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The *rax* homeobox gene is mutated in the *eyeless* axolotl, *Ambystoma mexicanum*

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Abstract

Background: Vertebrate eye formation requires coordinated inductive interactions between different embryonic tissue layers, first described in amphibians. A network of transcription factors and signaling molecules controls these steps, with mutations causing severe ocular, neuronal, and craniofacial defects. In *eyeless* mutant axolotls, eye morphogenesis arrests at the optic vesicle stage, before lens induction, and development of ventral forebrain structures is disrupted.

Results: We identified a 5-bp deletion in the *rax* (retina and anterior neural fold homeobox) gene, which was tightly linked to the recessive *eyeless* (*e*) axolotl locus in an F2 cross. This frameshift mutation, in exon 2, truncates RAX protein within the homeodomain (P154fs35X). Quantitative RNA analysis shows that mutant and wild-type *rax* transcripts are equally abundant in *E*/*e* embryos. Translation appears to initiate from dual start codons, via leaky ribosome scanning, a conserved feature among gnathostome RAX proteins. Previous data show *rax* is expressed in the optic vesicle and diencephalon, deeply conserved among metazoans, and required for eye formation in other species.

Conclusion: The *eyeless* axolotl mutation is a null allele in the *rax* homeobox gene, with primary defects in neural ectoderm, including the retinal and hypothalamic primordia.

Keywords

Ambystoma ; anophthalmia; eye morphogenesis; genetics; homeodomain; hypothalamus; leaky scanning; lens induction; mutation; optic vesicle; pituitary; Rax; ribosome; Rx; salamander; transcription factor; urodele

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Erik S. Davis: Investigation; writing-original draft. **Gareth Voss:** Investigation; writing-review and editing. **Joel B. Miesfeld:** Investigation; supervision; writing-review and editing. **Juan Zarate-Sanchez:** Investigation; writing-review and editing. **S. Randal Voss:** Conceptualization; data curation; funding acquisition; investigation; supervision; writing-review and editing. **Thomas Glaser:** Conceptualization; data curation; funding acquisition; investigation; supervision; writing-original draft; writing-review and editing.

1 | INTRODUCTION

Eye formation in vertebrates occurs via a stepwise process, involving regionalization, induction, and differentiation. Following gastrulation, the anterior neural plate is patterned into discrete regions, which are specified by eye field transcription factors (EFTFs).[1] These homeodomain (HD) proteins are expressed in overlapping sectors of the prospective eye field, and orchestrate its morphogenetic transformation into bilaterally symmetric optic vesicles, which emerge from the ventral diencephalon during neurulation.[2] In a series of classical experiments, first performed in urodele amphibians 120 years ago, Spemann, Lewis, and others showed that the optic vesicles induce formation of a lens placode (thickening) on each side of the head, as they contact the overlying surface ectoderm and involute to form bilayered optic cups.[3–5] These ectodermal tissues subsequently differentiate to form the lens and mature retina, with inner neural and outer pigmented layers.[6] Apart from critical EFTFs, such as Rax (retina and anterior neural fold homeobox) and Pax6 (paired box family), eye development is mediated by transmembrane and secreted protein factors, such as BMP4, which transmit inductive signals bidirectionally between the optic vesicle and surface ectoderm.[7,8] These morphogenetic steps were defined during the past century through studies involving amphibian embryos, which are easy to maintain, monitor and manipulate; conventional genetic models, such as laboratory mice, which allow comparative molecular analysis of mutants, phenotypes, and transgenes; and more recently, 3D retinal organoid cultures.[9,10]

Eyeless phenotypes, including clinical anophthalmia in humans,[11] may result from mutations that disrupt early patterning or partitioning of the eye fields, lens induction, migration of ocular neural crest cells, or periocular mesenchyme signaling—or disrupt the growth, separation and/or differentiation of the retina, pigmented epithelium (RPE), optic stalk or lens.[6] Eye regression can also occur via positive evolutionary selection, in species adapted to life in troglobitic (caves) or subterranean environments, with a complex genetic basis.[12,13]

The axolotl (Nahuatl *āxōlōtl*), *Ambystoma mexicanum*, is the oldest continuously studied model organism.[14] An endangered neotenic salamander, the axolotl is indigenous to Xochimilco and interconnected lakes that once surrounded Tenochtitlan (Mexico City).[15] This species has an aquatic life cycle, with most individuals retaining gills after metamorphosis and sexual maturation.[16,17] Axolotls have been an important model in the history of experimental embryology, and are capable of regenerating limbs and other tissues.[18–20] Existing laboratory colonies and inbred lines trace their ancestry to a cohort of live specimens imported to Paris in 1863.[21] Several Mendelian traits have been described, affecting organogenesis or physiology.[22] However, molecular studies have been limited by the large size (C-value) of urodele genomes, which are the largest among tetrapods. The axolotl genome has 14 chromosomes and is 32 Gb, more than 12 times the size of the mouse genome.[23] Recently, a chromosome-scale axolotl genome assembly was reported, and is the largest to date, providing a framework for gene discovery.[23,24]

Eyeless axolotls were first identified by Rufus Humphrey at Indiana University, in an inbred strain (Wistar) with a *white* (*d*/*d*) genetic background.[25] Homozygous *eyeless* mutants (*e*/*e*)

lacked eyes and were darkly pigmented. They had hypothalamic defects and immature gonads and were consequently sterile. The eye phenotype was initially attributed to the absence of optic vesicles,[26,27] but in later studies, small or delayed optic vesicles were described, which failed to progress.[28]

The dark pigmentation across the dorsal head region is caused by dispersion of melanin granules within large dendritic chromatophores, and is a secondary pleiotropic effect. This phenotype can be reproduced in wild-type (WT) animals by removing both eyes, which increases the level of melanocyte stimulating hormone, MSH.[29] Normal pigmentation was restored to *e*/*e* animals by grafting a single functioning eye, but these axolotls remained infertile.[30] Sterility has been attributed to hypothalamic brain defects, and the absence of gonadotropin releasing hormone (GnRH). Chimeric animals with WT heads and *eyeless* trunks are fertile, with mature gonads, but reciprocal chimeras are sterile.[26,31]

Epp and van Deusen proved that the *eyeless* gene acts within the forebrain ectoderm and not in the surface ectoderm that forms the lens, or inner mesoderm that transiently induces the eye fields during gastrulation.[26,27,32] They showed this by reciprocally grafting neural ectoderm between *eyeless* and WT embryos. WT grafts led to eye formation in *e*/*e* recipients, but reciprocal grafts failed. Thus, in *eyeless* mutants, the primary inductive interaction between surface and neural ectoderm layers, first described over a century ago,[4] fails to occur. The molecular basis for this defect has been unknown. In this report, we demonstrate linkage between *eyeless* and the *rax* homeobox gene, and identify the causative molecular lesion.

2 | RESULTS

2.1 | *eyeless* mutants

The embryonic development, adult phenotypes, and transmission genetics of the *eyeless* (*e*) axolotl have been reported, with major primary defects noted in eye, hypothalamic and pituitary development.[27,33] In *e*/*e* embryos, the optic vesicles fail to emerge from the ventral diencephalon or are significantly delayed; they do not progress to form bilayered optic cups or induce lens formation, and subsequently degenerate.[34] Likewise, the anterior hypothalamus develops abnormally. There is no preoptic recess or extension of PAF+ neurosecretory fiber tracts; the neurohypophysis appears atrophied and the anterior pituitary gland (adenohypophysis) is dysmorphic.[35] Consequently, *e*/*e* animals are sterile, lacking GnRH, and gonadotropins, and hyperpigmented, with expanded chromatophores in the absence of light perception and environmental feedback.[33]

To further demonstrate these phenotypes, we collected stage 42 larvae from an F2 cross. In *e*/*e* samples, anatomical defects were apparent in exterior photographs (Figure 1A), coronal immunofluorescence micrographs, stained with β3-neurotubulin (Tubb3) and Pax6 antibodies (Figure 1B), and histological sections stained with hematoxylin and eosin (H&E; Figure 1C–F). These findings are consistent with a primary patterning, identity or signaling defect in the anterior neural ectoderm. In some *e*/*e* larvae, small dysmorphic optic rudiments were observed below the surface ectoderm, with variable features suggestive of retina, lens and/or RPE (Figure 1E,F), as previously noted in *eyeless* axolotls at hatching[34] and targeted

rax mutant *Xenopus*.[36] These rudiments do not progress and subsequently degenerate during larval life.

2.2 | F2 segregation test

We initially considered two candidate genes—*pax6* and *rax*, critical EFTFs whose spatiotemporal expression, biological functions, and mutant features in other vertebrates are consistent with *e*/*e* phenotypes.

Pax6 is required for eye and brain development in diverse metazoans, and has deeply conserved structure, expression, and developmental functions.[37,38] It contains paired and homeobox DNA-binding domains, acts near the top of a hierarchy for eye development, and is notably sufficient to induce ectopic eyes in *Drosophila* and some vertebrates.[39,40] Loss-of-function mutations have been reported in humans, mice, zebrafish and fruit flies with anophthalmia and major brain malformations, including defects in the cortex, hypothalamus and pituitary[41–44] or cognate structures.[45,46] Null mutations are typically lethal in vertebrates, owing to defects in the brain and pancreatic islets, but hypomorphic and regulatory alleles are known.[47–50] Heterozygotes have milder phenotypes (haploinsufficiency). Pax6 mRNA is expressed throughout the optic primordia. In mutant mice, eye development arrests at the optic cup stage, before lens induction.[51] Previous in situ hybridization analyses show reduced cephalic *pax6* mRNA in axolotl *e*/*e* embryos—a potential cause or effect of the *eyeless* phenotype.[52]

The Rax (also Rx) homeobox gene is likewise required for eye and ventral brain development in chordates.[53–56] It acts earlier than Pax6, but has a more limited pattern and phenotype.[57,58] Rax is strongly and broadly expressed in the diencephalon, within the anlagen giving rise to the hypothalamus, pineal, optic vesicles and stalk. Spontaneous or engineered *Rax* mutations cause eyeless phenotypes in medaka and zebrafish, frogs, mice, and humans,[36,55,59–63] with limited progression past the optic vesicle stage. Homozygous *Rax*-null mice die at birth with brain and craniofacial malformations[64]; however, inbred eyeless mice carrying a hypomorphic allele (*ey1*, Rax^{M10L}) are viable and fertile.[65] There are two vertebrate Rax families, which arose through ancestral whole genome duplications, WGD.[66,67] In partially tetraploid species (*Xenopus laevis*, teleost fish), these paralogs are further duplicated. In zebrafish, for example, *rx3* is expressed in the anterior neural plate, optic vesicles, and forebrain, and corresponds to mouse *Rax*, whereas *rx1* and *rx2* are mainly localized to the neuroretina.[68] The orthologous gene (raxL, RxL, RAX2) promotes photoreceptor differentiation in chick, *Xenopus* and humans,[69–73] but was lost in at least five mammalian lineages, including rodents.[67] *RAX2* coding mutations cause retinal degeneration in humans[74,75] and an *rx1* regulatory deletion underlies the large natural variation in cone opsin expression among Lake Malawi cichlid species with diverse visual sensitivities.[76]

To test these candidates, we intercrossed carriers and prepared genomic DNA from F2 larvae, of which approximately 25% were eyeless. We then genotyped a subset of eyeless (*e*/*e*) and WT (*E*/−) offspring by PCR sequencing. We amplified *pax6* and *rax* 3′UTRs and scored single-nucleotide polymorphisms (SNPs). For *pax6*, we scored a G/A variant, heterozygous in each parent, and observed 1:2:1 genotype ratios in both phenotypic classes

(Table 1). *pax6* thus segregates independently of the *eyeless* trait and is excluded ($P = 0.33$, $\chi^2 = 2.22$, $df = 2$, $n = 23$). Consistent with these data, *pax6* cDNAs from *e*/*e* and *E*/*E* embryos have identical sequence (TG and RL Maas, unpublished).

For *rax*, we scored a 1-bp insertion (insG) that was heterozygous in the male parent only. In this case, we observed a 2:1 genotype ratio among 86 WT larvae, and no heterozygotes among 55 eyeless larvae (Table 1), with 1:1 ratios expected in each class for independent assortment. *rax* is thus tightly linked to *eyeless* ($P = 3.30 \times 10^{-13}$, $\chi^2 = 53.02$, $df = 1$, $n = 55$) and maps within 1.5 cM (95% CI).

2.3 | *rax* frameshift mutation in *eyeless* axolotls

To identify the causative mutation, we amplified the three *rax* exons and four conserved noncoding elements (CNEs 0–3) from archived *E*/− and *e*/*e* samples, with flanking PCR primers designed using the 32 Gb genome assembly from wild axolotl stocks[23,24] as a guide. Exons 1 to 3 and internal CNE2 (intron 2) span 16.1 kb of contig AMEXG_74394 (50.7 kb) on chromosome 6p (Figure 2A), and were identified through BLAST homology searches with known vertebrate *rax* sequences as query. This contig maps inside AMEXG_0030000955 (6.5 Mb), within a 51 kb assembly gap, flanked by CNE1 and CNE3 on opposite sides. This scaffold encompasses seven genes (*pmaip1*, *ccbe1*, *lman1*, *cplx4*, *rax*, *grp*, *sec11c*), with conserved synteny and order among tetrapods (Figure 2A). The *rax* CNEs are well characterized as *cis* regulatory enhancers in mice and frogs.[77–79] These seven features, which comprise the *rax* gene and regulatory unit, span about 500 kb in axolotls, compared to 15 kb in mice, consistent with the enormous evolutionary expansion of salamander (urodele) gene models.[80] In this case, the expansion is greater than average (33-fold vs 12-fold genome-wide).

The *rax* coding sequence extends from exons 1 to 3, and includes conserved octapeptide (OP), paired-class homeobox, RX (PPXY), and OAR (orthopedia, aristaless, rax) domains. In the exon 2 sequence chromatograms, we observed a 5-bp deletion (ΔCGGAC) in *e*/*e* samples, and mixed profiles in *E*/*e* samples, compared to the WT reference (Figure 2B). The *eyeless* deletion causes a translational frameshift at codon Pro154, in the HD. It is predicted to truncate the protein prematurely in exon 3, after 35 nonsense codons (P154fs35X).

2.4 | Genetic linkage between *eyeless* and *rax*

To further establish linkage between *rax* and *eyeless*, we genotyped 100 F2 larvae from cross 13673 for the HD deletion in exon 2 and UTR variant in exon 3. To our surprise, 51 offspring appeared homozygous for the Δ5bp mutation in exon 2; these include 29 eyeless larvae, lacking the insG UTR allele (as noted above), and 22 WT larvae, heterozygous for the insG allele, with well-developed eyes (Table 2). Reduced penetrance is theoretically possible, as an explanation for this discordance, and occurs in mice with the hypomorphic Rax^{M10L} mutation[65]; however, we expected these 22 axolotl larvae to exhibit some disruption of eye morphogenesis, if they were truly homozygous for a *rax* deletion allele.

Alternatively, the male parent may carry a different WT allele (E') that failed to amplify with ex2 primers and is coupled in *cis* to the ex3 insG variant (E' haplotype). To test this hypothesis, we amplified ex2 segments from discordant F2 offspring using multiple PCR

primer pairs (Figure 2C). By moving the 5′ primer closer to exon 2, we were able to amplify both alleles (WT and Δ5). In this way, we mapped a 1 kb transposon insertion 102 bp upstream from ex2 in the E' haplotype. This L1 retroelement[81] inhibits amplification of E' products in a competitive genotyping PCR. Using a WT-specific primer spanning codon 155, we amplified long E' products from E'/e carriers spanning the insertion site (Figure 2C). After regenotyping all available F2 samples with informative PCRs (Table 2), we found that the *rax* Δ5bp mutation and *eyeless* trait are fully concordant, with 29 eyeless (Δ5/Δ5, e/e) offspring and 71 WT offspring, of which 16 were +/+ (E'/E) and 55 were Δ5/+ (33 E/e, 22 E'/e). This final linkage result is highly significant ($P = 1.69 \times 10^{-20}$, $\chi^2 = 95.18$, $df = 3$, $n = 100$, with LOD = 19.8). The *eyeless* trait is thus caused by a *rax* frameshift mutation.

2.5 | Effects on *rax* mRNA and protein

Premature translation termination typically causes mRNA degradation through the nonsense-mediated decay pathway, unless the premature stop codon is encountered by the pioneer ribosome in the last 1–2 exons.[82] The *eyeless* P154fs35X mutation in exon 2 and premature stop in exon 3 are thus not expected to alter *rax* mRNA stability. To test this prediction, we performed quantitative fluorescence RT-PCR on pooled heterozygous (E/e) stage 22 to 25 embryos with primers in exons 1 and 3, and directly compared the abundance of Δ5bp and WT transcripts (Figure 3). At this stage, *rax* is highly expressed in eye and brain rudiments, which develop normally in E/e heterozygotes. The molar ratio of RT-PCR products was 1.14 (*eyeless* to WT). We conclude that mutant *rax* mRNA is normally expressed, spliced and stable.

Since axolotl *eyeless* mRNA is abundant, it should be translated, producing a truncated RAX protein with an abnormal C-terminal peptide (Figure 4A). This predicted protein should not bind DNA, since the HD is disrupted (Figure 4B), and may have a relatively short half-life. However, the altered polypeptide does not elicit significant toxicity, since E/e heterozygotes are phenotypically similar to WT. Therefore, the *eyeless* mutation is most likely a *rax* null allele.

In the HD amino acid alignment, we also show axolotl Rax2 protein (Figure 4B). This paralog, on chromosome 1p, was identified in our original BLAST search. It maps on scaffold AMEXG_0030005363 (2.7 Mb), between *matk* and *apba3*, and is likely to activate transcription in the developing retina and ciliary marginal zone during photoreceptor differentiation.[72,73] Like other tetrapod Rax2 proteins, it has a truncated N-terminus and no OP motif to mediate groucho repression.[66,72,73]

Interestingly, axolotl Rax shares a unique N-terminal feature with all other gnathostome orthologs—dual AUG start codons separated by 8 to 10 triplets (Figure 5), with translation initiating via a conserved leaky scanning mechanism.[65,83] This pattern was also found among Rax2 proteins in jawed fish. In each case, the first AUG occurs in a poor context, with mismatched nucleotides at core positions −3 (purine) and +4 (guanine), whereas the second AUG conforms well to the consensus sequence for optimal translation initiation in eukaryotes.[84]

3 | DISCUSSION

The linkage and molecular data in this report establish *rax* (P154fs35X) as the causative mutation for *eyeless* axolotls. This study was sped by the assembly of the *Ambystoma mexicanum* genome.[23,24] Likewise, a small number of classical Mendelian loci, involving axolotl pigmentation, limb and cardiac development, were recently mapped or identified using the emerging genome database as a framework.[23,85] Our finding of two *rax* haplotypes may reflect admixture, at the AGSC, of Wistar (*E*,*e*) and divergent (*E*′) laboratory populations.[14,86] Individuals in the AGSC cohort also harbor a significant fraction of North American tiger salamander (*Ambystoma tigrinum*) DNA in their genomes (5.8 ± 1%), which were cointroduced with the albino trait,[87] but these introgressed segments do not overlap the *rax* 1p region.[85]

3.1 | Molecular hindsight

Our results are consistent with studies involving *Rax* mutants in other species, and allow us to reinterpret older work on *eyeless* with molecular hindsight. First, the ocular, brain, pigmentation and fertility defects in *e*/*e* axolotls closely resemble the malformations reported in spontaneous *rx3* mutant (*chokh*) medaka[63] and zebrafish,[59,60,62] and *Xenopus tropicalis* with CRISPR engineered *rax* mutations.[36] In these mutants, notably, anterior forebrain territories do not segregate, and the eye fields are transfated into an expanded telencephalon and diencephalon.[88] However, unlike *eyeless* axolotls, *rax* mutant *Xenopus tropicalis* die at metamorphosis.[36] Among mammals, *Rax* knockout mice have no eyes or pituitary, and die at birth with craniofacial, oropharyngeal and brain defects that interfere with breathing and feeding.[64,89] In contrast, mice homozygous for a hypomorphic allele (Rax^{M10L}) are viable and fertile, but lack eyes[65]; they have disturbed circadian rhythms and abnormal hypothalamic morphology,[90,91] with expression of these traits dependent on modifier loci. Humans with biallelic *RAX* mutations are generally anophthalmic but viable, as their alleles allow partial activity.[61,92–95] In each case, the inheritance is recessive, but heterozygous phenotypes have been suggested.[96,97] Likewise, severe eye malformations have been reported in heterozygous (*E*/*e*) axolotls carrying the renal insufficiency (*r*/*r*) mutation, which modifies the penetrance of *eyeless*.[27,34]

Second, the constellation of defects in *e*/*e* axolotl embryos matches the *rax* mRNA expression patterns in mice, frogs and fish, beginning in the anterior neural plate, and continuing in the diencephalon and optic vesicles, which give rise to the neural retina, RPE, optic stalk, hypothalamus, and some cortical regions.[53,57,68,98,99] Likewise, mesenchymal cell disruptions in *e*/*e* axolotls[100] are reminiscent of neural crest migratory defects in zebrafish *rx3* mutants.[101] In the mouse hypothalamus, *Rax* is most strongly expressed in the rostral mediobasal region (preoptic, ventromedial, arcuate, and suprachiasmatic nuclei), associated with GnRH secretion, metabolic regulation and circadian rhythmicity,[102–104] consistent with the hypogonadal phenotype in *e*/*e* animals.

Third, the stage of eye developmental arrest in *e*/*e* mutants fits the timing of *rax* action in neural ectoderm. In *e*/*e* embryos, optic vesicles form, but fail to progress, similar to mouse and *Xenopus Rax* mutants.[36,55] However, *rax* endocrine phenotypes differ significantly between species. *e*/*e* axolotls lack gonadotropins (LH, FSH), but retain other pituitary

functions and anatomy.[27,35] They grow normally, develop limbs and are hyperpigmented—reflecting the action of hormones secreted by somatotropes (GH), thyrotropes (TSH), and corticotropes, respectively, although as facultative paedomorphs, WT and *e*/*e* axolotls are relatively hypothyroid.[17,105,106] In contrast, *Rax*-null mice lack all pituitary lobes—through a combination of cell-autonomous and indirect effects.[89] In *Rax* −/− mouse embryos, neurogenesis and dorsoventral patterning of the hypothalamus is profoundly disrupted[107]; consequently, the diencephalon does not extend an infundibulum (neurohypophysis), so Rathke's pouch is not induced to form the anterior pituitary gland (adenohypophysis). These disparate phenotypes may reflect fundamental differences in pituitary ontogeny between taxa,[108] or a smaller role for Rax in patterning the hypothalamus of urodeles compared to mammals.

Fourth, older studies of *e*/*e* embryos, involving blastomere chimeras,[26] transplantation,[27,109,110] ex vivo tissue culture[34] (Cuny and Malacinski) and parabiosis,[26,111] showed that the *eyeless* defect is cell-autonomous and intrinsic to the anterior neural ectoderm—consistent with a nuclear transcription factor, such as Rax, which acts to define the retinal fields and repress alternative forebrain identities.[36] In reciprocal embryo grafts, neural plate tissue from WT donors could induce lens formation in the head ectoderm of *e*/*e* recipients, but not vice versa.[27] Other grafting studies, however, suggested that an inhibitory signal from head epidermis is responsible for the *eyeless* phenotype.[112] Importantly, WT eyes transplanted into *e*/*e* heads developed optic nerves with topographic axon projections to the tectum, and restored functional vision with behavioral and pigmentary responses to light.[30,109] In retrospect, this developmental plasticity is not surprising, considering the remarkable ability of axolotls to regenerate limb and eye tissues.[20] Knowledge of the *eyeless* gene may help future studies investigating the molecular basis for ocular regeneration in urodeles.

3.2 | Dual start codons

While their ultimate effects on expression (kinetics and levels) and evolutionary fitness are unknown, the dual Rax start codons are separated by the footprint width[113,114] of one 40S subunit (24–30 nucleotides) and may interact alternately with ribosomes, in two different modes via leaky scanning. As ribosomes bypass the first AUG codon and engage the second (favorable) AUG, forming an initiation complex, they would impede progress of later ribosomes scanning from the 5′ mRNA cap.[115] These queued ribosomes would then be forced to pause over the first (unfavorable) AUG and initiate. This tiered mechanism may allow higher levels of translation when *rax* mRNA is limiting and precise tuning of protein output.[116] It would also create long and short Rax isoforms, with a variable N-terminal protein extension, and may thus modulate docking of the nearby OP (eh1, engrailed homology) domain to groucho/TLE1 corepressors.[117,118] Apart from tetrapod Rax2 paralogs, which lack an OP motif,[73] every gnathostome Rax protein we examined has dual AUG start codons in a "leaky" configuration—reflecting strong evolutionary conservation (with emergence before the 2R WGD) or convergence. The surrounding amino acid residues are notably dissimilar.

3.3 | Recurrent mutations

Rax is commonly mutated in eyeless laboratory models, which share a singular striking phenotype. In a similar way, convergent mutations in one gene, encoding tyrosinase, are responsible for sporadic cases of true albinism in most vertebrate species, including axolotls.[85] Accordingly, *RAX* mutations underlie a portion of clinical anophthalmia cases,[11] and *rx3* cosegregates with an ocular regression QTL (quantitative trait locus) under positive evolutionary selection in *Astyanax* cavefish,[12] suggesting the workings of a shared developmental pathway.

4 | EXPERIMENTAL PROCEDURES

4.1 | Animals

Axolotl embryos were provided by the AGSC (Ambystoma Genetic Stock Center) at Indiana University (in 1992) and the University of Kentucky (in 2013 and 2020). Eyeless and WT embryos (spawn nos. 8146, 8209, 13673, 15428) were generated from F2 crosses between heterozygous carriers of the *eyeless* gene (*e*/+) that descended from Rufus Humphrey's original colony.[25] The *eyeless* mutation was considered lost in 2009, but reappeared in 2013 in another AGSC stock. Embryos were maintained in modified ×0.2 Holfretter's solution at 4°C to 19°C and staged using developmental landmarks.[119] For F2 linkage analysis, eye phenotypes were scored at hatching (stage 42).

4.2 | Genomic DNA analysis

DNA was extracted from embryos (degelled in 3% cysteine), adult tail clips, hatchling larvae, or deparaffinized tissue sections by digestion in TNE (100 mM NaCl 10 mM Tris 1 mM EDTA pH 7.4) with 0.5% Na dodecyl sulfate and 250 μg/mL proteinase K at 55°C for 16 hours with agitation, isopropanol precipitation, and resuspension in TE.[120] We initially amplified 3′ UTR segments of *pax6* (accession AF169414.1) and *rax* from F2 samples with PCR primers designed from cDNA data (Table 3), and scored polymorphisms by sequencing the products.

We identified three *rax* exons and four associated CNEs on contigs AMEXG_74394 and AMEXG_0030000955 of the assembled axolotl genome[23,24] using vertebrate *rax* orthologs as query in BLAST homology searches. Segments were amplified from archived *e*/*e* and *E*/− samples using flanking primers (Table 3), GoTaq polymerase (Promega, Madison, Wisconsin) and a touchdown PCR protocol: 5 minutes initial denaturation at 94° C; followed 15 cycles of 20 seconds denaturation at 94° C, 30 seconds annealing at 62° C (decreasing 0.5° per cycle to 55° C), and 60 seconds extension at 70° C; followed by 28 further cycles with 55° C annealing; followed by final 7 minute extension at 70° C. Products were assessed by agarose gel electrophoresis and Sanger sequencing. To score the *eyeless* deletion in F2 offspring and map the *E*′ insertion, exon 2 segments were amplified using a combination of primers, including some specific to WT or mutant alleles (Table 3).

4.3 | Statistics

Linkage between *eyeless* and *rax* was assessed using the chi-squared test, with independent assortment as the null hypothesis. Confidence limits for linkage distance were calculated as described for the special case of complete concordance.[121]

4.4 | mRNA analysis

Total RNA was isolated from whole stage 22 to 24 embryos by affinity chromatography (RNA miniprep spin columns, Zymo Research, Irvine, California). To compare the abundance of *rax* transcripts, we performed reverse transcriptase (RT) PCR on pooled *E*/*e* embryos, which were genotyped using 2 mm of tail tissue. For each sample, we synthesized cDNA from 500 ng RNA template using iScript reagents (Bio-Rad, Hercules, California) with blended oligo-dT/random dN_6 priming, and amplified PCR products using primers in exons 1 and 3 (Table 3), with a 6-FAM (carboxyfluorescein) end-label on the reverse primer. The ratio of WT and *eyeless* (Δ5bp) *rax* products in the *E*/*e* pool was determined from fluorometric peak areas using Peak Scanner software (Thermo Fisher) after capillary electrophoresis.

4.5 | Histology and immunostaining

Degelled embryos or larvae were rinsed in phosphate-buffered saline (PBS) and fixed overnight in 4% paraforaldehyde 0.1M $NaPO_4$ pH7.4 at 4° C. Fixed embryos were cryopreserved stepwise in 10% to 30% sucrose PBS at 4° C and frozen in optimal cutting temperature compound (Thermo Fisher) or were dehydrated through a graded ethanol series, equilibrated with xylenes, and embedded in paraffin (Paraplast Plus, Thermo Fisher). Paraffin sections (10 μm) were stained for histology with H&E, stabilized in Permount and imaged at ×20 magnification as stitched composites using a Zeiss ApoTome (White Plains, New York).

Cryosections (10 μm) were blocked in Tris-buffered saline 0.1% Tween-20 (TBST) with 3% bovine serum albumin, 1% normal goat serum, and 1% normal donkey serum, processed for indirect immunofluorescence, and counterstained with 1μg/μL 4′,6-diamidino-2-phenylindole as described.[122] Primary antibodies were mouse anti-Tubb3 (clone 2G10, Sigma, 1:500) and rabbit anti-PAX6 (PRB278P, Covance, 1:50, and secondary antibodies were anti-mouse IgG2a and anti-rabbit IgG (AlexaFluor 488 and 647 conjugates, Jackson Immuno-Research, 1:500). After mounting in Prolong Gold (Thermo Fisher), sections were imaged with fluorescence optics using a Leica SPE confocal microscope (Wetzler, Germany) and displayed using RGB indexed color.

4.6 | Bioinformatics

For HD and AUG translation start site alignments, sequences were deduced from nucleotides of Rx/Rax cDNAs with accession numbers as follows—*Aedes aegypti* (mosquito): AaeRx, XM_021841460; *Anolis carolinensis* (anolis lizard); AcaRax, XM_008118598; *Apis mellifera* (honey bee): HbeRx, XM_001119966; *Aplysia californica* (sea hare): ApcRx, XM_013086395; *Astyanax mexicanus* (cavefish): AmeRx1, AF264703; AmeRx2, XM_022678823; AmeRx3, XM_007246033; *Branchiostoma floridae* (amphioxus): BflRx, JX101655; *Callorhinchus milii* (elephant shark): CmiRx3, XM_007903126; CmiRx1,

XM_007908006; *Crassostrea gigas* (oyster): CgiRx, XM_011429408; *Danio rerio* (zebrafish): DreRx1, AF001907; DreRx2, AF001908; DreRx3, NM_131227; *Drosophila melanogaster* (fruit fly): DmeRx, NM_166413; *Dugesia japonica* (planaria): DjaRx, AM942442; *Eptatretus burgeri* (hagfish): EbuRax, ENSEBUT00000011203.1; *Gallus gallus* (chicken): GgaRax, NM_001243724; GgaRax2, NM_204104; *Homo sapiens* (human): HsaRax, NM_013435; HsaRax2, NM_032753; *Latimeria chalumnae* (coelacanth): LchRx2, XM_014490274; LchRx3, XM_006005788; *Macaca mulatta* (rhesus macaque): RmaRax, XM_015122061; *Monodelphis domestica* (opossum): MdoRax, XM_007487510; MdeRax2, XM_001373844; *Mus musculus* (mouse): MmuRax, NM_013833; *Nannospalax galili* (mole rat): NgaRax, XM_008840033; *Nanorana parkeri* (Tibetan frog): NpaRax, XM_018570456; *Nematostella vectensis* (sea anemone): NveRx, XM_001634160; *Oikopleura dioica* (tunicate): OdiRx, AY705677; *Ornithorhynchus anatinus* (platypus): OanRax, XM_007659835; OanRax2, XM_001516307; *Oryzias latipes* (medaka fish): OlaRx1, XM_004068380; OlaRx2, NM_001104903; OlaRx3, NM_001104904; *Pan troglodytes* (chimpanzee): PtrRax, XM_001142510; *Petromyzan marinus* (lamprey): PmaRax, PIZI01000102v1; *Rattus norvegicus* (rat): RnoRax, NM_053678; *Rhinatrema bivittatum* (caecilian): RbiRax, XM_029586501; *Saccoglossus kowalevskii* (acorn worm): SkoRx, NM_001164903; *Strongylocentrotus purpuratus* (sea urchin): SpuRx, XM_777214; *Tribolium castaneum* (flour beetle): TcaRx, XM_968375; *Trichinella spiralis* (nematode): TspRx, XM_003371992; *Xenopus laevis* (African frog): XlaRx1a, NM_001088218; XlaRx1b, DQ360108;XlaRax2; NM_001088220.

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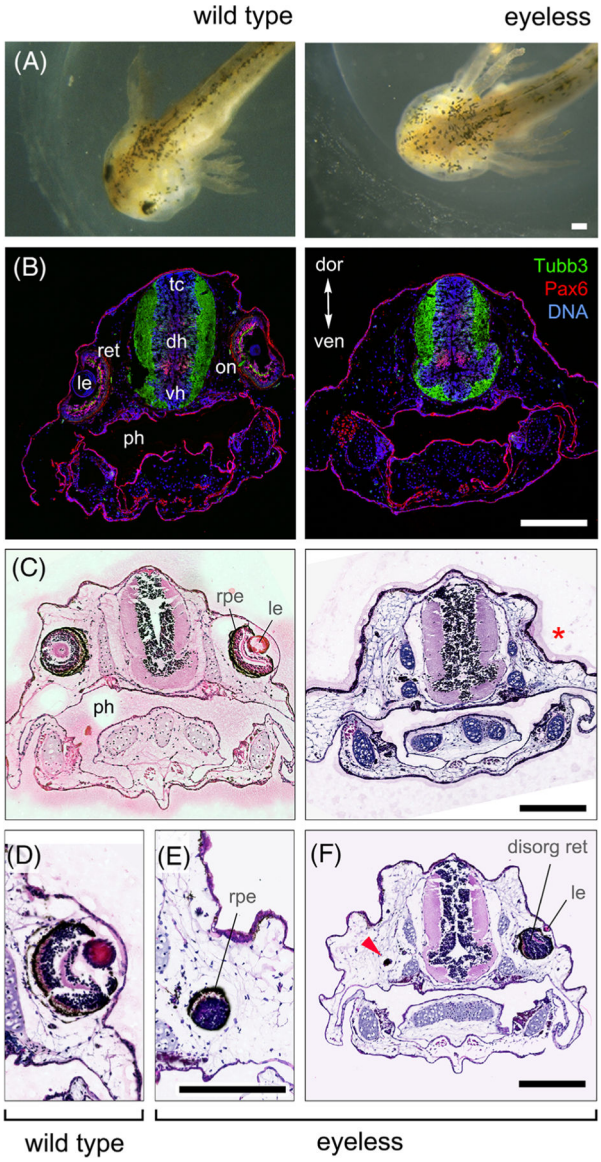

FIGURE 1.

eyeless phenotype at hatching (stage 42). A, *E*/− (wild-type) and *e*/*e* (mutant) axolotls in vitelline membranes. B, Immunofluorescence micrographs of coronal (transverse) sections through the head showing neural tracts (Tubb3, green), retinal layers and brain nuclei (Pax6, red), and absent eyes in *e*/*e* animals (*right* panel), with 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (blue). The rabbit antibody (red) cross reacts with tissue edges, highlighting morphology. C-F, Hematoxylin and eosin (H&E) stained coronal sections showing the absence of eyes in most *e*/*e* mutants (red asterisk, *right* panel in C), with abortive optic rudiments in some cases (red arrowhead in F, magnified example in E). These rudiments contain variably dysmorphic retinal and lens tissues, and pigmented cells. dor, dorsal; ven, ventral; dh, dorsal hypothalamus; le, lens; on, optic nerve; ph, pharynx; ret, retina; rpe, retinal pigment epithelium; tc, tectum; vh, ventral hypothalamus; disorg ret, disorganized retina; scale bars, 200 μm

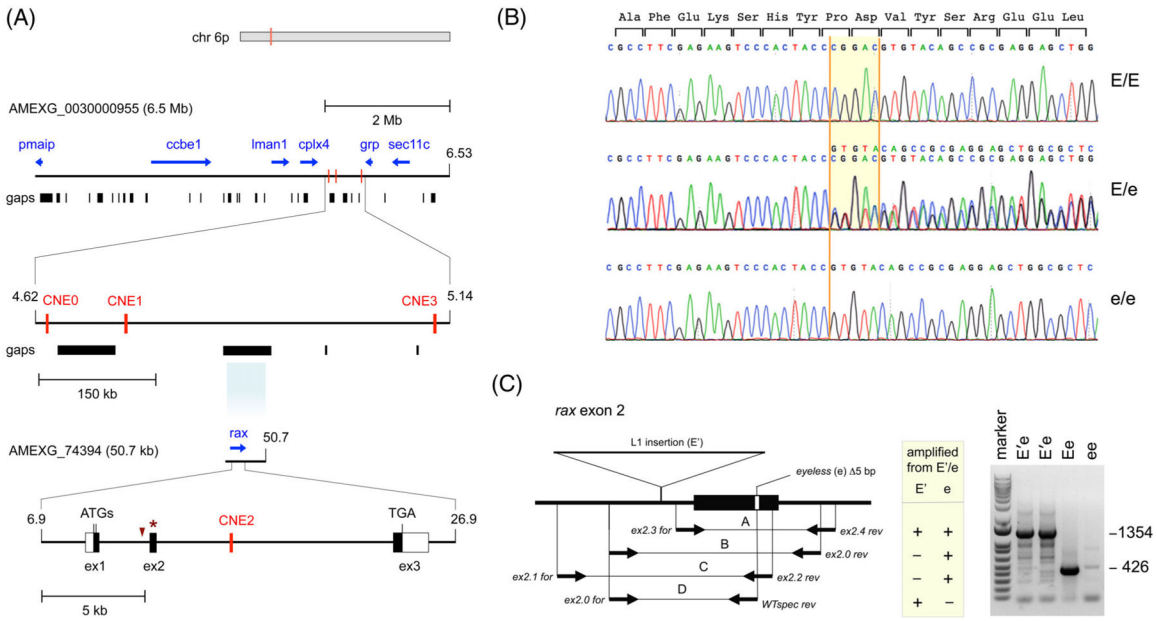

FIGURE 2.

rax gene structure and *eyeless* mutation. A, Layered maps of *rax* exons and CNEs, with relevant chr 6p scaffolds in assembly Amex_PQ.v4, showing flanking genes, coding sequence, Δ5bp mutation (*) and *E′* transposon insertion site (arrowhead). B, Sanger chromatograms of genomic PCR products, showing exon 2 profiles from *e*/*e*, *E*/*e*, and *E*/*E* genotypes. The reading frame and Δ5 mutation are indicated. C, Magnified view of exon 2, showing diagnostic PCRs, and results from *E′*/*e* genomic DNA (right column). The agarose gel shows ex2 PCR products amplified with a wild-type (WT)-specific primer overlying codon 155 (amplicon D^{WT}). The large product in *E′*/*e* lanes contains a transposon insertion

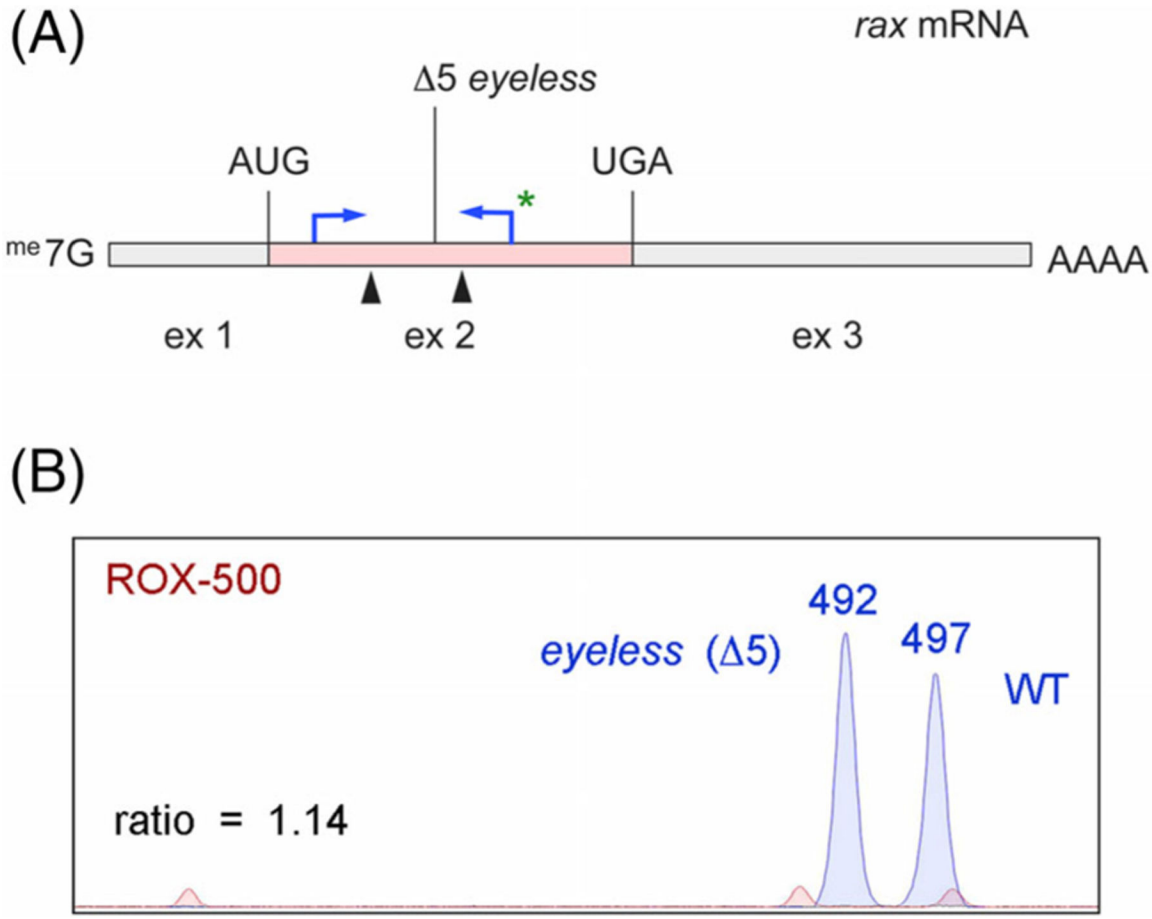

FIGURE 3.

rax mRNA analysis. A, RT-PCR design. B, Capillary electrophoresis profile showing equimolar wild-type (+) and *eyeless* mutant (Δ5) products coamplified from *E*/*e* cDNA in relation to ROX-500 size standards (*n* = 3 pooled embryos)

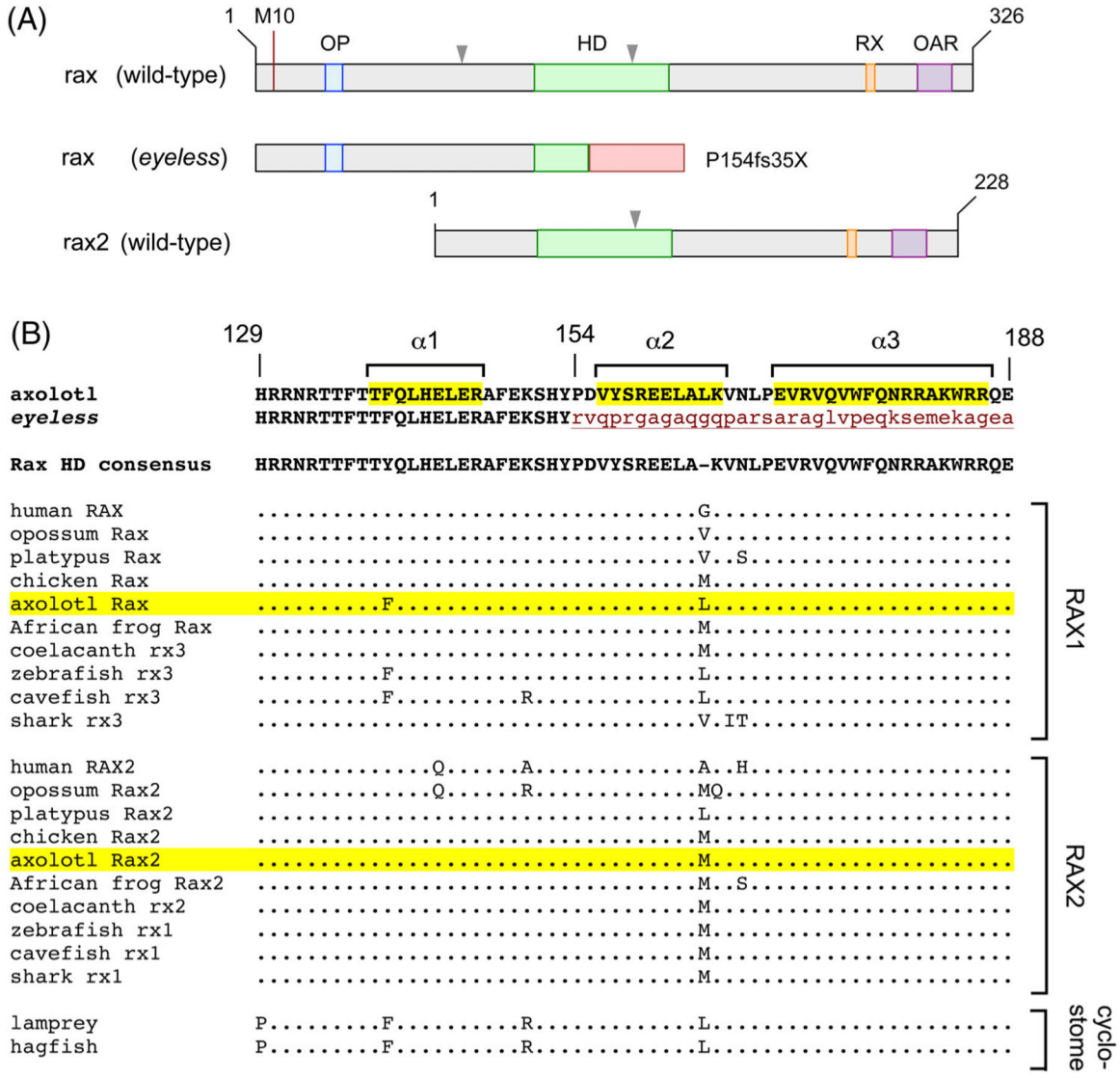

FIGURE 4.

Protein effects of the *eyeless* mutation. A, RAX polypeptide maps showing octapeptide (OP), homeodomain (HD), RX, and OAR domains, dual start codons, exon junctions (arrowheads), the predicted *eyeless* protein with Δ5 frameshift mutation and C-terminal nonsense peptide (red). B, Vertebrate RAX homeobox amino acid alignment, showing sequence identities, three critical α-helices for DNA binding, and *eyeless* HD

FIGURE 5.

Conserved dual start codons with leaky scanning. Left: N-terminal alignment of Rax proteins, numbered in relation to axolotl (mouse), with methionines (red) and gaps (−) indicated. The presence of a methionine at positions 8 to 12 is a conserved feature among gnathostome Rax proteins containing an OP motif, including axolotl Rax. Right: Comparison of M1 and M4–12 start sites in Rax mRNAs. The optimal sequence for translation initiation[84] is shown below each alignment, with nucleotide matches indicated in red. For scanning ribosomes to initiate, AUG sites must have a −3 purine (R) or +4 guanine (G). In every case, the M1 site is weaker than the downstream AUG. Predicted leaky scanning (LS) and initiation codons (start) are indicated

TABLE 1

eyeless F2 screen (male *E*/*e* × female *E*/*e* cross)

(A) *pax6* 3′ UTR SNP (G/A × G/A)

	F2 offspring	
Genotype	***e/e***	**WT**
GG	3	6
GA	12	7
AA	8	8
sum	23	21

(B) *rax* 3′UTR SNP (insG/+ × +/+)

	F2 offspring	
Genotype	***e/e***	**WT**
insG/+	0	51
+/+	55	35
sum	55	86

Abbreviation: SNP, single-nucleotide polymorphism.

TABLE 2

rax—*eyeless* linkage (male *E′/e* x female *E/e* cross)

***rax* genotype**				**F2 offspring**		
L1 ins	**ex2 Δ5**	**3′UTR insG**		***e/e***	**WT**	**Interpretation**
L1/+	+/**Δ**5	insG/+		0	22	*E′/e*
+/+	**Δ**5/**Δ**5	+/+		29	0	*e/e*
L1/+	+/+	insG/+		0	16	*E′/E*
+/+	+/**Δ**5	+/+		0	33	*E/e*
			sum	29	71	

Dev Dyn. Author manuscript; available in PMC 2022 March 09.

TABLE 3

Axolotl PCR primers and amplicons

(A) Initial UTR screen

Amplicon	WT (bp)	FORWARD primer	REVERSE primer
pax6	885	pax6 UTR for 5′-CAGTGAACCTGACATGTCTCAATAC	pax6 UTR rev 5′-TATAGCTAAGAACAAGGGACGTCTG
rax	562	rax UTR for 5′-AGACTATTTCAAAGCCTGGCATAC	rax UTR rev 5′-TGACTGCAGAGTAACCCAAAAACA

(B) Rax genomic analysis (with *eyeless* deletion indicated)

Amplicon	WT (bp)	FORWARD primer	REVERSE primer
CNE0	155	axorax CNE0 for 5′-AAGCTCAGGCTGTCATCCCA	axorax CNE0 rev 5′-TGACTGCACACAAGAGGCTG
CNE1	732	axorax CNE1 for 5′-AGACAAGTGAGTGTGGGTCG	axorax CNE1 rev 5′-TTGCTAATTAGTGCCGGCCA
CNE2	720	axorax CNE2 for 5′-TCCACCGTTTTACCAAGCGA	axorax CNE2 rev 5′-TTTCCAGCACTGCTCAGGAG
CNE3	283	axorax CNE3 for 5′-ATGGAGAGTGCCTTCCGACA	axorax CNE3 rev 5′-TGGCTGTAAACGTGGACTCT
3′UTR1	892	axorax 3′UTR1 for 5′-TTCCCAGTAGCTACACCCCA	axorax 3′UTR1 rev 5′-AGAGTCTGCCACCAATGCTG
3′UTR2	787	axorax 3′UTR2 for 5′-TGGTTGCACCCACTTGTTCT	axorax 3′UTR2 rev 5′-TCCACAGCATCACCACTCAC
5′UTR	705	axorax 5′ UTR for 5′-TGTGGTGTCAGGAGTGGGTA	axorax 5′UTR rev 5′-AAGATGGCCTCGATGCTGTG
ex1	467	axorax ex1 for 5′-GTAGTTCAGCCGACTGCACT	axorax ex1 rev 5′-GCACTCACCTTCGAAGCTCT
ex2 (A)	602	axorax ex2.0 for 5′-AAGGCGCTGGTTTGTGACTA	axorax ex2.0 rev 5′-GCTACTGCCAGGCTCTGAAA
ex2 (B)	613	axorax ex2.1 for 5′-ATCATTCAAACGGCAGGCAG	axorax ex2.2 rev 5′-TTCGGGCAGGTTGACCTTGA
ex2 (C)	448	axorax ex2.3 for 5′-TGCCTCTTGTGTCAGTGCAC	axorax ex2.1 rev 5′-ATGCTAAGCTATGCCGGTGT
ex2 (D^{wt})	426 (*E*) 1354 (*E′*)	axorax ex2.0 for 5′-AAGGCGCTGGTTTGTGACTA	axorax WTspec rev 5′-GTACAC**GTCCG**GGTAGTGGGA
ex2 (D^{mut})	429 (*e*)	axorax ex2.0 for 5′-AAGGCGCTGGTTTGTGACTA	axorax MUspec rev 5′-TCGCGGCTGTACAC|GGTAGTG
ex3	784	axorax ex3 for 5′-TCAGTGTCCGCATTGCTCTT	axorax ex3 rev 5′-AATGCCTGTCCAGTTACCCG

(C) Rax cDNA analysis (with *fluorescent end-label)

Amplicon	WT (bp)	FORWARD primer	REVERSE primer
cDNA ex 1–3	497	rax cDNA ex1 for 5′-ACAGCATCGAGGCCATCTTG	rax cDNA ex3 rev *5′-TGAGTCCTGCAGCTTCATTG