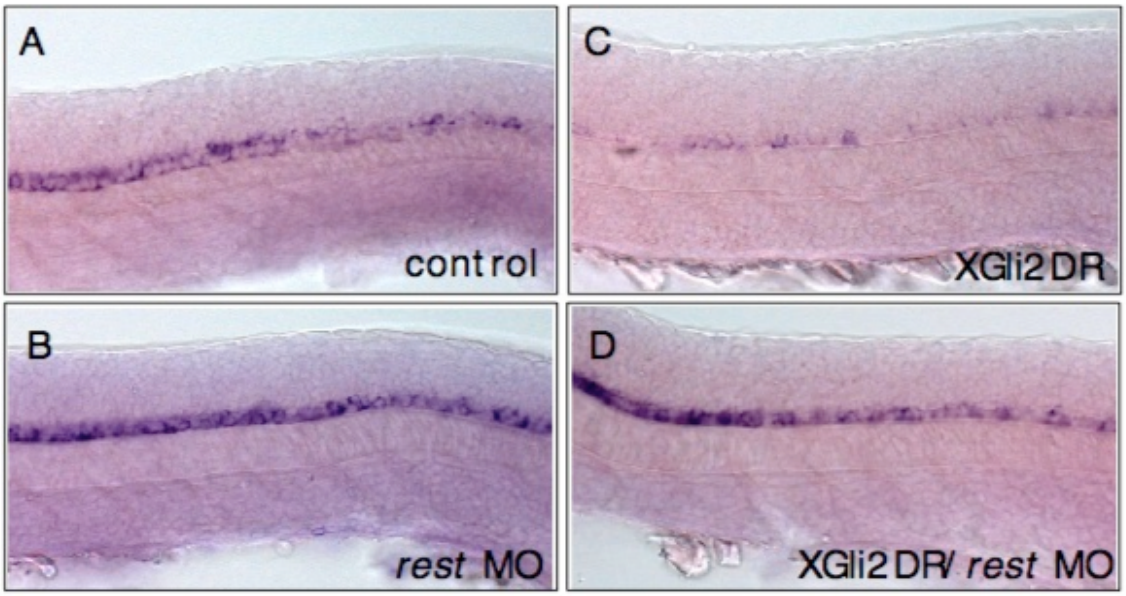

Figure 4.16. Restoration of XGli2DR induced downregulation of *nkx2.2a*. Lateral view, of anterior trunks of embryos injected with rest MO (B), mRNA of Xenopus dominant negative Gli2 (XGli2DR,C), or both (D), and stained for *nkx2.2a*. (C) Injection of XGli2DR mRNA interferes with Gli activator function resulting in decreased *nkx2.2a* expression. (D) Simultaneous knockdown of Rest partially compensates for this interference.

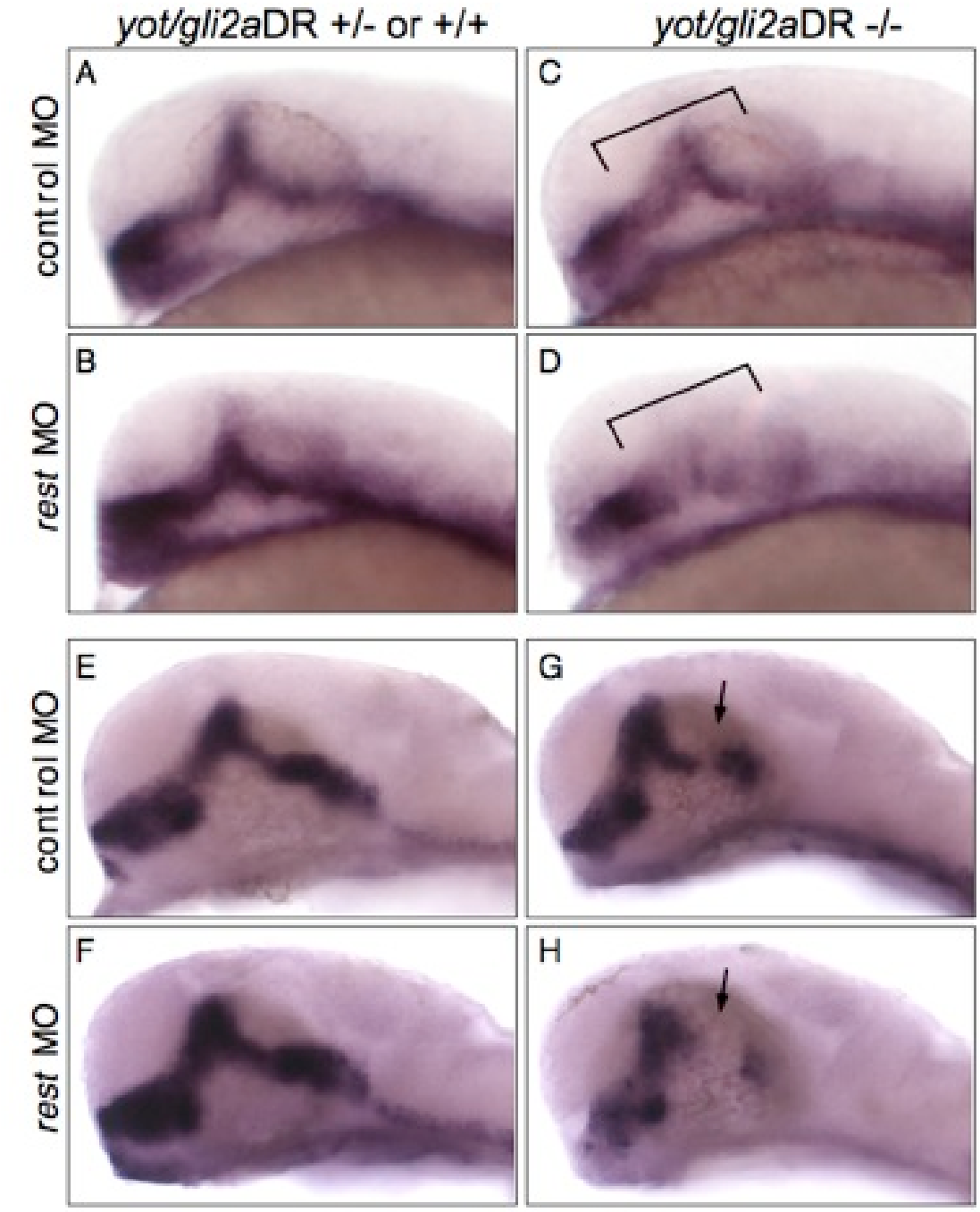

Figure 4.17. **Rest knockdown in *yot/gli2a* mutants represses Hh target gene expression**. Lateral view, 30hpf embryos stained for *ptc1* (A-D) or *nkx2.2* (E-H) mRNA. *rest* knockdown in wild-type embryos (B, F) results in modest enhancement of *ptc1* and *nkx2.2a* expression (compare B with A, and 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/diencephalon region (indicated by brackets, D and arrows, H) than in control injected mutants (C, G).

4.2.13 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., 2000b).

Embryos from a *dtr* +/- incross were injected with *rest* MO and assayed for *nkx2.2a* expression at 24hpf. *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. 4.18B). *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.4.18C, 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. 4.18D), 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.

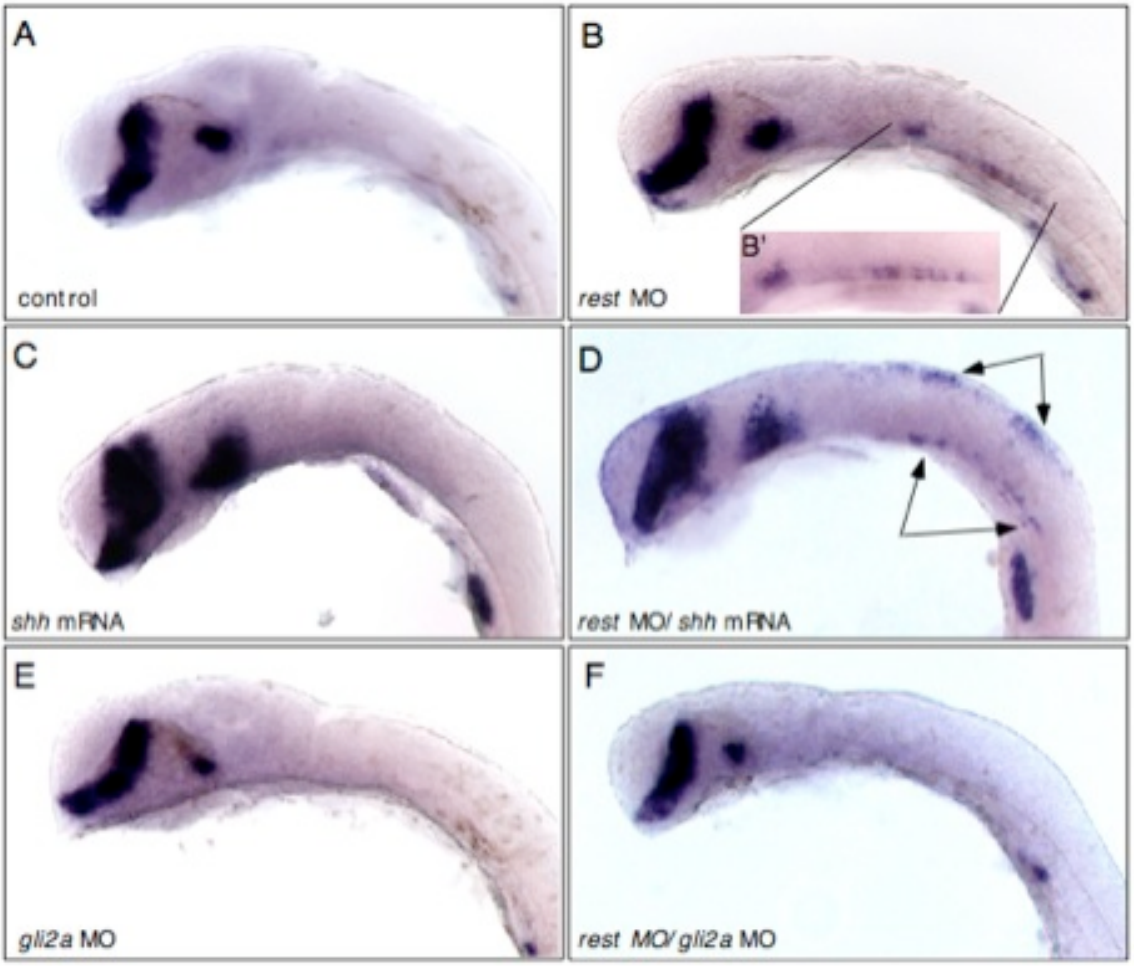

Figure 4.18. Rest mediated repression of Hh target gene expression in *gli* mutants requires Gli2a. *dtr/gli1* mutants, lateral views, head and hindbrain, stained for *nkx2.2a* by whole mount RNA *in situ* hybridization. Embryos were injected with control MO (A), *rest* MO (B), *shh* mRNA (C), *rest* 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.

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 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. 4.18B), this expression was suppressed by Gli2a knockdown (10/15 absent, 5/15 reduced in *dtr-/-* embryos, Fig. 4.18F, P value from chi square test .<.0001).

Confidence in the ability of Gli2a knockdown to negate Rest knockdown effects is bolstered by examining the *dtr* +/- embryos. *dtr* heterozygotes (Fig 4.19A) appear near wildtype, but can be easily identified by a significant reduction of *nkx2.2a* in the spinal cord (27/48) asterisks, Fig 4.19A). However, Rest knockdown resulted in restoration of this expression (Fig. 4.19B), as only 5/51 embryos could be identifiable as heterozygotes. In contrast, *dtr* +/- embryos injected with *gli2a* MO (14/35) show a further reduction in spinal cord expression (Fig 4.19C asterisks, compare to 4.19A) and a characteristic loss in the hindbrain. Rest knockdown could not compensate for this phenotype, as *dtr*+/- *rest/gli2a* double morphants (Fig. 4.19D, 35/62) resemble the *gli2a* morphants in this respect. This provides further evidence that the enhancement of the Hh response seen in *rest* morphants is dependent on Gli2a activator function. These epistatic experiments demonstrate that restoration of Hh target gene expression in *dtr*-/- and dtr+/-by Rest knockdown requires Gli2a.

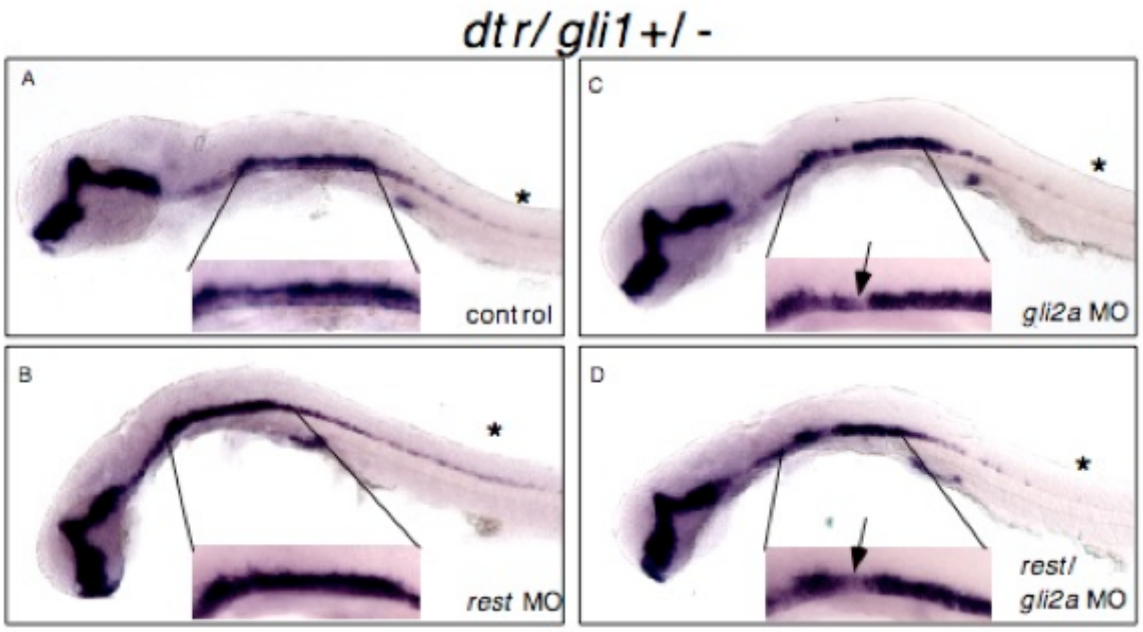

Figure 4.19. Gli2a knockdown negates *rest* MO restoration of *nkx2.2a* in *dtr/gli1* heterozygotes. Lateral view, 26hpf *dtr*+/- identified by reduced *nkx2.2a* expression in spinal cord (asterisk).(B) *rest* morphants show only subtle reductions. (C) *gli2a* morphants have more severe reduction in spinal cord, and a stereotypical hindbrain decrease. (D) Co-injection of *gli2a* MO reverses the effects of rest MO (Compare D with B)

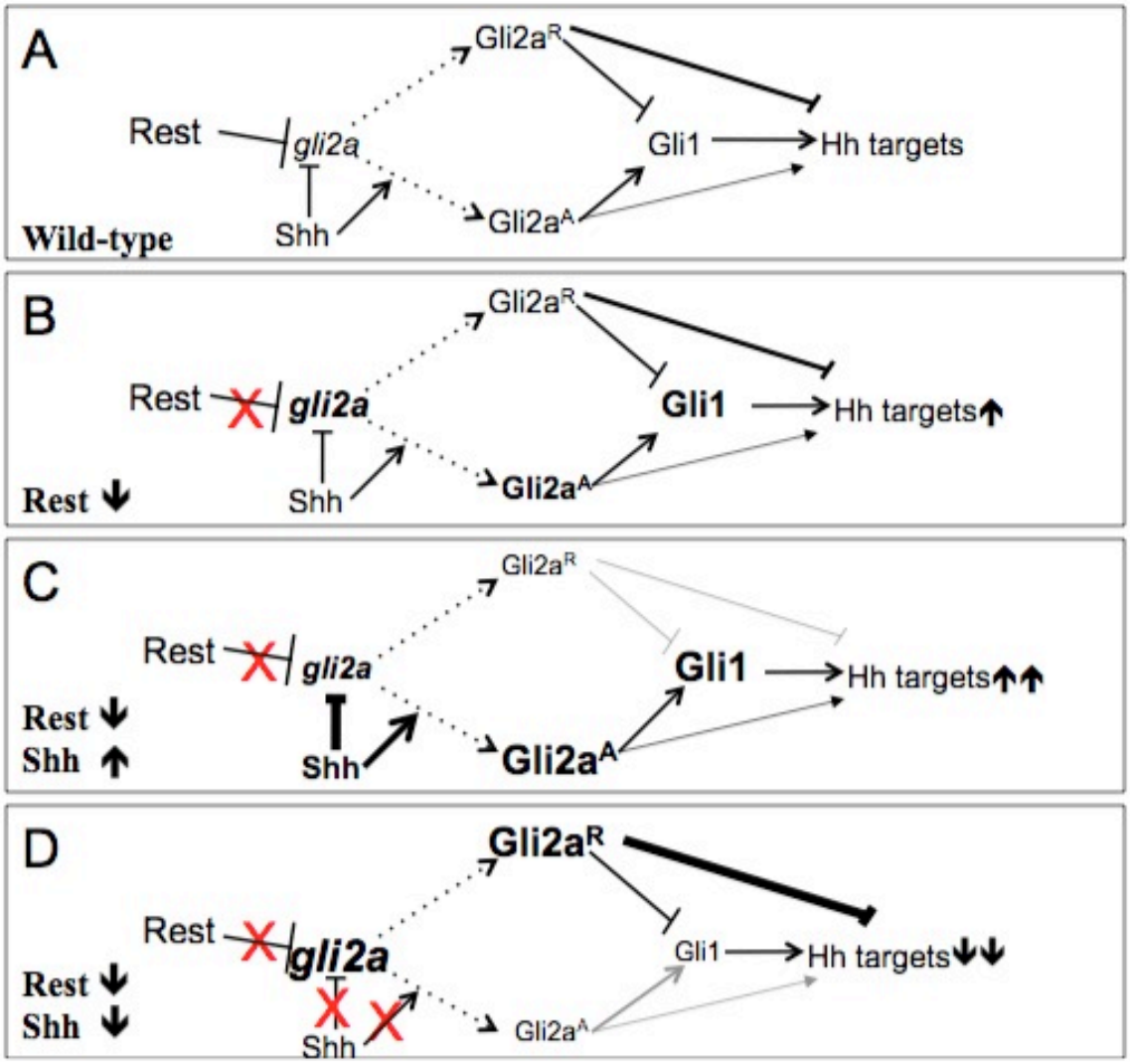

Figure 4.20. Model for Rest interaction with the Hh pathway. *gli2a* transcription is directly or indirectly repressed by Rest. When Rest activity is attenuated, more Gli2a protein is produced. Depending on the level of Shh, Gli2a is processed to activator or repressor forms. An alternate model where Rest regulates nuclear transport of Gli^A and Gli^R is also consistent with our data.

4.3. Discussion:

4.3.1 Summary

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. This study provides *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 I 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.

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.

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. 4.20). 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(Fig. 4.20C). In CyA treated *rest* morphants, the excess Gli2a would be converted to $Gli2a^{R}$, which would repress Hh target gene expression (Fig. 4.20D). 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{Borycki, 2000 #361)}. 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.

4.3.2. 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 Shh pathway regulates both neural and pancreatic development. We also find that Rest knockdown enhances expression of *nkx2.2a* in the developing zebrafish pancreas (Fig. 4.11). 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 knock-down, not a knock-out 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. This study demonstrates that Rest acts as a biphasic 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. The Rest dependent downregulation of *gli2a* in zones of Hh signaling may constitute a mechanism to tune down Hh signaling activity. 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.

4.3 Materials and Methods

4. 3.1 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}.

4.3.2 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).

4.3.3 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., 1993) Constructs used to synthesize the following probes have been described previously: *nkx2.2a* (Barth and Wilson, 1995) *axial*/*foxA2* (Macdonald et al., 1995) *nkx6.1* and *olig2* (Guner and Karlstrom, 2007) *pax6a* (Krauss et al., 1991) *pax3* 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) *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. Live embryos were mounted in 3% methyl cellulose contained in a well made with cold water surf wax (Mr. Zogs Sex Wax, Carpenteria, CA) on a glass slide.

4.3.4 Microtome sections

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

4.3.5 Chromatin immunoprecipitation

ChIP using cell lysate derived from whole embryos was done using a protocol modified from Havis et al, 2006 as follows.

Protease inhibitors:
1 mM PMSF
1μg/mL aprotinin
1μg/mL leupeptin

Homogenization/cell lysis buffer (HCLB)
2.2 M sucrose
3 mM $CaCl_2$
10 mM Tris-HCl, pH 7.5
0.5% Triton X-100
Protease inhibitors

Sucrose resuspension buffer (SRB)
.25 M sucrose
3 mM $CaCl_2$
10 mM Tris-HCl, pH 7.5
Protease inhibitors

SDS lysis buffer-from Milipore Kit
(50 mM Tris HCl pH 8.0, 10 mM EDTA, 1% SDS plus protease inhibitors)

1 cell embryos were mildly pronase, so that chorions remain on after washes but come off easy at later stages. 60 embryos per sample were collected at the desired stage, and dounce homogenized on ice in 1mL of HCLB. The homogenized lysate was transferred to ultracentrifuge tubes and spun at 100,000 x g for 3hrs @ 4˚ C. (Beckman Optima TLX Ultracentrifuge. TLA-120.2 Fixed angle rotor, 1 mL tube, 50,000 RPM). This step eliminates the vitellus and isolates the nuclei.

The following steps were done on ice:

The pellet was resuspended in SRB, spun at 5xg, 10min at 4˚ C. Cross linking was done in 1% formaldehyde in SRB . (resuspend pellet in .75 mL of SRB, then add .25mL of 4% PFA). Incubated 10min at room temperature, then 20m on ice. Spun 5xg for 5min, then resuspended in .4ml NLB. This was flash frozen, and placed at -80˚C at least 1hr. Once in NLB, standard ChIP protocol from Millipore ChIP assay kit was followed. The thawed NLB was sonicated on ice using a double step 3mm microtip, as follows: 10sec pulses at 150W, 12 times with 1min between pulses.

4.3.6 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 50-100pg (*dnPKA*, *shh*) or 500pg (*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 .2M KCl and

phenol red from a 34mg/ml stock to 8-10mg/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.

4.3.7 Cyclopamine treatments

Cyclopamine (CyA) (Calbiotech) was diluted in EM from a 10mM stock dissolved in DMSO. Embryos were incubated in the desired concentration of CyA media from 4 hpf (Fig. 4.13) or at shield stage (Fig. 4.12) on, with control embryos in the equivalent concentration of DMSO in EM.

Chapter 5: Rest knockdown and neurogenesis

5.1 Introduction.

Given the current model of REST function, my initial experiments focused on the role of Rest in zebrafish neurogenesis. While REST was initially associated with terminal differentiation genes, more recent studies implicate REST as an important factor at different steps of lineage commitment. *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). The apparent normal early patterning of the null mutant raises the possibility that the role of REST in CNS development is most crucial at stages after neural induction occurs. The fact that lethality occurs around the developmental stage that positive regulators of neurogenesis begin to increase suggests a later role for REST in counteracting transcriptional activator function. In the Chen et al. study, mosaic inhibition of REST (to circumvent lethality) by electroporation of a D/N REST construct resulted in derepression of several known REST targets in non neural tissue and progenitors. However, as in the mouse, overt ectopic differentiation of precursors was not seen. A recent study using a similar mosaic expression method in chick was more revealing (Bergsland et al., 2006). Here, electroporation of a D/N REST in chick spinal cord resulted in the premature expression of proneural effector genes Sox11 and Sox4, which work downstream of proneural factors to promote differentiation. Subsequent activation of some terminal differentiation genes was observed, including the terminal differentiation marker TUBB3. TUBB3 is a direct REST target shown in several *in vivo* studies to respond to

manipulations of REST function. Most revealing was that the ability of the D/N REST to derepress TUBB3 was blocked when upstream activators Sox4 and Sox11 were blocked. This implies loss of REST activity was insufficient by itself for upregulation of the target gene; Transcriptional activators with targets in common with REST must also be present in sufficient concentration.

Sox11 and Sox4 were also upregulated following interference with REST, but not shown to be directly regulated. It is possible this was due to proneural activators (upstream of Sox11 and Sox4) themselves being upregulated. However, REST regulation of proneural factors is controversial. *in silico* data suggests REST regulates Mash and NeuroD, among others(Ballas et al., 2005; Otto et al., 2007). *Rest* is highly expressed in ES cells (cortical progenitors) and occupies an RE1 49kb downstream of the transcriptional start site of proneural bHLH transcription factor *Mash1* in those cells. *Mash1* is not expressed in those stem cells but is in neural progenitors, where REST presence on the *Mash1* putative promoter was reduced 5 fold. However, another study (Jorgensen et al., 2009a) reported proneural genes like *Mash1* and *Neurogenin1* were not upregulated in ES cells following interference with REST.

By knockdown of Rest in the developing zebrafish embryo, we hoped to gain an understanding about how Rest function relates to the proper spatiotemporal expression of neural and proneural genes. My hypothesis was that the consequences of reduced levels of Rest would not be apparent until sufficiently high levels of activators build up. This study examines embryos with reduced levels of Rest during accelerated periods of neurogenesis, and finds that neither proneural or neural markers are prematurely or ectopically expressed. Instead, there appears to be a decrease in proneural gene expression and later born neurons. While these results do not conform to the dominant paradigm, they may reflect the more nuanced roles demonstrated for REST.

5.2 Results

5.2.1 Gross morphology of morphant

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. (Fig 5.1B) The stage at which delayed development begins is dose dependent. Embryos injected with 4ng of splice MO have delay beginning around 14-18 hpf. 5ng of splice blocking MO (or 9ng of translation blocking MO) usually causes delayed development starting around 5-6 hpf. For assaying phenotypes at periods around 24 hpf, embryos are stage matched by morphology, not absolute time. *rest* morphants typically take 2-4 hours longer than control around 24 hpf to reach comparable stages . For periods around 48 hpf, rest morphants are typically allowed to develop 4-8 hours longer. (See Fig. 5.6 for example)

5.2.2 Apoptosis in morphants

Rest morphants also experience mild apoptosis within the neural tube, as indicated by acridine orange assays. (Fig 5.1D) The cell death appeared confined to the neural tube, in hindbrain and spinal cord. Hindbrain labeling revealed a segmented pattern, possibly due to cell death occurring in the neurogenic centers of each rhombomere. While mouse *rest* -/- are also developmentally delayed and cell death is apparent, I have no evidence that this phenotype in zebrafish *rest* morphants is specific to reduced levels of Rest. As discussed above, developmental delay and p53 mediated apoptosis are common side effects of MO (Eisen and Smith, 2008).

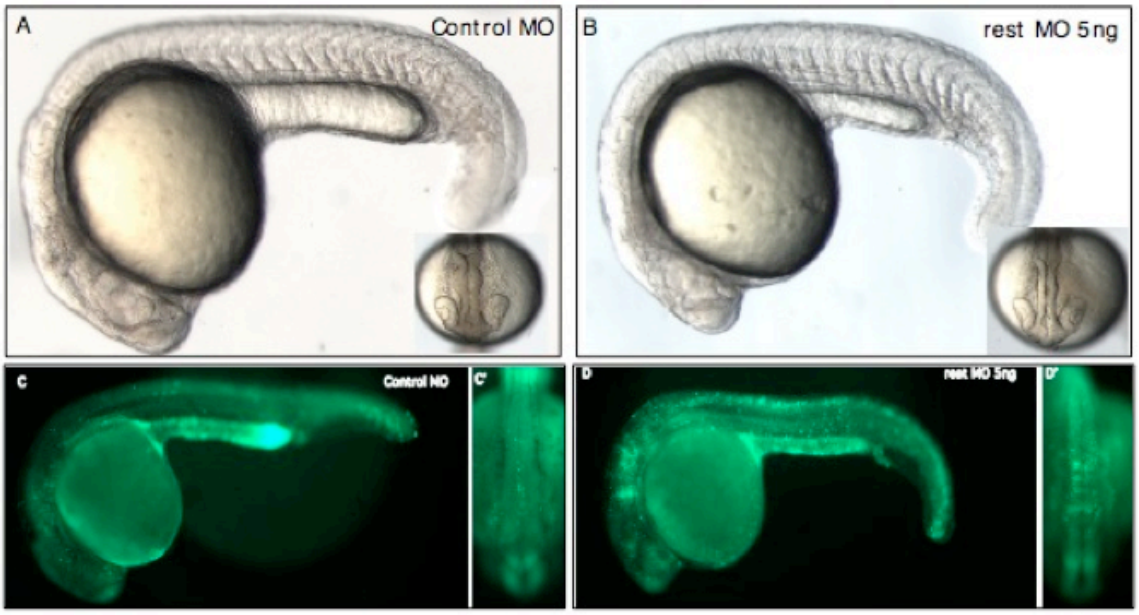

Figure 5.1. Injection of rest morpholino results in delayed development and apoptosis. Live images of control (A, C) and rest morphants (B, D). (A,B) DIC images, lateral and dorsal anterior (inserts) views of 22hpf live embryos. (C,D) Fluorescent images, lateral and dorsal hindbrain (C', D') views of 24hr live embryos incubated in Acridine Orange media. (A, B) Age matched rest morphants show normal gross morphology but are developmentally delayed. (D, D') Acridine orange labels apoptotic cells, which are evident as bright punctuated cells in the hindbrain and spinal cord.

5.2.3 Reduced Rest levels do not result in accelerated neurogenesis

My approach was to assay changes in gene expression upon Rest knockdown. Based on several studies (Ballas et al., 2005; Bergsland et al., 2006; Su et al., 2004), I predicted premature and/or ectopic expression of putative targets. Although there are many putative zebrafish REST targets, the lack of zebrafish Rest antibodies has prevented us from verifying Rest binding. Several consensus RE1s located in genes homologous to known REST targets were discovered by scanning the zebrafish genome using a software designed to detect RE1 sites. (Otto et al., 2007) Changes in transcript levels of conserved targets such as *snap25a*, *spop*, and *neuroD* have been checked by Real Time -PCR with cDNA from *rest* MO injected embryos, but results thus far have been inconclusive (LM personal communication).

Real Time-PCR may in any case be unable to detect local, domain specific upregulation. Rather, in situ hybridization or the use of transgenic fish with neural specific reporters are the preferred assays. To check for more general effects on neurogenesis induced by Rest knockdown, morpholino injected transgenic *elavl3*-GFP embryo's were monitored at different stages of development. *elavl3* is a marker of postmitotic neurons, and the *elavl3* locus contains a consensus RE1. *rest* morphants at early neurogenic period (~14 hpf) displayed excess GFP in the midbrain/anterior hindbrain region. (Fig. 5.2) However, no excess neurons were observed at this or any stage. At 24h, some background GFP- possibly corresponding to low levels of GFP in mitotic cells is evident (Fig. 5.3B). However, it is clear that there are no extra neurons. Importantly, *elavl3* transcript upregulation was not seen by *in situ* hybridization (Fig. 5.3D). The excess GFP at 14S (Fig. 5.2B) and the diffuse low level present at later stages (Fig. 5.3B) might thus be an experimental artifact of the *elavl3*-eGFP line. The eGFP in these transgenics is a calcium-sensitive derivative of GFP (cameleon, (Higashijima et al., 2003) that becomes more intense with greater calcium influx. This in turn could be due to apoptotic cells (see Fig. 5.1D), which are associated with excess calcium influx (Rizzuto et al., 2003).

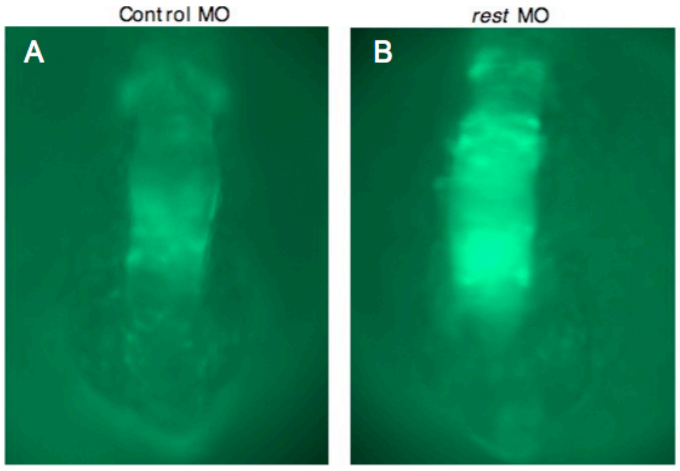

Figure 5.2. *rest* morphant show expansion of elavl3-GFP at early neurogenic stage. Dorsal views, anterior down. Head and hindbrain of transgenic *elavl3*-eGFP embryos injected with control (A) or *rest* (B) morpholino. *rest* morphants show an increase in GFP intensity in early stage midbrain neurons.

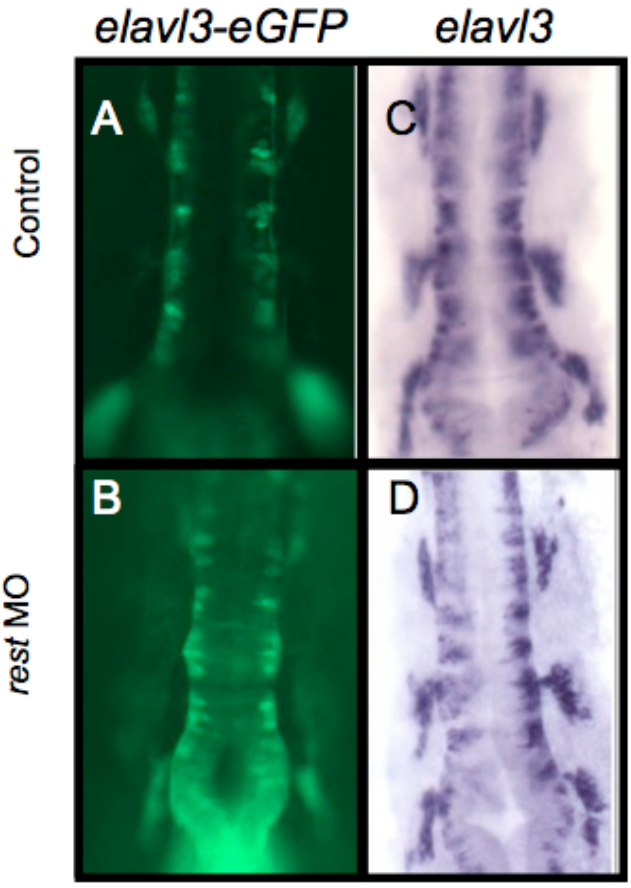

Figure 5.3. Neurogenesis is not enhanced in *rest* morphants. Dorsal views, anterior down. Hindbrains of control (A, C) or rest (B, D) morpholino injected elavl3-eGFP fish (live, A, B) or WT fish stained for endogenous *elavl3* transcript (fixed, C, D). (B) rest morphants show diffuse low level ectopic elavl3-GFP expression.(D) in situ hybridization reveals endogenous elavl3 transcript is not upregulated in rest morphants.

5.2.4 Proneural genes are not upregulated in *rest* morphants

I next examined the status of neural progenitors in *rest* morphants. The zebrafish proneural gene *asc1a/zash1a* is the homolog of *Mash1a*, a possible REST target (Ballas et al., 2005) and so may be upregulated upon Rest knockdown. *neurogenin1* was also assayed for more general effects on proneural fields. These genes might be expressed early even if they are not direct targets, since Rest knockdown may result in premature or accelerated neurogenesis. Control embryos were fixed at 20 hpf and 27 hpf, morphant

embryos were stage matched by gross morphology. Embryos were then assayed by *in situ* hybridization for changes in expression levels. Neither *ngn* nor *asc1a* expression were upregulated at 20 or 27 hpf but rather showed a mild decrease in expression. (Fig 5.4) This decrease appeared to be progressive, as the loss was more evident in later stage embryos.

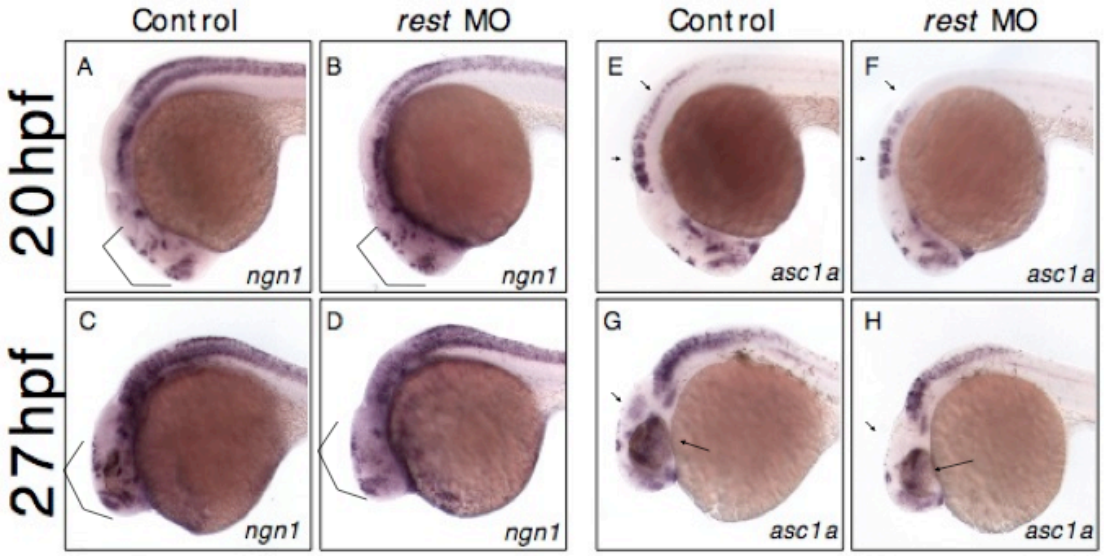

Figure 5.4. Proneural genes are not upregulated in rest morphants. Lateral views of 20hpf (A, B, E, F) and 27hpf (C,D, G, H) stained for ngn1 (A-D) o /asc1a/zash1a (E-H). rest morphants (B, F,) staged matched to 20 hpf controls (A,E) show a mild decrease in proneural genes. *rest* morphants (D,H) staged matched to 27 hpf controls (C.G) show progressive reduction of proneural genes , most evident for *ngn1* (D, brackets)

5.2.5 Loss of later born neurons

Examination of several cell types in neural tube of older embryos reveals that later born cell types are reduced compared to earlier born cells Early born cranial motor neurons (Islet-GFP positive) are mostly normal at 2 dpf (Fig. 5, A, B). In contrast, later born cn IV motor neurons, labeled by the Neurolin antibody, are decreased in *rest* morphants. (arrows Fig. 5C, D) *sox10* and *olig2* positive cells mark precursors and migrated oligodendrocytes, respectively, at 48 hpf in *sox10-GFP* and *olig2*-GFP lines. These cell types arise from the same progenitor

pool as motor neurons, but are born later, and are decreased in *rest* morphants (Fig. 5E-H). These deficits in late born neurons and glial cell types may result from a number of causes, such as mis-fating, precocious differentiation or progressive cell death. In support of the latter possibility, the *islet*-eGFP+ cranial motor neurons are relatively decreased by 4 dpf compared to 48 hpf embryos. (Compare Fig. 5.5B to 5.5J). A progressive decrease in the progenitor pool resulting from cell death in the neural tube over time could therefore account for the decreases. Further analysis of neural subtypes undertaken on *rest* morphants must take into account this possibility of loss due to cell death.

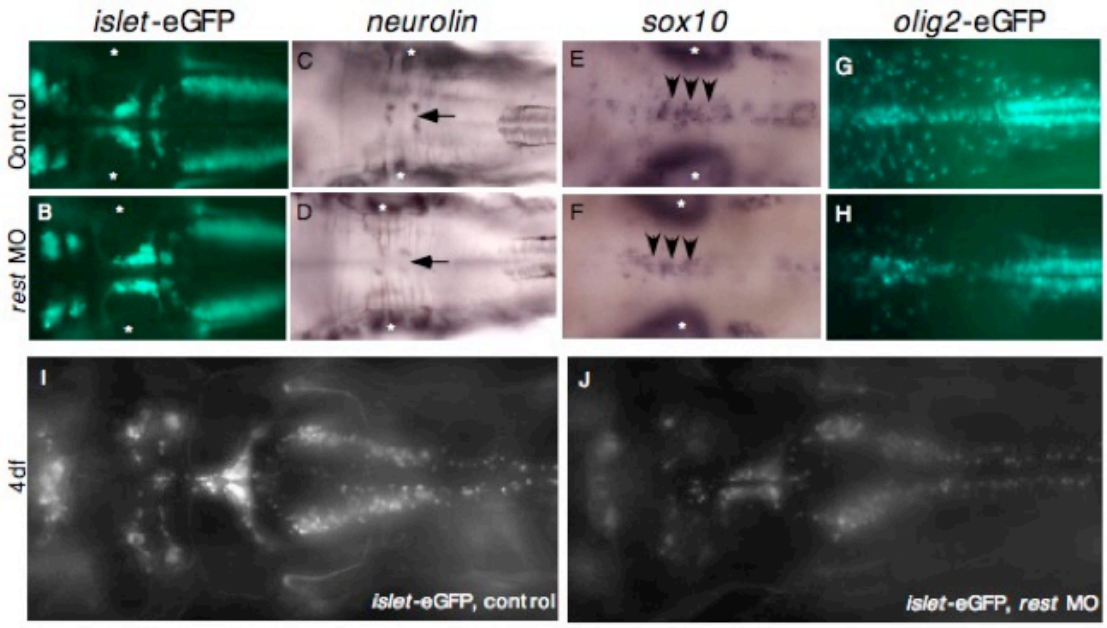

Figure 5.5. Post embryonic rest morphants show loss of diverse neural tube cell types. Dorsal views, hindbrains. (A-H) 2dpf control (A, C, E, G) or stage matched rest morphants (B, D, F, H).(I, J) 4dpf embryos. (B, J) rest morphants do not show loss of early born cranial motor neurons (islet-eGFP) at 2df, (B, compare with control A) but do show a reduction by 4dpf (J, compare with 4dpf control, I, and 2dpf rest morphant, B). (C, D) Late born cranial motor neurons labeled by antibodies for neurolin (arrows C, D) are reduced in rest morphants. (E-H) Sox10 and olig2 precursors and migrated populations of oligodendrocyte labeled by sox10 probe and tg[*olig2*-eGFP], respectively, are reduced in *rest* morphants

5.2.6 Proliferation marker is increased.

This loss of neurons and overall retarded growth of the *rest* morphant could be due to ongoing apoptosis, but decreased proliferation would also account for this phenotype. However, proliferation markers are instead increased. *nmyc* labels some populations neural progenitors, and like *rest,* the transcript is progressively downregulated as the embryo develops (Loeb-Hennard et al., 2005) and Fig 8A-E). Stage matched rest morphants show stronger expression at all stages examined, a phenotype that is consistent with upregulated Hh signaling (Koudijs et al., 2005).

5.2.7 Rest knockdown does not enhance neurogenic phenotype of notch mutant embryos.

Knockdown in wildtype embryos suggested that Rest knockdown did not accelerate neurogenesis, but rather appeared to hamper it. As Rest knockdown sensitized embryos to shh overexpression, it was possible that Rest role may be revealed if a second negative regulator of neurogenesis were simultaneously removed. Activated Notch signaling negatively regulates neuronal differentiation (Louvi and Artavanis-Tsakonas, 2006) by suppressing proneural gene expression. Deregulation of this pathway alongside Rest knockdown might therefore exacerbate phenotypes produced by either one alone. I therefore took advantage of the neurogenic mutant *mindbomb* (*mib*). The *mib* mutant has defects in Notch signaling pathway, and exhibits uncontrolled neurogenesis due to a lack of lateral inhibition. I reasoned that *mib* -/- embryos might become more sensitive upon Rest knockdown, because the morphant/mutant would lack two negative regulators of the differentiation pathway. Additionally, *mib* +/- embryos, which have no discernible neurogenic defects, might show a phenotype with

reduced levels of Rest. However, examination of proneural markers *ngn1* and *asc1a* at early stages did not reveal any differences between control and *rest* MO injected embryos. *elavl3*-GFP/mib +/- incross embryos were also assayed for premature neurogenesis at 24hpf but I was unable to discern any differences after MO induced knockdown. This study, while not exhaustive, suggests that the defective notch signaling in these embryos is not hampered by having reduced levels of Rest.

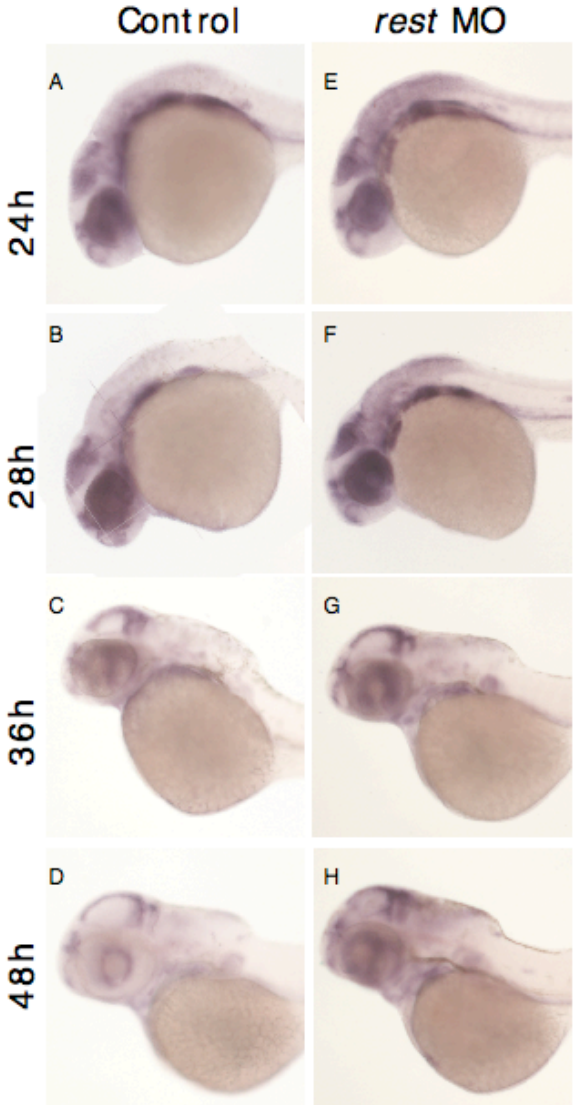

Figure 5.6. Proliferation marker *nmyc* is increased after Rest knockdown. Staging series, Lateral views, head and hindbrain of embros stained with *nmyc* antisense probe. (A-E) control embryos from single injection experiment collected at indicated times to left. *nmyc* is progressively downregulated as development proceeds. (F-J) *rest* morphants collected at 4hrs (F-H) 8hrs (I) or 12 hrs (J) later than control embryos on left. *nmyc* expression is stronger at all stages in these stage matched embryos.

5.2.8 upregulation of *islet* in hatching glands and axon guidance defects

Aside from the neurogenic aspects studied, two characteristics were repeatedly observed in transgenic lines upon Rest knockdown. A consistent effect in both splice and atg MO injected embryos is excess *islet*-GFP+ hatching gland cells.(Fig. 5.7) Upregulation of endogenous *islet* mRNA in hatching gland cells can also seen by *in situ* (Fig. 5.7D) . Hatching gland cells are endoderm derived secretory cells, and so it is possible this increase may relate to regulation of neurosecretory phenotype by REST (Bruce et al., 2006) A second observation was that *elavl3*-GFP fish showed specific axon guidance defects (Fig. 5.7F) a phenotype confirmed by collaborators (Oni map, personal communication).

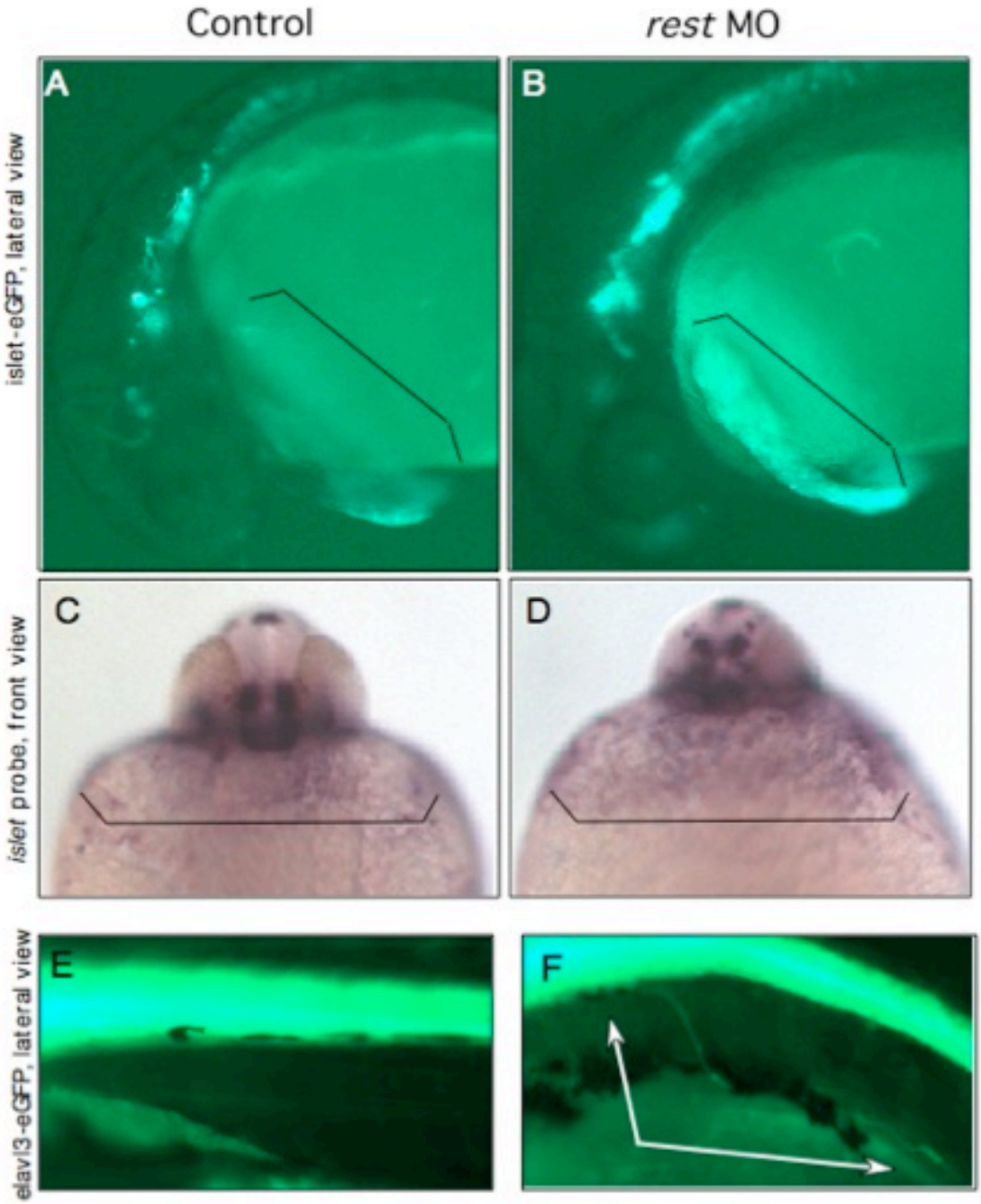

Figure 5.7. *islet* is upregulated hatching gland cells after Rest knockdown. control (A, C, E) or *rest* morphants (B, D,F) (A-D) Hatching gland cells (A-D, brackets) labeled in tg[*islet*-eGFP] fish or by endogenous *islet* transcript in situ. Injection of *rest* MO increases strength of islet expression by both labels. (E, F) Close up of anterior trunk in tg[*elavl3*-eGPF] (F) Lateral line axon migration defects in a *rest* morphant.

5.3. Discussion

The mouse knockout study and findings from Bergsland et al., suggest that the level of activators present was a crucial factor in determining whether REST target genes were expressed in loss of function assays. If relative activity levels between proneural transcriptional activators and Rest were key, embryos should be most sensitive to loss of Rest during accelerated period of neurogenesis. However, I found no conclusive evidence that Rest knockdown affected the differentiation process at any stage in zebrafish. Overall, this data demonstrates a trend of overall decrease in proneural and neural genes. This phenotype runs contrary to expected, based on the most familiar models of REST. However, there is solid evidence for a role of REST as a tumor suppressor, and morphant embryos exhibit prolonged expression of a proliferation marker, nmyc. Alternatively, Chapter 4 establishes a relationship between Rest and the Hh pathway. Several mutants with functions as negative regulators of the Hh pathway also show mild proliferation defects (Koudijs et al., 2008; Koudijs et al., 2005).

The apoptosis observed must be proven to be specific to Rest knockdown and not a side effect of the morpholino injections. If the apoptosis can be shown to be a direct effect of reduced Rest levels, some interesting parallels can be made with the mouse knockout. Additionally, cell death and loss of neural markers was seen in cultured ES cells after interference with REST function (Sun et al., 2008).

These findings are preliminary, and confounded by the possibility of nonspecific effects of morpholinos, developmental delay and cell death. Additionally, proneural genes such as *ngn1* and *zash1a/asc1a* have dynamic, complex expression patterns, which show variance even within wildtype embryos. Future comparisons of these subtle and inconsistent phenotypes will

require careful stage matching and control of cell death. Whether or not the downregulation was a specific effect, no indication of upregulation was observed in embryos with reduced levels of Rest. The lack of phenotype in this regard, despite significant reductions in *rest* levels in wild type and neurogenic mutant *mindbomb* embryos, suggests that Rest function is at least partially redundant in the networks and pathways controlling neuronal differentiation in zebrafish.

5.4 Materials and Methods

5.4.1. Zebrafish stocks and embryo maintenance

Embryos maintained as previously described in 3.3.1. Stage matching rest morphants to control embryos was done by gross morphology including head angle, tail development and the prominence of the cerebellum. Transgenic stocks used were tg[*islet-eGFP*] ((Higashijima et al., 2000), tg[*elavl3-eGFP*] (Park et al., 2000a), tg[*olig2-eGFP*] (Shin et al., 2003).

5.4.2 Morpholino injections

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 .2M KCl and phenol red from a 34mg/ml stock to 8-10mg/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'-CCTCTTACCTCA GTTACAATTTATA-3') were used in all experiments. At the appropriate stage, embryos were fixed in 4% paraformaldehyde for *in situ* hybridization or placed in TRIzol reagent (Invitrogen) for RNA extraction.

5.4.3. 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., 1993). Constructs used to synthesize the following probes have been described previously: *asc1a/zash1a* (Kudoh et al, 2001,) *neurogenin1, ngn1* (Korzh et al., 1998) and *elavl3/HuC* (Kim et al., 1996). After *in situ* hybridization, embryos were mounted in 75% glycerol and photographed using a Zeiss Axiocam mounted on a Zeiss Axioplan microscope.

Chapter 6. Significance and future directions

6.1 Significance of Rest regulation of the Hh/Gli pathway

While attempting to elucidate the nature of Rest function during neurogenesis, a role for modulating the Hh pathway was revealed. While this was unexpected, it is consistent with reports that indicate functions unrelated the model of REST as a repressor of differentiation. It is possible that this tie to the Hh pathway serves to connect some of the more seemingly disparate reports concerning roles in other pathways in development and disease.

Aside from revealing this role for Rest, this study highlights the idea that regulation of *gli2* transcription is important for controlling Hh signaling, a subtle mechanism that has received little attention.

6.1.1 The of role *gli2* transcriptional regulation in determining Hh output

This study centers on Rest function, but also says something about how Hh signaling controls output of pathway activation. The ability of Hh to tune down it's own signaling is an important characteristic of the pathway. Pathway feedback inhibition takes place through several distinct mechanisms, including ligand sequestering extracellularly and cytoplasmic sequestering of the Glis. This study implicates transcriptional regulation of *gli2a* as a likely additional mechanism. A clear relationship can be observed when Hh levels are manipulated ectopically in zebrafish. Overexpression of Shh leads to general downregulation of *gli2a* (Tyurina et al., 2005; Vanderlaan et al., 2005) Fig 4.15.), and blockage by CyA leads to its upregulation (Vanderlaan et al., 2005), not shown). Endogenous downregulation in a wildtype environment appears to reflect a temporal element, rather than simply being a function of strength of

signaling. Early in development, *gli2a* is expressed throughout the dorsoventral extent of the neural tube and so Gli2 and Gli3 are exposed to Hh signaling. A similar pattern is seen for *GLI2* in mouse, at least up to E9.5 (Sasaki et al., 1997), reflecting its early activator function in both species. As embryonic zebrafish development proceeds, the domain of *gli2a* expression become downregulated in areas of pronounced Hh signaling. (Fig. 6.1). Thus an early transducer of Hh signaling is being removed ventrally, and Gli1 takes over as the primary activator of Hh targets. Whether downregulation of *gli2* induced by prolonged Hh exposure occurs in other species is not clear, as data on the dynamics of *gli2* expression is limited. Gli2 is thought to be the main activator in mammals (Bai et al., 2002), so it would be interesting from a standpoint of evolution to see if transcriptional regulation has evolved accordingly.

In this study, I present evidence that there are context dependent consequences for a failure to properly downregulate *gli2a*. When this ventral downregulation of *gli2a* fails in a wildtype background, the effect is mild. In the wild-type neural tube, excess Gli2a forms would reflect the endogenous high (ventral) to low (dorsal) Shh gradient, leading to positional dependent $Gi2a^{Act}$ and $Gli2a^{Rep}$. Because of the biphasic response to increased and decreased Hh signaling in *rest* morphants, it might be expected to observe dual effects within the same embryo, between areas of high and low Hh signaling. However, there is no indication in the *rest* morphant of excessive Gli^{Rep} activity in areas of low Hh. In rest morphants*. ptc1* and *gli1* expression is stronger and *pax 3* is reduced dorsally, signifying increased pathway activation. This may be because while prolonged ventral activity of Gli2a represents an ectopic situation, *gli2a* is already expressed dorsally. Therefore the effect of excess $Gli2^{R}$ levels dorsally may be negligible.

While Rest may regulate both activator and repressor forms of Gli2, control of Gli^{Act} activity is most prominent in the wildtype setting. However, the *rest* morphant phenotype is not severe like mouse (Bai and Joyner, 2001) or

zebrafish (Koudijs et al., 2008) *ptc1* mutants, which have phenotypes similar to that produced by Shh or dnPKA overexpression. However, it is similar to the mild phenotypes of the zebrafish *sufu*, *ptc2* and *hip* mutants, all which have partially redundant functions in Hh autoinhibitory circuits (Koudijs et al., 2005). The weak activation of the Hh pathway in these mutants is partly attributed to negative feedback by *ptc1*, because *ptc1/ptc2* and *ptc1/hip* double mutants show a greater activation of the pathway than either alone (Koudijs et al., 2008). *ptc1* expression is strongly upregulated in *rest* morphants (Fig.4.10), and this likely dampens further effects. The high degree of degeneracy within such a complex negative feedback system can allow for stability upon perturbations of one or more pathway components. The function of otherwise redundant genes may be revealed with gain or loss of function in several pathway components, such as is common in tumor formation.

6.1.2 REST involvement with pathways antagonistic to Hh.

Rest regulation of the Hh/Gli signaling pathway is a novel finding. However, this discovery has interesting precedents. In two other *in vivo* studies, REST has been implicated in the BMP and Wnt pathways, both of which are known to antagonize Hh signaling. BMP signaling from the roof plate is thought to work in opposition to Hh signaling from the floor plate (Liem et al., 2000; Liem et al., 1995). In frog, it has been reported that blocking REST function mimics some aspects of decreased BMP signaling (Olguin et al., 2006). This implies Xenopus REST has a role in promoting BMP function, which is compatible with zebrafish Rest function in antagonizing the Hh pathway. This coincidence deserves a closer look, although there are important differences between the studies. The frog study focuses on early role of BMP in defining the ectodermal fate, not dorsoventral patterning of the neural tube. Further, the role of BMP in dorsal patterning of the zebrafish neural tube is poorly understood. In zebrafish, Wnt signaling from the roof plate is thought to be an

important determinant of dorsal neural cell type specification by working in opposition to Hh signaling (McFarland et al., 2008). The connection between Rest and Wnt has been demonstrated in several reports. In chick spinal cord, REST has been shown to be a directly upregulated by the Wnt activated B-catenin/ TCF complex (Nishihara et al., 2003). *Rest* is also upregulated by Wnt signaling in human teratocarcinoma cells (Willert et al., 2002). REST may in turn regulate Wnt signaling, as Wnt pathway components constitute a significant number of REST targets in ES cells (Johnson et al., 2008). Thus REST may be an important point of interaction between signaling pathways. The intracellular environment of cells exposed to these opposing morphogen gradients will determine how they respond, and REST may be an important determinant of that regulatory state.

6.1.3 REST and Gli2 in Cancer

Zebrafish Rest function was revealed most robustly upon knockdown combined with induced Hh signaling. (Fig. 4.5 and 4.6) In tumor progression, it is frequently a combination of loss of tumor suppressors and activated oncogenes that enable transformation. REST has been identified in several studies as a tumor suppressor, and the Glis are well known oncogenes (Kasper et al., 2006; Ruiz i Altaba et al., 2007). Decreased REST levels or interference with function have been found to result in higher rates of proliferation in several non-neural tumors. For example, REST was identified as a tumor suppressor by the ability of RNAi induced knockdown of REST to transform human epithelial cells (Westbrook et al., 2005). The study found a mutation that results in a C- terminal truncated form of REST is common in colorectal cancers. Tumor suppressor activity found in other nonneural tissue includes small cell lung cancer (Coulson et al., 2000; Gurrola-Diaz et al., 2003), prostate (Dhanasekaran et al., 2001) and lung (Garber et al., 2001). Interestingly, decreased REST activity has also been associated with

proliferation of mouse aorta smooth muscle cells (Cheong et al., 2005). Declining REST levels in this case results in upregulation of target gene KCNN4 that encodes the Kca3.1 potassium channel, a driving force for proliferation in these cells. In this case, it is clear why REST LOF may result in cell proliferation. Otherwise, the idea of REST as a tumor suppressor runs counter to the most familiar model of REST as inhibitor of differentiation. The interaction between Rest and the Hh pathway offers another avenue for investigation.

The findings here report that the oncogene Gli2 is central to the synergistic response to Hh pathway manipulations, and its observed transcriptional upregulation could hypothetically account for this response. However, transcriptional upregulation remains only an intriguing correlation, and Rest knockdown may affect Gli2 activity in other ways. In Westbrook et al., it was shown that transformation of human mammary epithelial cells (HMECs) due to REST loss of function was dependent on Insulin-like growth factor(IGF)/ phosphoinositide-3-kinase (PI(3)K) pathway activation. Interference with REST resulted in an increase in the intensity and duration of PI(3)K signaling. How REST interacts with the PI(3)K pathway is not known, although the IGF1 receptor is a putative REST target (Otto et al., 2007). How does this relate to the Hh pathway? Like Rest knockdown, activated PI(3)K signaling has been found to potentiate Hh signaling. In particular, Shh induced tumor formation is synergistically enhanced by PI(3)K pathway activation (Rao et al., 2004). Even more relevant, this positive regulation of the Hh pathway is accomplished through stabilization of the Gli2 activator form (Riobo et al., 2006).

Thus, it is possible that Gli2 stabilization through activated PI(3)K signaling is the mechanism for the synergistic response seen when Hh is misexpressed in rest morphants. More importantly, the interaction of Rest and Gli2 demonstrated here provides a potential clue toward understanding tumor suppressor properties of REST.

6.2. Future directions

A proper level of Gli2 activity was demonstrated to be a required for the response to Hh in *rest* morphants, but details of how Rest regulates Gli2 remain to be determined. On a different front, Rest knockdown caused a decrease in neural and proneural markers. This should first be confirmed as a specific effect of Rest knockdown, and the cause can then be investigated.

6.2.1 Components that regulate Gli2 protein

Investigating the PI(3)K pathway is a potentially important direction that offers possibilities concerning understanding details of the Rest-Gli2 interaction and beyond. As a first step, it should be determined if Rest knockdown activates the IGF/PI(3)K pathway in zebrafish. Several other scenarios involving post translational regulation of Gli2 could be also investigated. Transcriptional control over cytoplasmic components that regulates stability, processing or nuclear shuttling of the Glis, and Gli2a in particular, remain plausible mechanisms to explain the phenomena described. Possible interactions influencing Gli activity include mRNA stability, protein modification or intercellular localization. This may involve cytoplasmic components such as Sufu, Talpid3, or Iguana (Davey et al., 2006; Sekimizu et al., 2004; Wang et al., 2007; Wolff et al., 2004) that regulate both activator and repressor forms of the bifunctional Glis. For example, while Sufu is best characterized as a negative regulator of Hh signaling because of its role in the cytoplasmic retention of Gli activator, it may also retain the Gli repressor form as well (Flynt et al., 2007; Wolff et al., 2003). In that case, enhancement of any negative regulator of Sufu, such as Fu or miR-214 (Flynt et al., 2007), might result in nuclear accumulation of either the activator or repressor form depending on the state of Hh signaling. Another cytoplasmic component that may regulate both Gli forms is the zinc finger protein Iguana (Sekimizu et al., 2004; Wolff et al., 2004). Iguana is thought to be involved in nuclear shuttling of

the Glis, and zebrafish *igu* mutants show evidence of impairments to both Gli activator and repressor functions. Intraflagellular transport (IFT) proteins are other factors downstream of smo that may both positively and negatively regulate the Hh pathway, as shown in the complex Hh defects in zebrafish without cilia (Huang and Schier, 2009)

Analysis of transcript changes in *rest* morphants of such factors would be the first step in determining involvement. In particular, derepression of miR-214 should be checked, since overexpression of miR-214 in Flynt et al., mimics some aspects of the rest morphant. Epistatic studies involving Rest loss of function in a background with gain or loss of function of these factors may also prove informative.

6.2.2 The directness of Rest transcriptional regulation of *gli2a*

Indirect regulation of Gli2a, by regulation of components that regulate Gli2 activity, is consistent with our findings. However, the evidence we currently have is for *gli2a* transcriptional regulation, whether direct or indirect. Inappropriate expression of *gli2a* ventrally could account for the phenotype, and *gli2a* upregulation is not associated with Hh pathway activation. This is consistent with *gli2a* upregulation as a cause, and not an effect, of enhanced Hh signaling. It is reasonable to hypothesize that Rest is preventing inappropriate expression of target genes before their time. Downregulation of Rest in that case would provide a permissive cue for their expression. As such, it would be expected that expression of *rest* and a given target gene would be at least somewhat complementary. Even when not taking into account the post translational regulation of Rest, comparison of *rest* and *gli2a* expression patterns does not provide for an easy correlation between the two genes. Examination of the two genes reveals a spatiotemporal partial overlap of expression zones. Both genes are co-expressed throughout the dorsoventral extent of neural tube early in development. Both are downregulated in the ventrolateral neural tube as

development proceeds and neurons begin to differentiate there. Coexpression continues in the dorsal lip of the hindbrain, as this region remains proliferative or undifferentiated for the first two days of development. (Fig.6.1) At these stages, complementary expression can be seen in the midline ventricular zone. *gli2a* becomes increasingly confined to the most dorsal regions there while *rest* is still expressed down to the floor plate. It is this midline region where *gli2a* expression persists in *rest* morphants but not in wildtype embryos (Fig. 4.14) Can Rest be directly responsible for this late downregulation of *gli2a* in the midline, considering the earlier co-expression? It seems likely that a second transcription factor regulating *gli2a*, working in conjunction with Rest, would need to be posited in a model where Rest directly regulates *gli2a* expression.

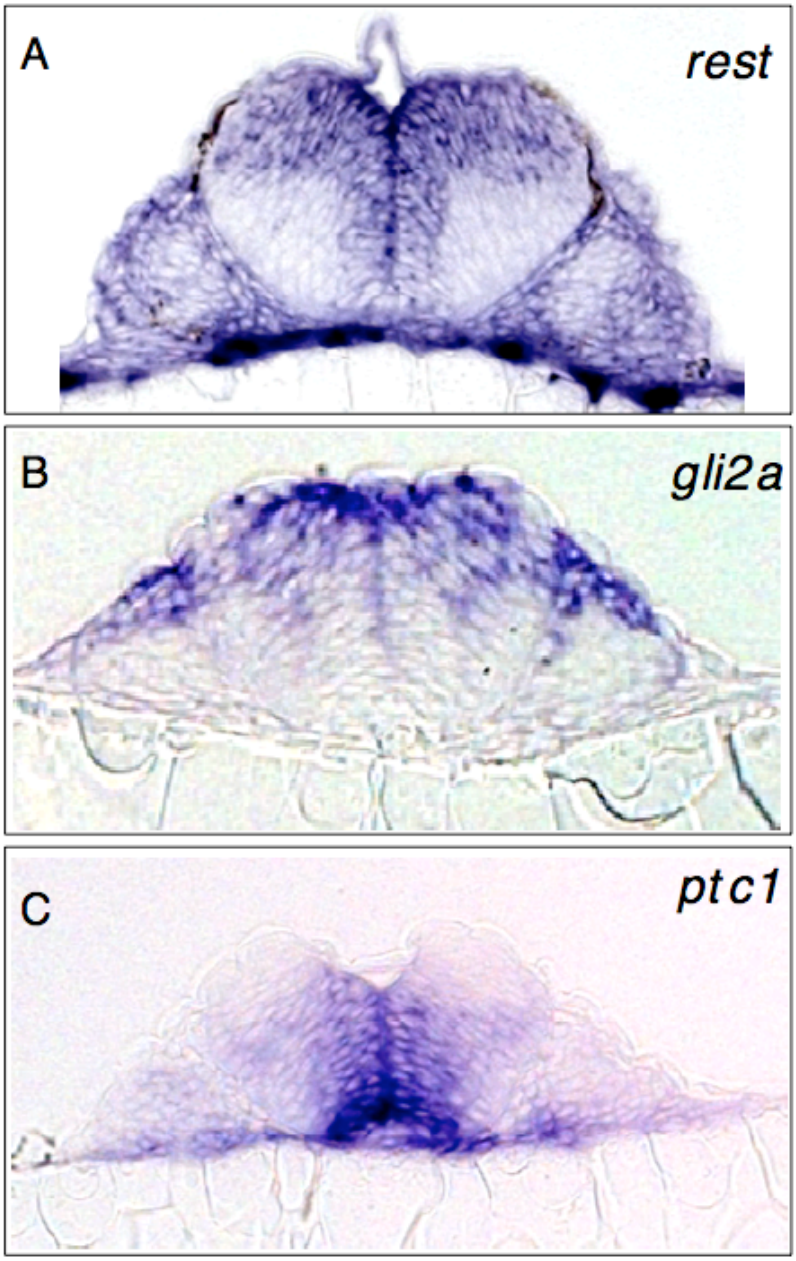

Figure 6.1. Comparison of *rest and gli2a* expression patterns.) (A-D) Transverse sections of hindbrains from one day old embryos. *rest* and *gli2a* expression partially overlaps in dorsal lateral regions of the rhombic lip, and both are absent from the ventrolateral region of postmitotic neurons. *rest* is present in the midline ventricular zone, where *gli2a* is has been largely cleared . This region is exposed to Hh signaling, as evidenced by ptc1 expression in (C).

Clearly, regulation of *gli2a* expression involves factors other than Rest. The endogenous pattern of downregulation appears to be Hh related, as expression is roughly complementary to *ptc1* expression. (Fig. 6.1C) Hh induced downregulation of *gli2a* was hampered by loss of Rest, but only partially (Fig. 4.15). Embryos with reduced levels of Rest combined with elevated levels of Hh have *gli2a* expression intermediate between embryos with either manipulation by itself. This additive response suggests that Rest and the Hh pathway operate in parallel to regulate *gli2a* levels.

Ultimately, establishing whether Rest directly regulates *gli2a* will require ChIP analysis. Ideally, Rest occupancy on RE1 sites at the *gli2a* locus at a time and place when *gli2a* is being downregulated would be determined. Current barriers to this process, mentioned in 4.2.11, will need to be overcome. Direct regulation may also be indicated by determining whether putative RE1s found in the *gli2a* locus are functional, using standard promoter-reporter analysis techniques.

The transcriptional regulation by Rest is just as likely to be indirect. In addition to the negative influence of Hh shown in zebrafish, positive transcriptional regulation of *Gli2* by FGF signaling in frog (Brewster et al., 2000) and Wnt signaling in quail (Borycki et al., 2000) has been reported. The pathway components involved in this regulation are not known in either case. Given that Rest is a repressor and *gli2a* is upregulated upon knockdown, an indirect regulation would include either Rest repression of an activator of gli2a, or Rest repression of a negative regulator of a repressor of *gli2a.* The possibilities quickly become unwieldy, so analysis should begin by determining whether manipulation of either pathway affects *gli2a* expression in zebrafish.

My preliminary experiments in this regard were inconclusive. Both gli2a and rest expression were assayed after stimulation of Wnt signaling by

incubation in LiCl. No changes in either were seen in the most controlled sets of experiments. However, more careful design may still yield conclusive results on this front. In checking for expression changes by *in situ*, both the *gli2a* and *rest* probes are poor ones, and variation of expression between siblings raised in the same condition is common. Real Time PCR with large numbers of samples might be the best solution to assay overall changes in expression after pathway manipulations. Both genes are also downregulated as development proceeds, so developmentally delayed embryos may show higher expression of these genes regardless of how they came to be developmentally delayed.

6.2.3 Rest and neurogenesis

While the model of Rest interaction with Hh pathway is novel, it is well supported by the data presented. In contrast, the results obtained concerning the more familiar model of Rest controlling neurogenesis must be considered preliminary. Rest knockdown resulted in a decrease in proneural and neural markers amid developmental delays and apoptosis. This phenotype shows similarities to the mouse knockout and the report from Sun et al., 2007 in cell culture. However, these are also known side effects of morpholinos and must be controlled carefully. When downregulation of genes occurs, especially ones with dynamic pattern like proneural markers, possible side affects of cell death and developmental delay must be ruled out. In the case of Hh, the upregulation and simpler pattern of Hh response genes allowed for greater confidence in the result. Some phenotypes, in particular the restoration of *nkx2.2a* in *dtr/gli1* mutants (Fig. 4.18), are harder to ascribe to non-specific effects. More importantly, specificity was shown with a second morpholino and mRNA add-back experiments. If further work on neurogenesis role is done using *rest* morpholinos, careful control of cell death and developmental delays will need to be undertaken. Ideally, the *rest* knockout fish will be available for this.

Preliminary findings of the current batch of zinc finger knockouts reveal only the mildest phenotypes. However, I encourage testing the knockout in different contexts to better reveal what might be the otherwise subtle functions of Rest.

6.3. Conclusion

This *in vivo* study, aimed at gaining a broader understanding of the role played by REST in the developing nervous system, adds yet another layer of complexity to the picture of the enigmatic REST. A novel role in the modulation of Hh signaling was revealed, and the principle point of interaction with the pathway was identified. Though bioinformatic approaches and others studies implicate REST in various pathways and networks, this is the first functional study to demonstrate such involvement within a developing embryo. It is doubtful that such a role would have been revealed, without exposure to the full range of development signaling in the living embryo. Whether this study in zebrafish embryos is relevant to mammalian REST function remains to be seen. Functional studies done in mammalian cell cultures have produced several intriguing, sometimes conflicting, theoretical roles of REST. Yet how these models may relate to the embryonic lethality in the mouse knockout is not known. It may well turn out that a role for Rest in modulating the Hh pathway during normal embryonic development is species specific. However, the interaction may still reveal itself in contexts such as tumor formation, in those cases where both Rest and Gli function are perturbed.

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Appendix

A. 1. Introduction

A1.1 CoREST background

CoREST was originally discovered through two hybrid screen designed to find REST interacting proteins (Andres et al., 1999), and is best characterized in its capacity as a corepressor for REST. Consequently, it has been discovered that CoREST is far more conserved during evolution than REST. Structurally and functionally similar homologs are found in fly and C.elegans, and it is highly conserved within vertebrates (Dallman et al., 2004; Jarriault and Greenwald, 2002). CoREST has also been discovered in various independent screens for HDAC interacting proteins and through analysis of other complexes. (Battaglioli et al., 2002; Hakimi et al., 2002; Humphrey et al., 2001; You et al., 2001). Notably, the CoREST complex was also found in an analysis of the core CtBP complex (Shi et al., 2003). Also found in this complex were CoREST3, putative oncogene ZNF217, and FGF effector Sip1. CoREST interaction with ZNF217 was shown to be direct, and this complex, with CtBP, repressed e-cadherin (Cowger et al., 2006). The CtBP interaction is important to this study's findings because CtBP is a known corepressor with mammalian notch target RBP-Jk/Su(H)(Oswald et al., 2005). It is also relevant that screens were done in non-neuronal lines, such as HeLa cells, and REST was not found associated with CoREST there. CoREST, but not REST, is also found in invertebrates. In the fly, CoREST represses neural genes with a different transcription factor (Dallman et al., 2004). There is also evidence that Drosophila CoREST may be a corepressor with notch target Su(H). (J.Dallman, personal communication) The C. elegans homolog, SPR-1, was discovered in a screen for mutations that suppressed an egg laying defect caused by defective LIN/12 notch signaling (Jarriault and Greenwald, 2002). In the proposed model, a non-functional SPR-1 allowed derepression of the necessary gene, previously repressed due to inactivity of LIN12/notch. Human CoREST was conserved enough to be used to restore the defective phenotype. The conservation implies CoREST carries out vital functions independent of REST in vertebrates as well, with a wider role than has been described.

A1.2. The CoREST complex

The different repressive states found between nonneural and neural stem cells does not appear to be due to repressor activity differences between CoREST and Sin3A, but rather between the different CoREST complexes assembled. Although many factors have been identified that associate with CoREST, the core complex is generally found to consist of HDAC1/2, LSD1 and BHC80. Histone Deacetylases 1/2 are closely related enzymes that remove acetyl groups from histones, resulting in the DNA being bound tighter, which is associated with transcriptional repression. LSD1 specifically demethylates lysine residue 4 of histone 3 (H3K4). Di- and Tri- methylated H3K4 is associated with active transcription so their demethylation to mono-methylation results in repression. The significance of tri- verses di-methylated H3K4 is not clear, but the difference may result in subtle changes in levels of repression. (Ballas and Mandel, 2005). The role of BHC80 in this complex is currently not clear, but it binds directly to all components of the CoREST complex, suggesting a scaffolding role, and has also been proposed to inhibit LSD1 function (Iwase et al., 2004).

CoREST functional domains have been studied thoroughly. The REST interaction domain (Repressor Domain 1) was mapped to CoREST N-terminal amino acids 102-195. (Ballas et al., 2001). CoREST contains N-and C- terminal SANT (SW13/Ada2/NCoR/TFIII B) domains. SANT 1 is needed for HDAC interaction, and stimulates deacetylase activity. (You et al., 2001). Repressor Domain 2 (Amino acids 321-442), (Ballas et al., 2001), includes the LSD1 interaction site (Yang et al., 2006) and the SANT2 domain . CoREST SANT2 interacts weakly with DNA and disruption of SANT2 diminishes CoREST-dependendent demethylation of histones by LSD1. (Yang et al., 2006).

A1.3. Lateral inhibition and activator model of neurogenesis

This study suggests CoREST appears to play a role in the neural determination step independent of REST, as a component of the Notch pathway. In the neural plate and tube, proneural fields, are induced in neural stem cells by prepatterning factors such as members of the Iroqouis families. Proneural fields are clusters of cells expressing proneural factors such as neurogenin (ngn) or Ash1A. Individual neurons arise within these proneural fields via Notch mediated lateral inhibition (Diez del Corral and Storey, 2001). The Delta/Notch signaling pathway arises within clusters of ngn positive cells and results in an upregulation of ngn in subset of these cells. Ngn upregulates the notch ligand delta, resulting in activated notch signaling in the neighboring cells. The activated Notch intracellular domain (NICD) displaces the corepressor complex associated with the transcription factor Suppressor of Hairless (Su(H)), and converts Su(H) from

repressor to activator. In the absence of notch signaling, Su(H) represses anti-neural genes such as bHLH transcription factor HES1 via a HDAC complex that has been shown to include CtBP (Bray, 2006). The Su(H)/NICD complex activates HES1, which represses the proneural genes at the transcriptional level, and by forming non-functioning heterodimers with the proneural bHLH proteins. Decreased Ngn activity results in a downregulation of *Delta* in that cell. This reduces Notch activity in the neighboring cell, so HES1 remains repressed, and *Ngn* is upregulated. The overall effect is that Ngn upregulates itself in one cell, leading to neural determination, and downregulates itself in the neighboring cell, which remains uncommitted. In these determined cells, upregulated proneural factors such as Ngn or Ash1A then promote cell cycle exit and induce initial differentiation steps by activating neurogenic factors such as NeuroD or Sox4/11. These neurogenic transcription factors in turn activate terminal differentiation genes.

A. 2. Results

A.2.1. Conservation of CoREST/*rcor1* in zebrafish.

CoREST studies outside of mediating REST function are limited. Xenopus CoREST was identified in a screen for genes regulated by the Wnt activated Xiro1, a homeobox gene of the iroquous family involved in neural prepatterning (de la Calle-Mustienes et al., 2002). XCoREST / mRNA was found colocalized with neural markers at early stages of CNS formation. At later stages in the neural tube, expression was found in the intermediate zone, where it was posited to play a role in differentiation. A mammalian model may be lacking because knockout mice were embryonic lethal. (N.Ballas, personal communication) This suggests a knockdown as opposed to knockout may be required for *in vivo* functional studies.

The CoREST/*rcor1* (zebrafish CoREST gene/mRNA hereafter referred to as Rest Corepressor1, *rcor1*) clone was obtained from a cDNA vector library. CoREST clones were sequenced and our predicted proteins from these sequences improved the alignments with their respective human homologs over the alignments using the predicted proteins from the database. The important SANT 1 and SANT 2 domains are conserved 95 and 100%, respectively. Two CoREST paralogs found only in vertebrates, are also conserved in zebrafish, suggesting conserved functions, although there is virtually nothing is known about these.

CoREST expression in Xenopus is not seen until 12.5, at the end of gastrulation. In mouse, early expression is restricted to the anterior early until becoming more widespread at E11.5. Zebrafish expression of *rcor1* was analyzed by *in situ* hybridization, and compared to expression of *rest.*

The early expression of *rcor1* was not examined extensively, but appears ubiquitous until 24-30 hpf. *rcor1* expression patterns become more dynamic as the rate of neurogenesis increases throughout the embryo. Differential expression patterns emerge between proliferating and neurogenic regions.

Similar to *rest, rcor1* is present in non-neural tissue and proliferative and undifferentiated populations of the neural tube, as in the undifferentiated optic tectum (Fig. 1B). In contrast, in areas of the neural tube undergoing differentiation *rcor1* staining reveals a dynamic expression pattern. *rcor1* transcript is found upregulated around the lateral edge of proliferative midline in neurogenic regions, at the lateral border of rest expression. This zone adjacent to the ventricular zone contains a population of cells exiting mitosis (Nguyen et al., 2001). This is of interest since in mouse ES cell culture, CoREST was found part of RARE complex repressing Rest in freshly postmitotic cells (Ballas et al., 2005)

Expression in the tegmentum and hindbrain (Fig 1B-D) cis reduced in the cells closest to the ventricular zone compared to the cells immediately adjacent to those. The same pattern is evident throughout the hindbrain, which is undergoing a similar rate of neurogenesis at this stage. This population of cells, located intermediate between the vz and the differentiated lateral populations, also express neural progenitor markers such as sox3, as well as cell cycle exit factor cdkn1c (Park et al., 2005). hdac1 also shows a similar pattern, where in zebrafish it has been shown to play a role in promoting neurogenesis (Cunliffe, 2004) *rcor1* is largely reduced in populations of the most lateral, differentiated neurons, although present in isolated cells there.

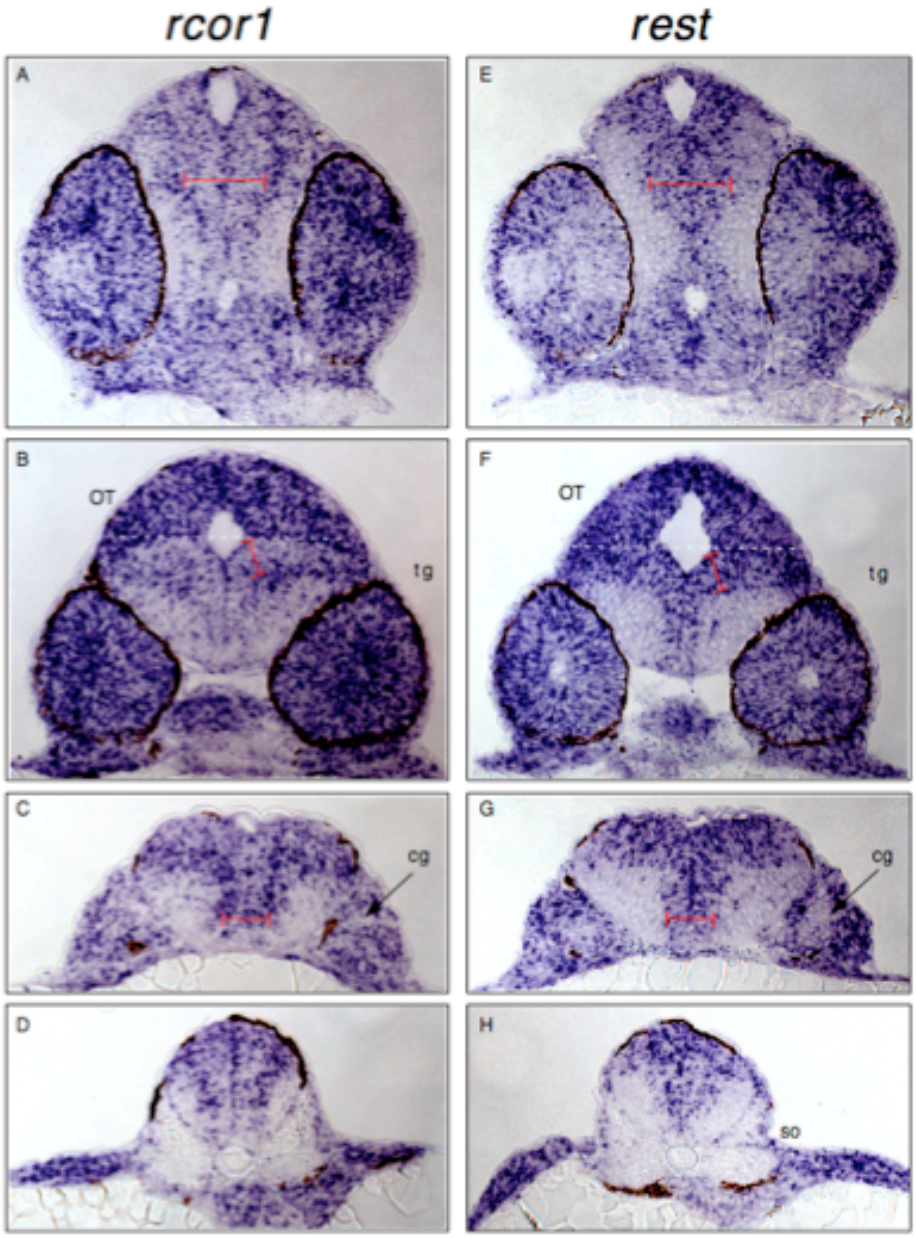

Figure A.1. *rest* and *rcor1* differentially expressed in zones of active neurogenesis. transverse sections, 42 hpf embryos stained for *rest* (A-D) and *rcor1* (E-H). sections taken at levels of forebrain (A, E) midbrain (B, F) hindbrain at anterior rhombomere 4 (C, G) and anterior trunk (D, H). (A, B, E, F) The largely undifferentiated eyes and dorsal midbrain, (the optic tectum, OT) display widespread expression of both *rest* and *rcor1* (eyes in A, E, eyes and optic tectum in B, F). r*est* expression remains largely confined to the proliferative midline (ventricular zone, vz) throughout the embryo. *rcor1* becomes weaker in these most medial regions while the intermediate regions, between the midline proliferative and lateral differentiated regions, show an upregulation of *rcor1*. Tegmentum tg, cranial ganglia cg

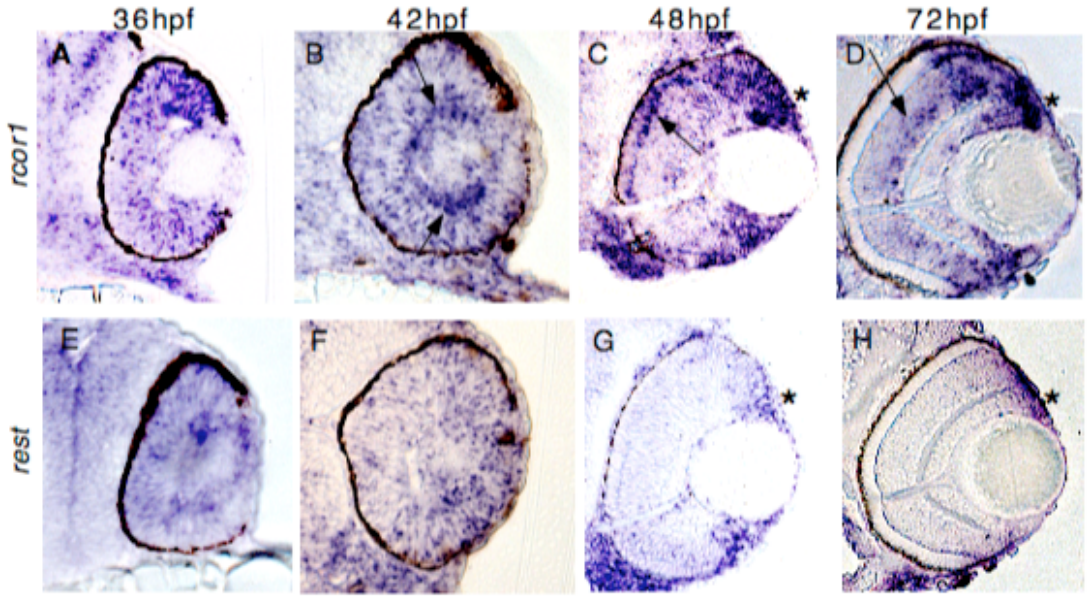

Figure A2. *rest* and *rcor1* in the developing eye. Transverse sections taken at mid eye level of 36hpf (A, E) 42hpf (B, F) 48hpf (C, G) and 72hpf (D, H) *rest* expression is coincident with proliferative cells as most of the eye remains undifferentiated at 36 and 42 hr and then becomes confined to the proliferative center (asterisks G, H) as the eye differentiates. *rcor1* remains in those proliferative regions (arrows C, D) but becomes upregulated in regions poised to differentiate, as in rgc (B, arrow), outer nuclear layer (C, arrow) and inner nuclear layer D, arrow)

Retinal neurogenesis occurs in a well-characterized pattern, and examination of the developing eyes is revealing on the differences between expression patterns of *rest* and *rcor1*. *rest* expression is mostly uniform in the still undifferentiated 36 hpf retinas, and remains present throughout development at the proliferative temporal center, (Fig. A1) as are other proliferative markers such as *notch1A* and *nmyc*. (Loeb-Hennard et al., 2005; Mueller and Wullimann, 2002). Like r*est*, *rcor1* is present in the proliferative temporal region throughout these stages, but shows a highly dynamic pattern in other regions of the retina. *rcor1* is significantly upregulated in specific populations of cells at different stages (Fig. A2). The dynamics of neurogenesis in the zebrafish eye have been described in (Li et al., 2000a; Li et al., 2000b; Link et al., 2001; Livesey and Cepko, 2001). At 42 hpf, *rcor1* is observed strongest in the retinal ganglion cell layer, while at 48 hpf *rcor1* is upregulated in the outer nuclear layer. The stage specific upregulation correlates with the timing of differentiation in each population. At 72hpf, *rcor1* expression shifts to the inner nuclear layer, in a specific band that excludes the differentiated amacrine cells and the horizontal cells located on the outer layer of the inner nuclear layer. This region includes bipolar cells which are also thought be undergoing differentiation at this time. Thus, *rcor1* is strongly expressed in those layers that are next to become differentiated at 42 and 48hpf and possibly at 72hpf.

Thus, *rcor1* expression is dynamically regulated. In proliferative regions where neurogenesis has yet to occur, expression is similar to *rest* . In regions undergoing neurogenesis, *rcor1* is most strongly expressed in those populations of cells that are exiting mitosis. This suggests that CoREST is acting independently of Rest at that stage. CoREST function there could involve the promotion of cell cycle exit. Alternatively, it could maintain repression of terminal differentiation genes .

A.2.2 *rcor1* morphant phenotype characterized by cell death.

CoREST is best characterized in its role with REST, so a simple prediction would be ectopic neural gene expression in neural progenitors and other nonneural cells. This assumes CoREST knockdown primarily affects REST function, but REST functions independently of CoREST in some contexts (Ballas et al., 2005; Grimes et al., 2000; Jepsen et al., 2000) and as shown above, expression patterns are not overlapping during the course of development. (see Fig. A1, (Grimes et al., 2000) Therefore, it is highly likely that CoREST acts as a corepressor in other complexes as well.

Morpholinos designed against the translation start site (ATG MO) and an exon-intron boundary (splice MO) were both used in the following assays. All controls were embryos injected with a standard control morpholino. These embryos do not differ from uninjected embryos under the same conditions. Low doses of morpholinos were used which allowed proper axis formation and neural tube development. At higher concentrations, defects are apparent beginning at early gastrulation with cell movement convergent and extension defects, result in severe epiboly defects and, later massive cell death. This severe phenotype precludes neurogenesis studies and will not be considered in these preliminary results.

rcor1 morphants display an outward phenotype marked by severe necrosis and apoptosis (Figure A3). Morphants created with the ATG morpholino are more severe than those injected with the splice morpholino. The brownish, opaque tissue seen in the head and hindbrain region (Figure A.3. C) is assumed to be necrotic tissue. Acridine Orange (Fig. A.3 E-H) is thought to label apoptotic cells exclusively. Apoptosis is evident by heavy labeling by Acridine Orange at the 12 somite stage, before necrosis is not apparent (not shown).

A.2.3 Co-injection of *rest* MO reduces cell death in *rcor1* morphant

While investigating the effect of knocking down CoREST and Rest together, an unexpected phenotype emerged. Intriguingly, co-injection of 5ng *rest* MO alongside 1.5ng ATG *rcor1* MO resulted in a reduction of cell death. (Fig. A.3 E-H). The opaque, brown by-product normally seen in *rcor1* morphants was largely

eliminated. AO staining also revealed decreased apoptosis in the head and sensory ganglia relative to the *rcor1* morphant. This was surprising because CNS cell death is a common side effect of morpholinos, thought to involve the p53 pathway. (Eisen and Smith, 2008). Neural degeneration also occurs in a majority of induced mutants (Furutani-Seiki et al., 1996). Inhibiting cell death, however, is not a common side effect. The Rest knockdown in *rcor1* morphants may influence specific developmental processes that are perturbed in the *rcor1* morphant, such as neurogenesis (see below), that subsequently lead to apoptosis in the *rcor1* morphant. However, the necrosis and/or apoptosis seen in the embryos injected with the ATG MO may be a largely non- specific side effect that is not specific to reduced levels of *rcor1*. This is demonstrated by morpholinos that do not explicitly target any zebrafish genes producing patterns of cell death similar to that seen in the *rcor1* morphant. (Fig. A.4). Also, higher

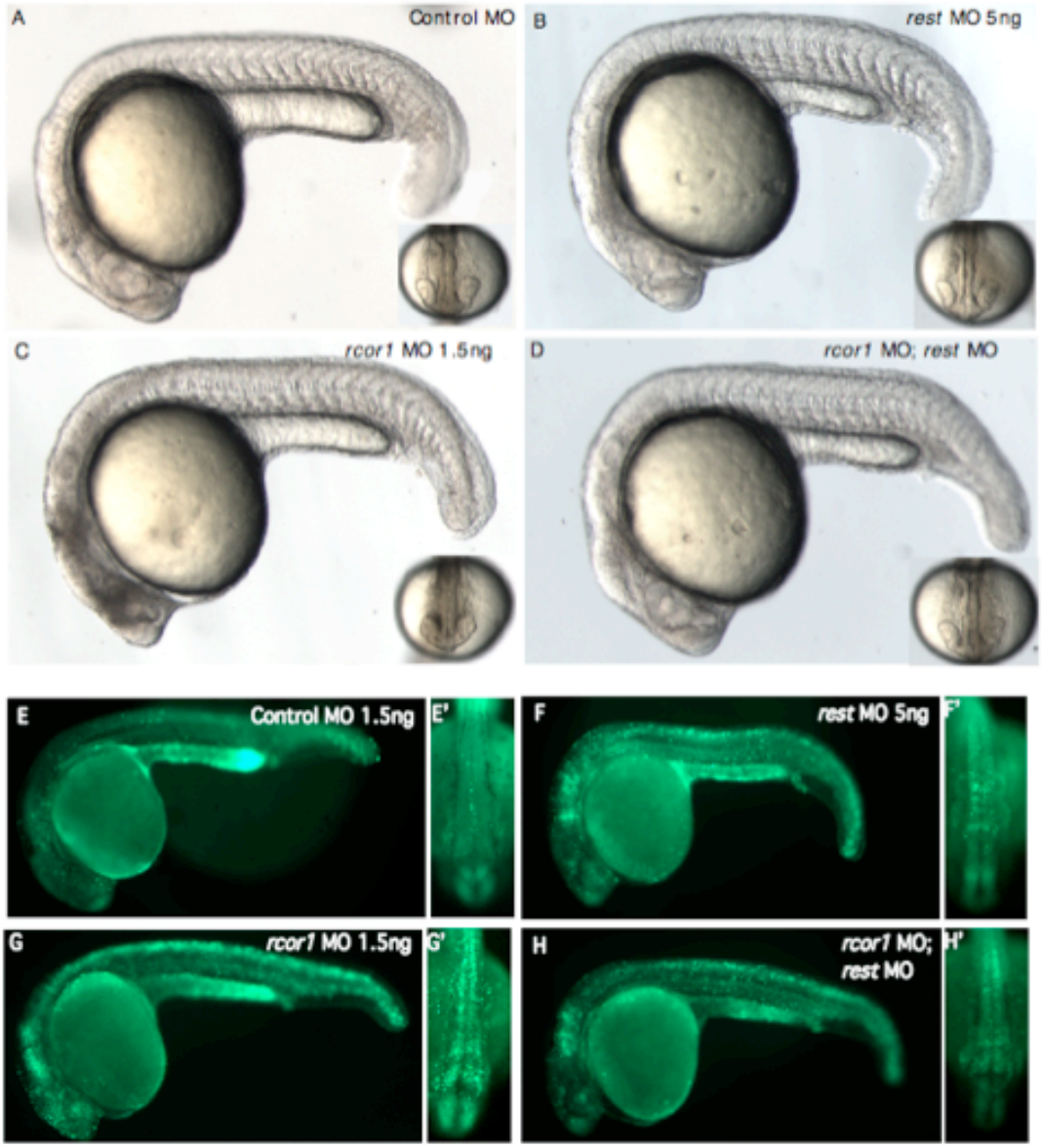

Figure A3. Rest knockdown reduces apoptosis in *rcor1* morphant.
Live images of control (A, E) rcor1 morphants (C,G) rest morphants (B, F). and double morphants (D,H) (A-D) DIC images, lateral and dorsal anterior (inserts) views of 22hpf live embryos. (E-H) Fluorescent images, lateral and dorsal hindbrain (E'-H') views of 24hr live embryos incubated in Acridine Orange media. Acridine orange labels apoptotic cells, which are evident as bright punctuated cells (G-H) (C) rcor1 morphants show normal gross development but opaque, presumably necrotioc, tissue is evident in head and hindbrain regions, in CNS and adjacent tissue..(G) Intense acridine orange labeling throughout embryo reveals widespread apoptosis. (D, F) the rest morphant is developmentally delayed and displays apoptotic cells confined to the posterior CNS. (D, H) necrosis and cell death are reduced in rcor1 morphants when rest MO is co-injected.(Compare H to G) The double morphants have apoptosis patterns more similar to the rest morphant (Compare F to H).

doses of *rcor1* MO result in severe defects earlier and massive cell death. This cell death is also eliminated by co-injection with *rest* MO, The embryo is not rescued in any other sense, as development appears largely halted at an early somitigenesis stage (not shown). This implies that Rest knockdown effect is due to a general inhibition of cell death. Although there is some apoptosis in the *rest* morphant, individual apoptosis inhibitors are known to work on specific cell death pathways but not others. The Rest knockdown in the *rcor1* morphant seems to affect the head mainly, whereas the hindbrain cell death that remains resembles that of the *rest* morphant alone.

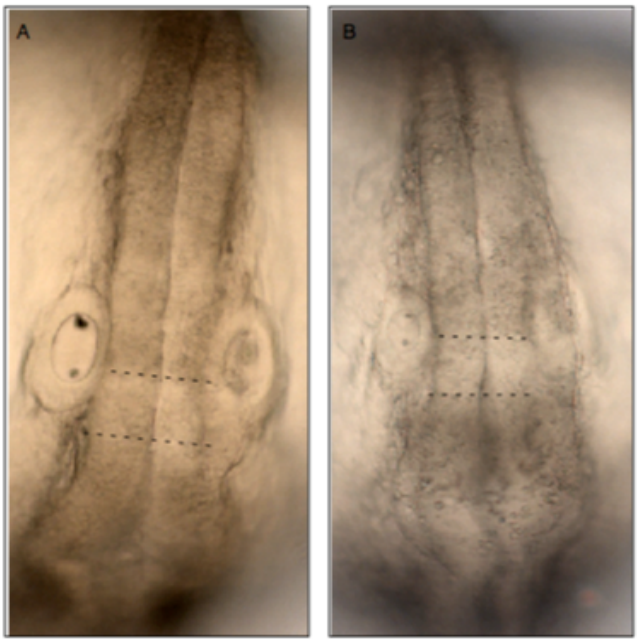

Figure A4. Neural necrosis as a morpholino side effect. DIC image, dorsal view , anterior down of hindbrains of live 24hpf embryos. rcor1 morphants with stereotypic cel death pattern, as indicated by darker, opaque tissue. Rhombomere 4 (between dashed lines) is typically relatively free of necrosis. (B) This same pattern can be obtained by injection of a morpholino designed against a Xenopus target with no known sequence similarities in zebrafish.

The degree to which putative non specific cell death obscures the effects of CoREST knockdown has not been established. This problem is highlighted in assays that follow proliferation at different stages after knockdown. At early stages, *rcor1* morphants have increased proliferation rates, as indicated by phosphorylated Histone H3 (pH3) antibody labeling (Fig.A.5). Sibling embryos fixed at a later stage, when neural degeneration becomes evident, show a relative decrease in pH3 staining compared to control. The embryos then exhibit recovery at subsequent stages, despite ongoing cell death. The outcome of the higher proliferation rates in cells at the intermediate stage is obscured by what may be off target effects.

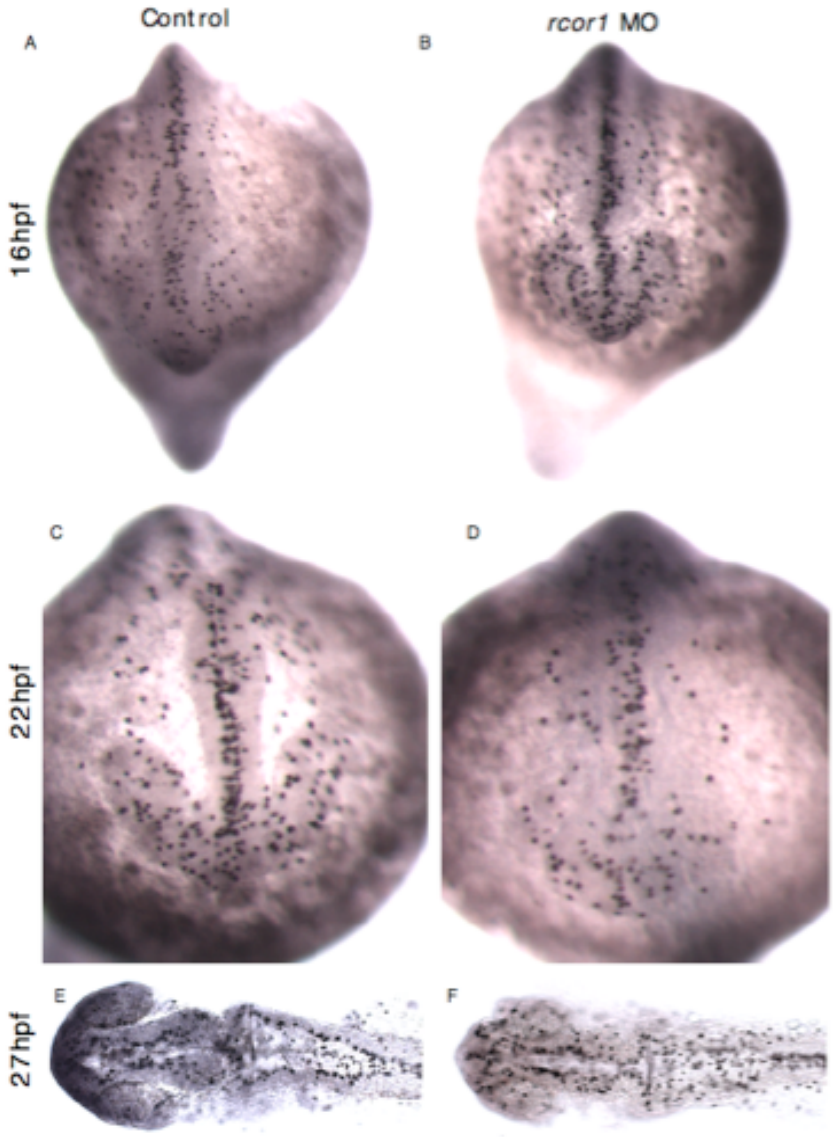

Figure A5. Early stage *rcor1* morphants show high rates of proliferation.
Embryos at different stages with mitotic cells labeled by PH3 immunostaining. (A-D) front view. (E, F) dorsal view, flat mounted embryos. (A,B) *rcor1* morphants show higher rates of proliferation than control embryos at 16hpf. (C, D) The number of mitotic cells is reduced compared to control 6 hours later. (E, F) rcor1 morphants recover at later stages and have comparable rates of proliferaton.

A.2.4 CoREST knockdown results in loss of proneural and neural markers

After cell death, the most prominent phenotype is a loss of proneural and neural markers. Proneural markers *ngn1* (Fig. A.6) and *zash1a* (not shown) are both reduced in head and hindbrain, whereas spinal cord staining was generally less affected. Pan-Neural marker *elavl3* is also severely reduced (Fig. A.7), and preliminary rescue attempts show this effect to be specific to CoREST knockdown. Co- Injection of expression plasmid containing full length *rcor1* resulted in partial restoration of *elavl3* expression. Examination of the live embryos demonstrates that adding back *rcor1* in this manner did not relieve the tissue degeneration phenotype (Not shown). This suggest that while at least some of the reduced neural markers can be specifically attributed to reduced *rcor1* levels, the necrosis may be a side effect of the morpholino.

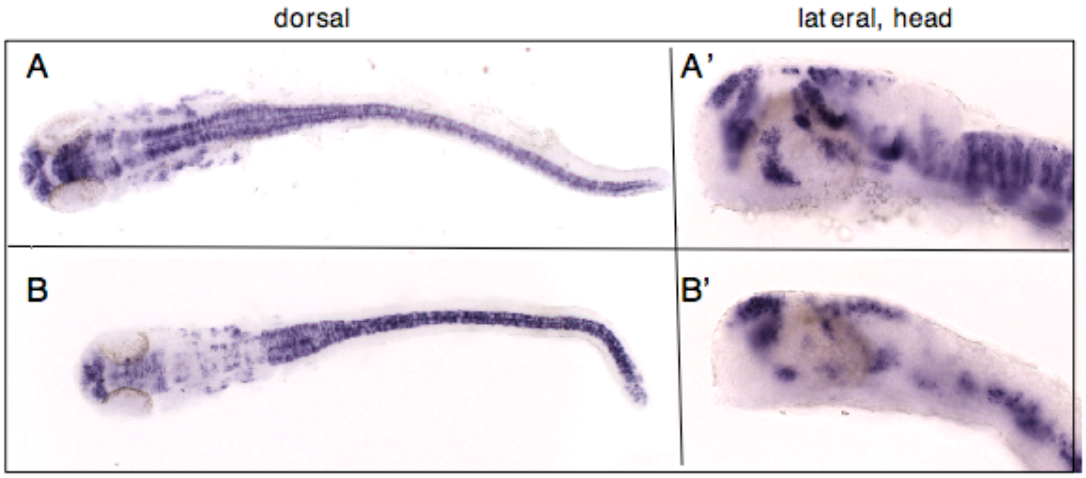

Figure A.6. CoREST knockdown results in loss of proneural gene *ngn1*. Dorsal (A, B) and lateral (A', B') views of 27 hpf control (A, A') and *rcor1*(splice) morphants. (B, B'). *rcor1* morphant shows a significant loss of *ngn1* expression in head and hindbrain. Sample in B is a milder representative and posterior regions .

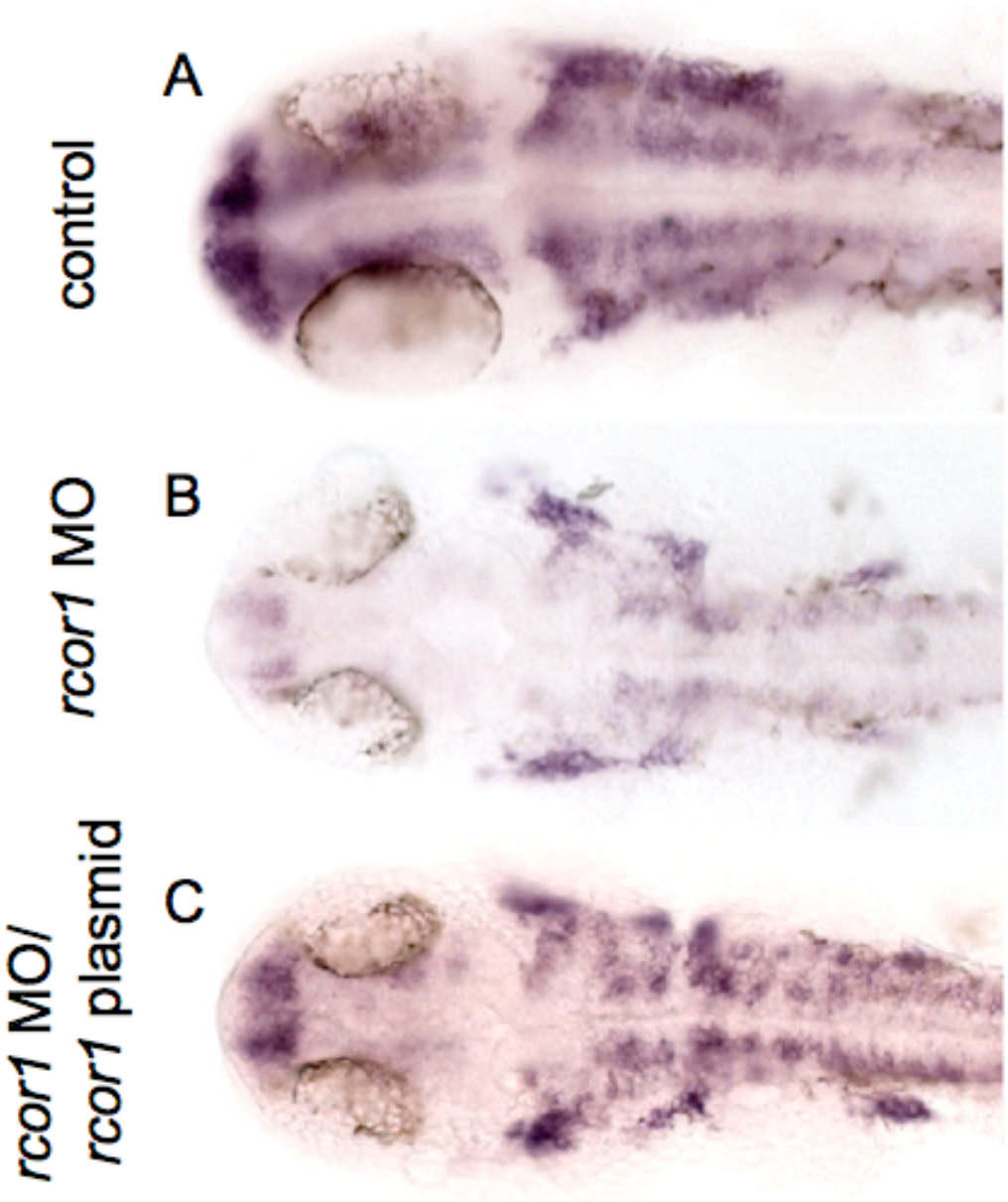

Figure A.7. Loss of Neural markers is specific to rcor1 knockdown. Dorsal views, flatmounted embryos stained for *elavl3/HuC*. (A) control embryos displaying wildtype elavl3 expression. (B) Embryos injected with *rcor1* MO and control plasmid show a significant decrease in this neural marker. (C) Embryos injected with *rcor1* MO and r*cor1* expression plasmid exhibit a partial restoration of *elavl3* expression (compare C to B) despite a similar cell death phenotype evident in live embryos (not shown).

A.2.5 Notch target *her6* is upregulated after CoREST knockdown

The loss of *elavl3* may be due to a depletion of ngn and zash progenitors caused by the cell death observed. The loss of proneural and neural markers may also be stem in part from the persistent expression of the notch target *her6*, a transcription factor that represses proneural targets. In control, *her6* expression is progressively restricted as wide scale neurogenesis occurs. (Fig. A.8). The morphant, however, shows failure to downregulate *her6* in several areas. In general, earlier expression persists even as the morphant makes appropriate morphological progression. (compare 27hr control embryos with 36hr morphants in Fig. A.7). The corresponding decrease of *elavl3* in sibling embryos suggests that Notch signaling may be enhanced in *rcor1* morphants.

A.2.6 CoREST functions downstream of Notch signaling

The morphants showed signs of increased Notch signaling, and CoREST has been shown to associate with Su(H) corepressor CtBP. It was therefore reasonable to hypothesize that CoREST in zebrafish functions as a corepressor of Su(H) in repressing *her6* expression. The *mind bomb (mib)* mutant has defective Notch signaling. Without activated Notch, Su(H) remains a repressor, and *her6* expression is constitutively repressed (Fig. A.9). If CoREST was a necessary part of the complex repressing *her6* , then *her6* may be derepressed even in the absence of activated Notch signaling. 1.5ng of *rcor1* ATG MO was injected into heterozygote *mib* in-cross embryos, and *in situ* hybridization was performed on the controls and morphants. After *in situ*, samples from the embryos were genotyped by PCR to identify the mutants. Fig. A. 9 shows that in *rcor1* morphants/*mib* mutants, *her6* was upregulated compared to control mutants. Thus, CoREST functions downstream of Notch signaling.

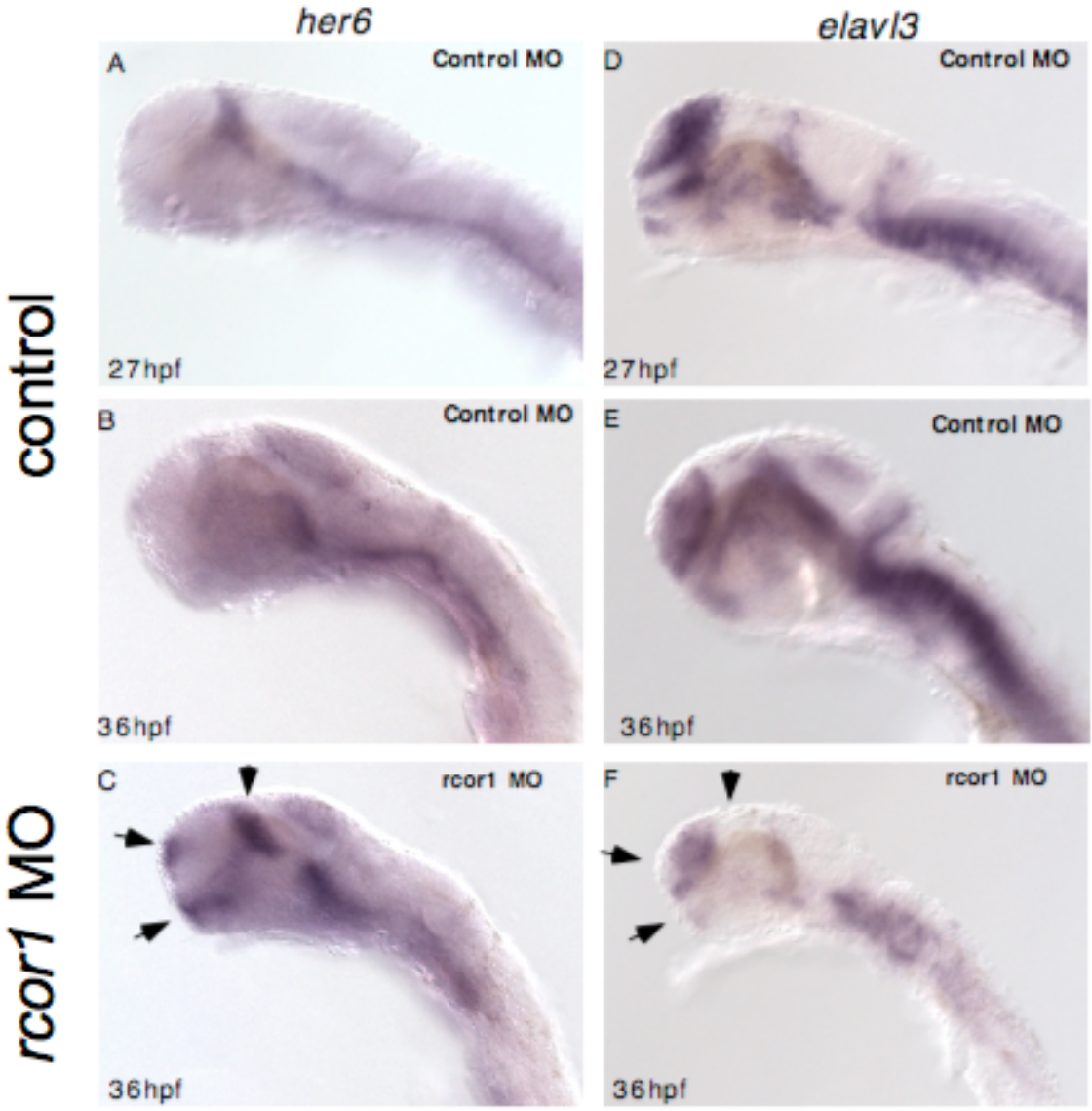

Figure A.8. *rcor1* morphants have increased notch signaling and decreased neural markers. Lateral view heads, of control (A-D) and rcor1 morphants stained for her6 (A-C) or elavl3 (D-F) (A, D) 27 control embryos shown for staging purposes. her6 expression decreases (compare B to A) and elavl3 staining becomes more abundant (compare E to D) as development proceeds. (B, C) 36hpf rcor1 morphants show normal morphology (note head shape) but increased her6 expression, indicative of excess notch signaling. (E, F) 36hpf rcor1 morphants show a severe decrease in panneural marker elavl3, even compared to much younger embryos (Compare F to D, E)

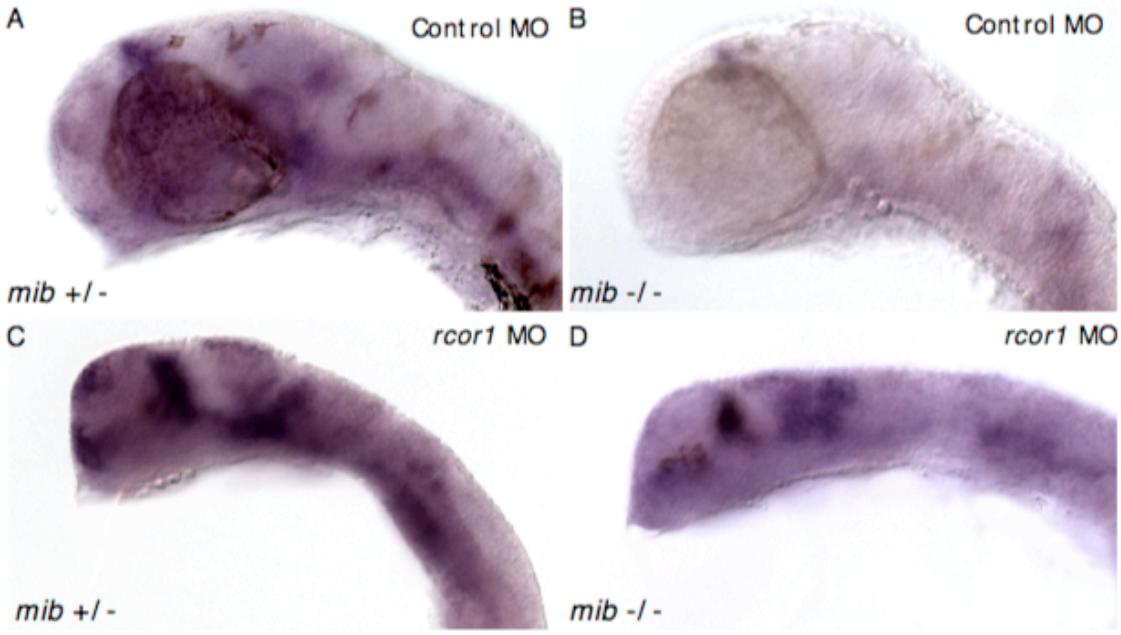

Figure A.9. CoREST acts downstream of Notch signaling to repress *her6*.
Lateral head views of 24hpf *mindbomb* mutants (B,D) or siblings (A, C).
(B) Defective notch signaling in *mindbomb* leads to Notch effector CBF1/Su(H) remaining in repressor mode, and target *her6* being suppressed. In mindbomb embryos with reduced levels of *rcor1* (D), this suppression is far less severe, indicating CoREST is a corepressor of CBF1 on the *her6* gene.

A. 3. Discussion and future directions.

CoREST is best characterized as corepressor to REST and so it was expected that a LOF phenotype would be similar to what expect for a Rest LOF . Based on the most familiar models of REST/CoREST function, loss of either might result in premature or ectopic neural markers. However, the preliminary data shows proneural and neural markers are in fact decreased. This may be due to perturbed Rest function (see Chapter 5), but could also be partly due to upregulated *her6*. In preliminary trials, Rest knockdown did not induce *her6* expression (not shown). This, and the mindbomb data(A.2.6) indicates a Rest-independent role for CoREST in antagonizing Notch signaling and promoting differentiation. As mentioned in the introduction, preliminary studies in fly may prove this to be a conserved function.

The expression data (A.2.1) also lends support to this idea. *rcor1* becomes

downregulated in proliferating population (the vz) then upregulated in the newly postmitotic population. A similar pattern of Xenopus rcor1 in the zone intermediate between proliferating and differentiated cells was interpreted as a role in promoting differentiation. Genes that are expressed exclusively in that intermediate zone of the developing optic tectum function in the promotion of cell cycle exit (Nguyen et al., 2001). Additionally, the cycle kinase inhibitor *cdkn1c*, a homolog of *p57*, is expressed in that zone (Park et al., 2005). This function of promoting differentiation would also be consistent with findings from Ballas et al., 2005, in which a NCoR complex containing CoREST is found repressing *Rest* expression in differentiating cells. The same study finds CoREST repressing REST targets after REST has been degraded, preventing premature expression of terminal differentiation genes. So, CoREST may have opposing roles at this stage.

The analysis is complicated by significant neural degeneration, which may stem from non-specific effects. However, preliminary rescue data is encouraging in that loss of the proneural and neural may be independent of this cell death. Additionally , the loss of proneural and neurogenic markers cannot wholly be attributed to cell death, because cell death and loss of markers does not necessarily correspond. In the trunk and tail, for instance, there is considerable cell death but no significant loss of *ngn* and *elavl3*. The rescue will need to be repeated for the markers shown. For future experiments along this line, the splice morpholino appears less toxic than the ATG morpholino, but shows the same reduction in proneural and neural markers and so is preferred. Morpholino induced apoptosis can also be controlled by co-injection with a p53 morpholino.

A. 4. Materials and methods

A.4.1. 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. mindbombta52b mutants, described in (Bingham et al., 2003) from heterozygote in-crosses were identified by PCR using the following primers .
Fwd 5'-GATGGATGTGGTAACACTGATGACTC-3'
Rev 5'-GGTGTGTCTGGATCGTCTGAAGAAC-3'

A.4.2. Zebrafish rcor1 cDNA isolation and alignment

Zebrafish rcor1 clone was bought from library. The full length rcor1 with 3' UTR was subcloned into pFRM 2.1 vector under the control of a carp beta-Actin promoter.

A.4.3 morpholino and plasmid microinjections

A splice inhibiting morpholino (MO) against the intron-exon boundary of zebrafish *rcor1* exon X
(5'-AGCCATTTCACTTACCATCTGCTGG -3') and a translation blocking MO (5'-TCTCGGTGGTGCCCTTCTCTAACAT -3') were synthesized by Gene Tools (Philomath, OR). Prior to microinjections, embryos were dechorionated in 1 mg/ml pronase (Sigma-Aldrich). Morpholinos were diluted in .2M KCl and phenol red from a 34mg/ml stock to 8-10mg/ml. MO was injected at the one cell stage, using 1.5 ng of ATG experiments or 5 ng of the splice MO. Equivalent amounts of the standard control morpholinos were used. 1.5pg carp beta actin-*rcor1* full length plasmid Equivalent amounts of carp beta actin- DS-Red plasmid was injected as control

A.4.4. 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., 1993). After *in situ* hybridization, embryos were mounted in 75% glycerol and photographed using a Zeiss Axiocam mounted on a Zeiss Axioplan microscope.

A.4.5. Microtome sections

After whole mount RNA *in situ* hybridization, embryos were dehydrated in ethanol, infiltrated and embedded in JB-4 resin (Ted Pella). For orientation of the embryos, embryos were embedded twice, as follows: For the first embedding, the infiltrated embryos were placed in a standard 2mL microcentrifuge tube with .750mL of embedding media. The tubes were closed and placed upside down so that the embryo lay flat on the bottom of the well located on the inside of the tube lid. This was allowed to harden overnight at 4°C. These samples were cut out and re-embedding in a BEEM embedding capsule (Ted Pella), and oriented so that the sample faced forward toward the tip of the tube. 10μM sections were obtained using an ultramicrotome (LKB 8800 ultratome III; Bromma).

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