Zebrafish rest regulates developmental gene expression but not neurogenesis

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SUMMARY
The transcriptional repressor Rest (Nrsf) recruits chromatin-modifying complexes to RE1 ‘silencer elements’, which are associated with hundreds of neural genes. However, the requirement for Rest-mediated transcriptional regulation of embryonic development and cell fate is poorly understood. Conflicting views of the role of Rest in controlling cell fate have emerged from recent studies. To address these controversies, we examined the developmental requirement for Rest in zebrafish using zinc-finger nuclease-mediated gene targeting. We discovered that germ layer specification progresses normally in rest mutants despite derepression of target genes during embryogenesis. This analysis provides the first evidence that maternal rest is essential for repression of target genes during blastula stages. Surprisingly, neurogenesis proceeds largely normally in rest mutants, although abnormalities are observed within the nervous system, including defects in oligodendrocyte precursor cell development and a partial loss of facial branchiomotor neuron migration. Mutants progress normally through embryogenesis but many die as larvae (after 12 days). However, some homozygotes reach adulthood and are viable. We utilized an RE1/NRSE transgenic reporter system to dynamically monitor Rest activity. This analysis revealed that Rest is required to repress gene expression in mesodermal derivatives including muscle and notochord, as well as within the nervous system. Finally, we demonstrated that Rest is required for long-term repression of target genes in non-neural tissues in adult zebrafish. Our results point to a broad role for Rest in fine-tuning neural gene expression, rather than as a widespread regulator of neurogenesis or cell fate.

KEY WORDS: Neurogenesis, REST (NRSF), RE1/NRSE

INTRODUCTION
Context-dependent regulation of gene expression is key to the generation of cell-type diversity during development. RE1-silencing transcription factor (Rest; also known as Neuron-restrictive silencing factor (Nrsf)) is thought to play a central role in the transcriptional repression necessary to achieve neural-specific gene expression (Chong et al., 1995; Schoenherr and Anderson, 1995). The ~23 bp repressor element 1/neuron-restrictive silencing element (RE1/NRSE) is the binding site for Rest and is associated with hundreds of neural genes in mammals (Bruce et al., 2004; Lunyak et al., 2002). Analysis of other vertebrate genomes, including zebrafish, has revealed that many possess rest homologs and comparable numbers of RE1 sites (Mortazavi et al., 2006).

Rest alters chromatin structure by mediating the assembly of multiple chromatin-modifying complexes. The N-terminal repressor domain of Rest recruits Sin3, which dynamically interacts with a wide range of repressor proteins including the methyl-DNA-binding protein MeCP2 and histone deacetylases (HDACs) 1/2 (Grzenda et al., 2009; Naruse et al., 1999). The Rest C-terminal domain interacts with CoREST (Ballas et al., 2001; Grimes et al., 2000) to assemble complexes that include HDACs 1/2 and histone H3 K9 methyltransferases (Lunyak et al., 2002; Roopra et al., 2004; Shi et al., 2003). The full repertoire of genes under the control of Rest is not regulated in unison and the context-specific requirements for Rest-mediated repression are unknown.

Initially, the chief function of Rest was considered to be the repression of neural genes in non-neural tissues. Recently, a more complex understanding of Rest has emerged. Rest also modulates neural gene expression within developing neurons and embryonic stem (ES) cells (Ballas et al., 2005; Singh et al., 2008). In these contexts, repression is transient, and Rest is proposed to delay target gene expression until the proper time. Consistent with this proposal, the transition from ES cell to neural progenitor to differentiated neuron is accompanied by reduction in Rest levels (Ballas et al., 2005; Guardavaccaro et al., 2008; Westbrook et al., 2008). However, the function of Rest in cultured stem and progenitor cells is controversial. Studies have reached opposing conclusions on the requirement for Rest as a regulator of stem cell pluripotency. Some have concluded that Rest is a key regulator of pluripotency or of the emergence of the neural lineage from stem cells (Singh et al., 2008; Sun et al., 2008; Gupta et al., 2009), whereas other studies differ in this view (Jørgensen et al., 2009a; Jørgensen et al., 2009b; Singh et al., 2008; Westbrook et al., 2008). Because a variety of factors influence the ability of cells to divide and differentiate, the outcomes of such experiments vary depending on the cell line and culture conditions employed.

Rest loss-of-function analysis has yet to provide a clear view of its role during embryogenesis. In the mouse Rest knockout, widespread cell death accompanies lethality by E11.5 (Chen et al., 1998). Expression of a few neural genes was observed in non-neural tissues, but the early lethality precluded further analysis;
specific molecular and cellular defects underlying the lethality remain unknown. In chick, inhibiting Rest with a dominant-negative construct resulted in ectopic expression of a few neural genes in non-neural tissues, but not in alterations of fate (Chen et al., 1998). Blocking Rest activity in Xenopus resulted in ectodermal patterning defects and the derepression of target genes (Olguín et al., 2006a). Previously, we knocked down zebrafish Rest using morpholinos and observed alterations of Hedgehog signaling (Gates et al., 2010).

To circumvent caveats associated with knockdowns and dominant-negative constructs, we employed zinc-finger nucleases to disrupt zebrafish Rest. Our in-depth analysis of the rest mutant established that expression of RE1-containing genes is enhanced in the mutant, and that blastula stage repression of some target genes requires maternal rest. Unexpectedly, neurogenesis progresses largely normally in rest mutants, although abnormalities were observed in oligodendrocyte precursor cells and in facial branchiomotor neuron migration. Analysis of an RE1/NRSE transgenic reporter system revealed that Rest represses gene expression in mesodermal derivatives including muscle and notochord, as well as within the developing nervous system. Although most rest homzygous zebrafish mutants die during larval stages, some survive to maturity. In these fish, some RE1-containing genes are inappropriately expressed in adult organs, demonstrating a long-term role for Rest in the repression of target genes in non-neural tissues. Together, these results establish Rest as a repressor of gene expression during embryogenesis and in adult non-neural tissue, but not as a broad regulator of cell fate or differentiation.

MATERIALS AND METHODS

Zinc-finger nucleosome targeting

ZiFiT (http://www.zincfingers.org/software-tools.htm) was used to identify zinc-finger nucleosome (ZFN) target sites that exclusively comprise GXX sequences. Two zinc-finger arrays were designed against each ‘half-target’ site (supplementary material Table S1). Conventional cloning strategies were employed to assemble arrays from clones in the Addgene ZF Kit. Each ZF array was cloned into KpnI/BamHI sites of the FokI-RR/FokI-DD vectors (Addgene). ZFN mRNA was synthesized using the mMESSAGE mMachine Kit (Ambion) and 10-25 pg of mRNA was injected into blastomeres of one-cell embryos. At 24 hours postfertilization (hpf), embryos were screened by PCR using primers 181 (5′-CTGA-GGGAAAGCAGATGATG-3′) and 184 (5′-TGTTGCTTGGTGATCT-3′). Quantitative PCR

Total RNA was extracted from tissues or pools of five embryos using Trizol (Invitrogen). SuperScript II reverse transcriptase (Invitrogen) was used to synthesize cDNA from 0.2-0.5 μg of total RNA. Quantitative (q) PCR was carried out with a LightCycler 480 (Roche) using 2× FastStart SYBR Green Master (Roche) or Quanta SYBR Green (Quanta Biosciences). Total RNA from each sample was normalized to 18S (ro data from this tissue or organs). Each experiment, three pools of five to three embryos or one adult organ were run in duplicate. Primer pairs are listed in supplementary material Table S3.

TSA treatments

Pronased embryos were placed in 40 nM Trichostatin A (TSA) or DMSO at the eight-cell stage and collected at 4 hpf for mRNA isolation.

Whole-mount in situ hybridization and immunostaining

Whole-mount in situ hybridization was performed as described by Thiese et al. (Thiese et al., 1993). Rest immunostaining was performed as described (Mapp et al., 2011).

RESULTS

Targeted rest disruption with zinc-finger nucleases

We disrupted zebrafish rest (Fig. 1; supplementary material Tables S1, S2) using zinc-finger nucleases (ZFNs) (Doyon et al., 2008; Kim et al., 1996; Meng et al., 2008) and recovered ten mutations in the first exon of rest. The majority of our studies utilized the rest[svbu29] allele (a 7 bp deletion), but we also examined viability of the rest[svbu34] allele (a 4 bp insertion). Both mutations produce similar frameshifts upstream of the DNA-binding domain and are likely null alleles. Unlike the mouse Rest knockout, rest[svbu29] and rest[svbu34] embryos appear grossly normal throughout embryogenesis and early larval stages [10 days postfertilization (dpf)]. However, adult mutants were not recovered at the expected Mendelian ratios. Of adult offspring of rest[svbu29] intercrosses, only 17/214 (7.9%) were homozygous mutants. Likewise, only 3/237 (1.3%) of adult offspring from rest[svbu34] intercrosses were homozygous mutants. A subset of the rest[svbu29] adults had posterior defects, while all three of the rest[svbu34] mutants were scrawny, had a severely curved axis and died by 3 months. Some surviving rest[svbu29] fish were fertile. Although we cannot exclude the possibility that the rest[svbu34] protein has antimorphic activity, it is likely that the phenotypic variations stem from genetic background variations within the wild-type strain that was used for targeting. The surviving mutants provided a unique opportunity to study the requirement for Rest in larval and adults.
RT-PCR analysis of rest sbu29/sbu29 mutants revealed that both wild-type and rest sbu29 messages are present at 6 hpf (early gastrula), but not at later stages (data not shown). The presence of wild-type rest mRNA in rest sbu29/sbu29 gastrulae reflects maternal rest expression and is consistent with our previous analysis (Gates et al., 2010). The lack of morphological defects in rest mutant embryos might be due to compensation by maternal rest mRNA. To eliminate maternal Rest activity, we generated embryos devoid of both maternal and zygotic rest (MZ rest sbu29/sbu29) by crossing homozygous rest sbu29/sbu29 females and males. To our surprise, these mutants also lacked overt morphological defects, demonstrating that Rest is dispensable for embryogenesis in zebrafish.

The nature of the rest lesions (indels) and their position within the first exon make it unlikely that either readthrough or alternative splicing can produce functional protein, as the first exon encodes zinc fingers that constitute part of the DNA-binding domain. To confirm the absence of Rest protein, we performed immunohistochemistry, which revealed abundant Rest protein in wild-type 6-hpf embryos but not in MZ rest sbu29/sbu29 mutants (Fig. 1C). This supports the conclusion that SBU29 is a null allele.

**Rest represses target gene expression during embryogenesis**

The zebrafish genome has ~1000 canonical RE1 sites (Mortazavi et al., 2006) (our unpublished results) and mammalian Rest binds teleost RE1 elements (Johnson et al., 2009; Tan et al., 2010). To determine whether loss of Rest affects target gene expression, we selected a set of RE1-containing genes with diverse cellular functions to analyze in the mutant. We studied their expression by qRT-PCR in MZ rest sbu29/sbu29 and wild-type embryos between 4 hpf (late blastula) and 16 hpf (mid-somitogenesis) (Fig. 2). During this time, expression of some Rest target genes was enhanced in MZ rest sbu29/sbu29. At 4 hpf, when the embryo comprises pluripotent blastomeres, the expression levels of most RE1-containing genes are either very low or absent in wild-type embryos (Fig. 2). At this stage, transcripts of gpr27, kcnh8 and snap25b can be detected in MZ rest sbu29/sbu29 mutants, but not in controls (Fig. 2A-C). Similarly, during somitogenesis (11 and 16 hpf), expression of these genes is low or absent in wild-type embryos, but much more robust in the mutant. Injection of morpholinos to impede rest translation also enhanced snap25b expression (data not shown).

Expression of bdnf, snap25a, grin1a, pcad (pcdh1g2) and cacng2 was enhanced in a stage-dependent manner in MZ rest sbu29/sbu29. However, we identified a group of Rest target genes (neurod, spop, gfp and bsp) that showed unchanged expression levels in MZ rest sbu29/sbu29 mutants, suggesting that both genomic context and cell type influence Rest activity. Consistent with our findings, previous studies have shown that Rest function is cell type dependent, and that blocking Rest does not derepress all target genes (Belyaev et al., 2004; Chen et al., 1998; Otto et al., 2007). Our findings provide the first evidence that Rest is required to suppress target gene expression in blastulae.
Maternal Rest function is required for early gene regulation

Our initial experiments did not distinguish the contributions of zygotic versus maternal Rest to the repression of target genes. To elucidate the role of maternal Rest in the regulation of RE1-containing target genes, we compared gene expression in progeny from \textit{rest\textsubscript{sbu29}}/\textit{+} females and wild-type males (maternal, \textit{M\textsubscript{restsbu29/\textsubscript{+}}}) to offspring of \textit{rest\textsubscript{sbu29/\textsubscript{+}}} females and wild-type males (zygotic, \textit{Z\textsubscript{restsbu29/\textsubscript{+}}}) as well as wild-type controls. Although \textit{Z\textsubscript{restsbu29/\textsubscript{+}}} embryos were used for this analysis, qRT-PCR demonstrated that loss of maternal Rest function increased levels of \textit{rest} transcript levels in wild-type, \textit{Z\textsubscript{restsbu29/\textsubscript{+}}} and \textit{M\textsubscript{restsbu29/\textsubscript{+}}} mutants, respectively, to wild types. Error bars indicate s.e. ND\textsuperscript{*}, not determined.

Four RE1-containing genes that are expressed at blastula stages were used for this analysis. qRT-PCR demonstrated that loss of maternal \textit{rest} function increased levels of \textit{snap25b}, \textit{bdnf} and \textit{pcad} at mid-blastula stage (Fig. 3A-C). In the reciprocal experiment, the expression levels of the RE1-containing target genes in \textit{Z\textsubscript{restsbu29/\textsubscript{+}}} embryos were more similar to wild-type levels (Fig. 3A-C). The expression levels of \textit{gpr27} expression in mid-blastula zebrafish embryos. Comparison of transcript levels in wild-type, \textit{Z\textsubscript{restsbu29/\textsubscript{+}}} and \textit{M\textsubscript{restsbu29/\textsubscript{+}}} embryos reveals derepression of target genes in \textit{M\textsubscript{rest}} but not \textit{Z\textsubscript{rest}} embryos. Fold comparisons are relative to \textit{M\textsubscript{rest}} transcript levels (defined as 1). Maternal (M) and zygotic (Z) embryos were obtained by crossing female \textit{M\textsubscript{restsbu29/\textsubscript{+}}} \textit{restsbu29/\textsubscript{+}} mutants, respectively, to wild types. Error bars indicate s.e. ND\textsuperscript{*}, not determined.

**Fig. 3. Maternal \textit{rest} suppresses target genes in mid-blastula embryos.** qPCR analysis of (A) \textit{snap25b}, (B) \textit{bdnf}, (C) \textit{pcad} and (D) \textit{gpr27} expression in mid-blastula zebrafish embryos. Comparison of transcript levels in wild-type, \textit{Z\textsubscript{restsbu29/\textsubscript{+}}} and \textit{M\textsubscript{restsbu29/\textsubscript{+}}} embryos reveals derepression of target genes in \textit{M\textsubscript{rest}} but not \textit{Z\textsubscript{rest}} embryos. Fold comparisons are relative to \textit{M\textsubscript{rest}} transcript levels (defined as 1). Maternal (M) and zygotic (Z) embryos were obtained by crossing female and male \textit{rest\textsubscript{sbu29/\textsubscript{+}}} mutants, respectively, to wild types. Error bars indicate s.e. ND\textsuperscript{*}, not determined.

REST-mediated histone modifications in blastulae are target gene dependent

Rest recruits chromatin-modifying enzymes to RE1/NRSE sites to alter histone acetylation and methylation states (Ballas et al., 2001; Huang et al., 1999). To determine whether effects on histone acetylation are central to the function of Rest during blastula stages, we examined the effects of the HDAC inhibitor TSA on target gene expression in wild-type and \textit{M\textsubscript{restsbu29/\textsubscript{+}}} embryos (Fig. 4). The difference between the effects of TSA on wild type versus \textit{rest} mutants reflects the relative contribution of HDAC-independent activities of Rest, namely histone methylation. At least three response profiles were apparent among the Rest target genes analyzed.

The expression levels of \textit{snap25a} and \textit{gpr27} in TSA-treated wild-type embryos were comparable to DMSO-treated \textit{M\textsubscript{restsbu29/\textsubscript{+}}} embryos (Fig. 4A,B). Alone, these findings suggest that HDAC recruitment might be the major component of Rest function at these loci. However, examination of transcript levels in TSA-treated \textit{M\textsubscript{restsbu29/\textsubscript{+}}} and \textit{Z\textsubscript{restsbu29/\textsubscript{+}}} embryos compared with DMSO controls. (C) TSA treatment of wild-type embryos produces a much smaller increase in \textit{snap25b} expression than does removing Rest function. This suggests that Rest-dependent histone methylation is central to repression of \textit{snap25b} expression. (D-F) HDAC inhibition has little effect on \textit{pcad}, \textit{spop} and \textit{grin1a} expression. Error bars indicate s.e.

**Fig. 4. Histone deacetylase contribution to Rest-mediated inhibition of target genes is locus specific.** qPCR analysis of RE1-containing gene expression at mid-blastula stage in wild-type and \textit{Z\textsubscript{restsbu29/\textsubscript{+}}} zebrafish embryos treated with DMSO or the HDAC inhibitor TSA. The fold differences are relative to transcript levels in \textit{M\textsubscript{restsbu29/\textsubscript{+}}} embryos (defined as 1). (A,B) TSA treatment enhances expression of \textit{snap25a} and \textit{gpr27} in both \textit{M\textsubscript{rest}} and wild-type embryos compared with DMSO controls. (C) TSA treatment of wild-type embryos produces a much smaller increase in \textit{snap25b} expression than does removing Rest function. This suggests that Rest-dependent histone methylation is central to repression of \textit{snap25b} expression. (D-F) HDAC inhibition has little effect on \textit{pcad}, \textit{spop} and \textit{grin1a} expression. Error bars indicate s.e.
observations reveal complex regulatory interactions at Rest target genes with histone deacetylation playing dramatically different roles at different loci. Surprisingly, the two zebrafish snap25 orthologs respond differently to TSA treatment, with repression of snap25b being almost entirely independent of HDAC activity.

We next asked whether key Rest co-factors were expressed in zebrafish blastulae. qRT-PCR analysis revealed that rcor1 (Corest1), rcor2 (Corest2), sin3aa, sin3ab and sin3b transcripts are present at blastula stage (supplementary material Fig. S1). Of these markers, only the expression of rcor1 was enhanced in MZrest<sup>pha29/bia29</sup> mutants (supplementary material Fig. S1). We analyzed rcor1 expression by RNA in situ hybridization and observed that many domains of expression overlap with rest expression (data not shown) and that rcor1 expression is slightly more robust in the mutant.

**Rest is not required for germ layer formation in zebrafish**

Studies from other organisms have not yielded a clear picture of the role for Rest during early development. In Rest knockout mice, midbrain head mesenchyme and myotomal cells in the somite are disorganized (Chen et al., 1998). However, overall germ layer formation and early patterning seem to be minimally affected (Chen et al., 1998). By contrast, perturbing Xenopus Rest expands the neural plate and reduces neural crest (Olguín et al., 2006b). To study whether Rest function is necessary for germ layer specification in zebrafish, we analyzed the expression of early patterning genes in MZrest<sup>pha29/bia29</sup> mutants. During gastrulation, expression of mesodermal (chd, ntl, myod1, foxa2), endodermal (foxa2) and neural (pax3) markers was not altered in MZrest<sup>pha29/bia29</sup> mutants (supplementary material Fig. S2). These results suggest that germ layer specification does not require Rest.

**Neurogenesis progresses normally in rest mutants**

Rudimentary observation of rest mutant locomotor behavior and gross neural anatomy did not reveal abnormalities. To further investigate the requirement for Rest in neurogenesis, the domains of proneural and pan-neural markers were assayed in MZrest<sup>pha29/bia29</sup> mutants by RNA in situ hybridization. Examination of the proneural markers ascl1a and neurog1 did not reveal abnormalities in MZrest<sup>pha29/bia29</sup> mutants (supplementary material Fig. S3). If Rest modulates the differentiation of neural progenitors, loss of Rest function might result in ectopic or premature neural differentiation. We examined expression of the pan-neural marker huC (elavl3) in MZrest<sup>pha29/bia29</sup> mutants between 10.5 hpf (2 somites) and 24 hpf. To our surprise, we did not detect inappropriate huC expression in MZrest<sup>pha29/bia29</sup> mutants (Fig. 5). We also examined the expression of an huC:GFP transgenic reporter in the MZrest<sup>pha29/bia29</sup> mutants at 1 dpf and 5 dpf, but did not observe alterations in expression (data not shown). From these results, we conclude that Rest does not play a fundamental role in zebrafish neurogenesis.

**olig2<sup>+</sup> oligodendrocyte precursors are reduced in rest mutants**

Neural fate determination appears grossly normal in rest mutants. To study subtle differences in neural subpopulations that lack functional Rest, we examined olig2<sup>+</sup> cells in MZrest<sup>pha29/bia29</sup> using an olig2:GFP transgene (Fig. 6). This transgenic line marks progenitors in the ventral spinal cord domain that give rise to motor neurons and oligodendrocyte precursor cells (OPCs). By 3 dpf, OPCs occupy distinct positions after migrating dorsally from the progenitor population (Park et al., 2002). The ventral spinal cord expression of olig2:GFP was not altered in rest mutants, but a deficit was apparent in the number of OPCs in the rest mutants at 50 hpf. On average, the rest mutants had only 13.4±2.4 OPCs (± s.e.; n=16) compared with 37.5±7.0 (n=8) OPCs in sibling controls. OPC deficiencies remain at 74 hpf (data not shown), but by 98 hpf the number of OPCs in the mutants had recovered to levels comparable to sibling controls (Fig. 6). Similar deficits were observed in OPCs following Rest knockdown using morpholinos (data not shown).

As in other species, the zebrafish OPC population is tightly regulated and compensatory mechanisms can overcome deficits (Kirby et al., 2006). The zebrafish rest mutant phenotype resembles the mouse Olig1 knockout, in which early deficits of OPCs are subsequently overcome (Lu et al., 2002). Recent studies have provided evidence that Rest regulates glial differentiation, including emergence of oligodendrocytes (Abrajano et al., 2009; DeWald et al., 2011; Soldati et al., 2012). Our in vivo results implicate Rest-regulated gene expression in the development of the OPC lineage in zebrafish.

**Facial branchiomotor neuron migration is impaired in rest mutants**

Facial branchiomotor neurons (FBMNs) are a subset of the cranial branchiomotor neurons common to all vertebrates. Zebrafish FBMNs are born in rhombomere (r) 4 of the hindbrain and migrate tangentially to r6-r7 (Chandrasekhar, 2004). Migration begins after the first neurons are born at 16 hpf and is complete by 48 hpf, when the FBMNs have reached their final destination in r6-r7. Rest is expressed in FBMs (supplementary material Fig. S4) and inhibition of Rest function by morpholino knockdown or a
dominant-negative approach was previously shown to cause defects in FBMN migration (Mapp et al., 2011). Using an islet1-GFP transgene, we examined rest mutants for defects in FBMN migration (Fig. 6). Following an intercross of rest\textsuperscript{uju29/+} and rest\textsuperscript{uju20/+}; islet1-GFP fish, we observed three phenotypic classes. Out of 59 embryos, ten had wild-type FBMN positioning (Fig. 6E). Genotype analysis revealed that nine of these were wild type for rest and one was a heterozygote. A second class showed very subtle changes in FBMN migration, exhibiting minor disorganization of neurons in r6 and r7 and a slight increase in the number of FBMNs remaining in r5 at 48 hpf (Fig. 6F). All 40 embryos in this class were rest\textsuperscript{uju20/+}; rest\textsuperscript{uju29/+} heterozygotes. The final phenotypic class showed more severe migration defects and seven out of nine of these embryos were rest\textsuperscript{uju29/uju29} homozygous mutants. In the mutants, many neurons were unable to migrate properly into r6, remaining instead in the r4-r5 region (Fig. 6G). These phenotypes closely resemble previously described Rest morphant phenotypes, and confirm a requirement for Rest function in control of FBMN migration.

Repression of RE1-containing genes by Rest at late embryonic and larval stages

Having observed derepression of Rest target genes in the mutant prior to 13 hpf (Fig. 2), we sought to establish whether Rest continues to repress target genes at later stages. As the embryo begins to mature and neurons differentiate, the overall expression levels of Rest target genes increase in wild-type embryos. We compared expression levels of RE1-containing genes in MZrest\textsuperscript{uju29/uju29} mutants and wild types by qRT-PCR between 1 and 19 dpf. Expression levels of nearly all target genes were comparable between mutants and wild types (supplementary material Fig. S5). Notable exceptions were gpr27, which showed elevated expression in rest mutants at all stages, and kcnh8, bdnf and pcad, which were elevated at 24 hpf in the mutants. We also employed more sensitive approaches to assay snap25a expression. However, neither in situ hybridization nor a new snap23a-GFP transgenic reporter line (data not shown) revealed evidence of ectopic expression.

Monitoring Rest activity with a transgenic reporter system

Although Rest-mediated repression of target genes in vitro is well studied, little is known about the spatial or temporal requirements for Rest in intact animals. Analysis is complicated by the nature of Rest activity: epigenetic chromatin modifications mediated by Rest may endure once Rest protein is degraded. To dynamically map tissues in the developing embryo where Rest activity is required, we crossed the rest\textsuperscript{uju29} allele into four transgenic enhancer trap lines [Et(NRSE-cfos:KalTA4), hereafter NRCK (X.X. and J.S.M., unpublished)] that each contain an RE1/NRSE element adjacent to a minimal c-fos promoter upstream of KalTA4, an optimized Gal4-VP16 driver (Distel et al., 2009).

Activity of these lines was monitored by crossing them to a 14xUAS:Nfsb-mCherry transgenic reporter line that expresses a fusion protein between nitroreductase (Ntr) and a monomeric red fluorescent reporter, mCherry [Tg(UAS-E1b:Nfsb-mCherry);c264, hereafter 14xNtrCh] (Davison et al., 2007). The UAS-linked mCherry reporter is dependent on KalTA4 activation. Therefore, the spatial and temporal expression of mCherry depends on the genomic context of the KalTA4 enhancer trap insertions. Owing to the influence of the RE1/NRSE element, NRCK lines express KalTA4 primarily in the nervous system, which in turn restricts expression of mCherry to the nervous system in double transgenics. Two of the lines, NRCK\textsuperscript{gmc606} and NRCK\textsuperscript{gmc607}, drive expression principally in the nervous system and weakly in the mesoderm. Two additional lines, NRCK\textsuperscript{gmc632} and NRCK\textsuperscript{gmc641}, display expression patterns that are almost exclusively restricted to the nervous system. In all cases, expression of mCherry reporters was altered in rest mutants.

The first line, NRCK\textsuperscript{gmc606}, drives mCherry expression in the spinal cord, a few notochord cells and in an occasional muscle fiber (Fig. 7A). The progeny from crosses between rest\textsuperscript{uju29/+}; NRCK\textsuperscript{gmc606}; 14xNtrCh;c264+ and rest\textsuperscript{uju29/+} fish fell into two phenotypic classes. The first class expressed mCherry in the expected predominately neural NRCK\textsuperscript{gmc606} pattern (n=33/50) and comprised only wild-type and rest heterozygous embryos (Fig. 7A). By contrast, the remaining embryos (n=17/50) had significantly expanded mCherry expression, with many notochord cells and numerous muscle fibers expressing mCherry, and were all rest mutants (class 2, Fig. 7B).

The NRCK\textsuperscript{gmc607} line expresses mCherry within the spinal cord, but also in a few muscle fibers and enteric neurons (Fig. 7C). Among progeny of rest\textsuperscript{uju29/+}; NRCK\textsuperscript{gmc607}; 14xNtrCh;c264+ and rest\textsuperscript{uju29/+} crosses, a phenotype (class 2) emerged that showed expanded neural expression, widespread expression in muscle of the trunk and head and in notochord (Fig. 7D). Genotyping revealed that 11/13 (84.6%) class 2 embryos were rest mutants (Fig. 7J). Conversely, only 4/51 (7.8%) of class 1 embryos, which displayed the expected expression pattern, were rest mutants. In summary, analysis of the NRCK\textsuperscript{gmc606} and NRCK\textsuperscript{gmc607} lines...
demonstrates that Rest acts on RE1/NRSE elements to repress expression of neighboring genes in mesodermal derivatives including muscle and notochord between 3 and 6 dpf. To our knowledge, this is the first evidence for Rest activity in the notochord.

Two additional lines, NRCK gmc632 and NRCK gmc641, are expressed almost exclusively within the nervous system. The NRCK gmc632 line labels sparse cells within the spinal cord and brain. At 4 dpf, the progeny of rest sbu29/+; NRCK gmc632/+; 14xNtrChc264/+ and rest sbu29/+ crosses again fell into two phenotypic classes (Fig. 7E,F). The majority of embryos (65/85) displayed the expected pattern of expression (class 1). The second class had broader expression within the spinal cord, including numerous interneurons (Fig. 7F). All 23 class 2 embryos that had broader expression were rest mutants (Fig. 7K). Class 1 comprised 64 wild-type and heterozygous embryos and a single rest mutant.

The neural-specific NRCK gmc641 line expresses primarily in secondary motor neurons. The progeny of rest sbu29/+; NRCK gmc641/++; 14xNtrChc264/+ and rest sbu29/+ crosses fell into three phenotypic classes at 3 dpf. In class 1, mCherry expression was observed in two parallel rows of cells along the spinal cord (Fig. 7G,G’/H11032). Class 2 (n=33) had similar expression except that mCherry-positive cells were frequently observed in the midline (Fig. 7H,H’). Genotypic analysis of class 2 revealed that mutants were predominant, representing 66.7% (22 embryos), whereas only 3% (1 embryo) was wild type (Fig. 7L). Conversely, mutants comprised only 9.2% (6/65 embryos) of class 1 (Fig. 7L). In class 3 (n=31), fewer cells expressed mCherry and it was not possible to assess the midline of these individuals. Genotype analysis of this class revealed no enrichments of any genotype.

The NRCK gmc641 results suggest that either mutant neurons prematurely express the RE1/NRSE transgene or that newly differentiated mutant neurons fail to migrate properly. By 5 dpf we could no longer distinguish between class 1 and class 2 specimens, as both had many mCherry-positive midline cells (data not shown).

Although we did not observe alterations in the subset of Rest target genes that we studied at larval stages, analysis of the reporter lines verified that Rest suppresses the expression of target genes between 3 and 6 dpf in muscle and notochord. In addition, analysis of these lines demonstrated that Rest is also required within the developing nervous system to repress gene expression. Taken together, these data suggest that Rest-mediated repression is highly context dependent.

Rest is required to maintain the repression of target genes in adult tissues

To determine whether Rest mediates long-term repression, we isolated brain, muscle, liver, heart, spleen and pancreas from adult wild-type and rest sbu29/+; snap25b/+; grp27/+; grin1a/+; bdnf/+ and neurod/+ transcript levels (Table 1). In wild-type animals, each of these markers showed variable expression between animals over many months. Although this variability might have obscured some effects, clear trends emerged nonetheless. In rest mutants, expression of snap25b was derepressed in pancreas, spleen and liver, whereas grin1a was derepressed in pancreas and muscle (Table 1). Expression of bdnf was enhanced in rest mutant spleens, but changes in neurod expression were not apparent in any of the organs tested. It has been suggested that Rest functions as a transcriptional activator in
Table 1. Derepression of RE1-containing genes in adult tissues of rest mutants

<table>
<thead>
<tr>
<th>Marker</th>
<th>Muscle</th>
<th>Pancreas</th>
<th>Heart</th>
<th>Brain</th>
<th>Spleen</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>gpr27</td>
<td>+</td>
<td>++</td>
<td>n.t.</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>snap25b</td>
<td>+++</td>
<td>+++</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>++</td>
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<tr>
<td>bdnf</td>
<td>+</td>
<td>--</td>
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<td>++</td>
<td>++</td>
<td>n.a.</td>
</tr>
<tr>
<td>grn1a</td>
<td>+++</td>
<td>++</td>
<td>--</td>
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</tr>
<tr>
<td>neurod</td>
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All markers were run on three independent tissue samples. Plus signs indicate the number of samples in which the given marker was derepressed in the mutant. n.t., not tested; n.a., no amplification.

Although some rest<sup>shu29/shu29</sup> adults produce viable offspring, their fertility and fecundity often wane more rapidly than those of wild-type fish. We therefore examined the expression of RE1 target genes in ovaries and testes (Fig. 8). Strikingly, snap25b expression was near the threshold of detection in wild-type ovaries, but expression was more abundant in rest mutant ovaries. In addition, levels of gpr27, kcnh8 and pcad expression were elevated in the mutant ovaries. To determine whether this effect was specific to female reproductive tissue, we examined all the markers in testes, but only kcnh8 expression was enhanced (Fig. 8). These observations demonstrate a high degree of tissue and locus specificity in the effects of Rest on target genes.

**DISCUSSION**

To examine the requirement for Rest in the context of an intact animal, we disrupted zebrafish rest using ZFNs. Based on previously proposed roles for Rest, we anticipated that this would cause inappropriate or failed neurogenesis. Contrary to this expectation, the mutants underwent relatively normal neurogenesis, although defects were observed in OPCs and in the migration of the FBMNs (Fig. 6). We observed inappropriate expression of genes and reporters in the mutant (Figs 2, 3, 6 and 7), but no clear alterations of cell fate (Fig. 5; supplementary material Figs S2, S3). Although our findings do not reveal any requirement for Rest in early embryos, paradoxically, some of the largest changes in gene expression that result from Rest disruption occur during blastula stages (Fig. 2). Under some conditions, this failure to restrain inappropriate transcription might influence the proliferation or developmental potential of these cells.

**Does Rest function differ in zebrafish and mouse?**

Mouse Rest mutants undergo widespread cell death and die by E11.5 (Chen et al., 1998). By contrast, zebrafish rest mutants survive for at least 2 weeks. The disparity between the zebrafish and mouse phenotypes might stem from distinct compensatory mechanisms present in zebrafish, or from alterations in rest function or targets between species. It is plausible that mechanisms that compensate for the lack of Rest function in zebrafish and that are less prominent in mammals. The most likely of such mechanisms would involve a second rest or rest-like gene, as gene duplicates are common in zebrafish (Postlethwait et al., 2000). Functional similarities have been proposed between rest and several invertebrate genes including spr-3, spr-4, charlatan and tramtrack (Dallman et al., 2004; Lakowski et al., 2003; Tsuda et al., 2006). A zebrafish homolog of one of these factors might also compensate for loss of Rest function. We extensively searched the zebrafish genome for rest-related genes and have not detected a second rest ortholog or another rest-like gene. In addition, we found that snap25a promoter-GFP transgenic lines that contain a scrambled RE1 element generally mirror expression of the wild-type

![Fig. 8. Rest target genes are depressed in germ tissue of adult rest mutants. qPCR analysis of RE1-containing gene expression in wild-type and rest<sup>shu29/shu29</sup> mutant ovary and testes (three examples of each are shown). All fold differences are relative to the average of the transcript levels in the three mutants (defined as 1). (A-D) Levels of snap25b, kcnh8, gpr27 and pcad are enhanced in the rest mutant ovary. (E) Only kcnh8 expression was enhanced in rest mutant testes.](https://example.com/image-url)
type promoter (data not shown). If additional factors act on the snap25a RE1 site, mutating the site should have caused inappropriate expression of the reporter.

We favor the model that the phenotypic differences in mouse and zebrafish rest mutations stem from differences in target genes. Zebrafish have a comparable number of RE1 sites to other vertebrates (Mortazavi et al., 2006). Fish RE1/NRSE elements have been shown to bind mammalian Rest (Johnson et al., 2009) and the mammalian and zebrafish Rest DNA-binding domains exhibit a high degree of conservation (Gates et al., 2010). However, a key difference might be in the positioning of RE1/NRSE sites. Many genes have RE1/NRSE sites that are conserved between fish and mammals, but some sites are unique. Because regulatory elements are thought to be prime targets for evolutionary change (King and Wilson, 1975), such differences are expected. The cause of the lethality of the mouse rest knockout remains unknown, but it precedes neurogenesis. The rampant cell death that is observed in the mouse knockout could potentially result from misregulation of a single locus that is not under the control of Rest in zebrafish.

While this manuscript was in preparation, a report was published showing that a neural-specific conditional Rest knockout does not alter neurogenesis, although effects were noted in cultured cells isolated from mouse (Aoki et al., 2012). Although further analysis of rest conditional knockouts is required, these findings support our conclusion that Rest is not essential for neurogenesis and highlight similarities between Rest function in zebrafish and mice.

What is the role of Rest?
Given the size and frequency of RE1 sites, strong selective pressure must maintain their presence throughout the zebrafish genome. Although we identified embryonic requirements for Rest in OPC development and in cranial nerve migration, these deficits are less severe than would be expected for a master regulator of neurogenesis. We also detected changes in the expression of RE1-containing genes during embryogenesis and early larval development. These changes were most apparent during blastula and somite stages and markedly reflected precocious gene expression. The large fold differences between rest mutants and wild type during these stages reflects low or absent expression in wild type and more easily detectable expression in the mutant. Our data provide the first in vivo evidence that Rest regulates gene expression in ES cells (blastomeres). However, no alterations in cell fate were observed in the mutants. Future analysis of the mutants might reveal biases in the developmental potential of Rest-deficient blastomeres, but our analysis does not support a broad role for Rest in cell fate determination. In this context, Rest might promote the fitness of stem cells by suppressing the premature production of target transcripts.

During later stages of embryogenesis, many of the loci we analyzed appeared to be largely unaffected by loss of Rest. Our analysis of target gene expression during late embryonic and larval stages relied primarily on qPCR, and subtle alterations in gene expression might have gone undetected. However, we also examined expression of a snap25a-GFP reporter line in the mutant and failed to detect changes in expression in the absence of Rest at these stages (data not shown). Simpler reporter constructs (the NRSE KalTA4 enhancer trap lines) revealed substantial Rest activity in mesodermal derivatives including muscle and notochord, as well as within the nervous system (Fig. 7). These findings suggest that Rest-mediated repression of RE1/NRSE sites is highly context dependent and such activity might be more apparent in the milieu of the NRSE-basal promoter constructs. From these observations, we suspect that the chief function of Rest at these stages is to fine-tune the expression of RE1-containing genes rather than as a robust silencer of expression.

Analysis of adult tissues from surviving rest mutants provided the first proof that Rest is required in non-neural tissues for long-term suppression of target gene expression (Fig. 8, Table 1). These effects were most pronounced in the germ tissue. Our anecdotal observations suggest that rest mutants tend to have reduced fertility compared with wild types. These observations suggest that Rest might have a role in the maintenance of the adult germline or simply contributes to overall fitness.

Rest has been proposed to function within the nervous system, but we did not observe changes in gene expression in rest mutant brain. However, many Rest targets are robustly expressed in the brain and are involved in a host of essential neural processes. Discrete alteration of expression of these genes might have profound consequences for higher-order neural functions. In addition, recent studies of a conditional Rest knockout in the mouse revealed a requirement for Rest in adult neurogenesis (Gao et al., 2011). Regulation of gene expression in the adult brain may be a primary Rest function. Future studies will explore the behavioral consequences of Rest depletion.

Regulation of neural migration may be a principal function of Rest. Abnormalities in FBMN migration were observed in our rest mutant. In addition, the OPC deficiencies and secondary motor neuron defects (in the NRCK^mc641 reporter line) might also stem from altered migration. Adhesion molecules are abundant among Rest targets (Otto et al., 2007), and Rest has been implicated in the regulation of radial migration of mouse neocortical progenitors (Mandel et al., 2011). Together, these data suggest a key role for Rest in neuronal migration.

In conclusion, the zebrafish rest mutant is a valuable tool that will help to broaden our understanding of Rest function. Our analysis reveals that although Rest represses the expression of RE1-containing target genes in several contexts, the effects often appear to modulate the timing or levels of expression during development rather than to silence vigorous expression. Although we identified discrete requirements for Rest during development, we found no evidence for a role as a fundamental regulator of neurogenesis or cell fate during development. Examination of the mutant also demonstrated that Rest-mediated repression of target genes persists in non-neural tissues in adult animals. Additional studies of the mutant will clarify the functions of Rest within the nervous system and in non-neural tissue to establish a more comprehensive view of Rest regulation of an expansive set of target genes.

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Competing interests statement
The authors declare no competing financial interests.


