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CoRest1 regulates neurogenesis in a stage-dependent manner

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Abstract

Background: Developmental processes, including neuronal differentiation, require precise regulation of transcription. The RE-1 silencing transcription factor (Rest), is often called a "master neuronal regulator" due to its large number of neural-specific targets. Rest recruits CoRest (Rcor) and Sin3 co-repressor complexes to gene regulatory sequences. CoRest not only associates with Rest, but with other transcription regulators. In this study, we generated zebrafish rcor1 mutants using transcription activator-like effector nucleases (TALENs), to study its requisite role in repression of Rest target genes as well as Rest-independent Rcor1 developmental functions.

Results: While rcor1 mutants have a slight decrease in fitness, most survived and produced viable offspring. We examined expression levels of RE1-containing genes in Maternal Zygotic rcor1 mutants (MZrcor1) and found that Rcor1 is generally not required for repression of Rest target genes at early stages. However, MZrcor1 mutants undergo more rapid neurogenesis compared to controls. We found that at gastrula stages, Rcor1 acts as a repressor of her gene family, but at later stages, many of these genes were increased in the MZrcor1 mutant.

Conclusions: Based on these findings, the central role of CoRest1 in neurogenesis is likely due to a Rest-independent role rather than as a Rest co-repressor.

Keywords

CoRest1; Rest/NRSF; neurogenesis; zebrafish

Introduction

The nervous system arises from pools of stem and progenitor cells through the highly regulated processes of neurogenesis. Tight control of gene expression is required to promote the emergence of neurons from undifferentiated cells. One key regulator of neural gene transcription is the RE-1 silencing transcription factor/Neuron restrictive silencing factor (Rest/Nrsf) (Chong, Tapia-Ramirez et al. 1995, Schoenherr and Anderson 1995). Rest is a transcriptional repressor that binds to a highly conserved ~23 nt DNA element called the RE1 site (Kraner, Chong et al. 1992, Lunyak, Burgess et al. 2002) to regulate neurogenesis, inhibit cellular differentiation, fine-tune expression of neural genes, and suppress expression of target genes in non-neural tissues (Ballas, Grunseich et al. 2005, Lunyak and Rosenfeld 2005, Gao, Ure et al. 2011, Aoki, Hara et al. 2012, Kok, Taibi et al. 2012, Moravec,

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Samuel et al. 2016). Co-repressor proteins recruited by Rest alter chromatin landscapes and modulate gene transcription include CoRest, Sin3, MeCP2, LSD1, and HDACs (Huang, Myers et al. 1999, Battaglioli, Andrés et al. 2002, Lunyak, Burgess et al. 2002, Cunliffe 2008).

Association of chromatin modifying factors with the C-terminal Rest repressor domain is mediated by CoRest proteins, which are a highly conserved family of co-repressors (Andrés, Burger et al. 1999). CoRest family members lack site-specific DNA binding activity but associate with DNA through interactions with other proteins. CoRest1, together with lysine-specific demethylase 1 (LSD1) and histone deacetylases (HDACs) 1/2, forms a powerful repressor known as the LCH complex (Gocke and Yu 2008, Sáez, Gómez et al. 2015). LSD1 binds to the second SANT domain of CoRest and HDACs 1/2 to the ELM2 and first SANT domain (You, Tong et al. 2001, Shi, Matson et al. 2005, Yang, Gocke et al. 2006). LSD1 removes methyl groups from histone 3 at lysine 4 or 9 (H3K4/H3K9) (Lee, Wynder et al. 2005), while HDACs remove acetyl groups from histone tails (Jenuwein and Allis 2001).

There are three vertebrate CoRest genes: *rcor1*, *rcor2*, and *rcor3*, with CoRest1 (Rcor1) being the first one identified (Tontsch, Zach et al. 2001, Zeng, Kong et al. 2010). When compared to CoRest1, CoRest2 and 3 have decreased repressor activity (Barrios, Gómez et al. 2014). CoRest2 repressor activity is not dependent on HDACs as alterations in the first SANT domain curtail interactions with HDACs. In comparison with CoRest1, CoRest3 is a less potent activator of LSD1 (Upadhyay, Chowdhury et al. 2014). These functional differences led us to speculate that CoRest1 is the primary Rest co-repressor and the other family members play secondary roles as Rest co-repressors.

Unlike *rest, rcor1* is found in invertebrates (Jarriault and Greenwald 2002, Bruce, Donaldson et al. 2004, Dallman, Allopenna et al. 2004), which suggests both ancient and Rest-independent functions of Rcor1. Like *rest* mutants (Chen, Paquette et al. 1998), murine *rcor1* mutants are embryonic lethal (Yao, Goldman et al. 2014). In both vertebrate and invertebrate models, Rcor1 appears important in early stages of neurogenesis. Early in mouse development, *rcor1* is expressed in neural tissue but decreases near birth (Tontsch, Zach et al. 2001). CoRest1 is also expressed at early neurulation in areas of active neurogenesis in *Xenopus* embryos (de la Calle-Mustienes, Modolell et al. 2002). In mouse cortex, CoRest is required to maintain progenitor pools and limit the number of differentiating neurons (Monaghan, Nechiporuk et al. 2017).

Although CoRest1 was isolated through its association with Rest, Rcor1 interacts with other transcription factors including C-terminal binding protein (CtBP), selected mouse cDNA on the X (SMCX)/Jumonji AT-rich interactive domain 1C (JARID1C), chromodomain on Y-like (CDYL), insulinoma-associated 1 (INSM1), Serine carboxypeptidase-like (Scpl), and Specificity protein 3 (Sp3) (Hakimi, Bochar et al. 2002, Shi, Sawada et al. 2003, Khalil, Guttman et al. 2009, Monaghan, Nechiporuk et al. 2017). Studies examining the independent role of the LCH during development are further parsing out the roles CoRest plays in neural processes and neural fate determination.

We generated a zebrafish *rcor1* mutant to determine whether Rcor1 is the key Rcor family member required for Rest repression of target genes during early development and to assess the requirement for Rcor1 during neurogenesis. We found that expression of most Rest target genes was unaffected in *rcor1* mutants early in development, indicating a locus-specific requirement for Rest-mediated repression. Examination of pro and pan-neural gene expression in *rcor1* mutants revealed increases in neural progenitors and early differentiating neurons, respectively. Accordingly, we observed misregulation of *her* genes in M*Zrcor1* mutants in a stage-specific manner suggesting that Rcor1 regulates the rate of neurogenesis through Rest-independent complexes.

Materials and Methods

Fish husbandry

Fish were housed, maintained, and genotyped as previously described (Kok, Taibi et al. 2012, Moravec, Li et al. 2015, Moravec, Samuel et al. 2016). Adult fish were genotyped and separated by sex into 1.8L tanks at 3 months of age, housed in groups of eight.

TALENs-generated mutations

TALENs were designed to target the first exon of *rcor1* using the zfit TALEN algorithm (Reyon, Khayter et al. 2012). Target sequences were AACAATTCATGGGAGGA and TCAAGTGATGACGAGCA. TALENs were synthesized by Genescript and cloned in the JDS70 vector (Sander, Cade et al. 2011). mRNA corresponding to the two TALENs was synthesized using the Message Machine kit (Ambion) and microinjected into 1-cell wild-type embryos. At 24 hours post fertilization (hpf); embryos were collected and mutations were screened by PCR. Primer pairs used for screening and subsequent genotyping: RCOR1 F2 (5’-GAGGGGCAGGAACTCTGTAA-3’) R2 (5’-GAACCCGAACTTCCTTCCTC-3’).

Quantitative RT-PCR

Total RNA was extracted from pools of eight sphere staged embryos and pools of six shield (6 hpf) and bud (10 hpf) stage embryos using Trizol (Invitrogen) and ZYMO Research Direct-zol RNA MiniPrep. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was performed with a Light Cycler 480 (Roche) using QuantaSYBR Green (Quanta Bioscience). Transcript levels from each sample were normalized to β-actin and error bars represent standard error. Each experiment consisted of three pools of embryos run in duplicate with mutants set to 1. Primer pairs used were previously described in (Kok, Oster et al. 2007, Kok, Taibi et al. 2012, Moravec, Samuel et al. 2016).

Whole mount RNA *in situ* Hybridization

Embryos were fixed in 4% PFA at 4°C overnight, washed with PBT, then stored in methanol at −20°C (~15-20 embryos per tube). Whole mount RNA *in situ* hybridization protocol was adapted from (Thisse, Thisse et al. 1993); digoxigenin labeled probes were made using T3, T7, and Sp6 RNA polymerase. For heterozygous crosses genotypes were determined post-hoc by PCR. Measurements of domain expression were taken and compared by Student’s t-test with error bars representing standard error.

Live imaging

rcor1$^{sbu54/+}$ allele was crossed with an *elavl3*:GFP transgenic reporter background. Embryos from the heterozygous cross were placed in 1-phenyl 2-thiourea (PTU) during segmentation to inhibit pigmentation. 26hpf, 50hpf, and 98hpf embryos were anesthetized with MS-222 (Tricaine) and mounted on 3% methyl cellulose. 12-15 lateral and dorsal images were acquired at each stage during one imaging session, and embryos were genotyped post-imaging. Average fluorescence was quantified for the olfactory bulb/forebrain and midbrain/hindbrain regions of the larva using ImageJ. Averages were compared by Student's t-test with error bar representing standard error.

Behavior

6 days post fertilization (dpf) larval behaviors were recorded using a Zebrabox imaging system (Viewpoint Life Sciences, France) constantly illuminated by infrared light and tracked with automated video-tracking software (Zebralab; Viewpoint Life Sciences, France). All experiments were conducted during the hours of 12 to 6 p.m. The visual-motor behavior paradigm consisted of 20 minutes of acclimation, 15 minutes in the light followed by a stimulus (light change), and 15 minutes in the dark. At 24hpf, larvae were placed in 24-well plates, with one larva placed per well. The software tracked parameters such as small and large movements, distance, duration, spatial preference, and stimulus-evoked movements upon stimulation (change from light to dark). Data was assessed in 1-minute bins for the analysis of spontaneous movement and 1 second bins to capture acute response to stimulus. The sex of the larvae cannot be determined at this stage and therefore could not be taken into consideration.

Movements were analyzed using repeated measures ANOVA with the independent variable being genotype and the repeated measure being time on SPSS. Significance was defined as $p < 0.05$ for all tests and error bars represent the standard error of the mean. A Student's t-test was used to compare average number between wild-type and mutant. This work was approved and conducted in accordance to the Stony Brook University Institutional Animal Care and Use Committee.

Results

rcor1 is expressed in neurogenic regions in larval zebrafish

Whole mount RNA *in situ* hybridization was used to determine the dynamic expression pattern of *rcor1* expression during early zebrafish development. At early stages, *rcor1* is widely expressed throughout the embryo, but becomes gradually restricted to the head (Figure 1A-E). This pattern is similar to *rest* expression at comparable stages (Gates et al. 2010). By 36 hpf, *rcor1* expression is enriched in the eyes, posterior optic tectum, and midline (Figure 1D, E) with these domains becoming further refined by 57 hpf (Figure 1F-I).

rcor1 expression was also studied in transverse sections of 42 hpf wild-type larva in four regions: forebrain, midbrain, hindbrain, and anterior trunk. *rcor1* is expressed in non-neural tissue, proliferative, and undifferentiated populations of the neural tube, including undifferentiated regions of optic tectum (Figure 1K). *rcor1* is also enriched in areas where

the neural tube is undergoing differentiation, revealing a dynamic expression pattern. Expression is robust in proliferative zones in the brain including along the midline of the forebrain, midbrain, and hindbrain (black bracket, Figure 1J-L). Expression of *rcor1* in the tegmentum and hindbrain (Figure 1K-M) is lower in the cells closest to the ventricular zone compared to the adjacent cells. The same pattern is evident throughout the hindbrain, which is undergoing a similar rate of neurogenesis at this stage.

rcor1 mutants are viable

To determine the requirement for CoRest1 during development, we disrupted *rcor1* using TALENs. Five germline mutations in *rcor1* were isolated (Figure 2C). All of the lesions are predicted to produce frameshift mutations that result in premature stop codons and eliminate the bulk of the protein including most of the ELM2 domain and both SANT domains (Fig. 2A), which are required for association with HDACs and LSD1 respectively (Barrios, Gómez et al. 2014). PCR analysis of cDNA prepared from MZ*rcor1*$^{sbu54/sbu54}$ embryos at 10 hpf failed to detect the wild-type allele. Only the smaller amplification product corresponding to mRNA produced from the mutant allele was present (Figure 2E). Because the SBU54 (Δ7bp) lesion produced a strong disruption of Rcor1, we primarily utilized this allele for our studies. Prior to these experiments, the *rcor1*^{sbu54} line was outcrossed for four generations to minimize possible non-linked off-target TALEN lesions.

Offspring of *rcor1*$^{sbu54/+}$ intercrosses all appeared morphologically normal at 6 dpf. Although many homozygous *rcor1* mutants survived to adulthood, they were not recovered at the expected Mendelian ratios (83/423 fish, 19.6%, Fisher's exact test $p \leq 0.011$), suggesting a slight reduction of fitness of the mutants (Figure 2D). Surviving *rcor1*$^{sbu54/sbu54}$ adults appear morphologically normal and are fertile. This contrasts the effects of a conditional Corest1knockout in mice, where the mutants die by P7 (Yao, Goldman et al. 2014). Because zebrafish *rcor1* is supplied to the embryo as a maternal transcript (Kok, Taibi et al. 2012), we sought to determine whether fish lacking both maternal and zygotic *rcor1* mRNA (MZ*rcor1* mutants) presented with severe defects. Like the zygotic *rcor1* mutants, MZ*rcor1* mutants appear morphologically normal and survive to adulthood. Therefore, most of the experiments described below were performed on MZ*rcor1* fish because of the ease in obtaining large numbers of mutants.

Expression of Rest target genes in *rcor1* mutants

Among CoRest family members, Rcor1 forms the most robust repressor complexes and is therefore often assumed to be the main co-repressor for Rest, but this premise has not been rigorously evaluated. Because Rest represses RE1-containing genes in early zebrafish development, we assessed the requirement for CoRest1in the repression of Rest target genes in blastula stage embryos. For this analysis, we selected a varied subset of RE1-containing genes that are de-repressed in *rest* mutants (Kok, Taibi et al. 2012, Moravec, Samuel et al. 2016) and had a wide range of functions during development (Figure 3A). cDNA was prepared from MZ*rcor1* mutants and related wild-type embryos at 4 hpf (late blastula) shortly after the onset of zygotic transcription, thus minimizing potential secondary effects. In the absence of maternal and zygotic *rcor1*, qPCR revealed *snap25b* expression was enhanced (de-repressed) (Figure 3A), but no significant changes in gene expression of the

nine other Rest target genes were observed, while expression of *amphiphysin* and *gpr27* was reduced (Figure 3A). These results indicate that Rcor1 is only required for repression of one of the 12 Rest target genes analyzed at late blastula stage, *snap25b*.

To determine whether Rcor1 regulates *snap25* expression at later stages, we assayed *snap25a/b* expression in 24 hpf *MZrcor1* and related wild-type control embryos using RNA *in situ* hybridization. No difference in expression was observed for *snap25a* in *rcor1* mutants (Figure 3 B-E). However, ectopic *snap25b* expression was observed in the midbrain and hindbrain of *rcor1* mutants (arrowhead in Figure 3C, n=6/10 embryos). These results suggest that while Rcor1 is important for Rest-mediated repression of *snap25b*, repression of many other targets occur independent of Rcor1. Presumably repression of these Rest target genes relies on other Rcor family members or additional co-repressors such as Sin3. However, *rest, rcor2*, and *rcor3* by qRT-PCR showed no differences in their mRNA levels in the *rcor1* mutant (data not shown).

rcor1 fish are hypoactive

Snap-25 is a SNARE protein that promotes vesicle fusion at the cell membrane (Clary, Griff et al. 1990). Failure of Rest-mediated repression of *snap25a/b* contributes to altered locomotor behavior in MZ*rest* mutants and *snap25a* or *snap25b RE1* site mutants (Moravec, Samuel et al. 2016). Therefore, we asked whether similar locomotor defects are apparent in MZ*rcor1* larvae using a visual-motor behavior paradigm (Figure 4A). This test assesses spontaneous movement in both light and dark conditions as well as the evoked response of the fish to a stimulus (light change). Individual larvae were assayed in 24-well dishes at 6 dpf. For the entirety of the assay, the following parameters were recorded for each fish: number of movements made, duration of movement, distance traveled, and location within the well.

Overall the MZ*rcor1* larvae were hypoactive in both the light and dark conditions. MZ*rcor1* larvae (n=36) initiated 1835±119.352 movements in the light while the wild-type controls (n=36) made significantly more movements (2385±175.884 movements) (Figure 4C). During each minute of the assay, MZ*rcor1* mutants initiated fewer movements (Figure 4B). On average, MZ*rcor1* larvae initiate 122 movements a minute compared to wild-type that make around 159 movements a minute (Figure 4B). This pattern is also observed when comparing duration (mutants= 10 seconds, WT= 12 seconds) and distance traveled (mutants= 72 mm, WT=104 mm). In the dark, the MZ*rcor1* mutants continued to move less than the wild-type controls, with mutants producing 1832±67 movements on average (compared to 2485±98 from wildtypes Figure 4E). In addition to making fewer movements, MZ*rcor1* larvae spent significantly less time moving. During the light interval the mutants each spent less than 150 seconds in motion while wild-type moved ~184 seconds; in the dark, the same pattern is seen with wild-type spending more time moving than mutants (Figure 4E, K). The wild-type controls traveled a greater distance in both light and dark, 1555±122 mm and 1769±91 mm, compared to the MZ*rcor1* larvae that traveled less than 1200 mm in both lighting conditions (Fig. 4G, M).

Because MZ*rest* mutants display anxiety-like behaviors (Moravec, Samuel et al. 2016) and prefer the outer regions of the well, spatial preference was also analyzed for MZ*rcor1*

mutants. In total, the MZ*rcor1* mutants spent more time exploring the inner well compared to wild-type (180±21 seconds and 98±15 seconds, respectively), while the controls spent more time in the outer (767±20 seconds) well than mutants, in the light (678±27 seconds) (Figure 5A). Across both the light and dark periods, the MZ*rcor1* mutants made fewer movements, spent less time moving, and traveled less than the related wild-type controls. There was no significant difference between wild-type and mutant in response to an evoked stimulus (light change). The mutant larvae exhibited a startle response that was comparable to that of the wild-type controls, suggesting the MZ*rcor1* mutants do not have gross visual or motor impairments to hinder their movements (Figure 5B).

CoRest1 is required for normal progression of neurogenesis

Based on the locomotor defects in the MZ*rcor1* mutants (Figures 4,5) and the expression pattern of *rcor1* (Figure 1) in neurogenic regions, we sought to explore the role CoRest1 plays in neurogenesis. Therefore, we examined pan neural marker, *elalv3*, by RNA *in situ* hybridization and live-imaging in the mutant. In both assays, we observed enhanced expression of *elalv3* in the mutant. At 13hpf (segmentation), RNA *in situ* hybridization revealed increased expression of *elalv3* in the midbrain, tegmentum, and in the trunk (Rohon-Beard neurons) in 13/17 MZ*rcor1* mutants when compared to related wild-type controls (n=30) (Figure 6A-B). Enhanced expression of *elalv3* was also detected in MZ*rcor1* (22/26 embryos) at 24hpf with robust expression along the midline from midbrain to tail when compared to wild-type (n=16) (Figure 6 C-E).

The *rcor1*^{sbu54} allele was crossed into the *elavl3*:GFP transgenic reporter background (Park, Kim et al. 2000) to allow for dynamic visualization of differentiating neurons in living fish. Fish that were heterozygous for the *rcor1* mutation and the *elavl3* transgene were crossed to *rcor1* heterozygotes and larvae were collected at 26, 50, and 98hpf, then imaged using fluorescent microscopy. At 26hpf, a significant increase of *elavl3*:GFP fluorescence was observed in the midbrain/hindbrain of the *rcor1* mutants compared to wild-type siblings (Fig. 6 F, I, L) (*rcor1*$^{+/+}$ N= 3, *rcor1*$^{+/-}$ N=6, *rcor1*$^{-/-}$ N= 6). No significant difference was observed in *elavl3*:GFP fluorescence at 50 and 98hpf, in *rcor1* mutants (Figure 6 G, H, J, K, L). This data demonstrates that differentiating neurons emerge more rapidly in MZ*rcor1* mutants at early developmental stages than in wild-type controls, but this defect does not persist.

Modulation of *her* gene family expression by CoRest1

The effects of a lack of CoRest on the increased neurogenesis that we observed could be explained if Rcor1 regulated expression of *her* genes, which are Notch targets known to be involved in maintaining neural progenitor pools and determining fate decisions. To test this hypothesis, we examined expression of *her* genes in MZ*rcor1* mutants by qRT-PCR and RNA *in situ* hybridization. We performed qPCR at 6 and 10hpf on Notch targets: *her1, her6, her15, her4*, and *nort* (Jarriault, Brou et al. 1995, Takke, Dornseifer et al. 1999, Tsutsumi and Itoh 2007) (Figure 7). Expression of *her4* and *nort* are largely neural, while *her6, her1, and her15* are both neural and mesodermal (Bae, Shimizu et al. 2005, Chapouton, Webb et al. 2011).We found that regulation of *her* genes by Rcor1 are stage- dependent. At 6dpf in MZ*rcor1*$^{sbu54/sbu54}$ mutants *her1, her6*, and *her4* transcript levels are increased while *her15*

(p=0.06) and *nort* (p=0.08) levels trend upward compared to wild-type (Figure 7A, C, E, G, I). Levels of these genes were comparable in MZ*rcor1* mutants and controls at 10hpf (Figure 7J). In comparison, neural expression of *her6* by RNA *in situ* hybridization revealed decreased expression in MZ*rcor1* mutants in comparison to wild-type controls at 13, 24, and 36 hpf (Figure 8A-F, Q). At 13hpf, little *her6* expression is apparent in the hatching gland of *rcor1* mutants and there is a sizable decrease of expression in the brain. At 24 and 36hpf, the trend remains, with decreased expression of *her6* in the brain/head region of the embryo in MZ*rcor1* mutants when compared with controls.

Because *her* genes regulate expression of proneural markers (Jarriault, Brou et al. 1995) we examined expression of proneural genes *neurogenin* (*ngn1*) and *zash1a* in *MZrcor1* mutants using whole mount RNA *in situ* hybridization. Ectopic *ngn1* expression was detected in the hindbrain of MZ*rcor1* mutants at 13 hpf (13/16 embryos) and 24 hpf (6/10 embryos), but expression domains were restored to wild-type levels by 36 hpf (Figure 8G-L). *zash1a* expression was not detected 13 hpf (image not shown), but an increase of *zash1a* in MZ*rcor1* mutants is observed at 36 hpf (15/15 embryos), with an expansion of *zash1a* visible in the hindbrain and trunk of the mutant larva when compared to wild-type (Figure 8M-Q). Together these results reveal stage-dependent modulation of *her* genes by Rcor1, with Rcor1 serving as a repressor at early stages but having the opposite effect later in development. Activation of *her* genes, later in development, presumably accounts for the increased expression of proneural markers and an altered rate of early neurogenesis observed in MZ*rcor1* mutants.

Discussion

CoRest1 was the first member of the Rcor family identified. It has frequently been studied in association with the Rest repressor complex and has been thought to play a pivotal role in Rest repressor function. However, Rcor1 is also associated with other proteins that modulate neurogenesis. We found that CoRest1 plays a nuanced role in Rest-mediated transcriptional repression in early zebrafish development suggesting that other co-repressors must play leading roles at these stages. Nonetheless, modulation of *her* genes by Rcor1 regulates the progression of neurogenesis in a stage-dependent manner.

CoRest as a Rest co-repressor

Surprisingly, only one of the Rest target genes that we examined (*snap25b*) was derepressed in MZ*rcor1* mutants (Figure 3). All of the genes we assayed are increased in MZ*rest* mutants at blastula stages suggesting that these effects presumably stem from Rest repressive activity that is independent of CoRest1. In many contexts *snap25* is particularly sensitive to manipulations of Rest levels and large alterations of *snap25* levels are often observed (Bruce, Donaldson et al. 2004, Prada, Marchaland et al. 2011, Moravec, Samuel et al. 2016). In zebrafish, *rest* loss-of-function mutants both *snap25a/b* genes are de-repressed in the hindbrain of 24hpf embryos (Moravec, Samuel et al. 2016). We previously found that at blastula stages, treatment of wild-type embryos with the HDAC inhibitor, TSA, increased *snap25b* to levels comparable with MZ*rest* mutants (Kok, Taibi et al. 2012), which indicates that Rest-dependent HDAC activity (mediated by Rcor1) is key to repression of *snap25b* at

blastula stages. However, TSA had different effects on expression of other RE1-containing genes suggesting variation in Rest complexes between loci. This is consistent with our analysis of *rcor1* mutants which shows that Rcor1 is only required at a subset of Rest targets genes. Together, these findings support the model that Rcor heterogeneity contributes to differential regulation of targets. It is also likely that some degree of redundancy of function exists between family members even though Rcor2 or Rcor3 are insufficient to provide repression of *snap25b* in the absence of CoRest1. Studies of the effects on expression of Rest target genes in the absence of Rcor2 or Rcor3 or combinations of multiple Rcors will be required to sort out locus-specific requirements for CoRest activity in Rest-mediated repression. Analysis of mouse conditional *rcor1/2* double mutants revealed a much stronger phenotype in the double mutants than the single mutants, which is consistent with the notion of overlapping functions of *rcor* family members (Monaghan, Nechiporuk et al. 2017).

rcor1 dysfunction results in atypical swimming patterns

Because enhanced *snap25b* expression is associated with hyperactivity in zebrafish (Wei, Thatcher et al. 2013) we examined locomotor behavior of MZ*rcor1* mutants and found them to be hypoactive. This phenotype is similar to zygotic *rest* mutants, which retain maternal *rest* expression but not MZ*rest* larvae and *snap25b-RE1* site mutants which have more profound deficits in Rest repressor function at the *snap25b* locus and exhibit hyperactive spontaneous swimming patterns (Moravec, Li et al. 2015, Moravec, Samuel et al. 2016). Locomotion is a complex behavior governed by many genes. However, the observation that loss of maternal and zygotic *rcor1* and *rest* (MZ*rcor1* and MZ*rest*) produce opposite behaviors is consistent with our conclusion that Rcor1 is not the chief Rest co-repressor during early development. The movement deficits in MZ*rcor1* result from disruption of other Rcor1 dependent regulatory complexes.

CoRest1 regulates neurogenesis

In the developing zebrafish nervous system, *rcor1* is well positioned to regulate neurogenesis. *rcor1* transcripts are enriched in regions of neural differentiation (Figure 1). Likewise, in the mouse, *rcor1* is expressed in undifferentiated neural stem and progenitor cells (Abrajano, Qureshi et al. 2010, Welcker, Hernandez-Miranda et al. 2013, Monaghan, Nechiporuk et al. 2017). Comparison of zebrafish *rcor1* expression with *rest* expression at 42hpf (Gates et al. 2010) shows that though both are expressed in the proliferative zones, *rcor1* expression extends laterally into the adjacent zone containing cells exiting mitosis. These cells located intermediately between the ventricular zone and the differentiated lateral populations, also expresses neural progenitor markers such as *sox3*, as well as cell cycle exit factor *cdkn1c* (Park, Boyce et al. 2005). *hdac1* also shows a similar expression pattern, where it plays a role in promoting neurogenesis (Cunliffe 2008). Murine CoREST1 is associated with the RARE complex mediated repression Rest in freshly post-mitotic cells (Ballas, Grunseich et al. 2005).

Although *rcor1* mutants are generally viable and show no outward defects as adults, we detected accelerated rates of neurogenesis in early development. Presumably, regulatory mechanisms that control neuron numbers compensate for the loss of CoRest1 over longer time periods restore the number of neurons in MZ*rcor1* mutants to near wild-type

levels. The slight decrease in fitness of the mutants could stem from animals where this compensation is partial. Alternatively, Corest1 has been implicated in host defense systems to establish viral latency (Zhou, Du et al. 2013) and failure of immune related functions could result in decreased fitness. Because of the modest percentage of Rest target genes altered in MZ*rcor1* mutants and the more robust effects on *her* family members, the overt effects on neurogenesis likely stem from alteration of *her* gene expression. However, in mouse conditional *rcor1/2* double mutants, neurogenesis is enhanced in a Rest-dependent manner (Monaghan, Nechiporuk et al. 2017), which points to Rest-dependent influences of Rcor1 on neurogenesis. As we have only analyzed a small subset of Rest target genes, we cannot rule out direct or indirect effects of Rest on *her* gene expression during zebrafish neurogenesis.

Modulation of *her* genes by CoRest1

Our results show reduced *her6* and enhanced neurogenesis which is largely consistent with a positive influence of Rcor1 on *her6* expression. However, knockdown of Rcor1 in the mouse cerebral cortex increases *hes1* and decreases *neurogenin* expression (Lopez, Saud et al. 2016). This result appears to be diametrically opposite to our results that show decreases in *her6* and an increase in *ngn1* expression (Figure 8). At earlier stages, we observe that Rcor1 repressed several *her* genes (Figure 7). This demonstrates that stage and tissue impact the nature of the Rcor1 interaction with the *her* gene family. Furthermore, in the mouse knockdown experiments, residual Rcor1 may also impact the character of regulation of the *hes* genes and subsequent proneural markers. CoRest1 binds both LSD1 and HDAC1/2 forming a dynamic repressor complex; context-dependent activity of Rcor1 by regulation of chromatin modifiers could account for seemingly opposing effects of Rcor1 on the *her* gene family. While the exact mechanism has yet to be fully elucidated, these results indicate that Rcor1 modulates *her* in a highly stage and tissue-specific manner.

Her genes are highly regulated by the Notch pathway, which plays a pivotal role in neural differentiation. All of the *her* genes examined regulate neural fate, with some playing a dual role in both neural and mesodermal tissue. Of the many binding partners associated with CoRest, the C-terminal binding protein (CtBP) has been shown to complex with the RBP-J repressor, implying a role for CoRest1 in Notch signaling (Oswald, Winkler et al. 2005). However, opposing conclusions have been drawn on the effect of CoRest on Notch signaling. In *Drosophila*, CoRest is important for the mitotic-to-endocycle transition in follicle cells (Domanitskaya and Schüpbach 2012). The Notch target gene *hnt* and a transgenic Notch reporter are both downregulated in *Drosophila corest* mutants. This suggests positive modulation of Notch by Rcor1, possibly by destabilizing CtBP/LSD1 complexes or repressing another repressor of the Notch pathway. In contrast, both *in vitro* and *in vivo* studies in mice suggest that CoRest1 is a repressor of Notch target gene transcription (Lopez, Saud et al. 2016).

Our focus has been on the role of CoRest1 as a repressor of neural gene expression and impacts on neurogenesis. However, CoRest1 functions in diverse processes due to its many binding partners. Recent work has shown requirements for CoRest1 in hematopoiesis (Saleque, Kim et al. 2007), promoting viral latency following infection (Zhou, Du et al.

2013) and in a variety of cancers (Chan, Telenius et al. 2015, Kalin, Wu et al. 2018). Zebrafish *rcor1* mutants have decreased fitness, but their outward appearances appear to be healthy suggesting that the fish *rcor1* mutants compensate for lack of the protein. Our analysis of the mutants suggests that at most loci, Rcor1 is not required for Rest repressor activity (Figure 3) and therefore other co-repressors must recruit the needed chromatin-modifying factors. Nonetheless, we observed that Rcor1 modulates the rate at which neurons differentiate via stage-dependent influences on expression of *her* genes. Our study demonstrates that in the context of early development one of the key roles of CoRest1 is to modulate the progression of neurogenesis. Analysis of possible redundancy with other CoRest family members will be important in discerning the activities of Rcor1.

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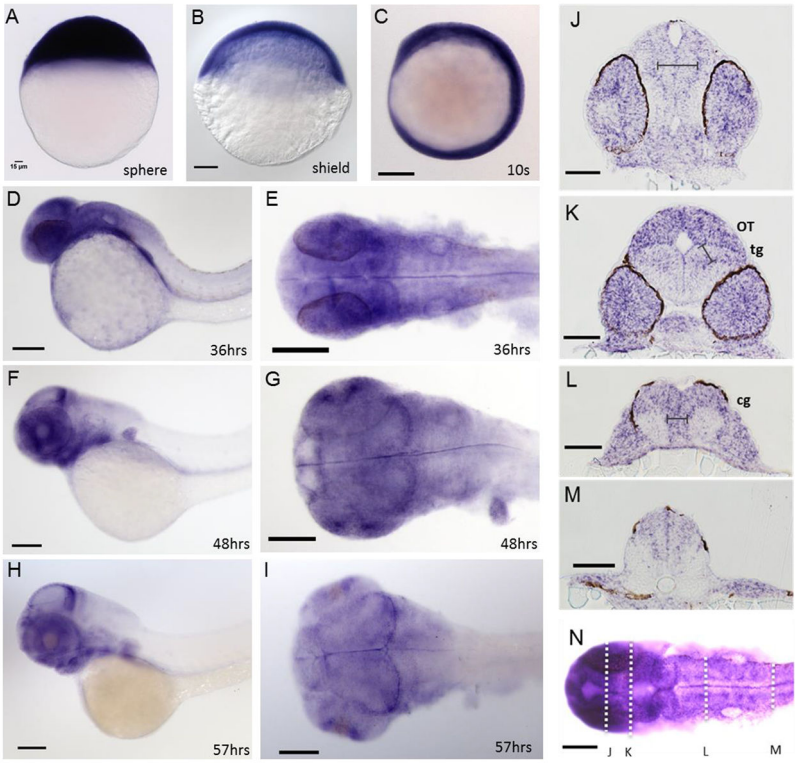
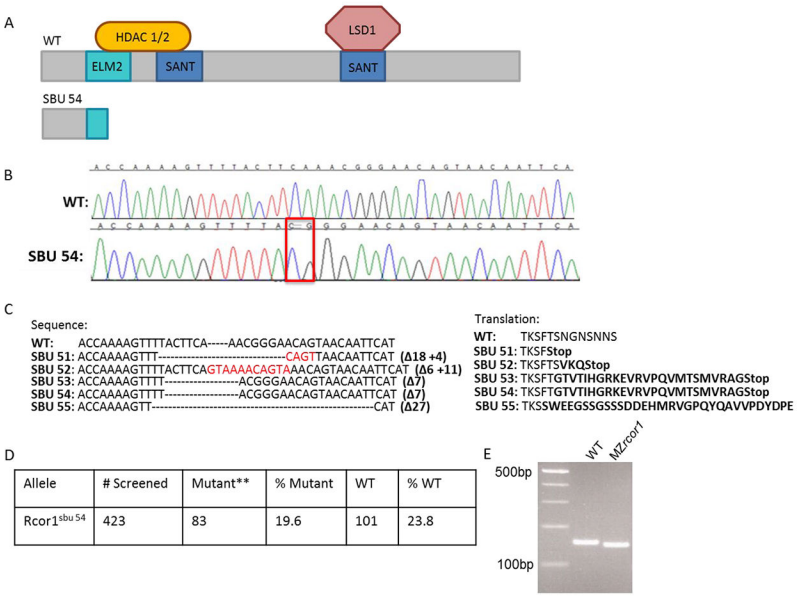


Figure 1. Expression pattern of *rcor1* in early zebrafish development.

(A-C) At 4hpf, 6hpf, and 13hpf, *rcor1* is ubiquitously expressed in the embryo. **(D, E)** Lateral and dorsal views of 36 hpf larvae reveal broad *rcor1* expression throughout the head. **(F-I)** Lateral and dorsal images of 48 hpf and 57 hpf, respectively, show expression of *rcor1* in the eye and posterior optic tectum. Transverse sections of 42 hpf larvae of the forebrain, midbrain, hindbrain and anterior trunk **(J-M).** Dorsal view of whole 42hpf embryo used for transverse sectioning **(N)**. Brackets outline the proliferative zone along the midline indicating regions of un-differentiated cell populations. Staining is seen in the eyes, OT, tg, and cg. OT-optic tectum, tg-tegmentum, cg- cranial ganglia. (Scale bar = 15 um)



Allele	# Screened	Mutant**	% Mutant	WT	% WT
Rcor1^{sbu54}	423	83	19.6	101	23.8

Figure 2. Generation of a zebrafish *rcor1* mutant.

(A) Schematic representation of CoRest1 domain structure including ELM2 and SANT domains. **(B-C)** Chromatogram with region of mutation indicated by red box and sequence alignment of wild-type and *rcor1* alleles with protein translation for each allele indicating stop codon. **(D)** Survival data on recovery of adult Rcor1$^{sbu54-/-}$ fish were not recovered at Mendelian ratios (**p <0.01). **(E)** RT-PCR of RNA extracted from bud stage indicating mutant mRNA in MZ*rcor1* embryos.

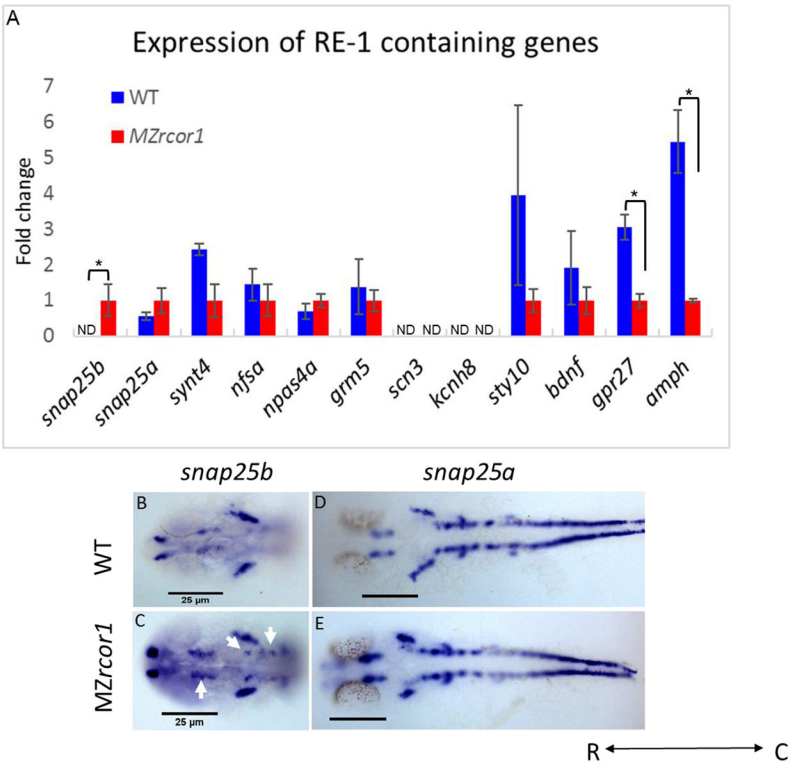


Figure 3. Expression analysis of Rest target genes in MZ*rcor1* mutants.

(A) qPCR analysis of RE-1 containing genes in MZ*rcor1* mutants compared to related wild-type control embyos at 4 hpf. Increased expression of *snap25b* is observed in MZ*rcor1* mutants, but no significant differences in expression level is seen in *snap25a, synt4, nfsa, npas4a, grm5, scn3, kcnh8, sty10*, and *bdnf*, while decreased expression of *gpr27* and *amph* is observed in MZ*rcor1* mutant embryos. MZ*rcor1* mutant expression is set to 1 because some markers are not detectable (ND) in wild-types. **(B-E)** RNA *in situ* hybridization at 24 hpf showing ectopic expression of *snap25b* in the hindbrain of MZ*rcor1* mutants (indicated by arrowheads) (seen in 6/10) **(B-C)** and comparable *snap25a* expression in MZ*rcor1* mutants and wild-type controls **(D-E).** (*p<0.05) (Scale bar = 25um)

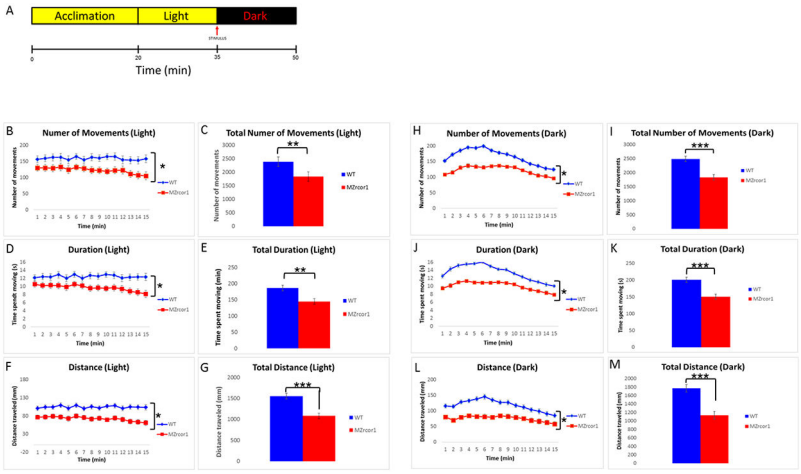


Figure 4. MZ*rcor1* mutants are hypoactive in comparison to sibling wildtypes.

Schematic of the visual-motor behavioral paradigm **(A).** Total counts (number of movements), total duration, and total distance were calculated for 15 minutes in light **(B-G)** and dark conditions **(H-M)** for MZ*rcor1* mutants and related wild-type controls. MZ*rcor1* mutants initiate fewer swimming movements than controls, which results in decreased distance travelled and duration of swims. Locomotion was assayed at 6 dpf. A Student's t-test was used to analyze the average between groups and a repeated measures ANOVA, along with a Tukey post-hoc, to assess the spontaneous movements at each of the 15 minutes of movement. (*p<0.05, **<0.01, ***<p<0.001) (n=36)

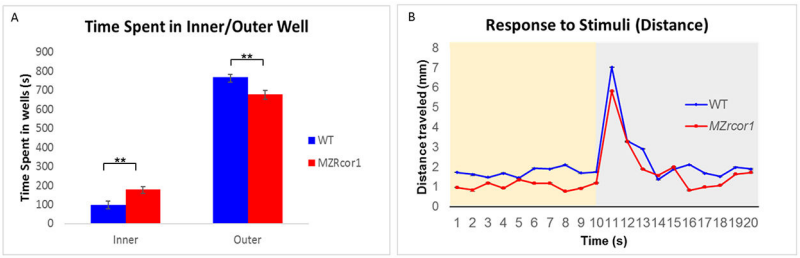


Figure 5. MZ*rcor1* mutant larvae show spatial preference.

Analysis of place preference showed that MZ*rcor1* mutants mutants spend more time in the inner well and less time in the outer well compared to related wild-type controls **(A).** Upon shift from light to dark conditions, the evoked responses of MZ*rcor1* mutants were comparable to related wild-type controls **(B)**. (*p<0.05, **<0.01) (n=36)

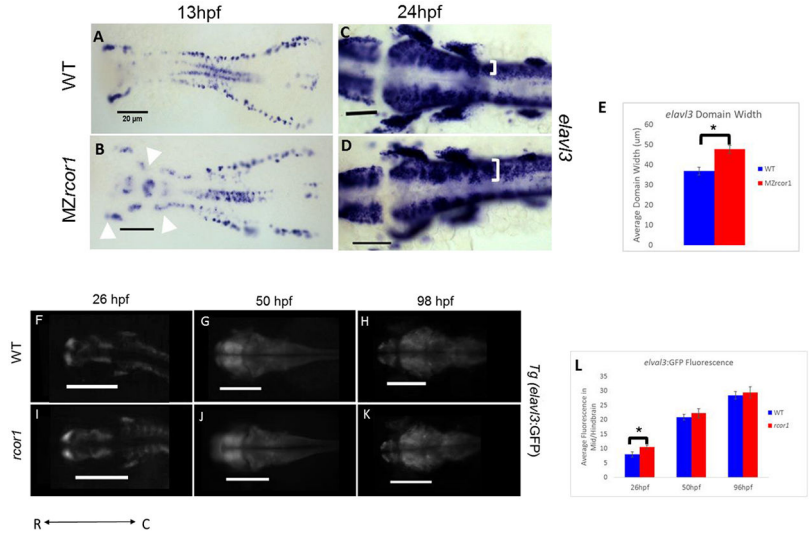


Figure 6. Enhanced neurogenesis in MZ*rcor1* mutants.

Whole mount RNA *in situ* hybridization of MZ*rcor1* and related wild-type embryos at 13 hpf **(A, B)** and 24 hpf **(C, D)** using *elavl3* probe reveals ectopic expression of *elavl3* in the midbrain and tegmentum at 13 hpf and increased expression along the midline from midbrain to tail at 24 hpf (as indicated by bar). Quantification of domain expression (indicated by bar graph). The average width of the *elavl3* domain in the MZrcor1 was 47.7±1.5 um compared to 36.9±2 um for the wild-type controls. **(E)**.Dorsal views of 26 hpf **(F, I)**, 50 hpf **(G, J)**, and 98 hpf **(H, K)** of *Tg(elavl3*: GFP);*rcor1*$^{sbu54/sbu54}$ mutants show increased differentiating neurons at 24 hpf in midbrain and hindbrain. **(L)** Quantification of fluorescence levels of *Tg(elavl3:GFP);rcor1*$^{sbu54/sbu54}$ larva and sibling controls. (*p<0.05) (Scale bar=20 um)

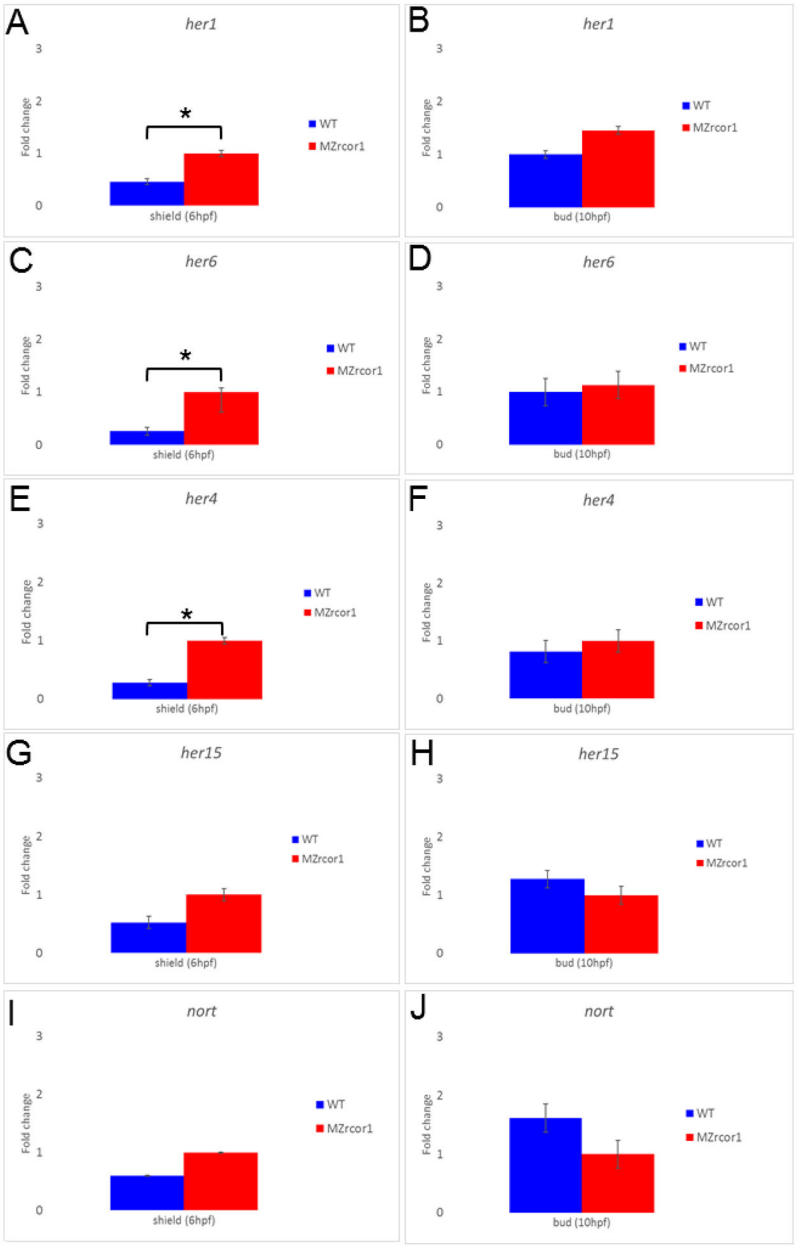


Figure 7. Expression levels of *her* genes in MZ*rcor1* mutants.

qPCR analysis of *her1, her6, her4, her15, and nort* expression levels at 6 hpf **(A, C, E, G, I)** demonstrates increased levels of *her1, her6*, and *her4* genes in MZ*rcor1* mutants when compared to related wild-type controls. However, at 10 hpf there was no significant difference in expression levels of *her* genes in MZ*rcor1* mutants **(B, D, F, H, J)**.

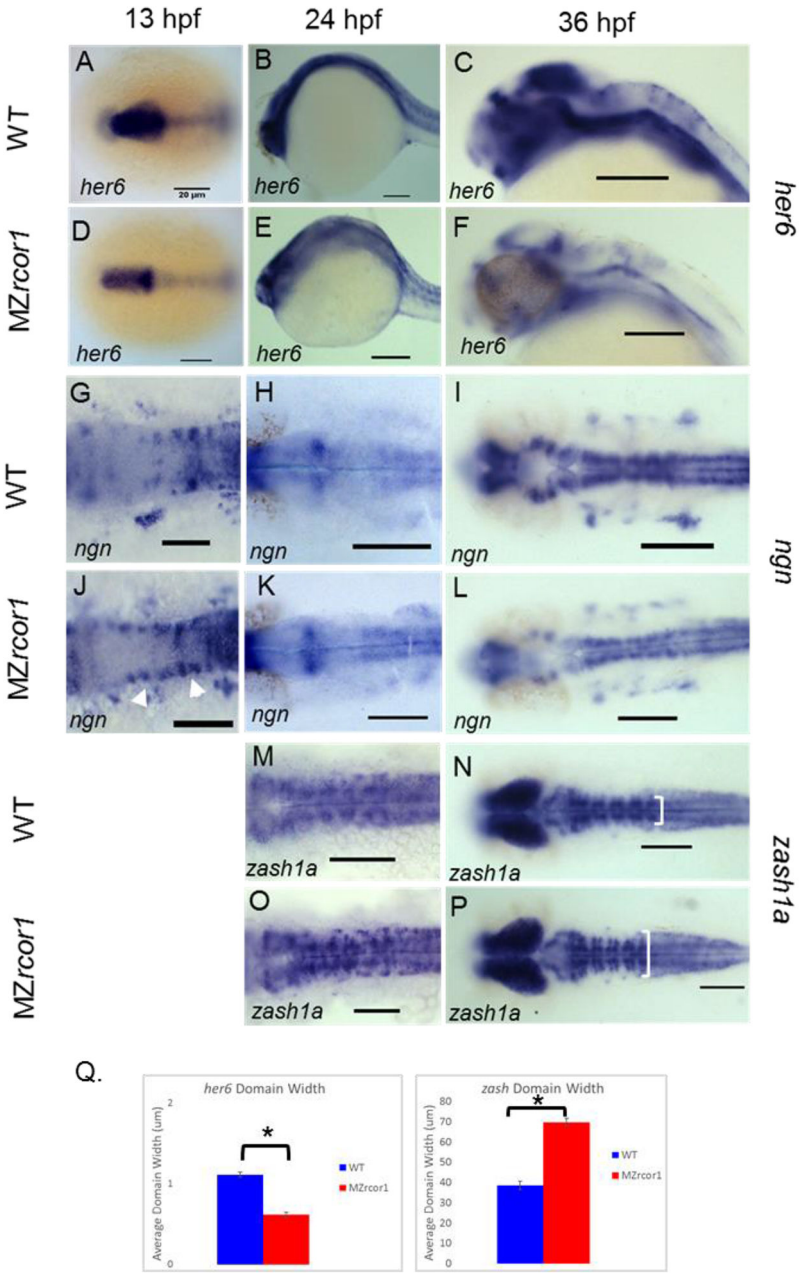


Figure 8. MZ*rcor1* mutants have increased pro-neural gene expression.

(A-F) RNA *in situ* hybridization of MZ*rcor1* mutants and related wild-type controls revealed that *her6*, has decreased expression pattern in the mutants at 13, 24, and 36 hpf. At 13 **(G, J)** and 24 hpf **(H, K)**, *ngn1*, expression is more robust in the hindbrain of MZ*rcor1* mutants when compared to wild-type control, while MZ*rcor1* mutants have increased *zash1a* expression at 36hpf **(N, P)** compared to wild-type controls. Quantification of expression domain at 13hpf of her6 indicated that MZrcor1 domain was 0.6±0.06 um in comparison to 1.11±0.03 for wild-type. Expression at 36 hpf of zash1a expression showed increased width

in the MZrcor1 mutants (69.78±3.5) when compared to controls (38.6±2) **(Q)**. (*$p<0.05$) (Scale bar = 20 um)