The lineage-specific gene ponzr1 is essential for zebrafish pronephric and pharyngeal arch development

Victoria M. Bedell1,*, Anthony D. Person2,*, Jon D. Larson2, Anna McLoon2, Darius Balciunas1,4, Karl J. Clark1, Kevin I. Neff1, Katie E. Nelson3, Brent R. Bill2, Lisa A. Schimmienti2, Soraya Beiraghi2 and Stephen C. Ekker1,2,‡

SUMMARY
The Homeobox (Hox) and Paired box (Pax) gene families are key determinants of animal body plans and organ structure. In particular, they function within regulatory networks that control organogenesis. How these conserved genes elicit differences in organ form and function in response to evolutionary pressures is incompletely understood. We molecularly and functionally characterized one member of an evolutionarily dynamic gene family, plac8 onzin related protein 1 (ponzr1), in the zebrafish. ponzr1 mRNA is expressed early in the developing kidney and pharyngeal arches. Using ponzr1-targeting morpholinos, we show that ponzr1 is required for formation of the glomerulus. Loss of ponzr1 results in a nonfunctional glomerulus but retention of a functional pronephros, an arrangement similar to the aglomerular kidneys found in a subset of marine fish. ponzr1 is integrated into the pax2a pathway, with ponzr1 expression requiring pax2a gene function, and proper pax2a expression requiring normal ponzr1 expression. In addition to pronephric function, ponzr1 is required for pharyngeal arch formation. We functionally demonstrate that ponzr1 can act as a transcription factor or co-factor, providing the first molecular mode of action for this newly described gene family. Together, this work provides experimental evidence of an additional mechanism that incorporates evolutionarily dynamic, lineage-specific gene families into conserved regulatory gene networks to create functional organ diversity.

KEY WORDS: Kidney development, Kidney evolution, Pharyngeal arch development, Zebrafish, Plac8

INTRODUCTION
The evolution of animal complexity has fascinated scientists ever since Darwin presented his ideas on embryology and ‘descent with modification’ (Darwin, 1890). Species’ adaptation to new environments required specific organ system modifications. The adaptation of fish from hyperosmotic saltwater to hypoosmotic freshwater environments is an extreme example of organ system adaptation. This movement required drastic modification of salt and water homeostasis mechanisms. Two organ systems essential for maintaining osmotic balance are the gills and kidney. In aquatic animals, the gills are gatekeepers for maintaining salt balance whereas the kidney is needed for maintaining both salt and water concentrations. Thus, maintaining homeostasis requires cooperation between these two systems. How differential adaptation of the gills and kidney to new environments is encoded in the genomes of diverse animals is not well understood.

Gross anatomical observations readily demonstrate kidney diversity across species. For over a century and a half, scientists have compared adult mammalian kidneys, cataloging both similarities and differences (Wagner and Tulk, 1845). These differences are illustrated when comparing the horse (Fig. 1A) and human (Fig. 1B) kidney. Despite such outward structural diversity among mammals, the building blocks of their kidneys, the nephrons, are nearly identical. However, comparing nephrons among chordates reveals clear differences (Dantzler, 1989; Smith, 1937). As early as 1937, key variances in nephron structure were noted to track with broad evolutionary changes in vertebrates (Smith, 1937). When examining the earliest developing kidney, the pronephros, variations are observed when comparing glomerular absence (aglomerular; Fig. 1C) or presence (non-integrated or integrated glomerulus; Fig. 1D,E), and by examining the complexity of tubules and ducts extending from the glomerulus. Aglomerular kidneys (Fig. 1C), found in a subset of teleosts such as Opsanus tau and Lophius piscatorius (Marshall, 1930), remove waste by tubule lumen secretion (Beyenbach, 2004). In organisms containing a glomerulus, two examples of glomerular pronephric evolution are the non-integrated and the integrated glomerulus. The non-integrated form of the pronephros is found in Xenopus, in which the coelom separates the glomerulus from the tubules and ducts (Fig. 1D, arrow) (Dressler, 2006). By contrast, the zebrafish pronephros (Fig. 1E) represents the most common teleost kidney type – a glomerulus integrated with the pronephric tubules and duct – and is similar to the nephron found in adult mammalian kidneys (Fig. 1F). The molecular basis underlying these developmental variations of the vertebrate pronephros is unknown.

Broadly, three related but molecularly distinct models have been proposed as the genetic basis of organ diversity. The first involves conservation of master regulatory genes (Carroll, 2005); the second outlines gene regulatory networks (GRNs) (Davidson and Erwin, 2006); and the third focuses on dynamic gene families (Demuth et al., 2006). First, the ‘master regulation’ model – whereby a handful of key conserved genes are necessary and sufficient for organ development – is the best characterized. Changes in number and regulation of key highly conserved genes is an important mechanism underlying evolutionary diversity (Carroll, 2005). For example, Hox genes specify variation in body segments, including

1Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905, USA. 2Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN 55455, USA. 3STEM Squad, Mayo Clinic, Rochester, MN 55905, USA. 4Department of Biology, Temple University, Philadelphia, PA 19122, USA.

*These authors contributed equally to this work
‡Author for correspondence (ekker.stephen@mayo.edu)

Accepted 15 December 2011
appendage development in insects (Lewis, 1978) and leg loss in snakes (Cohn and Tickle, 1999). Two demonstrated mechanisms for Hox-driven evolutionary diversity are copy number change and expression pattern alteration via variation in regulatory sequences (Force et al., 1999). These mechanisms are responsible for at least part of the diversity seen in body structure (Gilbert, 2000).

The kidney’s master regulatory gene, paired box 2 (pax2), is conserved from flies to humans (Cagan, 2003; Lun and Brand, 1998; Sanyanusin et al., 1995; Schimmer et al., 1997; Torres et al., 1995) and is both necessary and sufficient to specify renal tubule and ductal cell fate. However, the glomerulus has distinct evolutionary origins and does not require pax2 (Majumdar et al., 2000). Therefore, pax2 and its role in kidney diversity represent a strong model for exploring the mechanisms underlying how conserved genes are manipulated throughout evolution. Pax2 is a paired box transcription factor known to be required for pronephric development. Zebrafish pax2a mutants, no isthmus (noi) (Brand et al., 1996), show distinctive kidney phenotypes (Majumdar et al., 2000). Without pax2a, the podocyte marker wilms tumor 1a (w11a) shows caudally expanded expression at 25 hours post fertilization (hp) (Majumdar et al., 2000). Furthermore, cross-sections of noi mutants show improper pronephric tubule and duct formation but reveal intact glomeruli. Evidence from a detailed analysis of the structure and function of the pronephros by Howland and by Price (Howland, 1921; Price, 1910), along with the noi mutant data, suggests that the glomerulus originates and develops independently of the pronephric tubules and ducts (Drummond and Majumdar, 2003). The key question, then, is how pax2 works with other genes to code for glomerular innovation and kidney diversity.

The second model of diversity, the GRN model, also attributes evolutionary innovation to a set of conserved genes. As currently conceptualized, a GRN consists of four types of sub-circuits arranged hierarchically within a network (Davidson, 2010; Davidson and Erwin, 2006). In summary, GRNs consist of ‘kernels’ comprising a few genes and regulatory regions absolutely required to form a specific organ (Punzo et al., 2002; Silver and Rebay, 2005). Should any part of the kernel be lost, the organ does not form. Modules called ‘plug-ins’ provide subsequent layers of complexity (Cadigan and Nusse, 1997; Kingsley, 1994). Plug-ins, which interact with and regulate the kernel, are not required to be organ-specific. ‘Cis-regulatory linkages’ are considered input-output switches involved in regulation of the other sub-circuits (Bolouri and Nusse, 2002; Hersh and Carroll, 2005). ‘Differentiation gene batteries’ are the organ-specific genes that provide the final termination of the network (Gilchrist et al., 2006; Peter and Davidson, 2009). Thus, GRNs can encode diversity via changes in plug-ins and differentiation gene batteries because changes in these peripheral systems will not change the kernel.

Recently, current in-depth sequencing methods and innovative bioinformatic approaches have uncovered genes that do not follow traditional conservation models – ‘evolutionarily dynamic’ gene
families (Obbard et al., 2009). In particular, vertebrate-specific gene families have been discovered (Boutet et al., 2010; Katsube et al., 2009). One such example is the vertebrate-specific Ccn family of small, reactive, cysteine-rich proteins that are crucial for signaling of many vertebrate traits, including vasculogenesis and chondrogenesis (Katsube et al., 2009).

The master regulatory gene and GRN models exhibit substantial conceptual overlap, while each offers unique insight into mechanisms that can ultimately cooperate to encode evolutionary diversity. However, how these newly described, lineage-restricted gene families are specifically utilized by core, highly conserved genes and their corresponding signaling networks through evolution is still an open question.

Here, we molecularly and functionally characterize one member of an evolutionarily dynamic gene family, plac8 onzlin related protein 1 (ponzr1). Throughout development, ponzr1 expression localizes to the pronephros and pharyngeal arches. We show that ponzr1 functions downstream of pax2a and forms a feedback loop that also modifies pax2a expression. Morpholino knockdown reveals ectopic midline expression of pax2a and wta1 at 24 hpf. At 3 days post fertilization (dpf), ponzr1 knockdown results in a modified zebrafish kidney with loss of the glomerulus and disrupted podocytes. Despite the loss of the glomerulus, the resulting kidney in ponzr1 morphants unexpectedly reveals a functioning structure reminiscent of the simpler kidney found in agglomeran fish (Cagan, 2003; Vize and Smith, 2004). These data lay the foundation for a new model of kidney development in which pax2a signals for kidney differentiation in the pronephric ducts and tubules, and ponzr1 serves as a switch to signal for a more complex kidney that filters with an integrated glomerulus.

Examining the second organ system involved in osmotic homeostasis, we find that the wilms tumor 1b (w1t1b)-expressing pharyngeal arches, which will eventually develop into the gills, do not form in ponzr1 morphants. Finally, we show ponzr1 can function as a transcription factor or co-factor, providing the first mechanistic insight for this gene family. This protein gives us an additional mechanism of control for conserved genes using a member of a dynamic gene family to generate diversity.

MATERIALS AND METHODS

ponzr1 isolation and constructs

ponzr1 was isolated by RT-PCR from total RNA isolated from 2 dpf embryos. The reverse transcription reaction was performed with 5 μg total RNA, 100 ng of random hexamers (Invitrogen), and Superscript II (Invitrogen). PCRs was performed with the following ponzr1 primers: 5'-CGCGGTTAAACACATTTGCTG-3' and 5'-TATCAGCGATCACAA-3' with DIG-labeled riboprobe generated by in vitro transcription with T3 RNA polymerase (Roche) according to the manufacturer’s protocol. pGEM-3Zf(+) was used for EcoRI and DIG-labeled riboprobe was transcribed with SP6 as described (Krauss et al., 1991). For double in situ hybridizations, pax2a riboprobes were made by incorporating fluorescein-labeled UTPs instead of DIG. pBluescript KS+ was cut with SpeI and riboprobe transcribed with T7 RNA polymerase (Roche). cdi71, wta1 and podocin cDNAs were all isolated by RT-PCR from total RNA from 24 hpf embryos. cdi71 primers (5'-ACAG-CTGGAGACCCCTCAGA-3', 5'-GTCTGGAAAGCAGATGAAGC-3'), wt1 primers (5'-TGCTGGTGACACCTCCTCTCTG-3', 5'-TAGGGTTTCTCCTCCCTGTG-3'), podocin primers (5'-CAAGACCGAATTCTGTGAGGAGT-3', 5'-GTCCTGAAGGCAGATGAAGC-3'), transcribed with SP6 to generate DIG-labeled riboprobes. Whole-mount in situ hybridizations were as described (Thiase and Thiase, 2008).

Morpholino experiments

The following MOs were used in this study: ponzr1 MO1 5'-GAAGTCCCTTGTCTGTTGAGCAA-3', ponzr1 MO2 5'-CCGTAATGAAATGCTGGCATGAC-3', Control MO 5'-CAAGACCTTCTGTTGAGCAA-3'. Morpholino injections were performed as previously described (Nasevicius and Ekker, 2000).

Glomerular capillary experiments

Double transgenic larvae were generated by crossing the Tg(wt1b:EGFP) to Tg(gata1:dsRed) line. Embryos were imaged in fluorinated ethylene propylene tubing (Cole Parmer, USA, P/N EW-00244-ZU) as described by (Petzold et al., 2010) held in a custom-built capillary stage that allowed rotation of the capillary and rotation in the lateral plane about the focal point. Images and movies were taken using a Zeiss Examiner D1 stand, equipped with the W Plan-Apochromat 20x/1.0 objective (Zeiss, Germany, P/N 421452-9800) and a digital videocamera (Sony, Japan, HDR-HC9) capable of HD 1080i videorecording.

Alcian Blue staining

Alcian Blue cartilage staining was performed as previously described (Walker and Kimmel, 2007) with two modifications. First, we used a 0.01% Alcian Blue concentration to stain the larvae. Second, the larvae were stained for 6 hours then washed overnight in 20% glycerol in 0.25% KOH.

Zebrafish work

Acknowledgments

All zebrafish work was conducted under full animal care and use guidelines with prior approval by the local institutional animal care and use committee. Danio rerio pax2a null nos29,31-/- and pax2a hypomorphic strains nos21-/- used in our studies were described previously (Lun and Brand, 1998). Danio rerio Tg(wt1b:EGFP)-expressing strain was used...
Previously described (Perner et al., 2007), as was the mRFP-expressing line, GBT0046, used as a filtration assay (Clark et al., 2011; Petzold et al., 2009). Danio rerio transgenic lines were described previously: Tg(atf1a:4:GFP) (Liu et al., 2007), Tg(enpep:GFP) (Seiler and Pack, 2011) Tg(fil1:EGFP) (Traver et al., 2003) and Tg(gata1:dsRed) (Lawson and Weinstein, 2002).

Statistics
All histograms are the mean of two or three experiments. The error bars were calculated using the mean variance. Statistics was run using a one-way ANOVA and the Newman-Keuls multiple comparison test to look for statistical significance while still accounting for multiple comparisons. All statistical analysis was run on RCF Prism v50a.

RESULTS
The evolutionarily dynamic, chordate-specific Ponzr gene family
ponzr1 was isolated as a part of a forward genetic screen for novel genes required for zebrafish organogenesis (Pickart et al., 2006). ponzr1 encodes a 124-amino acid predicted protein with a plac8 domain, a protein motif of unknown function. The human genome encodes three known proteins containing this same motif: placenta-specific 8 (PLAC8), PLAC8-like 1 (PLAC8L1), and Cornifelin (CNFN). The signature family motif contains two conserved, cysteine-rich domains (domains 1 and 2) separated by a variable region (supplementary material Fig. S1A). Using the conserved sequence obtained from the human and zebrafish Ponzr1 alignment, we mined numerous animal genomes for Ponzr family members (supplementary material Fig. S1B). Ponzr family members are found in the extended vertebrate lineage (supplementary material Fig. S1A) and do not appear in the genomes of Drosophila melanogaster or Caenorhabditis elegans (data not shown). The Ponzr gene family is evolutionarily diverse. For example, several branches of the Ponzr family tree have no human or mouse orthologs (supplementary material Fig. S1B); other branches contain two human genes that have been truncated or are thought to be no longer functional (PLAC8 pseudogenes) that align to a Ponzr member from the invertebrate chordate and are thought to be no longer functional (PLAC8 pseudogenes) to an orthologous Ciona intestinalis (supplementary material Fig. S1B). Furthermore, even in animals with the same number of Ponzr family members, such as Ciona and Xenopus, the identified Ponzr genes are not orthologous. Zebrafish ponzr1 represents one of a dozen Ponzr genes in this teleost.

ponzr1 is expressed in the developing kidney and pharyngeal arches
Whole-mount in situ hybridization reveals that ponzr1 is expressed in the intermediate mesoderm, which forms the developing kidney (Fig. 2B,D). This pattern resembles the expression of the pronephric progenitor marker lhx1a (previously lim-1) (Toyama and Dawid, 1997; Toyama et al., 1995) at eight somites (8S) (Fig. 2A) and pax2a at 12S (Fig. 2C). Tissue-specific expression of ponzr1 is visible throughout pronephric development (Fig. 2E-J) and can be seen in the pronephric tubules and ducts at 24 hpf (Fig. 2K). Double in situ hybridizations at 24 hpf show ponzr1 expression in the pax2a-positive tissue of the pronephric tubules and ducts (Fig. 2N). ponzr1 expression persists in the pronephric duct and can be detected in the pronephric tubules at 48 hpf (Fig. 2L) and 72 hpf (Fig. 2M). Unlike pax2a (Wingert et al., 2007), ponzr1 is also expressed in the developing glomerulus (Fig. 2L,M). Furthermore, ponzr1 is expressed in the pharyngeal arches at 48 hpf (Fig. 2M) and 72 hpf (Fig. 2N).

ponzr1 functions downstream of pax2a
We asked whether ponzr1 is genetically downstream of the kidney master regulatory gene, pax2a, by performing gain-of-function and loss-of-function experiments. In gain-of-function experiments, overexpression of pax2a Isoform 2 mRNA results in formation of ectopic pronephric tissue that express the kidney-specific marker, cadherin 17 (cdh17) (Fig. 3A-C). This functionally demonstrates that Pax2a is a master regulatory factor sufficient to specify pronephric cell fate during embryogenesis. Overexpression of pax2a also results in commensurate ectopic ponzr1 expression in the ectopic ducts (Fig. 3D-F). To perform loss-of-function experiments, we used the pax2a mutant line, no isthmus (noi 29±/−) (Lun and Brand, 1998). In wild-type sibling embryos at 32 hpf, ponzr1 expression was detected throughout the developing pronephric tubules and ducts (Fig. 3G,H). By contrast, ponzr1 expression in noi 29±/− mutant embryos was not detected in the
anterior pronephric tubules at 32 hpf (Fig. 3I) and was substantially diminished in the posterior pronephric ducts (Fig. 3J). Together, these data provide evidence that ponzr1 is genetically downstream of pax2a.

**ponzr1 morpholino knockdown embryos exhibit altered kidney marker expression**

Using whole-mount in situ hybridization, we screened known markers of kidney development to ascertain whether ponzr1 knockdown affected kidney development. First, we assayed for altered expression of intermediate mesoderm markers during early pronephric development to determine the effect of ponzr1 knockdown on early kidney development. Between 4S and 12S, we noted no phenotype (supplementary material Fig. S2). At 18S, ponzr1 morphants exhibit expanded pax2a expression in the anterior pronephric tubules (Fig. 4B) compared with uninjected controls (Fig. 4A). Ectopic and expanded midline expression of pax2a was visible at 24 hpf in ponzr1 morphants (Fig. 4D) compared with controls (Fig. 4C). At the 21S intermediate developmental time point, deformation of the anterior kidney is apparent in ponzr1 morphants when assayed using pax2a expression (Fig. 4F,G, compared with 4E). The pax2a midline fusion is apparent at 3 dpf in ponzr1 morphants (Fig. 4I).

The markers of the future podocytes of the glomerulus, wt1a and podocin, are normally expressed in two distinct domains at 24 hpf (Fig. 4L,N). However, ponzr1 morphants exhibit ectopic wt1a and podocin expression in a single midline position (Fig. 4M,O). To ensure the phenotype was specific to ponzr1 loss of function, we tested for the pax2a and wt1a 24 hpf phenotypes with a second ponzr1 morpholino as well as a mismatch control (Fig. 4J,P) and found both morpholinos were significantly different from wild type and the mismatch control. Additionally, we noted a statistically significant rescue of the phenotype by coinjection of ponzr1 mRNA (Fig. 4K,Q). We tested ponzr1 MO knockdown efficiency using an artificial ponzr1 5’ UTR/green fluorescent protein (GFP) synthetic mRNA injected into zebrafish embryos [based on Chen et al. (Chen et al., 2004)]. Briefly, GFP intensity was quantified by fluorescent imaging. Each experiment was standardized with wild-type embryos as 0% (supplementary material Fig. S5A), and ponzr1 5’ UTR mRNA-injected embryos as 100% (supplementary material Fig. S5B). ponzr1 MO-injected embryos demonstrated a 94% knockdown (supplementary material Fig. S5C,D), whereas ponzr1 MO2-injected embryos displayed a 90% knockdown (supplementary material Fig. S5E,F). By contrast, the highest dose of ponzr1 MO1 mismatch oligo showed only a 50% knockdown (supplementary material Fig. S5G-I). Therefore, in ponzr1 MO-injected animals at 24 hpf, we saw ectopic midline expression of multiple kidney markers with expanded expression of pax2a.

The three markers pax2a, wt1a and podocin label the anterior kidney, specifically the developing glomerulus. Next, we examined the pronephric tubules and ducts in ponzr1 MO knockdown embryos. cdh17 staining in 48 hpf morphants demonstrated a widening of expression in the anterior pronephric duct (Fig. 4S) compared with controls (Fig. 4R). In the Tg(atplala:4GFP) (Liu et al., 2007) (supplementary material Fig. S3) and Tg(enep:GFP) (Seiler and Pack, 2011) (supplementary material Fig. S4) lines, no obvious phenotypes were noted at 28 hpf (A-H) and 48 hpf (I-P).

**Loss of ponzr1 results in a nonfunctional glomerulus but functional pronephros**

Because of the atypical 24 hpf phenotype in ponzr1 morphants, we hypothesized that at 3 dpf there would be an architectural and functional change after the pronephros was fully formed. We assessed the anterior kidney structure at 3 dpf using the Tg(wt1b:EGFP) line (Perner et al., 2007). In Tg(wt1b:EGFP) larvae, both the podocytes surrounding the glomerulus and the pronephric tubules proceeding distally and caudally from the podocytes (Fig. 5B) express EGFP. In ponzr1 MO1-injected embryos, a gap in fluorescence is noted where the podocytes should be encircling the glomerulus, and the pronephric tubule staining is largely lost (Fig. 5C,D, quantified in 5F). Furthermore, pax2a hypomorph embryos (nopax2a) (Lun and Brand, 1998) show an inhibition of podocyte migration from the pronephric tubules to the glomerulus with fluorescence running caudally (Fig. 5E) rather than concentric in the midline.

Because the 3 dpf kidney structure was altered, we hypothesized that the pronephros would be nonfunctional. To assay for kidney function, we used a zebrafish line that expresses an mRFP attached to a signal sequence. The mRFP is secreted into the blood and, as long as the glomerulus is functional, accumulates in the curvature of the pronephric ducts and tubules (Fig. 5H,I). Loss of pax2a results in a similar phenotype (Fig. 5J). The filtration phenotype is quantified in Fig. 5K.
As the \textit{wt1b} fluorescence showed altered architecture of the podocytes and pronephric tubules, we examined the glomerulus using serial sections stained with haematoxylin and eosin (H&E). In wild-type embryos, the glomerulus (arrowhead, Fig. 5L) is clearly visible, as are the flanking tubules (arrows, Fig. 5L) and pronephric ducts (Fig. 5O). In \textit{noitb21}–/– mutant embryos, the glomerulus is unaltered (Fig. 5M), but the pronephric ducts are overtly dilated (Fig. 5P). This phenotype is comparable to that noted in \textit{noitb29a}–/– mutant embryos (Majumdar et al., 2000). By contrast, \textit{ponzr1} morphants show a loss of the central glomerulus and a dilation of the pronephric tubules (Fig. 5N) as well as the ducts (Fig. 5Q). These data confirm that \textit{ponzr1} morphants lack a centralized glomerulus but are still able to retain some kidney filtration function.

To further assess the defect in the glomerulus, in \textit{ponzr1} morphant embryos we examined the glomerular capillaries, which make up the bulk of the cells within the podocytes. Because the vasculature is so abundant in the head, we were unable to visualize the capillaries using Tg(\textit{fli1}:EGFP) (Lawson and Weinstein, 2002) (data not shown). Moreover, confidence in focal plane alignment was low in that line. Therefore, we used a double transgenic Tg(\textit{wt1b}:EGFP) and Tg(gata1:dsRed) (Traver et al., 2003) to ask for...
whether the glomerulus had functional capillaries. We used an updated Specimen in a Corrected Optical Rotational Enclosure (SCORE) protocol (Petzold et al., 2010) combined with a 20X water-emersion, long working-distance lens to visualize the samples and a high-definition camera to record the data. The Tg(wt1b:EGFP) was used to focus on the glomerulus; then we monitored RFP fluorescence to visualize blood flow through the capillaries. In both uninjected embryos (supplementary material Movie 1; Fig. 6A,B) as well as mismatch MO controls (supplementary material Movie 2; Fig. 6C,D), blood cells could be seen moving through the capillaries. However, in ponzr1 MO-injected larvae, no blood cells could be seen flowing through the area where the glomerular capillaries should be located (supplementary material Movie 3; Fig. 6E,F). The loss of blood flow in the ponzr1 MO-injected larval glomeruli is significantly different from both wild-type and mismatch MO controls (Fig. 6G).

Fig. 5. ponzr1 is required for anterior kidney form and function. (A) A drawing of a larval zebrafish at 3-4 dpf. The green box is the area of the larva seen in images B-E, and the red box is the area seen in images G-J. (B) Tg(wt1b:EGFP) larvae (Perner et al., 2007) mark podocytes surrounding the glomerulus (arrowhead) and the pronephric tubules (arrows). (C,D) ponzr1 morphants show a loss of fluorescence in the area surrounding the glomerulus, except at the midline (brackets). The pronephric tubular staining is lost (arrows) in a dose-dependent manner (D: 1 ng MO1; E: 1.5 ng MO1). (E) noitb21−/−mutant embryos exhibit a loss of fluorescence around the glomerulus (brackets) and the pronephric tubule fluorescence runs posteriorly instead of laterally (arrows). (F) Two ponzr1 MOs induce a similar phenotype that is ameliorated in mismatch control MO injections. (G) mRFP appears bilaterally in the curvature of the pronephric tubules (arrows) in control embryos due to glomerular filtration. (H-J) In ponzr1 morphants, mRFP is collected in a more posterior part of the tubules – sometimes only one pronephric tubule collects fluorescence and sometimes fluorescence is seen in both (H: 1 ng MO1; I: 1.5 ng MO1). A similar phenotype is seen in noitb21−/−mutant embryos (J). (K) Percentage of embryos with the filtration phenotype. (L-Q) Larvae at 4dpf that have been sectioned and H&E stained. (L) Uninjected embryos show a glomerulus (arrowhead) with two pronephric tubules (arrows). (M) noitb21−/−mutant embryo has a glomerulus (arrowhead) but shows dilated tubules (arrow). (N) ponzr1 morphants show dilated pronephric tubules (arrows) with no glomerulus. (O) The posterior pronephric tubules in wild-type embryos (arrows). noitb21−/−mutant (P) and ponzr1 morphant embryos (Q) both show dilated tubules (arrows). **P<0.01, ***P<0.001
Pharyngeal arch loss in ponzr1 morphants

A second way in which aquatic animals manage their osmotic balance is through the gills. The gills form from the pharyngeal arches (Kimmel et al., 1995), which begin to show ponzr1 expression at 2 dpf (Fig. 2L). Additionally, wt1b is also expressed in the pharyngeal arches (Perner et al., 2007) in a pattern similar to that of ponzr1. Therefore, we examined wt1b expression in the pharyngeal arches at 3 dpf. Wild-type embryos showed three pharyngeal arches expressing wt1b (Fig. 7B). ponzr1 MO knockdown resulted in a complete loss of wt1b in the arches (Fig. 7C). This phenotype was seen with both ponzr1 morpholinos but not with control morpholinos or in control wild-type larvae (Fig. 7D).

To determine if the cartilages of the posterior pharyngeal arches are lost in ponzr1 knockdown embryos, we stained with Alcian Blue at 5 dpf. We observed complete loss of the posterior four pharyngeal arches in the ponzr1 MO1-injected larvae (supplementary material Fig. S6B) but not in wild-type or mismatch MO-injected larvae (supplementary material Fig. S6A,C).

Rescue of anterior pax2a mutant phenotype by ponzr1

Having demonstrated that ponzr1 is downstream of pax2a, we asked whether ponzr1 overexpression would be sufficient to rescue the noitb21–/– kidney phenotype at 24 hpf. We examined two known pax2a mutant phenotypes: ectopic, expanded wt1a expression in the anterior kidney (Majumdar et al., 2000); and truncated cdh17 expression in the posterior kidney. Injection of ponzr1 mRNA into pax2a mutant embryos results in a significant reduction in the frequency of embryos displaying the wt1a expression phenotype (supplementary material Fig. S7A). In contrast, addition of ponzr1 mRNA does not significantly alter the posterior cdh17 expression defect (supplementary material Fig. S7B). Therefore, ponzr1 is sufficient to rescue signaling for the anterior portion of the kidney but cannot replace pax2a under these conditions for the posterior kidney at 24 hpf.

ponzr1 genetically functions as a transcription factor or co-factor

Little is known about the mechanism of action for members of the Ponzr gene family. To determine whether ponzr1 is a terminally expressed gene or a member of a gene regulatory pathway, we asked whether ponzr1 can act as a transcription factor or co-factor. We used a previously described activator/repressor genetic test (Brickman et al., 2000; Conlon et al., 1996) to probe the ability of ponzr1 to interact with the transcriptional regulatory system. We made DNA constructs with ponzr1 fused to either the transcriptional activator herpes simplex virus protein 16 (VP16) (Sadowski et al., 1988) or to the transcriptional repressor engrailed repressor (EnR) (Jaynes and O’Farrell, 1991) (Fig. 8A). We then injected mRNA synthesized from ponzr1-VP16 or ponzr1-EnR, respectively, into zebrafish embryos and examined cdh17 and pax2a expression patterns at 24 hpf. Expression of ponzr1-VP16 fusion caused a severe truncation of cdh17 in the pronephric ducts (Fig. 8D) similar to,
but more extreme than, the overexpression of ponzr1 mRNA (Fig. 8C). The truncation phenotype is significantly increased compared with wild-type embryos (Fig. 8E). Injections of ponzr1-VP16 mRNA also resulted in a reduction in pax2a expression in the anterior pronephric tubules (Fig. 8G, J) compared with controls (Fig. 8F, I). By contrast, expression of the ponzr1-EnR mRNA resulted in enhanced pax2a expression (Fig. 8H, K), resembling the change of pax2a noted in ponzr1 morphant embryos (Fig. 4B). However, we did not observe a cdh17 phenotype in ponzr1-EnR injected embryos (Fig. 8E). The pax2a phenotypes seen in the ponzr1-VP16 and ponzr1-EnR injections are significantly different from wild type (Fig. 8L). Together, these data strongly suggest that ponzr1 normally functions as a transcription factor or co-factor during kidney development.

**DISCUSSION**

**The mysterious glomerulus**

The conserved regulatory gene, pax2, plays a conserved role in kidney biology from flies to humans. However, because pax2 is a key kidney regulatory gene, it is found in both glomerular and aglomerular vertebrates, presenting a conundrum regarding the mechanism underlying differences in kidney biology between

---

**Fig. 7. ponzr1 is required for wt1b-expressing pharyngeal arches.** (A) A drawing of a 3 dpf larval zebrafish showing where the pharyngeal arches are located. The green box is the area seen in images B and C. (B) Tg(wt1b:EGFP) larvae 3 dpf lateral view shows three pharyngeal arches express wt1b. (C) When ponzr1 MO is injected, the pharyngeal arches are lost. (D) The loss of pharyngeal arches phenotype is significantly different in the two ponzr1 MOs compared with both the wild type and mismatch controls. **P<0.01, ***P<0.001

---

**Fig. 8. ponzr1 can function as a transcription factor or co-factor.** (A) Diagrams showing the transcriptional activator and repressor mRNAs injected into zebrafish. The NLS was included to facilitate nuclear access of the fusion proteins. (B) Dorsal view of wild-type cdh17 expression in the pronephric ducts at 24 hpf. (C) ponzr1 mRNA-injected embryos reveal cdh17 expression loss at the cloaca (arrow). (D) ponzr1-VP16 mRNA-injected embryos show a severely truncated cdh17 expression in the pronephric ducts (arrows). (E) cdh17 phenotype in ponzr1- and ponzr1-VP16-injected embryos are significantly different from control, whereas ponzr1-EnR-injected embryos are not. (F) Wild-type pax2a expression in the anterior pronephric ducts at 24 hpf (magnified I). (G-K) ponzr1-VP16 mRNA-injected embryos (G) show reduced pax2a expression (magnified J) whereas ponzr1-EnR mRNA-injected embryos (H) demonstrate expanded pax2a expression in the anterior pronephric ducts (arrows) (magnified K) at 24 hpf. (L) Quantification of pax2a phenotypes standardized to wild-type pax2a at 100%. Percentage of embryos with reduced pax2a is seen as below 100% (as seen in ponzr1VP16-injected embryos) and expanded pax2a expression is seen above 100% (as seen in ponzr1EnR-injected embryos). Both ponzr1-VP16 and ponzr1-EnR are significantly different from wild type. ***P<0.001
vertebrate lineages. The glomerulus is a complex organ structure whose development requires multiple cell types of diverse origin (Dittrich, 2005; Kramer-Zucker et al., 2005). Over the past few years, great strides have been made in discovering the molecular signals for tubular and ductal development (Drummond, 2004; Drummond et al., 1998; Vize et al., 1997) as well as identifying signals directing individual cell types, such as the vasculature (Erema and Quaggin, 2004; Kitamoto et al., 1997; Majumdar and Drummond, 1999) and podocytes (Majumdar and Drummond, 2000; Quaggin and Kreidberg, 2008), to migrate into the glomerulus. However, localized, cell-type-specific development of a centralized glomerulus requires coordination through global signals that remain largely unidentified. For example, VEGF has been implicated in glomerular vascularization, but what regulates VEGF in this developmental setting is unknown (Erema and Quaggin, 2004; Kitamoto et al., 1998; Vize et al., 1997) as well as identifying signals directing tubular and ductal development (Drummond, 2004; Drummond et al., 2004; Kreidberg, 2008), to migrate into the glomerulus. However, pax2a alone is insufficient to specify the glomerulus. We demonstrate that the zebrafish embryo deploys ponzr1 to provide an additional layer of complexity beyond the regulatory gene pax2a, thus facilitating the formation of a glomerular pronephric kidney (Fig. 5). Therefore, we propose an addition to the current kidney development model: pax2a signals to downstream genes for the pronephric ducts but acts with ponzr1 in the zebrafish to develop additional filtration capabilities by the formation of a glomerulus (Fig. 9).

weightlifter is thought to antagonize pax2 function in kidney development (Ryan et al., 1995). ponzr1 morphants, however, display distinct effects on pax2a compared with weightlifter. ponzr1 morphants show altered pax2a expression; however, the overall expression of weightlifter appears largely unaffected. The net result – ectopic pax2a but normal weightlifter – appears to separate the interplay between pax2 and weightlifter in the zebrafish during kidney formation.

What is the cellular response to this separation between weightlifter and pax2 function? One hypothesis is that co-expression of pax2 and weightlifter in the developing glomerulus prevents the glomerular cells from differentiating properly and, therefore, from forming a functioning glomerulus. Within such a model, our data argue that the podocyte markers do not require this repression effect, but vascular invasion into the glomerulus does.

### Transcription factor ponzr1?

We have shown evidence that ponzr1 can act as a transcription factor or co-factor (Fig. 8). Can ponzr1 act as a DNA-binding protein? ponzr1 lacks any overt homology to known DNA-binding proteins. Alternatively, ponzr1 could bind to a DNA-binding protein and interact with the transcription machinery. This complex would enable ponzr1 to function with established, conserved proteins to influence the development of the glomerulus.

### ponzr1 and pax2a and the kidney GRN model

GRNs help visualize and conceptualize the dynamic interplay of the numerous genes and regulatory sequences needed to make an organ (Davidson, 2010; Davidson and Erwin, 2006). Our simplified pax2a/ponzr1 model is easily incorporated into the GRN model of organ development and innovation. Using this hybrid model, ponzr1 would be a component of a pronephric-specific plug-in that interacts with and regulates the pronephric kernel driven by pax2 and related, conserved regulatory genes. Using plug-ins that are lineage-specific or even species-specific provides another way to encode for diversity.

### ponzr1 as a pronephric gene

Studies in mammals as diverse as humans, rabbits and sheep have described the pronephros as ‘rudimentary’ (Moritz et al., 2008; Rouiller and Muller, 1969), and it is believed to be nonfunctional in these animals (Moritz et al., 2008; Rouiller and Muller, 1969). Furthermore, the most complete mammalian genomes, namely the human and mouse, do not yield any apparent ortholog to ponzr1 (supplementary material Fig. S1). However, the genome of an animal with a functional pronephros, *X. tropicalis*, does encode a putative ortholog (supplementary material Fig. S1). One intriguing question is whether ponzr1 is a zebrafish-specific gene or is needed for all vertebrates with a functioning pronephric glomerulus. Functional assessments of ponzr1 in diverse organisms like *X. tropicalis* will distinguish between these possibilities for ponzr1 function.

### Evolutionarily dynamic gene families

New in-depth sequencing of the genomes of diverse organisms and improved predictive and comparative bioinformatics approaches have facilitated the discovery of evolutionarily dynamic gene families (Boutet et al., 2010; Katsube et al., 2009) (this paper). How these families arose, their molecular functions and the biological capacities in which they serve have yet to be characterized. One mechanism for protein genesis includes de novo origination from non-coding sequences followed by subsequent gene duplication to give rise to a lineage-specific protein family (Cai et al., 2008; Knowles and McLysaght, 2009; Levine et al., 2006). Regardless of the molecular origin of these novel genes, however, examining a variety of model organisms at key points along the evolutionary timeline will be crucial to experimentally determine how these lineage-specific gene families operate in the context of well-studied ancient conserved pathways.
Do ancient pathways and lineage-specific genes lead to new structures? 

The data presented in this study represent functional evidence that members of evolutionarily dynamic gene families can provide an additional pathway to innovation. We find that one member of a dynamic family, *ponzr1*, is integrated into the ancient *pax2* pathway, facilitating innovation in the pronephros through the formation of the glomerulus. Whether other PonZr family members or additional dynamic gene families—only now being discovered through the extensive new comparative genomics projects—are responsible for diversity in additional organ systems is unknown. Do other lineage-specific genome additions function in ancient pathways to encode for innovation?

Acknowledgements

We thank Dr Englert for the Tg(+/+EGFP) fish line, Dr Drummond for the Tg(pol1a:egfp) fish line, Dr Pack for the Tglenpep4:GFP fish line, and Stephanie Westcott and Dr Keith Cheng for key manuscript revisions.

Funding

This work was funded by National Institute of General Medical Sciences (NIGMS) [GM63904]; National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) [YF3DK083219-01]; and National Institute on Drug Abuse (NIDA) [DA14546]. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.071720/-/DC1

References


