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***Xenopus* mutant reveals necessity of *rax* for specifying the eye field which otherwise forms tissue with telencephalic and diencephalic character**

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SUMMARY

The retinal anterior homeobox (*rax*) gene encodes a transcription factor necessary for vertebrate eye development. *rax* transcription is initiated at the end of gastrulation in *Xenopus*, and is a key part of the regulatory network specifying anterior neural plate and retina. We describe here a *Xenopus tropicalis rax* mutant, the first mutant analyzed in detail from a reverse genetic screen. As in other vertebrates, this nonsense mutation results in eyeless animals, and is lethal peri-metamorphosis. Tissue normally fated to form retina in these mutants instead forms tissue with characteristics of diencephalon and telencephalon. This implies that a key role of *rax*, in addition to defining the eye field, is in preventing alternative forebrain identities. Our data highlight that brain and retina regions are not determined by the mid-gastrula stage but are by the neural plate stage. An RNA-Seq analysis and *in situ* hybridization assays for early gene expression in the mutant revealed that several key eye field transcription factors (e.g. *pax6*, *lhx2* and *six6*) are not dependent on *rax* activity through neurulation. However, these analyses identified other genes either up- or down-regulated in mutant presumptive retinal tissue. Two neural patterning genes of particular interest that appear up-regulated in the *rax* mutant RNA-seq analysis are *hex1* and *fezf2*. These genes were not previously known to be regulated by *rax*. The normal function of *rax*

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Author contributions

M.B.F., T.N., M.F., N.H., A.C., R.R., S.C. and A.H. performed experiments. M.B.F., T.N., M.F., S.C., A.H., D.L.S. and R.M.G. analyzed data. M.B.F., T.N., M.F. and R.M.G. conceived the study and wrote the manuscript. M.B.F. and T.N. contributed equally as joint first authors.

Competing interests

The authors declare no competing financial interests.

is to partially repress their expression by an indirect mechanism in the presumptive retina region in wildtype embryos, thus accounting for the apparent up-regulation in the *rax* mutant. Knock-down experiments using antisense morpholino oligonucleotides directed against *hesx1* and *fezf2* show that failure to repress these two genes contributes to transformation of presumptive retinal tissue into non-retinal forebrain identities in the *rax* mutant.

Keywords

Xenopus tropicalis; forebrain patterning; retina formation

INTRODUCTION

A complex network of transcription factors activated in the early neurula contributes to forebrain and early eye patterning (reviewed by Ogino et al., 2012; Papalopulu, 1995; Shimizu and Hibi, 2009; Sinn and Wittbrodt, 2013; Zuber, 2010). The retinal anterior homeobox (*rax*) gene is one key factor in this network. *rax* is expressed in the anterior neural plate starting during late gastrula stages (St. 12; stages according to Nieukoop and Faber, 1994) in regions fated to form retina and hypothalamus and later appears in the pineal anlagen (Furukawa et al., 1997; Mathers et al., 1997). Mutations in the DNA-binding homeodomain of the zebrafish *rax* homolog *rx3* result in eyeless fish (Kennedy et al., 2004; Loosli et al., 2003), and the mouse *Rax* null mutant is also eyeless (Mathers et al., 1997; Voronina et al., 2005). Human *RAX* mutations result in anophthalmia in compound heterozygotes (Voronina et al., 2004). Morpholino (MO) knockdown experiments targeting *rax* in *Xenopus* also resulted in eyeless phenotypes (Andreazzoli et al., 2003).

To utilize the many advantages of *Xenopus* for studying early embryogenesis together with genetic manipulations (Harland and Grainger, 2011), we developed the N-ethyl-N-nitrosourea (ENU) mutagenesis protocol described here, which entails spermatogonial mutagenesis of *Xenopus tropicalis*, and the use of F1 mutagenized animals in a Targeting Induced Local Lesions IN Genomes (TILLING) screen to identify mutations in genes of interest. This study describes a new nonsense mutation in the *Xenopus tropicalis rax* gene, which prematurely terminates protein translation upstream of the *rax* homeodomain, consistent with this acting as a null mutation.

We performed an RNA-Seq analysis targeting just the affected tissue, the anterior neural plate, in *rax* mutants at the time of eye specification (stage 15, Saha and Grainger, 1992), which allowed us to greatly reduce background from a whole embryo analysis. This analysis identified differentially expressed genes in the *rax* mutant, revealing genes of interest not previously known to be regulated by *rax*.

In *rax* mutants the tissue that would normally be fated to become retina is transformed into both telencephalic and diencephalic tissue. This implies that a key role of *rax* is not only to help define the retina in a positive sense, but also to inhibit adjacent factors from specifying this region towards other forebrain fates, and highlights that regional determination of the brain and retina are not fixed at the time of onset of *rax* expression.

MATERIALS AND METHODS

ENU mutagenesis and TILLING

We have utilized spermatogonial mutagenesis, a widely used approach for vertebrate ENU mutagenesis, e.g. in mouse (Weber et al., 2000). Adult males were injected with ENU (100 mg/kg body weight) at weekly intervals for two or three weeks. Higher doses, or treatments at 100 mg/kg for more than three weeks, resulted in higher lethality and lower doses or single week treatments at 100 mg/kg were not effective. After recovering for several months when males were effectively sterile, F1 populations were generated for evaluation of recessive phenotypes or for TILLING studies.

A small-scale TILLING screen was performed by Sanger sequencing of a limited number of gene targets in an F1 population. The calculated mutation rate in the loci examined was 1 in 200 to 300 kb, varying among offspring of different mutagenized males, but in the range expected for efficient ENU mutagenesis in other vertebrates (Beier, 2000; Kettleborough et al., 2011), and similar to the initial *X. tropicalis* TILLING screen using direct mutagenesis of mature sperm (Goda et al., 2006). The *rax* mutation described here was the first nonsense mutation recovered during our screen that affected a key eye regulatory gene.

rax, *rax-EnR* mRNA and BAC injections

rax or *rax-EnR* (*rax-engrailed*) capped mRNA was synthesized from pCS2-Xrx1 or pCS2-Xrx1-EnR (kind gifts from Massimiliano Andreazzoli, Andreazzoli et al., 1999, 2003) using the mMESSAGING MACHINES[®] SP6 Kit (Invitrogen). 5 pg (or 5 pg and 10 pg for *rax-EnR* experiments) mRNA was injected into one dorsal blastomere at the 4-cell stage of *X. tropicalis* embryos along with 20 ng fluorescein-labeled dextran (FLDx) as tracer, where un-injected sides served as controls. The *rax*-containing *X. tropicalis* BAC clone ISB1-349A23 was recombineered and injected into *Xenopus* embryos as described (Fish et al., 2012).

RNA-Seq Analysis

Anterior neural plates (ANPs) from neural plate stage (St. 15) embryos were dissected from a *rax* mutant heterozygote cross, collected individually and frozen in multi-well dishes. The remaining embryonic tissue was processed for genotyping as described in Goda et al., (2006), using RaxGenoF and RaxGenoR primers for PCR amplification and subsequent Sanger sequencing: RaxGenoF: 5'-ACCAGGCACCTCTTTTTGTG-3'

RaxGenoR: 5'-CTGACCTCGGGCAAGTTTAC-3'

Homozygous wildtype and mutant ANPs were collected into three pools per genotype. Pooled tissues were lysed in Trizol (Invitrogen) and processed according to manufacturer's instructions. cDNA libraries were prepared using the Illumina mRNA kit, and sequenced using Illumina Solexa sequencing. RNAseq raw data from two experiments is available on the European Nucleotide Archive (ENA), study number ERP002095, (<http://www.ebi.ac.uk/ena/data/view/ERP002095>). The analysis used existing gene models in Ensembl and those generated by TopHat (Trapnell et al., 2009) and Cufflinks (Trapnell et al., 2010), to run a differential gene expression analysis, using Cuffdiff. Cuffdiff was run

with a GTF file, produced from Cuffcompare. RNAseq analysis data is published on Array Express, experiment number E-ERAD-130 (<http://www.ebi.ac.uk/arrayexpress/experiments/E-ERAD-130/>). A simplified table of statistically significant differentially expressed genes and their calculated read values, as generated by the Cuffdiff analysis, is available in the Supplementary Materials (Table S4).

Genotyping of *rax* mutant and wildtype embryos

Since we can easily distinguish between *rax* mutant and wildtype phenotypes after St. 24 due to differential morphologies of the eye region, we do not necessarily perform genotyping on embryos older than St. 24. In this case, the genotype of wildtype embryos is shown as (+/?). All embryos shown at earlier stages were genotyped. Embryos were lysed as described (Fish et al., 2012; Nakayama et al., 2013) followed by genomic PCR of the *rax* region (PCR primers are listed above). The PCR amplicon was purified and sequenced. In most experiments, phenotypes are indistinguishable between +/+ and +/-, and thus one representative embryo of either genotype has been chosen as a “wild-type” exemplar to image and labeled as (+/?).

Histology

For analysis of the mutant phenotype, embryos were fixed overnight in Bouin's fixative and embedded in JB-4 Plus® resin (Polysciences). 3 µm sections were cut and stained with toluidine blue.

For assessing expression domains of certain transcription factors following *in situ* hybridization, embryos were embedded in Paraplast® Plus and sectioned at 10 µm. Other embryos were embedded in 2% low melt agarose in 0.1 × MBS and vibratome sectioned at 30 µm.

DiI Labeling

Presumptive retina rudiments were labeled at St. 15 using DiI (Molecular Probes® Invitrogen). A 10mg/ml stock of DiI in DMSO was diluted 1:20 in 250 mM sucrose, loaded into a microinjection needle and applied to the surface of embryos. Injection sites were standardized using an ocular grid, measuring along the line of the inner margin of the anterior neural fold (x-axis) and a line running through the embryonic midline of the embryo (y-axis).

Tissue Transplantations

Small pieces of anterior neural plate containing the left eye rudiment (roughly equivalent to the *rax* expression domain) were removed at St. 15 from *rax* +/? and *rax* -/- embryos and transplanted to the posterior flank of same stage hosts. Hosts were allowed to develop to approximately St. 37 and fixed with MEMFA (100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO₄, 3.7% (v/v) formaldehyde) for *in situ* hybridization. Host and donor embryo pairs were raised in parallel so that the genotype of donor tissue could be determined by the phenotype of the eye on the un-operated side of the donor embryo.

Fluorescence microscopy and photomicroscopy

Fluorescent lineage tracer (FLDx) in embryos was observed using a Zeiss Discovery V12 microscope with fluorescent illumination and 470nm GFP filter set. Photos were taken with a Zeiss AxioCam Mrc5 CCD camera assembled on the microscope, using Axiovision 4.8 software.

In situ hybridization

Whole-mount *in situ* hybridization was carried out essentially according to the procedure in Sive et al.,(2000), though modified when genotyping was required. In these cases the acetic anhydride step and lengthy overnight fixation of post-stained embryos were omitted. Both steps interfere with the ability to accurately sequence DNA. After photographing embryos following this procedure, embryos were genotyped as described above. All antisense probes were labeled with digoxigenin (Roche) and detected by BCIP/NBT (Roche). See Table S1 for details of probe construction.

Morpholino antisense oligonucleotides and validation mRNA injections

See Tables S2 and S3 for injected-morpholino sequences and mRNA constructs used to assay morpholino efficiency.

Gfp Immunostaining

Embryos were fixed in MEMFA for 1 hour, then washed and stored in phosphate buffered saline (PBS) at 4°C. Vibratome sections were blocked in PBS with 0.1% Tween 20, 2 mg/ml BSA and 10% normal goat serum (PBST/NGS) and incubated overnight with primary anti-Gfp antibody (Invitrogen A11122- rabbit, 1:500). Sections were washed and blocked again, and then incubated overnight with secondary antibody (GARGG-AlexaFluor488, 1:500). Embryos were washed in PBST and then mounted on subbed slides in 70% glycerol/PBS.

RESULTS

A *Xenopus tropicalis* *rax* mutant results in an eyeless phenotype

A reverse genetic screen (as described in the Materials and Methods) performed in *Xenopus tropicalis* identified a nonsense mutation in the *rax* gene. The *X. tropicalis* Rax protein consists of 326 amino acids containing an octapeptide (OP) domain, homeodomain (which also contains 2 nuclear localization signals (NLSs), Voronina et al., 2004) and OAR (*otp*, *al*, and *rax*) domain (Fig. 1A). The OP domain may be involved in repression while the C-terminus OAR domain is thought to function as a transactivation domain (Furukawa et al., 1997; Pan et al., 2006). The nonsense mutation identified in our spermatogonial mutagenesis/TILLING screen occurs in the second of three exons, changing a glutamine codon into a stop codon, halting the translation of Rax protein after the OP domain and upstream of the homeodomain and OAR domain (Fig. 1A). Notably, a similar truncation previously described in human patients (Q147X) results in a protein product that cannot translocate to the nucleus and has no DNA-binding activity, as it does not contain an NLS and eliminates helices 2 and 3 of the homeodomain (Voronina et al., 2004). Since our mutant locus could potentially encode only a shorter protein (Q125X) lacking the entire

homeodomain (including two NLSs), the truncated protein, if it is present, we expect has no ability to interact with DNA or to localize properly to the nucleus and thus most likely behaves as a null. Animals homozygous for the mutant allele are eyeless (Fig. 1B, right and 1C, top). A small amount of retinal pigmented epithelium (RPE) is sometimes observed in mutant embryos (Fig. 1B and 1C, orange arrows). Embryos illustrating the range of observed RPE were chosen from one clutch in Fig. 1B; it should be noted that this range does not represent the observed ratios of RPE phenotypes, which is variable depending on the parental mating.

The eyeless phenotype can be rescued in approximately 40% of embryos by injecting homozygous mutants with low levels (5 pg) of mRNA encoding the wildtype Rax protein (Fig 1C, bottom, while another 40% showed partial rescue (i.e. microphthalmic eyes, not shown) as well as by the injection of a Bacterial Artificial Chromosome (BAC) clone that expresses a Rax-Gfp3 fusion protein (Fish et al., 2012, Fig. 1D). Both rescue experiments support the conclusion that the eyeless phenotype is indeed caused by the *rax* nonsense mutation and not by other background mutations induced by ENU mutagenesis.

The expression of key eye field transcription factors is unaffected in neurula stage *rax* mutant embryos

Originally, experiments performed in a mouse *Rax* mutant supported a regulatory network model placing key eye field transcription factors (EFTFs) (*Pax6*, *Six3*, *Lhx2*, and *Six6*) downstream of *Rax* because these genes are down-regulated in the mutant at early stages (Zhang et al., 2000; and reviewed in Bailey et al., 2004). We examined the activation of these genes by *in situ* hybridization in neural plate stage *Xenopus* embryos and did not detect any differences in expression of *pax6*, *otx2*, *six3*, *lhx2* or *six6* (Fig. 2A). This is consistent with previous examinations of the early expression patterns of *pax6*, *otx2* and *six3* in zebrafish *rx3* mutants (Loosli et al., 2003, Kennedy et al., 2004). By early tailbud stages, however, all of these genes are down-regulated in *rax* mutants in the presumptive retinal region (Fig. 2B). In the case of genes that are normally expressed both in the retina and other brain regions, such as *pax6*, *otx2*, and *lhx2*, only the retinal expression is affected in the *rax* mutant (Fig. 2B).

An RNA-Seq analysis was performed to compare the gene expression profiles of St.15 anterior neural plates of *rax* mutant and wildtype embryos. Although *rax* expression is initially detected in late gastrula *Xenopus* embryos, the neural plate (St.15) was chosen to capture genes that are directly affected by loss of functional Rax protein or indirectly regulated but part of the immediate network of genes associated with Rax activity. At this stage the retina is also determined, though it is not determined when *rax* is first activated (Saha and Grainger, 1992). In addition, the neural plate provides landmarks that make precise dissections possible and detailed fate maps exist for this stage (Eagleson and Harris, 1990; Eagleson et al., 1995). Our RNA-Seq analysis (Fig. 2C) supports the *in situ* hybridization results showing no significant expression changes in *pax6*, *otx2*, *six6*, or *lhx2*. The gene *six6* may be slightly, though not significantly, reduced, though no differences between wildtype and mutant are seen by *in situ* hybridization (e.g. Fig. 2A).

Morphological changes in the *rax* mutant can be detected as early as St. 21, prior to the down-regulation of key EFTFs

Histological sections were examined to look for the earliest morphological changes in mutant embryos. Although embryos of any genotype look similar at St. 20, by St. 21 a thinning of the posterior optic vesicle can be observed in wildtype embryos that is not observed in mutant embryos (Fig. 3A, red arrows). Interestingly, this morphological change is detectable before the down-regulation of key EFTFs, e.g. *pax6* and *lhx2*, begins to occur (Fig. 3B). One transcript of interest found to be significantly increased in mutant embryos before the morphological change at st. 21 is the *aristaless-related homeobox (arx)* gene. This transcription factor is expressed early in the developing diencephalon (posterior to the retina, El-Hodiri et al., 2003; Miura et al., 1997) and is important for forebrain patterning in *Xenopus*, mouse and zebrafish (Filippi et al., 2012; Kitamura et al., 2002; Rodríguez-Seguel et al., 2009). The expression of the transcriptional repressor *arx* in *rax* mutant embryos begins to expand from the initial diencephalic territory into the presumptive retina at St. 19 (Fig. 3C, blue arrows). By the stage where morphological changes are first detected (St. 21, Fig. 3C, red arrows), *arx* expression has further expanded from its posterior domain into the region where the optic vesicle normally forms in wildtype embryos.

We also examined the expression of the primarily telencephalic gene *foxf1*, both because expansion of the telencephalon is observed in zebrafish *rx3* mutants (Stigloher et al., 2006), and since it is a target of *hesx1* (previously *xanf*), which is also up-regulated in the *rax* mutant (see below), though *hesx1* is no longer active at stages we examine here. The transcription factor *foxf1* is an important integrator of signaling pathways patterning the forebrain and regulating telencephalon formation (Andoniadou et al., 2011; Danesin et al., 2009). We observe expansion of *foxf1* expression in mutant embryos as early as St. 21 (Fig. 3D, red arrows), with significant lateral expansion into the presumptive optic vesicle territory (white arrows) observed by St. 24. Divergence in eye morphology continues at later stages, and by St. 30 a well-formed optic cup is discernable in wildtype embryos (Fig. 3E, black arrows) and the lens is beginning to form (Fig. 3E, black arrowhead). These structures typically fail to form in *rax* mutants (Fig. 3E).

Retinal tissue in the *rax* mutant is transformed into tissue with diencephalic and telencephalic properties

Since the region fated to become retina appears to persist in some form, as illustrated in Fig. 3 (and substantiated below), we wanted to establish what the fate of the presumptive retina might be in *rax* mutant embryos, i.e., is it transformed, for example, into adjacent brain tissue types? The data mentioned above showing that the diencephalic gene *arx* is expanded into the retina region supports this proposal. Toward that end we examined expression of the gene *fezf2* in *rax* mutant embryos because it has significant diencephalic expression posterior to the retina, it is up-regulated in the mutant (as discussed further below), and it regulates *arx* activity (Rodríguez-Seguel et al., 2009). *fezf2* is a zinc-finger transcription factor that plays a key role in diencephalic and telencephalic fates (Jeong et al., 2007). In mouse knockout studies, it functions similarly and partially redundantly with closely-related *fezf1* to localize the zona limitans intrathalamica (ZLI), the signaling center that marks the boundary between the prethalamus and thalamus subdivisions of the diencephalon (Hirata et

al., 2006; Lavado et al., 2008). In *Xenopus*, *fezf2*, together with *fezf1*, suppresses formation of the caudal diencephalon as part of its role in ZLI localization (Shimizu and Hibi, 2009). Examining the *fezf2* expression pattern in *rax* mutant embryos at tadpole stages (St. 32 and 35) in both whole-mount (Fig. 4A) and sectioned embryos (Fig. 4B), we observed that it was greatly expanded in mutant embryos, extending laterally into the region where the retina would normally form (Fig. 4A-B, blue arrows).

In both whole-mount and sectioned embryos we also observed posterior and lateral expansion of the telencephalon (defined by *foxg1* expression) into the region where the retina would normally form (Fig. 4A-B, red arrows). In *rax* mutant tadpoles we also observed the persistence of low levels of *rax* expression in a small rudiment that remains (Fig. 4B and 5A, green arrows), which may be what remains of the optic stalk.

Embryos were either cut or sectioned in a parasagittal plane in the brain rather than in the more lateral optic vesicle in order to better visualize the early changes in the expression domains of these key genes in mutant embryos (Fig. 4C-F). We observed that *arx* expression expands ventrally toward and into the presumptive telencephalon (red outlines), which, more laterally, would be the anterior face of the optic vesicle. In addition, *arx* is expanded posteriorly into part of what is the secondary prosencephalon (yellow outlines) in mutant embryos (Fig. 4D), a region which is contiguous to the posterior face of the optic vesicle. Telencephalic marker *foxg1* expands less significantly dorsally (Fig. 4E), but as discussed above (Fig. 3D) expands laterally into the optic vesicle instead. In this plane of section the predominantly diencephalic and posterior secondary prosencephalic marker *fezf2* has expanded expression into the telencephalic territory (Fig. 4F) and, as discussed below, is in fact expanded throughout the optic vesicle. A three-dimensional model summarizes the proposed directions of expansion of *arx* and *foxg1* in the *rax* mutant. The expression of *arx* expands ventrally, both anteriorly and posteriorly in the presumptive optic vesicle in the mutant, while the telencephalic gene *foxg1* has a primarily lateral expansion into the anterior face of the optic vesicle (Fig. 4G).

To distinguish whether these forebrain tissues expand via proliferation into the space occupied by the eye field, or if the eye field tissue is transformed into these alternative fates, we utilized a transgenic line to label eye field cells. This line, which expresses *gfp3* under the control of the *rax* upstream promoter (*rax pro-gfp3*) in the same domain as endogenous *rax* (Hirsch et al., 2002), was crossed to our *rax* mutant line to create *rax* mutant heterozygotes with the *rax pro-gfp3* transgene. When intercrossed, animals from this line produce *rax* homozygous mutant embryos in which both endogenous *rax* and *rax pro-gfp3* transcription is eventually down-regulated and the eyes fail to form, whereas Gfp3 protein accumulates, remaining for days in cells where *rax* was initially transcribed because of its longevity compared to mRNA (Verkhusha et al., 2003). St. 32 embryos from such a cross were collected, sectioned and immunostained to detect Gfp3 protein, which labels tissues originally fated to contribute to the retina (Fig. 5B). In wildtype embryos, most of the labeling is observed in the retina, and the *gfp3* RNA expression also is high in the retina (Fig. 5C, left panel). By contrast, very little *gfp3* mRNA is seen in *rax* mutant embryos (Fig. 5C, black arrows). However, Gfp3 protein is still detected by immunocytochemistry (Fig. 5B). By comparing the mutant Gfp3-labeled brain regions with mutant embryos showing the

expansion of telencephalic marker *foxf1*, we can observe the overlap in expression domains, supporting the conclusion that at least some of the presumptive retina has been transformed into telencephalic tissue in the mutant (Fig. 5B and D). We also observe some Gfp3-labeled regions that are not coincident with *foxf1* expression (Fig. 5B, blue arrows) but include the expanded *fezf2* expression zone (Fig. 4B) indicating an enlarged region of diencephalic character within the original eye field.

In another experiment testing this transformation hypothesis, we labeled presumptive retinal tissue with DiI marker at St. 15, and observed where the marked cells were found in St. 32 embryos (Fig. 6A). In wildtype embryos labeled cells were usually seen in retinal and small patches of diencephalic tissue, while in mutant embryos we found labeled cells in tissues that overlap with the expanded diencephalic and telencephalic marker-expressing tissues (i.e. *fezf2* and *foxf1*, Fig. 4B).

Finally we tested the extent to which this fate switch was autonomously determined by St. 15 through a tissue transplantation assay. Putative retinal tissue from St. 15 homozygous *rax* mutant or wildtype embryos was transplanted to the posterior of St. 15 host embryos (Fig. 6B). These embryos were raised to St. 37, fixed and the telencephalic marker *foxf1* was assayed by *in situ* hybridization. Consistently, in wildtype transplants only small regions or no regions with telencephalic properties were detected (Fig. 6B) based on the low levels of *foxf1* expression. In *rax* mutant transplants, however, we saw large domains of *foxf1* expression in transplanted tissue, indicating that this tissue had at least in part already been transformed to an autonomously determined telencephalic character by St. 15.

Examination of genes differentially expressed in the *rax* mutant at St. 15 reveals the up-regulation of forebrain patterning genes and down-regulation of key eye genes

Our transplant experiment from neural plate stage embryos (St. 15) indicated that a fate transformation to non-retinal forebrain tissue has occurred by this stage, the same stage as the RNA-Seq analysis that was performed here. To identify which genes may be causing transformation of retina to brain in the mutant, genes identified as highly differentially expressed in the RNA-Seq analysis were examined by *in situ* hybridization (Fig. 7A-D; see Table S4 for all differentially expressed genes). Three transcripts identified as significantly increased in *rax* mutants were *fezf2*, *hesx1*, and *fzd4*. The wild-type expression pattern of all three genes is similar at St. 15, where transcripts are absent from the central eye field and detectable only in the periphery; the pattern forms a central domain lacking expression within the eye field (hereafter, we refer to this as an expression “hole”), which is not observed in *rax* mutants (Fig. 7B, arrows). Down-regulated genes were also examined, and in the cases of *myc*, *myb* and *foxn4*, among others, expression in the presumptive retina region is lost in *rax* mutant embryos (Fig. 7C, arrows). Although the *mab21l2* gene was not identified in the RNA-Seq data, we assayed its expression since it has been shown to be down-regulated in zebrafish *rx3* mutant lines and necessary for eye development (Kennedy et al., 2004; Yamada et al., 2004). Indeed, *mab21l2* expression is lost in the retina region of *rax* mutants (Fig. 7C, arrows). Finally, although we do not observe any heterozygote phenotype morphologically, or in the expression of most genes assayed via *in situ*

hybridization, in the case of *vsx1* we do observe reduced expression in heterozygous embryos and a loss of expression in homozygous mutants (Fig. 7D, arrows).

The genes *fezf2* and *hesx1* were chosen for further analysis because of their known roles in forebrain patterning (Ermakova et al., 1999; Shimizu and Hibi, 2009; Wang et al., 2011). A time-course of expression patterns for both genes reveals that they are initially activated more broadly in the region where the anterior neural plate will form at St.12, and at this stage their expression patterns look the same in wildtype and *rax* mutant embryos (Fig. 7E). By St. 13, however, the apparent repression of both transcripts in the central eye field can be detected resulting in the distinctive expression pattern ring; this repression does not occur in mutant embryos and therefore the expression “hole” is not observed (Fig. 7E). In the case of *fezf2* expression, the expanded expression domain in the mutant is observed through late neurula stages (St. 21) and beyond, and is strongly expressed in the putative retina region in *rax* mutants (Fig. 7E, Fig 4A, B). Increased expression of *hesx1* persists through St. 18, although by St. 21 the expression domain in wildtype and mutant embryos has receded to just the presumptive anterior pituitary gland (Fig. 7E). It is important to highlight then that the higher expression levels of *fezf2* and *hesx1* in the *rax* mutant, that were initially detected by both RNA-Seq of St. 15 ANPs, are a consequence of the failure in the mutant of earlier repressive interactions. The expression patterns of these two genes imply a localized transcriptional repression in the early central eye field that must occur after an initial, broad activation. It does not appear that Rax is directly acting as this repressor, however, based on two observations: 1) the expression domain of *rax* is larger than the repression region; and 2) the results of experiments where an obligate repressor form of Rax was expressed in *Xenopus* embryos (Fig. S1). This fused repressor, RaxEnR (containing the Rax DNA-binding domain fused to the *Drosophila* Engrailed repressor domain), was shown to function as a dominant repressor in *Xenopus* (Andreazzoli et al., 1999). When overexpressed in wildtype embryos, RaxEnR increased the expression of *fezf2* and *hesx1*, mimicking the *rax* mutant phenotype. RaxEnR mimics loss of function of Rax, and thus, wildtype Rax must function as an activator, not a repressor, in this context.

Knockdown of forebrain patterning genes *fezf2* and *hesx1* can reduce the expansion of telencephalic marker *foxf1* and diencephalic marker *arx* in *rax* mutant embryos

To examine the effects of the lack of regional repression of *fezf2* and *hesx1* in *rax* mutants on telencephalic and diencephalic expansion, we performed translation-blocking antisense MO microinjections to discern effects on *foxf1* and *arx* domain expansion. MOs were first tested for their efficiency (Fig. S2) and both individually and in combination were injected into one dorsal blastomere of 4-cell stage embryos to knockdown the expression of *Fezf2* and/or *Hesx1* in one lateral half of the embryo. To assay for *foxf1* expansion, *in situ* hybridization was performed to assess the effect of MO injection on expanded *foxf1* expression on the injected side, using the un-injected side as control for identifying mutant embryos (Fig. 8A). Under our experimental conditions, a control MO injection had little effect and close to 80% of injected mutant embryos showed the typical expansion of *foxf1* expression, whereas moderate doses of either *fezf2* or *hesx1*-targeted MOs reduced the expanded *foxf1*-expressing region significantly (Fig. 8A'). Furthermore, less than 20% of mutant embryos showed the typical expansion of the *foxf1* domain when injected with low

doses of both MOs together, whereas comparable doses of each individual MO injection still showed typical expanded *foxf1* expression in up to 60% of mutant embryos.

To examine the effects of increased *fezf2* and *hesx1* expression on diencephalic expansion, we performed a similar experiment, knocking down expression of *fezf2* and *hesx1*, and assaying instead for diencephalic marker *arx* expansion in *rax* mutant embryos (Fig. 8B). We similarly observed a reduction of the severity of the mutant phenotype (expanded *arx* expression) in a majority (60%) of MO-injected embryos (compared to 30% of control MO-injected, Fig. 8B').

These results suggest that the telencephalic and diencephalic expansions observed in *rax* mutant animals are likely mediated by many of the same genes, and that the inability to repress *fezf2* and *hesx1* in the central eye field, (most likely resulting from failure to express some repressive factor(s) downstream of *rax*), is a key mediator of the expansion of forebrain identities into the region where the retina would normally form.

DISCUSSION

Using the *Xenopus tropicalis rax* mutant line, we have gained significant insights regarding the role of *rax* in the gene regulatory network forming the retina. In addition, we have further elucidated the role of *rax* in preventing encroachment of non-retinal forebrain fates into the eye field, utilizing the synergy of working with a mutant line together with the many other experimental advantages of the *Xenopus* system for studying development.

EFTF genes are activated normally in the absence of functional *Rax*

RNA-Seq and *in situ* hybridization analyses support the conclusion that *rax* is not necessary to activate key eye field factors *pax6*, *lhx2*, or *six3* (Fig. 9A). In fact, these genes maintain comparable levels of expression to wildtype embryos in the *rax* mutant through St. 21, after morphological divergence in optic vesicle formation is first observed along with the increased expression domain of forebrain patterning gene *arx*, strengthening the argument that there are independent pathways controlling retina formation and highlighting the key role of *rax* in defining the eye field. Reduced expression of these EFTF genes can be observed by St. 24, and could be due to the reassignment of the putative retinal tissue to different identities that either fail to maintain EFTF expression or actively repress these factors. It is important to note that even with the normal expression of *pax6*, *lhx2*, *otx2*, *six6* and *six3*, morphogenesis of the optic rudiment is halted prior to full optic vesicle formation in the absence of *rax*, suggesting a master role of *rax* in retina formation.

Genes downstream of *rax*

We have confirmed key retinal factors previously identified as downstream of *rax*, such as *mab21l2* (Kennedy et al., 2004), as well as identifying new factors such as *myc*, *myb*, *foxn4*, and *vxx1* (Fig. 9A). The placement of *myc* expression downstream of *rax* has also been inferred from experiments using MO knockdown assays in *Xenopus*, although a direct interaction has not been shown (Terada et al., 2006). Instead, an intermediate factor, *xhmg3*, was proposed to mediate the activation of *myc* by *rax*. However, *xhmg3* is actually not differentially expressed in our RNA-Seq analysis; this is one of a handful of

discrepancies in genetic interactions observed between the *Xenopus rax* mutant phenotype and those reported in MO-based knockdown or overexpression studies (discussed in more detail below). Nevertheless, the role of the oncogene *myc* will be a focus for further study, as its dual roles in promoting proliferation and in determining cell fate are likely important but complex and not well understood (Bellmeyer et al., 2003; Rodrigues et al., 2008). Interestingly, *vsx1* expression is one example of the few instances where a heterozygote phenotype can be detected, and its transcription may therefore be highly sensitive to *Rax* levels.

More recent work on the zebrafish *rx3* mutant has implicated Eph/Ephrin signaling downstream of *rx3* as key to proper morphogenesis of the eye (Cavodeassi et al., 2013). Future analysis of our *Xenopus rax* mutant is planned to examine the fate of this signaling pathway as well.

Mechanism of creating the expression “hole” within *fezf2*, *hesx1*, and *fzd4* domains

Novel repressive interactions downstream of *rax* were identified over the course of these experiments: forebrain patterning genes *fezf2* and *hesx1* and Wnt-signaling pathway component *fzd4* each display a distinctive expression domain “hole” in the anterior neural plate of wildtype embryos that is not observed in the *rax* mutant. RNASeq and *in situ* hybridization of the expression patterns of many genes expressed in the eye region at early stages rule out the possibility that increased apoptosis or reduced proliferation resulting in elimination of tissues corresponding to the “hole” is involved in the process of losing the expression “hole” in *rax* mutants (if this were the case, all genes expressed in the retina region should be affected and at least several, e.g. *pax6* and *lhx2*, are not). Instead, our experiments support the conclusion that *fezf2* and *hesx1* are initially activated normally in the mutant, but are not properly repressed in the central eye field at neural plate stages, leading to improper expression of these factors and their downstream targets in the presumptive retinal region and causing expansion of alternative forebrain identities into the eye field (Fig. 9B). Experiments designed to clarify which repressor(s) downstream of *rax* are acting to prevent transcription of these genes in the repressed region (after their early, broad activation) have yet to identify convincing candidates, although several putative repressors down-regulated in the *rax* mutant have been tested, including *myc*, *foxn4* and *tbx3* (data not shown). Identification of the repressor or repressors active in this role will be an important future step in elucidating this portion of the eye field regulatory network. The possible role of *fzd4* in this network is also an intriguing line of inquiry, as discussed further below. Like *fezf2*, its expression, normally restricted to a ring in early forebrain, fills the presumptive eye field in the *rax* mutant (data not shown), thus potentially having a role in the observed phenotype.

Another role for *rax* gene in brain patterning

A second key role for *rax* revealed by these experiments is the prevention of alternative forebrain fates via repression of diencephalic and telencephalic patterning genes, in addition to the activation of eye field-promoting genes. Fig. 9C illustrates the proposed expansion of alternative brain fates. As noted above, expansion of the telencephalon is also seen in a *rx3* mutation in zebrafish (Stigloher et al., 2006). However, the expansion of the diencephalon

displayed in the *Xenopus* mutant is not observed in the zebrafish *rx3* mutant, and similarly *arx* gene expression is not expanded. One reason for this may be the duplication and subfunctionalization of *rax* genes *rx1*, *rx2*, and *rx3* in zebrafish (Chuang and Raymond, 2001; Chuang et al., 1999). Although *rx3* is the earliest expressed of the three genes, *rx1* is expressed soon after and appears initially unaffected in the zebrafish *rx3* mutant (Stigloher et al., 2006) and may impact the nature of the patterning changes seen in zebrafish. Like human (Wang et al., 2004), but unlike mouse, *Xenopus* also has a second *rax* gene, *rax2*, which is expressed later, after neurulation (Pan et al., 2006, data not shown), and could not have any possible redundant function with *rax* at early stages unlike the multi-gene family in zebrafish.

Fig. 9C and nomenclature in this paper follow the widely used fate map for *Xenopus* (Eagleson and Harris, 1990) suggesting that tissue posterior to the retina is diencephalic, though recent work argues that tissue immediately posterior to neural plate stage presumptive retina (and the retina) derives from secondary prosencephalon (Puelles, 1995; Puelles et al., 2013). After global neural patterning during gastrulation, fine-tuning of forebrain patterning occurs through later gradients created by local patterning centers in the anterior neural ridge (ANR), and, from the posterior, in the ZLI (Echevarría et al., 2003; Kiecker and Lumsden, 2012). These secondary organizers, along with roofplate and floorplate, create local gradients of Wnt, Shh, Bmp, and Fgf8 that define the boundaries of the major prosencephalic regions, the telencephalon, secondary prosencephalon (including retina and hypothalamus), and diencephalon (Bielen and Houart, 2012; Puelles et al., 2013). However, in the absence of Rax, the existing patterning gradients result in expansion of the forebrain tissues neighboring the presumptive retina. The effects of the loss of *rax* function on Wnt signaling may be of particular interest for further study, as both canonical and non-canonical Wnt signaling play key roles in patterning the eye field and diencephalon (Cavodeassi et al., 2005; Wilson and Houart, 2004). Studies in zebrafish have demonstrated that non-canonical Wnt signaling via Wnt11 and Fz5 can antagonize canonical Wnt signaling in the eye field; when non-canonical Wnt signaling is reduced, the eye field is lost and re-specified into caudal diencephalon (Cavodeassi et al., 2005). Our observation that *fzd4* expression is up-regulated in the *rax* mutant is intriguing in this regard and may reveal complex interactions, since the *fzd4* receptor has been shown to mediate both canonical and non-canonical Wnt signaling, and can act as both an activator or repressor of signaling (Swain et al., 2005).

Returning to the basic finding that properties of the brain both posterior to and anterior to the retina are expanded in the *rax* mutant, it is important to note that the retina region is likely not completely respecified toward telencephalic and diencephalic fates. We note that, as shown in Fig. 4, the expanded regions of *foxf1* and *arx*, indicators of telencephalic and diencephalic fate, respectively, overlap in the *rax* mutant, indicating that the respecified retinal tissue likely has a blended or incompletely transformed regional identity.

Timing of *rax* gene function

It is also important to note that the fate shift resulting from the *rax* mutation implies that when *rax* activation occurs in the late gastrula (St. 12), forebrain regional boundaries

including the presumptive retina are not yet determined and that the default fate of the retina-forming region would be to assume alternative forebrain identities. This lability is also supported by work showing that brain and eye regions in *Xenopus* are not determined within the anteroposterior neuroaxis at St. 11.5 (Saha and Grainger, 1992) though they are by the neural plate stage (St. 14). In addition, recent work (S. Louie, M. Fisher and R.M. Grainger, unpublished) argues that the *Xenopus* retina becomes determined by St. 12.5, suggesting a key role for *rax* activation in commitment. Consistent with this time frame for regional determination, the transplant experiment presented here shows that the fate of the presumptive retinal field in St 15 *rax* mutant embryos has already been stably transformed to non-retinal forebrain. Determination of diencephalic regions in zebrafish occurs at a similar stage (Staudt and Houart, 2007). Resolving the determination mechanisms here, both for the retina and brain regions, will be greatly aided by the many approaches the *Xenopus* affords for examining early development.

Comparison of knockdown strategies and genetic lesions for untangling gene networks during *Xenopus* embryogenesis

The studies performed with the *rax* mutant illustrate some clear advantages over transient methods for interfering with gene expression (MOs or dominant-negative constructs). The stability of using embryonic tissue resulting from a consistent genetic lesion allows one to perform manipulations, like the RNA-Seq or transplants performed here, that would be much more uncontrollable because of the genetic variation from embryo to embryo were an injection-based strategy (e.g. using MOs) used instead.

Schulte-Merker and Stainer (2014) report that there accumulated evidence in zebrafish that, for many genes, MO-mediated gene-knockdown phenotypes are not always the same as genetic mutants and in some cases may result from artifacts not accurately assessing the *in vivo* function of a gene. While *Xenopus* researchers have used MOs to reveal a multitude of important biological functions, the utility of direct gene mutations as described here allows not only an accurate assessment of gene function but also permits one to screen phenotypes throughout the life of an animal, i.e. with the limitations of MOs to examining early development. Our own observations also at least raise questions about the differences observed between these two methodologies and highlight potential advantages of using mutations for studying development. For example, although we have identified new targets downstream of *rax* in addition to confirming previously known targets as described above, our observations are not consistent with some interactions implied by experiments utilizing strategies to assay Rax function through MO-knockdown or expression of transcriptionally repressive forms of Rax (Andreazzoli et al., 1999, 2003; Giannaccini et al., 2013; Terada et al., 2006). Specific downstream genetic interactions inferred from such studies, such as placing *xhmg3* downstream of *rax* (Terada et al., 2006) or transcriptional repressors *crx*, *tle2* and *hes4* (Giannaccini et al., 2013), are not consistent with the RNA-Seq analyses presented here where we see that the expression levels of these transcripts are unaffected in *rax* mutant tissue at neural plate stages. Although it is possible that some of these previously implied downstream effects occur later in development, (i.e. after our RNA-Seq analysis was performed), these differences may also reflect some of the questions raised by Schulte-Merker and Stanier about inferring genetic connections from experiments using MOs (which

may also apply to overexpression of dominant-negative constructs). Thus, although MOs or dominant-negative constructs can be and have been very useful reagents for rapid loss-of-function studies, one needs to be aware that they may not always reveal native genetic interactions or at least need to be reconciled with genetic data.

This work highlights the value of generating mutations in *Xenopus* for studies of early embryonic patterning and organogenesis events that are less tractable in many other vertebrate model systems. While the TILLING technology used to generate the mutation studied here requires significant labor to produce mutations, new genome editing techniques developed for many model organisms (TALENs and CRISPR/Cas9) are now available in the *Xenopus* system (Blitz et al., 2013; Guo et al., 2014; Lei et al., 2012; Nakajima and Yaoita, 2013; Nakayama et al., 2013; Suzuki et al., 2013). These advances will allow researchers to combine the advantages of *Xenopus* with facile genetic approaches that will allow rapid generation of null mutations, providing the experimental framework for gaining a more accurate understanding of the gene networks involved in normal developmental processes along with the genetic alterations underlying many human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We identified an eyeless *X. tropicalis* *rax* mutant during a mutagenesis screen.
- RNA-Seq analysis identified novel genes downstream of *rax*.
- Key eye field transcription factors are expressed normally in this eyeless mutant.
- Eye tissue is transformed to non-retinal forebrain identities in *rax* mutants.
- Failure to locally repress *fezf2* and *hesx1* in mutants leads to forebrain expansion.

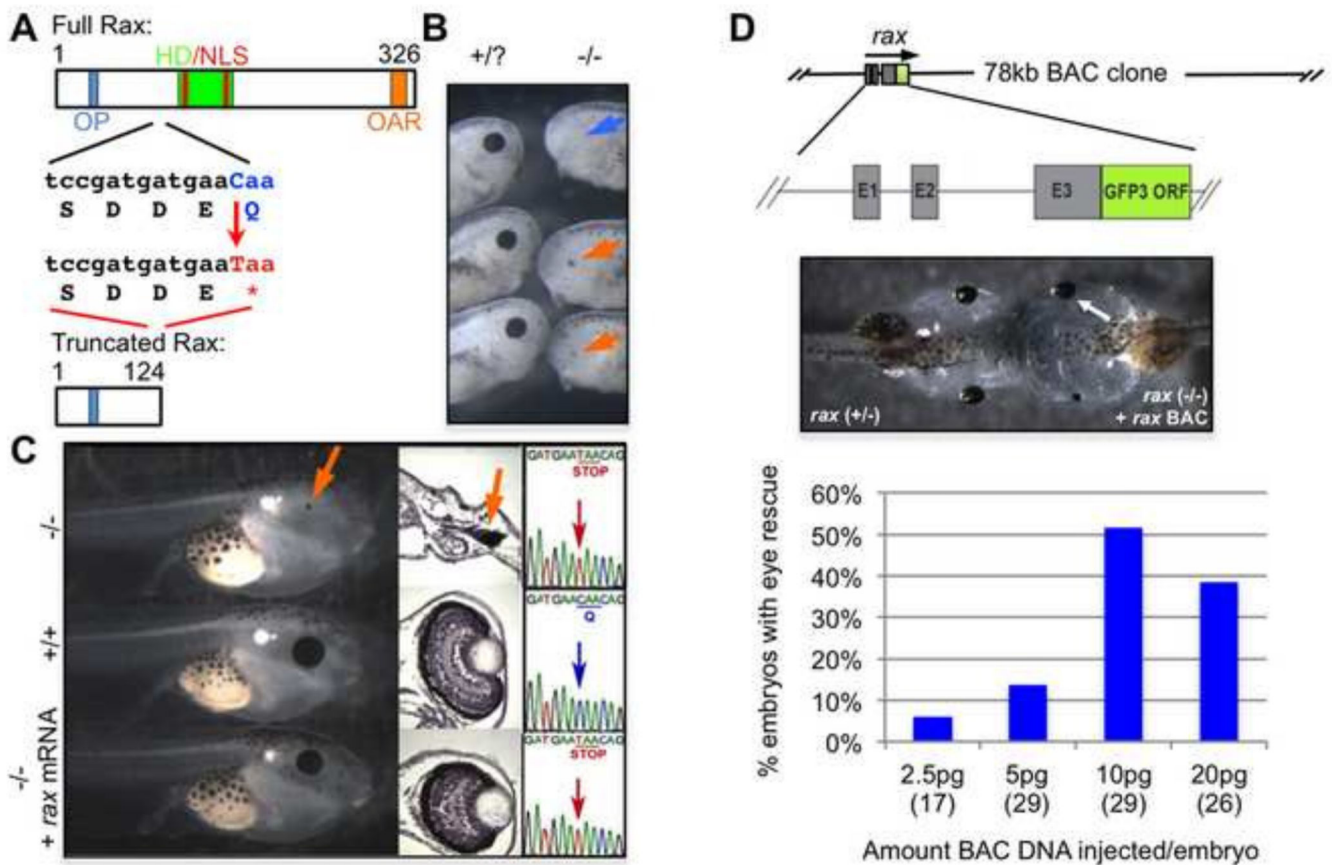


Figure 1. A nonsense mutation in the retinal anterior homeobox gene (*rax*) results in an eyeless phenotype

(A) The Rax protein structure includes an octapeptide domain (OA, blue), homeodomain (HD, green) and OAR domain (orange). Nuclear localization signals (NLSs, red) are found in the homeodomain. A nonsense mutation results in a truncation prior to the homeodomain and NLSs. (B-C) Tadpoles homozygous for the *rax* mutant allele fail to form eyes (B, right; C, top). A small amount of retinal pigmented epithelium (RPE) is sometimes seen in mutant animals (B, C, orange arrows) whereas some mutant embryos have no sign of RPE (B, blue arrow); this variation is observed within the same clutch of embryos. The eyeless phenotype can be rescued with the injection of 5 pg of *rax* mRNA (C, bottom, 10 out of 24 injected). Sections through the mutant, wildtype, and mRNA-rescued mutant tadpole eye regions are shown in the middle panel, illustrating the loss of eye tissue, except small residual RPE in some mutant embryos, and rescue of the retinal and lens structures in mRNA-injected mutant. Far right of (C), insets of Sanger-sequencing showing point mutation in exon 2, which results in a premature stop codon. (D) A *Xenopus tropicalis* BAC clone containing a *rax-gfp* fusion gene can rescue eye formation when transiently expressed in mutant embryos. Note that BAC injection tends to be effective dominantly (or only) in one side as described before (Fish et al. 2012). E1-E3 indicate *rax* exons; the *gfp3* open reading frame (ORF) has been fused to the 3' end of the final *rax* exon (E3) to produce a *rax-gfp3* fusion gene. Quality of rescue is highly dose-sensitive, with the best rescue observed with 5 pg mRNA (C, data not shown) and 10 pg of BAC DNA injected (D).

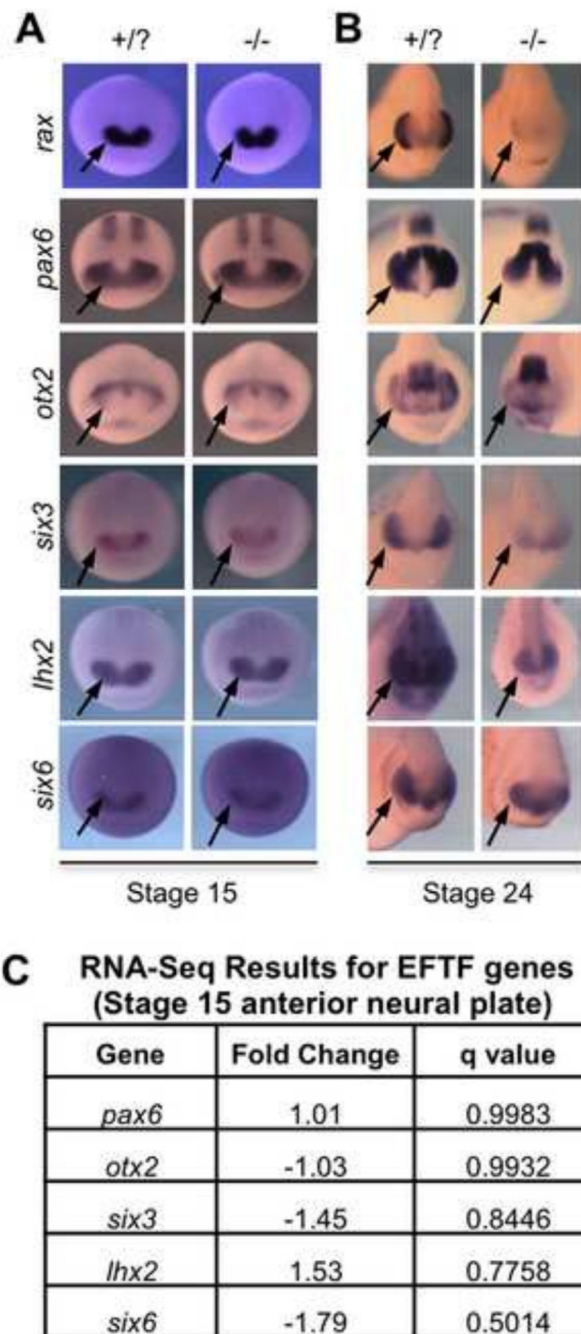


Figure 2. Key eye field transcription factor (EFTF) expression is unaffected in neural plate stage embryos

(A) The expression patterns and levels of *rax*, *pax6*, *otx2*, *six3*, *lhx2*, and *six6* assayed via *in situ* hybridization in neural plate stage (St. 15) embryos with at least one wildtype allele versus homozygous mutant embryos reveal that key EFTF expression levels are unaffected in the *rax* mutant. Presumptive retinal regions indicated (arrows). (B) By early tailbud stage (St. 24), down-regulation of EFTFs can be detected in regions where the retina normally forms (arrows). (C) RNA-Seq analysis comparing wildtype and mutant St. 15 anterior

neural plates confirms that EFTFs are not significantly affected in early stage *rax* mutant embryos. Fold change calculated using normalized read counts averaged from triplicate pooled samples. Q-values calculated using analysis utility Cuffdiff, part of the Cufflinks software package (Trapnell et al., 2010), with a False Discovery Rate (FDR) of 0.05. High q-values indicate that listed genes are not significantly affected in neural plate stage *rax* mutant embryos.

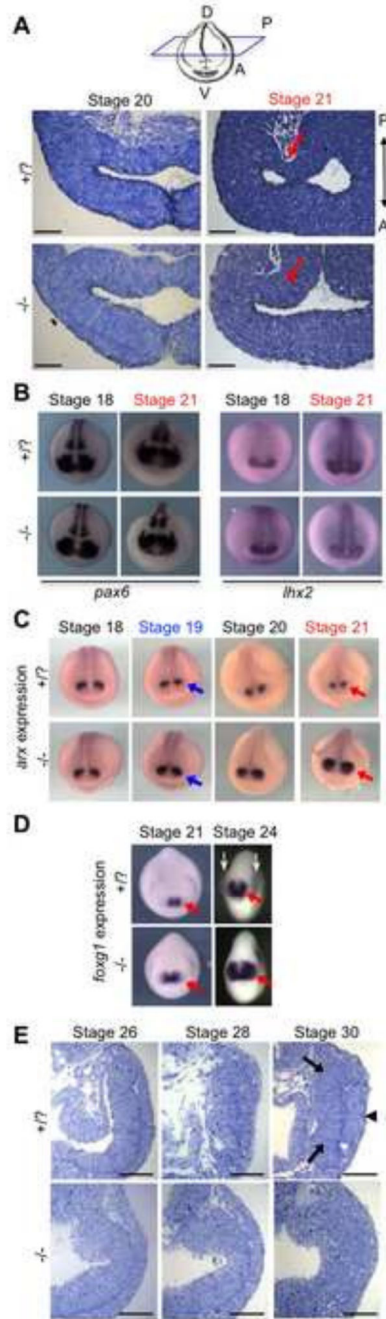


Figure 3. Morphological changes in *rax* mutant embryos can be detected by St. 21, prior to differential expression of key EFTFs, but following up-regulation of *arx*. Up-regulation of telencephalic marker *foxg1* is also observed by St. 21

(A) Histological sections through St. 20 and St. 21 embryos reveal no detectable change in optic vesicle morphology at St. 20, but a lack of thinning of the posterior optic vesicle wall in mutant embryos at St. 21 (red arrows). The plane of sectioning is indicated above, and is the same plane for all stages shown throughout (serial sections were carefully examined to correctly match the plane of sectioning). The anterior and posterior ends of sections are marked A and P, respectively. (B) Examination of key EFTFs (*pax6* and *lh2* shown) via *in*

situ hybridization at St. 18 and St. 21 reveals no detectable change in expression. **(C)** By St. 19, anterior expansion of the *arx* expression domain is observed in mutant embryos (blue arrows). This expansion becomes more pronounced at St. 21 (red arrows). **(D)** Examination of telencephalic marker *foxg1* at stages 21 and 24 reveals lateral expansion in *rax* mutant embryos (red arrows). At stage 24, lateral bulging of the optic vesicles can be observed in whole embryos from an anterior view (white arrows); these bulges are not observed in *rax* mutant embryos, and *foxg1* expression is expanded into a region of the territory the optic vesicle would occupy in wildtype embryos. **(E)** Histological sections at St. 26, St. 28 and St. 30 show the increasing divergence of wildtype and mutant morphologies. By St. 30, the optic cup and lens tissue are forming in wildtype embryos (black arrows and arrowhead, respectively), but typically fail to form in *rax* mutant embryos. In panels A and E, a minimum of three embryos for each stage and genotype were sectioned and consistently displayed the shown morphology. Scale bars in panels A and E measure 75 micrometers.

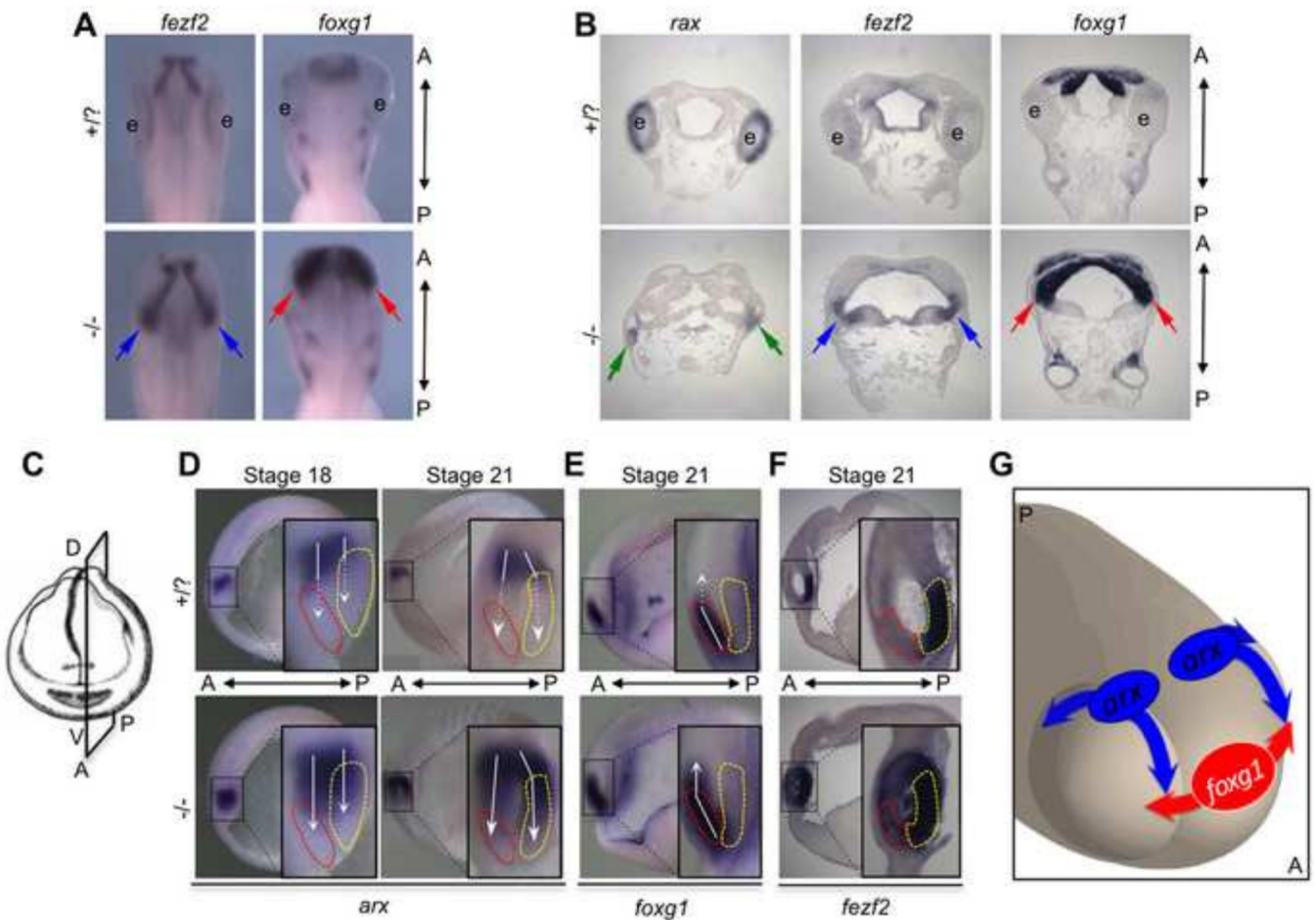


Figure 4. Diencephalic and telencephalic tissue is expanded in the *rax* mutant

(A) Dorsal views of St. 32 embryo heads assayed for diencephalic marker *fezf2* (left) or telencephalic marker *foxg1* (right) via *in situ* hybridization. Both *fezf2* (blue arrows) and *foxg1* (red arrows) expression is expanded into regions where the eyes would normally form in wildtype. (B) Frontal sections through St. 35 wildtype and mutant embryos detecting *rax*, *fezf2*, *foxg1* or *foxd1* expression by *in situ* hybridization. In *rax* mutant embryos (bottom row), green arrows indicate small remaining rudiment still expressing *rax*. In the absence of functional *rax*, expression of *fezf2* (blue arrows) and *foxg1* (red arrows) is expanded into the tissue where the retina would normally form. (C) Visualization of the parasagittal plane of sectioning performed on embryos in D-F. (D) Lateral views of parasagittally-bisected embryos at indicated stages stained via *in situ* hybridization for early diencephalic marker *arx*. In panels D-F, red dashed outlines indicate developing telencephalic tissue, and yellow dashed outlines indicate developing posterior secondary prosencephalon. White arrows cover the expression range of each marker, and dashed white arrows indicate into which region expression expands in *rax* mutant embryos (bottom row). *arx* expression is expanded ventrally in mutant embryos, and expands both anteriorly into the telencephalon and posteriorly into the secondary prosencephalon, with significant expansion observed by St. 21. (E) Parasagittally-bisected embryos at St. 21 show ventral expansion of telencephalic marker *foxg1*, which expands beyond the wildtype telencephalic domain in *rax* mutants. (F)

fezf2 expression in the wildtype marks the posterior secondary prosencephalon. In *rax* mutant embryos this expression expands into the telencephalon and optic vesicle. (Note: for clarification of dense staining, this embryo was thinly parasagittally-sectioned via vibratome, instead of simply bisected as in panels D-E. The plane of sectioning is the same in panels D-F). (G) A cartoon illustrating the 3-dimensional movements of *arx* and *foxf1* expansion into the presumptive optic vesicle regions of *rax* mutant embryos. Observed expansion of *arx* is moving ventrally, both anteriorly and posteriorly. Expansion of *foxf1* is primarily lateral. Movement of these marker boundaries (and others) likely results in the expansion of diencephalic and telencephalic markers observed in the later mutant embryos shown in A-B.

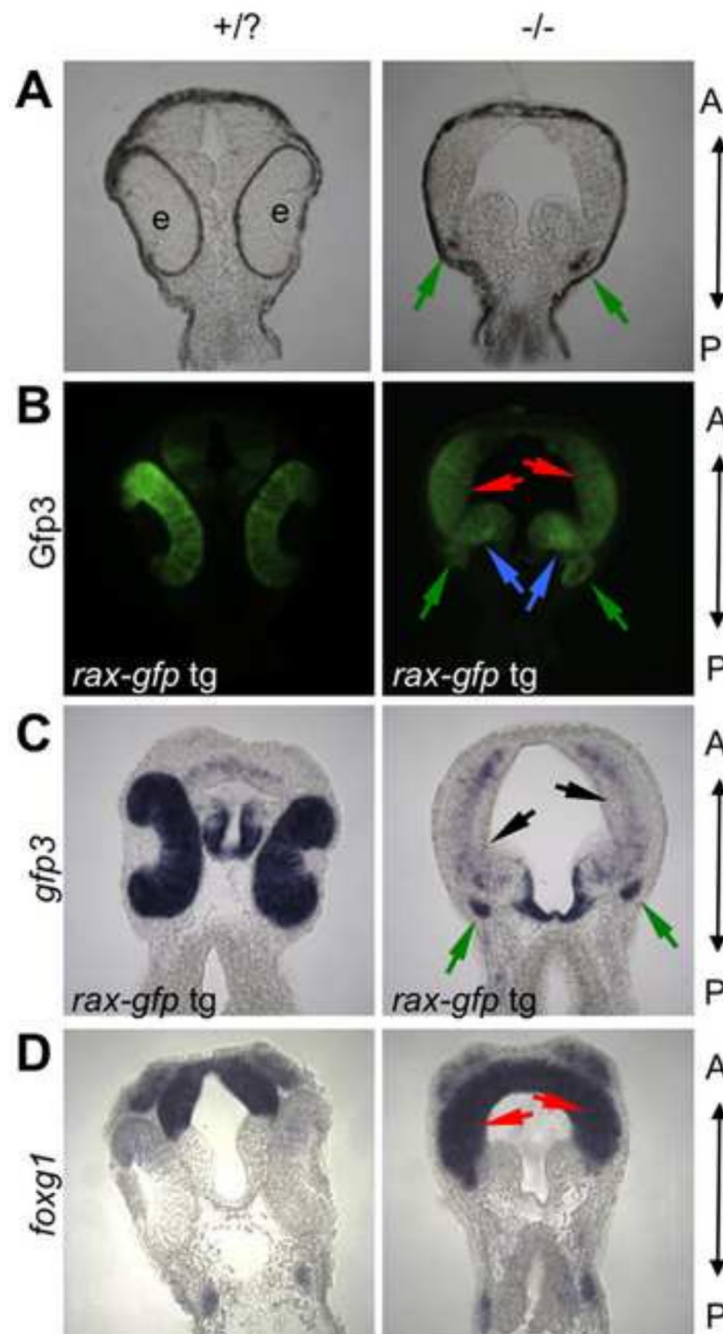


Figure 5. Presumptive retinal tissue is transformed into diencephalic and telencephalic tissue in the *rax* mutant

(A-C) Genetic labeling of retinal fated tissue in wildtype and mutant embryos. A transgenic line expressing *gfp3* under control of the *rax*-promoter was crossed into the *rax* mutant background. (A) Brightfield image of frontal sections from stage 32 embryos. (B) Gfp3 protein expression detected via immunostaining. (C) Transgene (*gfp3*) mRNA expression visualized via *in situ* hybridization. (D) Telencephalic marker *foxg1* mRNA expression visualized via *in situ* hybridization. In all panels, A marks the anterior and P the posterior.

Red arrows mark regions of telencephalic expansion, and blue arrows mark diencephalic expansion into eye region. Green arrows mark the small rudiment that is pigmented (A) in this specific section and continues to express Gfp3 (B-C). Although Gfp3 protein can still be readily discerned, the mRNA is mostly absent in the expanded telencephalic region (black arrows). Five embryos from each phenotype (wild-type or mutant eye morphology) and treatment were scored for these analyses; expression patterns were highly stereotyped within each phenotype. A minimum of two embryos for each phenotype and treatment were then chosen for serial sectioning and imaging.

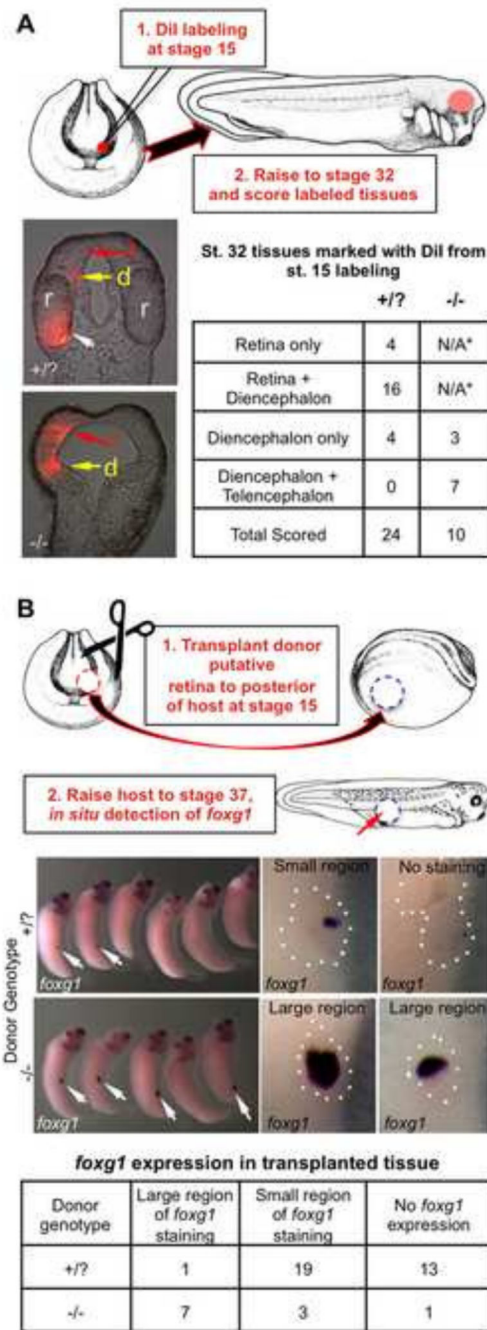


Figure 6. Presumptive retinal tissue is transformed into both diencephalic and telencephalic fates in *rax* mutant embryos

(A) Retinal rudiment was labeled in St. 15 embryos using DiI marking. Embryos were then raised to St. 32 and scored for the fate of labeled tissues. Frontal sections of St. 32 embryos show DiI marking the retina (r, white arrow) and small amount of diencephalon (d, yellow arrow) in wildtype embryos, but not the telencephalon (t, red arrow). In mutant embryos, labeled tissues are found extensively in both the diencephalon and expanded telencephalon. Results shown in the table. *Since eyes fail to form in *rax* mutants, DiI labeling in the retina

is not applicable (N/A). **(B)** Presumptive retinal tissue from St. 15 *rax* mutant or wildtype donor embryos was transplanted to the posterior of host embryos, which were raised to St. 37 and fixed. Donor embryos were allowed to develop in order to determine donor phenotype. *In situ* hybridization to detect telencephalic marker *foxg1* was performed on hosts with donor tissue to determine the extent of telencephalic character in donor transplants. Left panels show lateral views of whole embryos, white arrows indicate regions positive for *foxg1* expression; right panels show higher magnification view of transplanted region (outlined with white dots). Representative examples of each category (large or small region of transplant that is positive for *foxg1* expression domain relative to entire transplant size) are shown in right panels. Results from scoring are tallied in the bottom table.

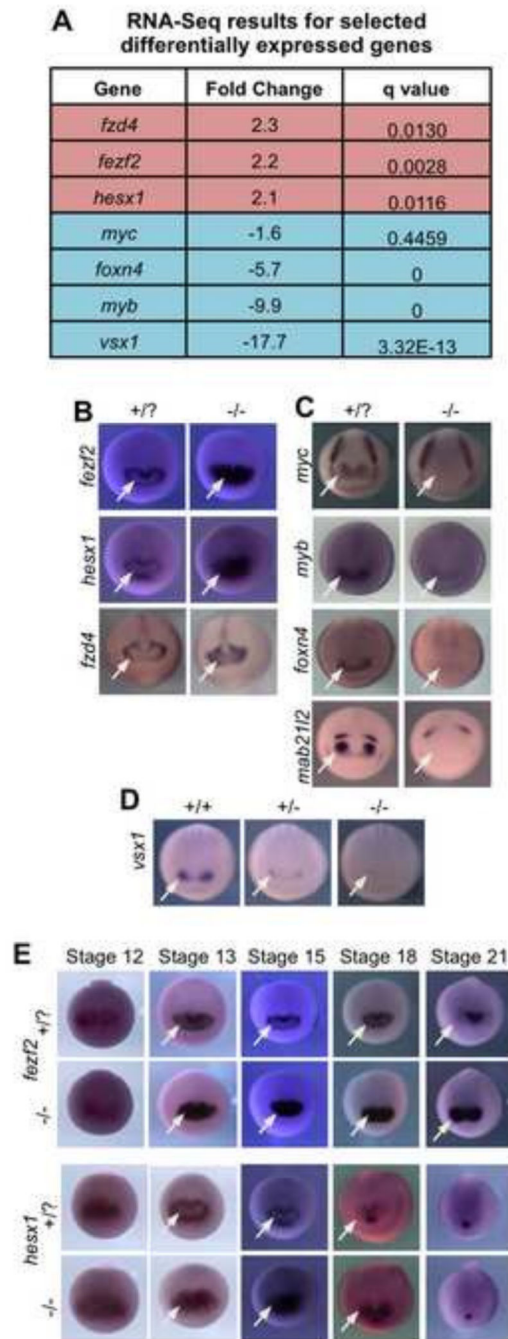


Figure 7. Genes with altered expression in *rax* mutants include those necessary for retina formation and forebrain patterning

(A) Table listing key up-regulated (red) and down-regulated (blue) genes from RNA-Seq analysis. (All significantly affected genes identified in this analysis can be found in Table S4) (B) *In situ* hybridization for up-regulated genes in St. 15 embryos. White arrows indicate regions where up-regulation is observed in mutants in the presumptive retinal field. (C) *In situ* hybridization for down-regulated genes in St.15 embryos. White arrows indicate regions in the eye field where expression is not detected in the mutant. (D) *vsx1* expression

(arrows) at neural plate stage is reduced in the heterozygote compared to wildtype and not detected in *rax* homozygous mutant embryos. **(E)** A time course of *fezf2* and *hes1* expression from late gastrula (St. 12) through neural tube stage (St. 21) in *rax* mutant and wildtype embryos. White arrows indicate regions where repression is seen in wildtype embryos and not observed in *rax* mutants.

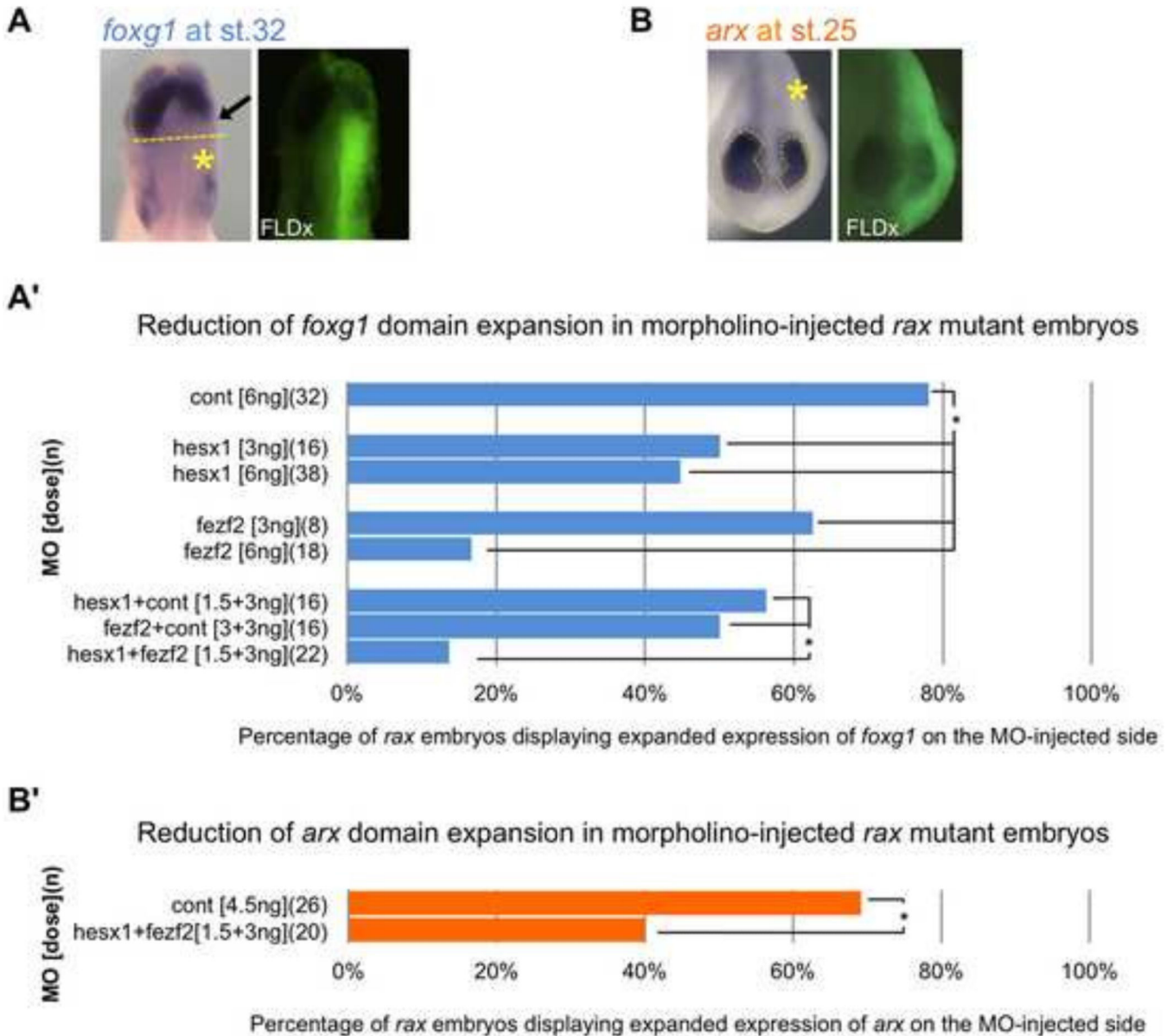


Figure 8. Knockdown of *fezf2* and/or *hesx1* function reduces expanded *foxg1*- and *arx*-expressing region(s) in *rax* mutant embryos

(A) Representative example of the dorsal view of St. 32 *rax* mutant embryos injected with *fezf2* and/or *hesx1* MO(s), and assayed for *foxg1* expression by *in situ* hybridization (left panel). Note that reduced signal is seen in the ectopically expanded *foxg1*-expressing region (arrow, left panel) on the injected side of mutant embryos (yellow asterisk, left panel), as visualized by FLDx tracer (right panel). (A') Percentage of embryos with mutant phenotype (expanded *foxg1* expression) on injected side after receiving MO(s) injection. Embryos were injected into one dorsal blastomere at the 4-cell stage with either control, *fezf2* or *hesx1* morpholinos, or a combination of two morpholinos, as indicated in the y-axis. (B) Representative example of the frontal view of a St. 25 *rax* mutant embryo injected with combined *fezf2* and *hesx1* MOs, and assayed for *arx* expression by *in situ* hybridization (left

panel). Dashed yellow outlines mark the region of *arx* expression, which is reduced on the injected side (marked with an asterisk, and visualized by FLDx tracer in right panel). **(B')** Percentage of embryos with mutant phenotype (expanded *arx* expression) on injected side after receiving MO(s) injection. Embryos were injected into one dorsal blastomere at the 4-cell stage with either control or combined *fezf2* or *hesx1* morpholinos. (A'-B') Injected doses of morpholinos is indicated in brackets, and numbers of embryos scored in parentheses. Asterisks indicate p-values of <0.05 by chi-squared test.

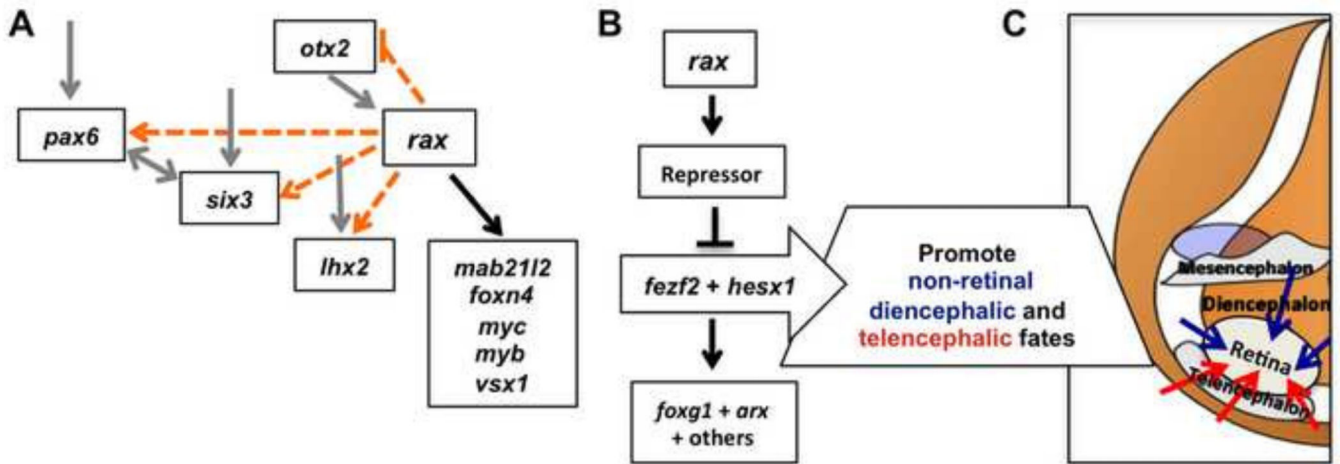


Figure 9. Models of *rax* function

(A) Analysis of the *rax* mutant indicates that *rax* function is not necessary for activation of key EFTFs *pax6*, *otx2*, *lhx2*, or *six3*. *rax* expression is necessary for the correct expression of other key eye genes *mab2112*, *foxn4*, *myc*, *myb*, *vsx1*, in addition to other factors. Dotted orange lines indicate putative, early interactions within the eye field gene regulatory network not supported by these analyses, although later, indirect effects of *rax* inactivation does affect their expression. Solid grey lines indicate previously described interactions not addressed in this work, but consistent with our data, and solid black lines indicate interactions supported by these analyses. (B) A key forebrain patterning event necessary for correct retina formation is the activation of a repressor(s) by *rax*, whose role is to down-regulate *fezf2* and *hesx1* in the anterior neural plate, creating a hole within their expression domains starting by late gastrula stages (St. 13). As shown in (C), when this repression of *fezf2* and *hesx1* fails to occur we propose that they contribute to establishment of non-retinal diencephalic (blue arrows) and telencephalic (red arrows) properties within presumptive retinal tissue, resulting in the expansion of these forebrain identities into the retinal region. Fate map in (C) adapted with permission from (Eagleson and Harris, 1990).