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Evolution of a tissue-specific silencer underlies divergence in the expression of *pax2* and *pax8* paralogues

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Recent studies underscore a role for the differential degeneration of enhancers in the evolutionary diversification of paralogue expression. However, no one has reported evidence for the involvement of innovative *cis*-regulatory changes. Here we show that silencer innovation diversified expression of the vertebrate paralogues, *pax2* and *pax8*. *pax2* shows multi-tissue expression, as does the ancestral amphioxus orthologue, *pax2/5/8*, whereas *pax8* expression localizes to a subset of *pax2*-expressing tissues. We reveal that both *pax2* and *pax8* retain ancestral enhancers capable of directing *pax2*-like, multi-tissue expression. However, a silencer within the *pax8* proximal promoter suppresses pleiotropic enhancer activity outside the *pax8*-expressing tissues. In contrast, the combination of the *pax2* proximal promoter with either the *pax8* or *pax2* enhancer recapitulates *pax2*-like expression, as in the amphioxus *pax2/5/8* promoter. We propose that silencer innovation, rather than enhancer degeneration, was crucial for the divergent expression of paralogues with pleiotropic enhancers inherited from their common progenitor.

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During chordate evolution, two rounds of whole-genome duplication (WGD1 and WGD2) occurred in the vertebrate ancestor, to be followed later by a third round (WGD3) in the teleost lineage[1,2]. These WGDs generated a number of duplicated gene pairs (known as paralogues) from the ancestral gene set, with members of sibling paralogues often showing overlapping, yet distinct, expression patterns in modern vertebrates. The evolutionary mechanisms underlying this divergence of expression can be explained by the duplication–degeneration–complementation model[3]. This model is based on the regulatory complexity of developmental genes that generally have an array of multiple enhancers for discrete expression domains. It assumes that degenerative mutations occur much more frequently than do innovative ones. The model predicts that, after the WGD(s), parts of duplicated enhancers were lost reciprocally from sibling paralogues because of degenerative mutations, but at least one enhancer copy will remain in either of the paralogues so that their resulting complementary expressions cover the original full expression of the progenitor gene, a process known as subfunctionalization. This model is supported by discoveries of complementary enhancer degeneration in teleost paralogues generated by WGD3[4,5]. For example, the mammalian *Pax6* gene has multiple *cis*-regulatory elements, including enhancers for the pancreas, telencephalon and diencephalon[6]. These enhancers are conserved in tetrapods, but were lost reciprocally from the duplicated zebrafish *pax6* genes because of degenerative mutations: one lost the pancreas enhancer and the other lost the telencephalon and diencephalon enhancers. However, although the WGDs are expected to have stimulated not only such degenerative *cis*-regulatory changes but also innovative changes, involvement of the latter phenomena—such as enhancer and/or silencer acquisition—remains unknown. In addition, it is unclear to what extent such degenerative *cis*-regulatory changes can account for the divergence in the expression of paralogues generated by the earlier WGDs (WGD1 and WGD2), whose *cis*-regulatory changes are generally thought to have been more significant than those in the teleost-specific paralogues.

The paired-domain transcription factor genes, *pax8* and *pax2*, are essential for the development of multiple organs, including the kidney[7,8]. These two genes and another related gene, *pax5*, arose from a single progenitor following WGD1 and WGD2[9–11]. *Pax8* is mainly expressed in the kidney, ear and thyroid gland during development[12,13], while *pax2* shows expression not only in the *pax8*-expression domains but also in other tissues such as the eye, pharyngeal arches, midbrain–hindbrain boundary (MHB), hindbrain and spinal cord[14,15]. The cephalochordate amphioxus, which diverged from the vertebrate lineage before the WGDs occurred and likely retains ancestral modes of gene regulation, possesses the single orthologue, *pax2/5/8*[9]. This gene shows pleiotropic expression in most of the tissues that are homologous to the vertebrate *pax2*-expressing tissues, implying that *pax2* retains most of the ancestral regulatory mechanisms.

In this study, we chose *pax8*, *pax2* and *pax2/5/8* as models for investigating regulatory diversification after WGD1 and WGD2. We found that both *pax8* and *pax2* have inherited ancestral enhancers from their common progenitor and that silencer innovation within the *pax8* proximal promoter, rather than enhancer degeneration, accounts for the divergent expression of this paralogue pair. This is the first evidence for the involvement of an innovative *cis*-regulatory change in the evolutionary diversification of paralogue expression.

Results

Conserved non-coding sequences in *pax8* and *pax2* loci. To explore the *cis*-regulatory evolution of *pax8* and *pax2*, we initially compared the genomic sequence of a 160-kb segment encompassing the human *PAX8* gene with the orthologous interval in mouse, frog (*Xenopus tropicalis*) and pufferfish (*Takifugu rubripes*) genomes using the MultiPipMaker alignment tool[16]. This analysis identified five conserved non-coding sequences (CNSs), CNS1–CNS5, as candidates for *pax8* enhancers (Fig. 1a; Supplementary Fig. S1). CNS1 and CNS2 are conserved from the human to pufferfish, whereas CNS3–CNS5 are found only in tetrapods. We also compared the *pax8* genomic sequences with the genomic sequences from the human, mouse, frog and pufferfish *pax2* loci, and found the conservation of a recognizable paralogous sequence of *pax8* CNS1 (*pax8*-CNS1) in the 5′-flanking region of *pax2* (Fig. 1a; Supplementary Fig. S1). Local alignment of such duplicated sequences, *pax8*-CNS1 and *pax2*-CNS1, revealed the conservation of a cluster of putative transcription factor-binding motifs (Fig. 1b). This finding implies partial conservation of ancestral regulatory mechanisms between *pax8* and *pax2*, which might account for their overlapping expression in the pronephros and otic vesicle during embryonic development (Fig. 1c,d).

***pax8* enhancers direct *pax2*-like pleiotropic expression**. In the functional analysis, we first focused on the *cis*-regulatory landscape of *pax8*. The *Xenopus laevis* system was chosen to examine the potential enhancer activity of the CNSs, because an efficient transgenesis technique for a non-mosaic founder assay is readily available[17]. Each *X. tropicalis pax8*-CNS was cloned into a green fluorescent protein (GFP) reporter plasmid carrying a β-*actin* basal promoter[18]. Each construct was then used to generate transgenic embryos, and their GFP expression was examined once the resulting embryos reached the tailbud stages (stages 30–32)[19]. The reporter constructs without a CNS showed no significant GFP expression (Fig. 2a). By contrast, the constructs carrying *pax8*-CNS1, *pax8*-CNS2, *pax8*-CNS3 or *pax8*-CNS4, but not that carrying *pax8*-CNS5, drove reproducible expression not only in the pronephros that expresses endogenous *pax8* but also in other tissues that do not express *pax8* at the developmental stages examined (Fig. 2b–f, compare with Fig. 1c; group pictures of transgenic embryos, scoring results and expression patterns are summarized in Supplementary Fig. S2, and Supplementary Tables S1 and S2, respectively). These tissues include the eye, pharyngeal arches, MHB and hindbrain, which are the *pax2*-expression domains (Fig. 1d). Moreover, when all the *pax8*-CNSs were assembled in a single reporter construct, its expression mostly recapitulated that of *pax2* (Fig. 2g). Similar *pax2*-like, pleiotropic expression was observed when the β-*actin* promoter of this construct was replaced with a β-*globin* basal promoter[20] (Fig. 2h,i), which indicated that *pax2*-like expression patterns depend absolutely on the enhancer activity of the *pax8*-CNSs.

Tissue-specific silencing by the *pax8* proximal promoter. The discrepancy between the pleiotropic expression driven by the conserved *pax8* enhancers and the more restricted expression of endogenous *pax8* suggests the presence of a tissue-specific silencer(s) that limits enhancer function. As the tissue-specific expression pattern of *pax8* is mostly conserved in vertebrates[11,13], this silencer activity is also expected to be conserved evolutionarily. However, no conserved regions other than CNS1–CNS5 were found near the *pax8* locus. Therefore, the proximal promoter region was examined for conserved regulatory function without apparent cross-species sequence conservation, as in the *in vivo* analysis of the *PHOX2B* gene combined with the *in vitro* data from the ENCODE project[21]. When the proximal promoter region of *X. tropicalis pax8* (nucleotides −2038 to +130) alone was linked to GFP, no significant expression was observed (Fig. 3a). When this proximal promoter region was flanked by the *Xenopus pax8*-CNS1 to *pax8*-CNS5, GFP expression was observed only in the pronephros, hindbrain and a small anterior part of the midbrain, making a striking contrast to the pleiotropic expression driven by the β-*actin* and β-*globin* basal promoters flanked by the same set of *pax8*-CNSs (Fig. 3b, compare with Fig. 2g,i). These results suggested that the *pax8* promoter harboured tissue-specific silencer activity, which partly explains

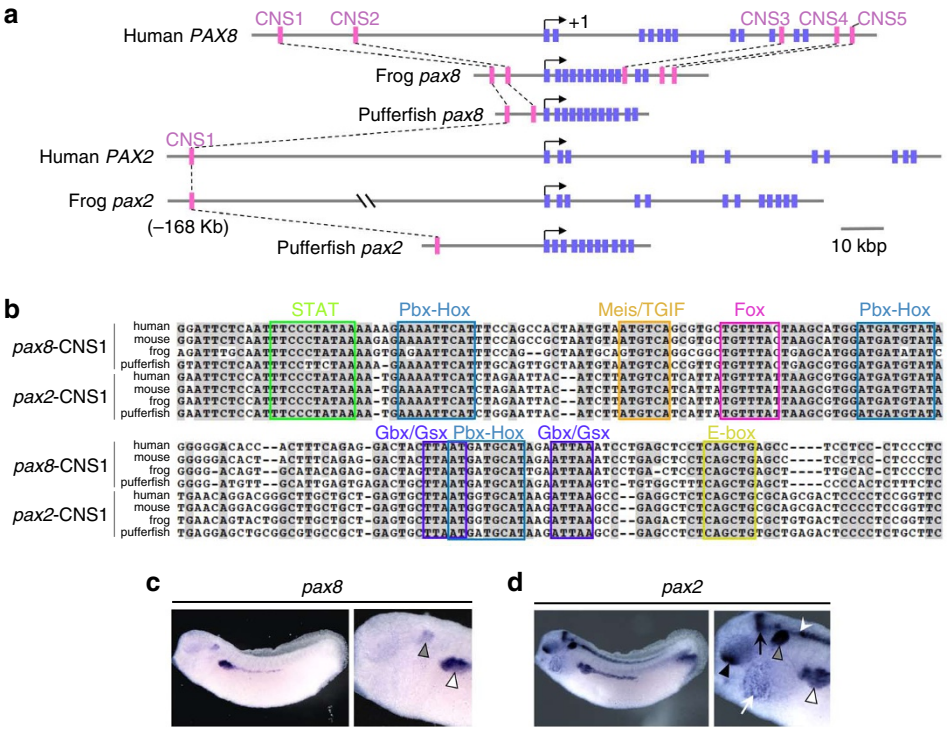

Figure 1 | The CNSs and embryonic expression of *pax8* and *pax2*. (**a**) Diagram of vertebrate *pax8* and *pax2* loci showing the position of CNSs. Magenta and purple boxes indicate CNSs and exons, respectively. For *pax2*, only *pax2*-CNS1—paralogous to *pax8*-CNS1—is shown. Broken lines indicate orthologous and paralogous relationships between CNSs. CNS lengths range from 123 to 407 bp in the frog genome. (**b**) Alignment of *pax8*-CNS1 and *pax2*-CNS1 sequences from the human, mouse, frog and pufferfish. Only their conserved core regions are shown. Nucleotides identical in more than six sequences are shaded in grey. Conserved transcription factor-binding motifs are boxed, although their functional significance was not examined in this study. (**c**,**d**) *In situ* hybridization analysis showing expression of *pax8* (**c**) and *pax2* (**d**) in *X. tropicalis* tailbud embryos (stage 30). White, grey and black triangles indicate expression in the pronephros, otic vesicle and eye, respectively. White and black arrows indicate expression in the pharyngeal arches and MHB, respectively. A white arrowhead indicates expression in the hindbrain. Note that the thyroid gland expression of *pax2* occurs later, from stage 34[13].

the restriction of the enhancer function to the correct expression domains of *pax8*, such as the pronephros. Given that endogenous *pax8* was not expressed in the hindbrain and midbrain but did show expression in the otic vesicle at the stages examined (Fig. 1c), *pax8* may have another silencer and enhancer for these tissues. This possibility is also supported by a reporter assay using a fosmid construct that covers the −24 kb to +12 kb region of *pax8* and faithfully recapitulates endogenous *pax8* expression (Supplementary Fig. S3). Deletion assays narrowed down the sequence responsible for the silencer activity to a 344-bp region (−214 to +130) in the proximal promoter (Fig. 3c,d). This 344-bp sequence suppressed the expression from heterologous promoters in an orientation- and distance-independent manner (Supplementary Fig. S4), satisfying the criteria for a silencer[22].

The functional cross-species conservation of both the pleiotropic enhancer and the proximal promoter-associated silencer was examined using the sequences from the mouse *Pax8* locus. As expected from sequence conservation, mouse *Pax8*-CNS1 drove *pax2*-like, multi-tissue expression from the β-*actin* promoter in *Xenopus* embryos as with the *Xenopus pax8*-CNS1 (Fig. 3e). A proximal promoter region of mouse *Pax8* (−1607 to +494) in itself drove no significant GFP expression, as with the *Xenopus pax8* proximal promoter (Fig. 3f). When this mouse *Pax8* promoter was flanked by the mouse *Pax8*-CNS1, GFP expression was observed only in the pronephros, otic vesicle, MHB and eye (Fig. 3g), indicating tissue-specific suppression of enhancer activity by the promoter. Note that mouse *Pax8* exhibits expression not only in the pronephros and otic vesicle but also in the MHB[8], in contrast to *Xenopus pax8* that does not exhibit expression in the MHB[13] (Fig. 1c). Thus, GFP expression driven by the mouse *Pax8*-CNS1 from the mouse *Pax8* proximal promoter mostly recapitulated the mouse *Pax8* expression in *Xenopus* embryos, although the ectopic expression remaining in the eye suggests the presence of another silencer in mouse *Pax8*.

Enhancer conservation and silencer innovation. The comparative genome sequence analysis identified the duplicated sequences, *pax8*-CNS1 and *pax2*-CNS1 (Fig. 1a,b), which implied the partial conservation of *cis*-regulatory mechanisms between *pax8* and *pax2*. This possibility was supported by the recapitulation of *pax2* expression by the *pax8* enhancers including *pax8*-CNS1 (Fig. 2). The *pax8*-CNS1 and *pax2*-CNS1 were cloned previously from the pufferfish and were shown to have enhancer activity in a transgenic zebrafish assay[23]. As the activity of *pax2*-CNS1 has not been examined in tetrapods, it was cloned from *X. tropicalis* for the present study and used in the transgenic *Xenopus* assay. The *pax2*-CNS1 drove GFP expression from the β-*actin* basal promoter in all tissues where the *pax8*-CNS1 was active (Fig. 4a, compare with Fig. 2b), indicating that their pleiotropic enhancer activities are conserved. We note that these expression domains include not only the *pax2*-expressing tissues but also the somite and midbrain, which suggests that *pax2* also has a silencer(s) somewhere in its regulatory region but the tissue range of this putative *pax2* silencer is much more restricted than that for *pax8*.

Next, we investigated whether the silencer element in the *pax8* proximal promoter could limit the enhancer functions of *pax8*-CNS1 and *pax2*-CNS1 in a similar tissue-specific fashion. To test

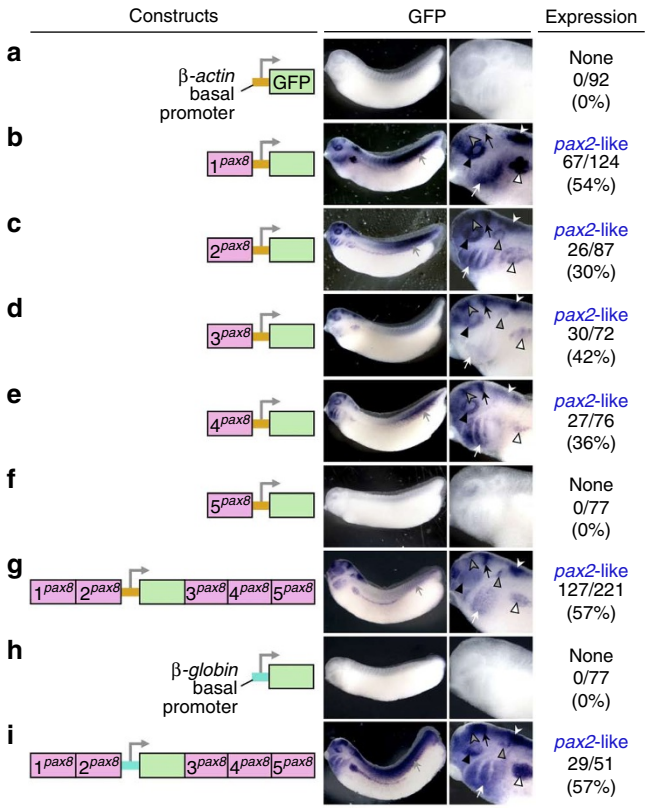

Figure 2 | GFP expression driven by *pax8*-CNSs recapitulates the pleiotropic expression of *pax2*. Transgenic *X. laevis* embryos were generated with the following reporter constructs and their expression was analysed using *in situ* hybridization. (**a**) The β-*actin* basal promoter alone was linked to GFP. (**b**–**f**) The β-*actin* promoter-GFP cassette was flanked by either *X. tropicalis pax8*-CNS1 (**b**), *pax8*-CNS2 (**c**), *pax8*-CNS3 (**d**), *pax8*-CNS4 (**e**) or *pax8*-CNS5 (**f**). (**g**) The β-*actin* promoter-GFP cassette was flanked by all the *pax8*-CNSs. (**h**) The β-*globin* basal promoter alone was linked to GFP. (**i**) The β-*globin* promoter-GFP cassette was flanked by all the *pax8*-CNSs. The *X. tropicalis pax8*-CNS1 to *pax8*-CNS5 are shown as 1^{pax8} to 5^{pax8}. Representative examples of GFP expression are shown together with the injected reporter constructs. The right-hand panels show high-magnification views of the embryonic head regions illustrated in the left-hand panels. GFP expression patterns are summarized on the extreme right with the scoring results. Numbers of embryos with the positive expression similar to the representative examples, and the total number of analysed embryos injected with each construct are indicated with percentages of the former cases. White, grey and black triangles indicate expression in the pronephros, otic vesicle and eye, respectively. White, grey and black arrows indicate expression in the pharyngeal arches, somites and MHB, respectively. White and grey arrowheads indicate expression in the hindbrain and anterior midbrain, respectively.

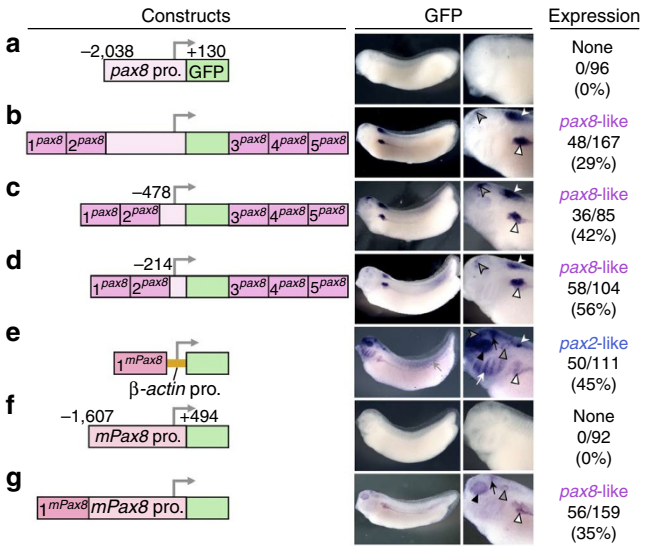

Figure 3 | Identification of tissue-specific silencer activity associated with the *pax8* proximal promoter. Transgenic *X. laevis* embryos were generated with the following GFP constructs. (**a**) The proximal promoter region of *X. tropicalis pax8* (−2038 to +130) alone was linked to GFP. (**b**–**d**) A deletion series of the *X. tropicalis pax8* proximal promoter (**b**, −2038 to +130; **c**, −478 to +130; and **d**, −214 to +130) was flanked by the *X. tropicalis pax8*-CNS1 to *pax8*-CNS5. (**e**) The mouse *Pax8*-CNS1 (shown as 1^{mPax8}) was linked to the β-*actin* basal promoter-GFP cassette. (**f**) The proximal promoter region of mouse *Pax8* (−1607 to +494) alone was linked to GFP. (**g**) The mouse *Pax8* proximal promoter was flanked by mouse *Pax8*-CNS1. White, grey and black triangles indicate expression in the pronephros, otic vesicle and eye, respectively. White, grey and black arrows indicate expression in the pharyngeal arches, somites and MHB, respectively. White and grey arrowheads indicate expression in the hindbrain and an anterior part of the midbrain, respectively.

this possibility, GFP reporter constructs, carrying either *pax8*-CNS1 or *pax2*-CNS1 with the *pax8* proximal promoter (−2038 to +130), were subjected to a transgenic assay. In the resulting embryos, GFP expression from both constructs was restricted to the pronephros, which mostly recapitulated endogenous *pax8* expression (Fig. 4b,c). We also performed experiments using the *pax2* proximal promoter region (−1109 to +615 of *X. tropicalis pax2*) in place of the *pax8* proximal promoter. This *pax2* promoter in itself drove weak GFP expression in the *pax2*-expression domains (Fig. 4d). When this *pax2* promoter was flanked by the *pax8*-CNS1 or *pax2*-CNS1, GFP expression mimicked *pax2* expression in the pronephros, pharyngeal arches, MHB and hindbrain (Fig. 4e,f) in contrast with the experiments using the *pax8* promoter. These results indicated that silencer activity uniquely associated with the *pax8* proximal promoter is the primary factor generating the divergence of expression between *pax8* and *pax2*.

Finally, we asked whether *pax8* acquired the silencer following the WGD event(s), or whether both *pax8* and *pax2* inherited the element from their common progenitor but only *pax2* has lost it. The pleiotropic expression observed for the amphioxus gene, *pax2/5/8*, suggests the first scenario[9]. To examine this possibility, a GFP reporter construct carrying the amphioxus *pax2/5/8* promoter (−1330 to +589, Supplementary Fig. S5) was generated and used in the transgenic assay. This construct drove GFP expression in the eye, pharyngeal arches, MHB, hindbrain and pronephros, and the expression was very similar to that driven by *pax8*-CNS1 or *pax2*-CNS1 from the *pax2* proximal promoter (Fig. 4g, compare with Fig. 4e,f). This pleiotropic activity of the *pax2/5/8* promoter supports the idea that the silencer is an evolutionary innovation in the *pax8* promoter.

Discussion

In this study, we have shown that both *pax8* and *pax2* retain the ancestral modes of *cis*-activation involving the duplicated enhancers, and that the innovation of a tissue-specific silencer in *pax8* has had a crucial role in generating divergence in the expression of these paralogues. A previous study in teleosts suggested the conservation of the duplicated enhancers between *pax8* and *pax2*[23]. However, the activities of this and other *pax8* enhancers to drive *pax2*-like expression and the mechanism for suppressing such pleiotropic activities

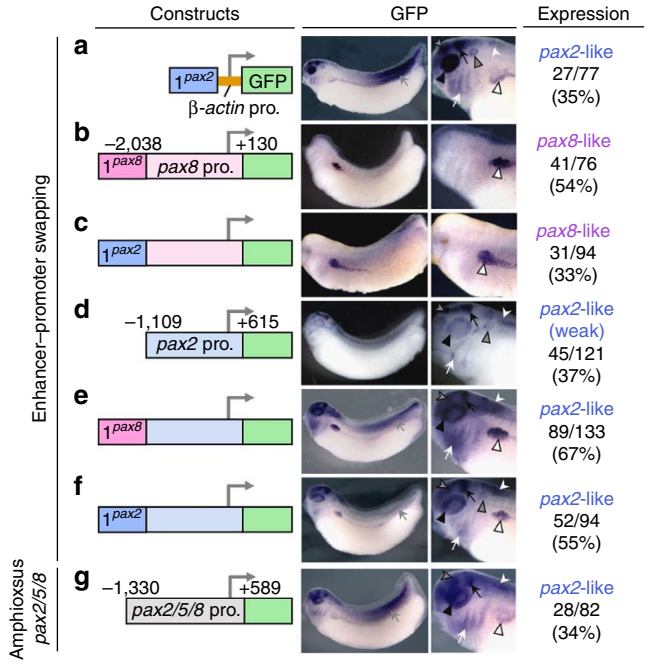

Figure 4 | Silencer innovation in *pax8* is crucial for generating divergence in the expression of *pax8* and *pax2*. Transgenic *X. laevis* embryos were generated with the following GFP constructs. (**a**) The *X. tropicalis pax2*-CNS1 (shown as 1^{pax2}) was linked to the β-*actin* basal promoter-GFP cassette. (**b**,**c**) The *X. tropicalis pax8* proximal promoter (−2038 to +130) was flanked by either the *X. tropicalis pax8*-CNS1 (**b**) or *pax2*-CNS1 (**c**). (**d**) The proximal promoter region of *X. tropicalis pax2* (−1109 to +615) alone was linked to GFP. (**e**,**f**) The *X. tropicalis pax2* proximal promoter was flanked by either the *X. tropicalis pax8*-CNS1 (**e**) or *pax2*-CNS1 (**f**). (**g**) The amphioxus *pax2/5/8* promoter (−1330 to +589) was linked to GFP. White, grey and black triangles indicate expression in the pronephros, otic vesicle and eye, respectively. White, grey and black arrows indicate expression in the pharyngeal arches, somites and MHB, respectively. White and grey arrowheads indicate expression in the hindbrain and in an anterior part of the midbrain, respectively.

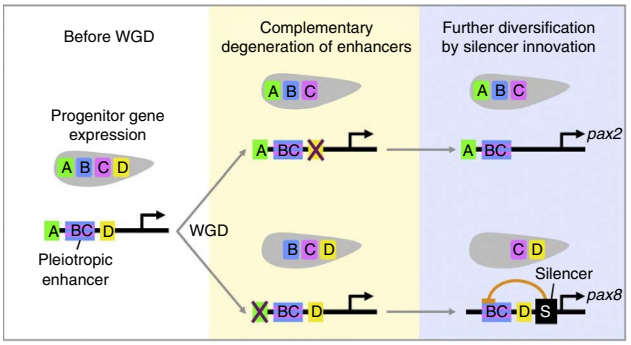

Figure 5 | A model for the regulatory diversification of *pax2* and *pax8*. To simplify the model, *pax5* is not depicted. The model is based on a progenitor gene with four expression domains (A, B, C and D) and three enhancers (A, BC and D), where the pleiotropic enhancer, BC, drives expression in both B and C domains (left panel). Following WGD1 and/or WGD2, the complementary loss of enhancers occurs in the duplicated genes: one (*pax2*) loses the D enhancer and the other (*pax8*) loses the A enhancer because of degenerative mutations ('×' in the middle panel). Silencer innovation then occurs in the proximal promoter of *pax8* (black box 'S'), to allow selective suppression of BC enhancer activity in the B domain (right panel, orange line).

outside the *pax8*-expressing tissues had remained unknown. Sequence analysis identified putative binding motifs of transcriptional repressors, REST (also known as NRSF)[24,25] and Nkx[26,27], in the silencer region of *X. tropicalis pax8* promoter and its orthologous region of mouse *Pax8*, but not in the homologous regions of *X. tropicalis pax2* or amphioxus *pax2/5/8* (Supplementary Fig. S6). The unique association of these repressor motifs to the *pax8* promoter implies their involvement in silencer activity.

In our transgenic assay, both the *Xenopus* and mouse *pax8* proximal promoters showed silencer activities but their tissue specificities were slightly different: the *Xenopus* promoter suppressed expression in the MHB but the mouse promoter did not (compare Figs 3g and 4b). Given that the *Xenopus* and mouse *pax8* enhancers (CNS1) are both capable of directing expression in the MHB, this difference in the silencer activities might account for the species differences in the MHB expression of *Xenopus* and mouse *pax8*.

It has been shown that the thyroid gland expression of *pax8* and *pax2* also shows species difference: thus, zebrafish expresses both *pax8* and *pax2*, whereas *Xenopus* and mouse exclusively express *pax2* and *pax8*, respectively, in the developing thyroid [12,13,28]. Although such overlapping or reciprocal expression implies the conservation of thyroid enhancers between *pax8* and *pax2* and species-specific modification of the silencers, our transgenic experiments did not detect any expression in the thyroid with either *pax8*-CNSs or *pax2*-CNS1 (Supplementary Fig. S7). Thus, a thyroid enhancer(s) might be present outside these CNSs in the *pax8* and *pax2* loci, and the involvement of any silencers remains unknown.

Given that only one pair of enhancers is conserved between *pax8* and *pax2*, complementary degeneration likely occurred in some of their duplicated enhancers after the WGD events. The silencer innovation in *pax8* promoter occurred either before or after the enhancer degeneration, and these two events have led to the resolution of their duplicated expression in modern vertebrates (Fig. 5). This process may also have involved the innovation of another *pax8* enhancer(s) and/or silencer(s), whose presence was suggested by the fosmid reporter assay. Possibly, the duplicated expression of *pax8* and *pax2* was advantageous for evolution of the kidney and ear but disadvantageous for other tissues. If *pax8* and *pax2* had inherited monofunctional enhancers rather than pleiotropic ones from their common progenitor, any disadvantages caused by duplication of their expression would have been fully dissolved by degeneration of the enhancers responsible, as predicted by the duplication–degeneration–complementation model. However, because pleiotropic enhancers such as *pax8*-CNS1 and *pax2*-CNS1 drove both advantageous and disadvantageous expressions, the silencer innovation was necessary to suppress the latter. The redundant regulatory system of *pax8* that involves additional enhancers, CNS2–CNS4, with overlapping activities might also have contributed to this silencer innovation by robust protection of the original expression pattern from degenerative mutations.

The two features present in *pax8* enhancers, pleiotropy and redundancy, have been found in the regulation of many developmental genes. For example, *Sox10* has three enhancers that drive overlapping expression in four discrete neural tissues[29], and *Sonic hedgehog* has two enhancers that drive overlapping expression in the notochord and tegmentum[30]. Recent studies in *Drosophila* have revealed that such redundant regulation involves 'shadow enhancers', which ensure robust gene expression under adverse environmental or genetic conditions[31–33]. Therefore, the redundant *pax8* enhancers might function as shadow enhancers, and silencer innovation could be a common evolutionary escape route for developmental genes that harbour such robust regulatory mechanisms.

Methods

Genome sequence analysis. The 160-kb genomic sequence of the human *PAX8* locus (hg18, chr2: 113,675,016–113,835,015) and its orthologous sequences in the mouse (mm9, chr2: 24,262,407–24,415,729), *X. tropicalis* (xenTro2, scaffold_30: 1,830,001–1,870,000) and *Takifugu* (fr2, chrUn: 75,566,029–75,596,029) were downloaded from the UCSC Genome Browser[34]. The genomic sequence of the human *PAX2* locus (hg18, chr10: 102,287,183–102,677,960) and its orthologous sequences in the mouse (mm9, chr19: 44,638,956–45,020,167), *X. tropicalis* (xenTro2, scaffold_204: 1,014,773–1,688,046) and *Takifugu* (fr2, chrUn: 158,920,197–159,034,770) were also downloaded from this genome browser. These sequences were aligned using MultiPipMaker[16]. Sequence alignments of the *pax8*-CNS1 and *pax2*-CNS1, amphioxus *pax2/5/8* promoters, and the *pax8*, *pax2* and amphioxus *pax2/5/8* proximal promoters were generated using ClustalW[35]. Searches for potential transcription factor-binding sites were performed with transcription factor-binding motifs collected from the TRANSFAC and JASPAR databases[36,37]. Putative transcription start sites of *X. tropicalis pax8*, *pax2*, mouse *Pax8* and amphioxus *pax2/5/8* were predicted with their previously identified complementary DNAs (Genbank accession numbers: NM001079301, AL969279, NM011040 and AF053762, respectively) and an eukaryotic promoter prediction program[38].

Plasmid and fosmid constructs. The reporter plasmid, actGFP, carrying a chicken β-*actin* basal promoter (−55 to +53), was previously described as βGFP[18]. The construct, gloGFP, was generated by replacing the β-*actin* basal promoter of actGFP with a human β-*globin* basal promoter (−37 to +12)[20]. Primer sequences used in this study are summarized in Supplementary Table S3. The CNSs and other promoter regions were cloned from *X. tropicalis*, mouse or Japanese amphioxus (*Branchiostoma japonicum*) genomic DNA by PCR and verified by sequencing. The sequence of the *B. japonicum pax2/5/8* promoter was deposited to GenBank (accession no. JQ902140). The reporter constructs carrying the *pax2*, *pax8* or *pax2/5/8* promoters were generated by introducing each fragment into the promoter-less GFP reporter plasmid, pBSSK+EGFP[18]. For generating the *pax8*–GFP fosmid construct, a GFP-flpe-*kan* cassette was introduced in-frame into a *X. tropicalis* fosmid clone, AOPZ250282 (European Xenopus Resource Centre), using homologous recombination technique[39]. After the recombination, the *kan* gene used as a selection marker was flipped out from the fosmid by arabinose-induced Flpe recombinase[40].

Transgenic reporter assay. Transgenic *Xenopus* embryos were generated by a sperm nuclear transplantation method with oocyte extracts[17,41]. The manipulated embryos were cultured until the tailbud stages, and all normally developed embryos were subjected to *in situ* hybridization to examine their GFP expression with maximum sensitivity[18]. The frequency of GFP expression varied depending on constructs, egg quality and preparation of the oocyte extracts. However, within any GFP-positive group, more than 80% showed an expression pattern consistent with the representative examples, and the remainder showed non-reproducible ectopic expression (Supplementary Fig. S2 and Supplementary Table S1).

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Author contributions

H. Ochi performed the molecular cloning and transgenic experiments, and participated in writing the paper. T.T. and H.N. performed the transgenic experiments and *in situ* hybridization analysis. A.K. and N.S. participated in the *in situ* hybridization analysis. H. Ogino designed the experiments and wrote the paper. All authors discussed the results and made substantial contributions to the manuscript.

Additional information

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