The enpp4 ectonucleotidase regulates kidney patterning signalling networks in *Xenopus* embryos

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The enpp ectonucleotidases regulate lipidic and purinergic signalling pathways by controlling the extracellular concentrations of purines and bioactive lipids. Although both pathways are key regulators of kidney physiology and linked to human renal pathologies, their roles during nephrogenesis remain poorly understood. We previously showed that the pronephros was a major site of enpp expression and now demonstrate an unsuspected role for the conserved vertebrate enpp4 protein during kidney formation in *Xenopus*. Enpp4 over-expression results in ectopic renal tissues and, on rare occasion, complete mini-duplication of the entire kidney. Enpp4 is required and sufficient for pronephric markers expression and regulates the expression of RA, Notch and Wnt pathway members. Enpp4 is a membrane protein that binds, without hydrolyzing, phosphatidylserine and its effects are mediated by the receptor s1pr5, although not via the generation of S1P. Finally, we propose a novel and non-catalytic mechanism by which lipidic signalling regulates nephrogenesis.
Vertebrate kidney organogenesis is orchestrated by numerous signalling pathways and transcription factors regulating the proliferation and differentiation of diverse cell types to form the functional kidney. Despite the differences in complexity and organization of the three vertebrate kidneys, pronephros, mesonephros and metanephros, there is a remarkable conservation of molecular mechanisms during their development\(^1\). The pronephros, the functional embryonic kidney in amphibians, is a simple, easily accessible organ, which displays structural similarities to the other more complex kidney forms. Therefore, it has become an ideal model system to study molecular regulation during nephrogenesis and renal pathologies\(^2\).

In mammals, lipidic and purinergic pathways regulate nephric physiology and their deregulation has been linked to acute renal injury and chronic kidney diseases including renal fibrosis polycystic kidney disease, renal cell carcinoma, nephritis or diabetic nephropathy\(^6\)–\(^8\). However, their potential roles during renal development have not been fully established, although the bioactive lipid sphingosine-1-phosphate (S1P) has been implicated during kidney branching\(^1\). Purinergic, mostly ATP and its derivatives, and bioactive lipids, S1P and lysophosphatidic acid (LPA), can function as extracellular ligands for G protein-coupled cell surface receptors\(^12\),\(^13\). Their availability for these receptors, in the extracellular space, is regulated by the activities of several membrane-bound enzymes, such as the ectonucleotidases, which are major regulators of renal health and disease\(^10\),\(^14\). The enpp (ectophosphodiesterase/nucleotide phosphohydrolase) proteins, which belong to the ectonucleotidase subfamily, are key regulators of both purinergic and lipidic signalling pathways with their dual enzymatic activities of hydrolysing purines and generating S1P and LPA bioactive lipids\(^15\). We have demonstrated that the pronephros is the major site of expression for the amphibian enpp genes family, in particular, enpp4 is highly expressed in Xenopus laevis pronephric tubules\(^16\). These data provided the first temporal and spatial embryonic expression profile for this evolutionally conserved enzyme which remains functionally poorly understood\(^17\)–\(^19\). In the present study, we investigated the function of enpp4 during pronephric development.

We demonstrate that Enpp4 function is crucial during kidney formation. While its knock-down leads to kidney formation defects, the overexpression of wild-type Enpp4, but not an inactive enzymatic protein, induces the formation of ectopic pronephros characterized mostly by the presence of proximal tubule markers but in rare occasion of more distal tubule markers. These effects are mediated by the lipidic receptor S1pr5 and we also show that Enpp4 specifically binds to phosphatidylycerine, implying a role for bioactive lipids in pronephrogenesis. Finally, we provide evidence that enpp4 misexpression alters the expression of members of the Notch, Wnt and RA signalling pathways and we propose a model for the mechanisms of action for Enpp4 and lipidic signalling in kidney development.

**Results**

**Overexpression of Enpp4 results in ectopic pronephric tubules formation.** To analyse potential functional roles of Enpp4 during pronephros development, we first undertook a gain of function approach by performing immunostaining with pronephric tubules specific antibodies\(^20\) on stage 41 embryos (Fig. 1a–o, and Supplementary Table 1 for raw data and statistical analyses). Enpp4 overexpression altered proximal pronephric tubules formation, in nearly 50% of the analysed embryos and induced ectopic (23%) and enlarged (18%) regions of the 3G8 staining domain (n = 91, Fig. 1b–e, Supplementary Table 1). Distal tubules were less affected, with 31% of the analysed embryos displaying abnormal 4A6 staining. Ectopic 4A6 staining was rare (2%), with enlarged more distal tubule staining being the predominant phenotype (20%). Enpp4-induced phenotypes are significantly different compared to those of LacZ controls (3G8: p < 0.001 and 4A6: p < 0.05). Ectopic pronephroi were observed only when injections were performed into regions fated to become the lateral region of embryos (V2 blastomere) (Supplementary Fig. 1a). Enpp4 overexpressing embryos displaying ectopic 3G8 staining (n = 5) were analysed by transverse section. Nine of the eight ectopic tubules sectioned had epithelial tubule structure complete with a lumen (Fig. 1f–j), which were similar to normal pronephric tubule structure (Fig. 1k, l). Similar significant renal phenotypes were observed following mouse Enpp4 mRNA injection (p < 0.001, n = 63; Fig. 1m). However, no ectopic pronephric tubules were observed upon overexpression of Enpp4 mutant constructs containing a point mutation in the putative catalytic domain (T72A, T72S) or metal cation binding domain (D36N, D189N) (Fig. 1n, o)\(^16\). These data suggest that ectopic proximal pronephric tissues formation caused by Enpp4 overexpression depends on its catalytic activity.

**Overexpression of Enpp4 disturbs proximal-distal patterning of pronephros.** To further investigate this phenotype, embryos injected with enpp4 mRNA were examined at stage 37 by whole-mount in situ hybridization using pronephric specific markers, slc5a1.1, slc12a1, clcnkb and gata3, which mark the various proximal/distal tubule segments\(^21\),\(^22\) (Fig. 1a and p–s, see also Supplementary Table 1). Interestingly, Enpp4 overexpressing embryos showed ectopic and enlarged staining of slc5a1.1 (proximal tubule marker, ectopic 30%, enlarged 14%; n = 57, p < 0.001) and slc12a1 (marker of intermediate tubules, ectopic 17%, enlarged 25%; n = 57, p < 0.001) domains (Fig. 1p–q). Injection of enpp4 mRNA failed to induce any separate ectopic clcnkb expression although the normal domain of expression (intermediate and distal tubule) was somewhat enlarged on the injected side (19%, n = 58, p < 0.01; Fig. 1r). The gata3 expression domain (distal and collecting tubules) was relatively normal, although its anterior limit of expression, determined relative to the somite number, was slightly more posterior in more than half of the injected embryos (58%, n = 43, p < 0.001; Fig. 1s). This might reflect a change in anterior/posterior patterning induced by Enpp4 overexpression.

Injection of enpp4 mRNA induced enlarged and reduced expression domains of both glomus marker wt1 and nphs1 at stage 33/34 but ectopic glomus staining was only observed in rare cases (Fig. 1t–u; Supplementary Table 1a). Although the statistical significance of these phenotypes was demonstrated (Supplementary Table 1b), we were not able to conclude on the exact Enpp4 effects on this structure.

Taken together, the results demonstrate that enpp4 mRNA injection altered pronephros formation, leading to enlarged expression domains of markers of the entire tubule segments and to ectopic pronephric structures containing mostly domains of proximal and, in rare occasions, distal tubules marker genes.

**Overexpression of Enpp4 upregulates early kidney markers expression without altering mesoderm formation.** Embryos injected with enpp4 mRNA were also examined by whole-mount in situ hybridization using early pronephros anlagen markers irx1, lhx1, pax8\(^23\),\(^24\) and compared to lacZ mRNA injected embryos (see Supplementary Table 1). At stage 28, expression of both lhx1 (61%, n = 51) and pax8 (70%, n = 50) was significantly (p < 0.001) expanded especially in posterior parts of pronephric anlagen, with areas of intense staining consistent with that of the more anterior presumptive tubules (Fig. 1v–w). At early neurula...
stages (Fig. 1x–y), the expansion of pax8 (17%, n = 70) expression domain was also observed (Fig. 1y). Furthermore, ectopic pax8 expression was also induced following enpp4 RNA injection at both stages analysed (neurula stage, 21%; stage 28, 2%; p < 0.001; Supplementary Table 1b). Lhx1 expression was also altered at early neural stages, but no ectopic lhx1 expression was observed (Fig. 1x, Supplementary Table 1b). Furthermore, irx1 expression domain was not altered following Enpp4 overexpression (Supplementary Fig. 1b).

Since normal somite development is a prerequisite for pronephros development, enpp4 mRNA injection was analysed by whole-mount in situ hybridization using the muscle marker myh4 at stage 33/34. The expression pattern was normal in all injected embryos (n = 55; Supplementary Fig. 1c; Supplementary Table 1). Enpp4 overexpression did not also alter the expression of pan-mesoderm marker, xbra at stage 10.5 (n = 46; Supplementary Fig. 1d and Supplementary Table 1). Therefore, we conclude that, enpp4 mRNA injection had no gross effects on mesoderm induction per se or on somite development.

**Morpholino knock-down of enpp4 results in smaller pronephros formation.** To determine whether Enpp4 is required for normal pronephros development, we have undertaken a loss of function approach using two specific anti-sense morpholino oligonucleotides (MOs) (Fig. 2, Supplementary Fig. 2a–c for specificity and efficiency of the MOs and Supplementary Table 2). The overall morphology of the embryos appeared normal in enpp4 MO1 injected embryos (myh4 100%, n = 47 and xbra 84%, n = 19; Supplementary Fig. 2d, e) suggesting that
14. The embryo shown in (uninjected side of each embryo. Blank arrowheads in (statistical analyses and Supplementary Figs. 2 and 3. Not statistically significant (\( p > 0.05 \)).

\[ n = 33/34 \text{ (wt1 expression domain were reduced after } enpp4 \text{ MO1 injection (53.5%, } n = 46; \text{ MO2 64%, } n = 46; \text{ MO2 64%, } p < 0.001; \text{ see also Supplementary Table 2). At stage 24, the expression domain of } lhx1 \text{ was clearly reduced (75%, } n = 20; \text{ Fig. 2i), suggesting involvement of } Enpp4 \text{ in early pronephros differentiation. Injection of } enpp4 \text{ MO2 caused similar phenotypes at both stages (stage 28, 30%, } n = 20; \text{ stage 24, 55%, } n = 20; \text{ Supplementary Fig. 2k–l). At early neurula stages, expression of } lhx1 \text{ was also reduced following } enpp4 \text{ MO1 or MO2 injection (MO1 37%, } n = 46; \text{ MO2 64%, } n = 61; \text{ } p < 0.001; \text{ see also Supplementary Table 2) and even absent after MO1 injection (MO1 31%, } n = 46; \text{ Fig. 2m and Supplementary Fig. 2m). The injection of both MO’s resulted in a stronger reduction of } lhx1 \text{ expression ( } n = 31; \text{ Fig. 2n). A reduction of } pax8 \text{ expression was also observed at the neurula stages following } enpp4 \text{ MO1 or MO2 injection (Fig. 2o and Supplementary Fig. 2n).}

These results suggest that enpp4 knock-down affected pronephric tubule, especially proximal and intermediate segments, differentiation rather than just the proximal-distal patterning of pronephric tubule segmentation.

To address potential Enpp roles during early phases of pronephros development, we tested by RT-PCR its expression at key stages during kidney development in dissected developing pronephric tissues (Fig. 3a). At later stages, enpp4 expression profile is in agreement with our published in situ hybridization data \cite{16}. However, weak expression is also detected in the embryonic kidney from stage 12.5 and is upregulated by stage 26. These data suggest that Enpp4 might be involved during early pronephric developmental phases. The expression domain of lhx1 and pax8 was altered following knock-down of enpp4 by MO injection (see Supplementary Table 2 for raw data and statistical analyses). At stage 28, expression of lhx1 was reduced especially in posterior elements of the pronephric anlagen (35%, \( n = 23; \text{ Fig. 2k), although its expression in presumptive proximal tubules was sometimes unaffected or expanded. At stage 24, the expression domain of lhx1 was clearly reduced (75%, } n = 20, \text{ Fig. 2i), suggesting involvement of Enpp4 in early pronephros differentiation. Injection of } enpp4 \text{ MO2 caused similar phenotypes at both stages (stage 28, 30%, } n = 20; \text{ stage 24, 55%, } n = 20; \text{ Supplementary Fig. 2k–l). At early neurula stages, expression of } lhx1 \text{ was also reduced following } enpp4 \text{ MO1 or MO2 injection (MO1 37%, } n = 46; \text{ MO2 64%, } n = 61; \text{ } p < 0.001; \text{ see also Supplementary Table 2) and even absent after MO1 injection (MO1 31%, } n = 46; \text{ Fig. 2m and Supplementary Fig. 2m). The injection of both MO’s resulted in a stronger reduction of } lhx1 \text{ expression ( } n = 31; \text{ Fig. 2n). A reduction of } pax8 \text{ expression was also observed at the neurula stages following } enpp4 \text{ MO1 or MO2 injection (Fig. 2o and Supplementary Fig. 2n).}

\[ n = 23; \text{ Fig. 2k) and was worsened when both MO were injected together (Fig. 2c). These results indicate that Enpp4 knock-down affected pronephric tubule formation.}

\[ n \text{ (wt1 expression on pronephric development (Fig. 2f and Supplementary Fig. 2k). At stage 33/34, lhx1 (k–n) at stages 28, 24 and 14, pax8 (o) at stage 14. The embryo shown in (f) was coinjected with 2 ng of mouse Enpp4 mRNA to rescue enpp4 knock-down phenotype. The asterisk denotes the control, uninjected side of each embryo. Blank arrowheads in (h) indicate the anterior limit of gata3 expression. See also Supplementary Table 2 for raw data and statistical analyses and Supplementary Figs. 2 and 3.}

An injection of enpp4 MO1 resulted in a significant reduction of expression of both 3G8 (65%, \( p < 0.001 \)) and 4A6 (28%, \( p < 0.05 \)) (\( n = 107; \text{ Fig. 2a, b; Supplementary Table 2), indicating that a smaller pronephros had formed. A similar phenotype was observed following enpp4 MO2 injection (3G8 49%, 4A6 24%, \( n = 87; \text{ Supplementary Fig. 2f, g) and was worsened when both MO were injected together (Fig. 2c). These results indicate that Enpp4 is required for both proximal and distal pronephric tubule development.}

Enpp4 knock-down in embryos showed a significant reduced expression of slc5a1.1 (58%, \( n = 64, p < 0.001 \)) and slc12a1 (56%, \( n = 75, p < 0.01 \)) with MO1 (Fig. 2d, e) as well as with MO2 (Supplementary Fig. 2h, i). Rescue experiments performed by coinjecting mouse Enpp4 mRNA (2 ng) with enpp4 MO1 or MO2 (10 ng each, \( n = 72 \) or 28, respectively) restore partially but significantly (\( p < 0.01 \)) the normal phenotype of slc12a1 staining domain confirming the specificity of the knock-down of enpp4 expression on pronephric development (Fig. 2f and Supplementary Fig. 2i). Ectopic slc12a1 expression was also observed in some embryos (11% and 50% with MO1 or MO2, respectively) consistent with Enpp4 overexpression phenotype. The clcnkb expression in the intermediate tubules and gata3 anterior expression domain were reduced after enpp4 MO1 (53.5%, \( n = 40 \) and 31%, \( n = 42 \), respectively) but these differences are not statistically significant (\( p > 0.05 \)) (Fig. 2g, h). Enpp4 knock-down does not affect somitogenesis, as the expression of wt1 and nphs1 was normal in most of enpp4 MO1 injected embryos at stage 33/34 (wt1 = 94%, \( n = 34 \) and nphs1 = 83%, \( n = 41 \)) (Fig. 2i, j).
Taken together, the Enpp4 knock-down and rescue experiments demonstrate that normal levels of \textit{enpp4} expression are required for normal pronephric development. \textit{Enpp4} misexpression phenotypes are distinct from those following \textit{enpp6} misexpression. To address if the ectonucleotidase \textit{Enpp6}, also expressed in the proximal pronephric tubules\textsuperscript{16}, can compensate for \textit{enpp4} loss of function, we performed single or double \textit{enpp4/enpp6} knock-down and rescue experiments (Supplementary Fig. 3 and Supplementary Table 2). \textit{Enpp6} depletion induced the formation of a smaller pronephros on the injected side, in the similar frequency than \textit{enpp4} knock-down (Supplementary Fig. 3b, c). The co-injection of \textit{enpp4} MO2 and \textit{enpp6} MO resulted in the formation of reduced 3G8 (64\%, \(n = 76\)) and 4A6 (62\%, \(n = 76\)) positive tissues and was not statistically different from the effects of \textit{enpp4} MO2, demonstrating that the \textit{enpp6} MO did not worsen the renal phenotype caused by \textit{enpp4} knock-down (Supplementary Fig. 3a). Pronephric formation was altered following \textit{Enpp6} overexpression, with reduced or absent, but never ectopic, pronephros observed and injection of \textit{enpp6} mRNA did not rescue the \textit{enpp4} MO2 phenotype (Supplementary Fig. 3d, e). Taken together, these data suggest that there is no functional redundancy between \textit{Enpp4} and \textit{Enpp6} ectonucleotidases.

Fig. 3 The \textit{enpp} and the lipidic receptors, the \textit{lpar} and \textit{s1pr}, gene family members are expressed in the pronephros. Developing pronephric anlagen or pronephric tubules were dissected as indicated, from whole \textit{X. laevis} embryos and total RNA extracted. RT-PCR was performed on pronephric dissected tissues and control whole embryos along with negative and linearity controls. a Comparative expression pattern of the \textit{enpp} genes and pronephric and muscle marker genes controlling the quality of the dissections. b Comparative expression profile of the \textit{lpa} receptors. c Comparative expression profile of the \textit{s1pr} genes.
Enpp4 misexpression affects expression of several components of the RA, Notch and Wnt signalling pathways. Since retinoic acid (RA), Notch and Wnt signalling pathways are involved in pronephros formation and patterning and the timing of the endogenous expression of many components of these pathways overlap, we hypothesized that Enpp4 might affect these pathways.

We therefore examined embryos injected with enpp4 mRNA (Fig. 4a–g) or enpp4 MO1 (Fig. 4h–n) by in situ hybridization for alterations in expression domain of representative members e.g. ligands (Dll1, Jag1, Wnt4) receptors (Notch1) and metabolic enzymes (Raldh1a2, Rdh10, Cyp26a1) of these three pathways (see also Supplementary Table 3).

Enpp4 overexpression induced ectopic and enlarged raldh1a2 and rdh10 expression domains in the pronephric region (p < 0.001, n = 37 and 34, respectively, Fig. 4a, b and Supplementary Table 3). Enpp4 knock-down reduced their expression in pronephric region of ~20% of analysed embryos but this phenotype is not significant (p > 0.05, n = 33 for each probe, Fig. 4h, i and Supplementary Table 3). Raldh1a2 expression was unaffected in the pharyngeal arches. In contrast, cyp26a1 expression was normal in the pronephric region following enpp4 mRNA injected embryos showed dl11 reduced expression (33%, n = 46, Fig. 4l). Ectopic (20%) and enlarged (44%) jag1 expression domains were observed following Enpp4 overexpression (n = 81, p < 0.001, Fig. 4d), while MO1 injection reduced its expression domain (in 38% of the analysed embryos, n = 42, Fig. 4m). These results suggest that Enpp4 also regulates members of the Notch signalling pathway and that jag1 expression is more affected by enpp4 depletion than dl11 expression. Since Rnf4 overexpression caused ectopic pronephric formation, we further addressed the link between Enpp4 and the Notch pathway by injecting enpp4 mRNA or MO2 in presence of rnf4 mRNA or MO. Our data show that modulation of notch-ligand interactions by Fringe proteins alters Enpp4 pronephric phenotypes, although differences are not significant (Supplementary Fig. 5 and Supplementary Table 3).

Finally, enpp4 mRNA injected embryos showed enlarged (32%) and ectopic (17%) wnt4 expression domains (n = 41, Fig. 4g) while expression of wnt4 was reduced in most of the enpp4 MO1 injected embryos (82%, n = 39, p < 0.001 Fig. 4n, Supplementary Table 3). These data suggest that Enpp4 is necessary and sufficient for promoting pronephric wnt4 expression.

Xenopus Enpp4 is localized to the plasma membrane. To address the cellular localization of the amphibian Enpp4 protein, we generated a specific polyclonal antibody against the full-length Xenopus protein (see Supplementary Table 4 for specificity evaluation of the antibody) and expressed Xenopus wild type (WT), T72S mutant and mouse Enpp4 cDNA in CHO cells by transient transfection. Xenopus Enpp4 WT protein was detected, by western blotting, in whole cells and in the membrane fractions, but not in the soluble fractions (Fig. 5a). Immunofluorescence experiments confirmed Enpp4 expression at the cell membrane (Fig. 5b). These results show that Xenopus Enpp4 is a transmembrane protein, as its mouse ortholog. Unfortunately, we failed to detect the endogenous Enpp4 expression in Xenopus embryos using the anti-XlEnpp4 antibody.
Phospholipid receptors are expressed in the developing pronephros along with the enpp4 gene. Based on sequence homology of Xenopus Enpp family, Enpp4 is more related to lipid-hydrolysing Enpp6 and 7 enzymes. We therefore hypothesized that the roles of transmembrane-bound Enpp4 during pronephrogenesis might be linked to the lipidic signalling pathway. To test if phospholipid receptors might mediate Enpp4 functions, we established the expression profiles of lpar and slp receptor family members previously identified by RT-PCR in kidney dissected tissues (Fig. 3b, c). All lpar receptors, except lpar3 and 5, are expressed in pronephric tissues at a similar level from the time of kidney specification to late differentiation, confirming their ubiquitous expression profile during Xenopus embryogenesis. The slp receptors display different expression profiles, with slp355 being the only family member to be expressed in the developing kidney at every stage analysed, particularly in the presumptive pronephric tissue at stage 12.5. No such renal expression was detected by in situ hybridization in our previous study, although expression in marginal zone of blastula embryos was detected by RT-PCR. These data suggest that the pronephric level of expression of these lipidic receptors, especially s1pr5, is relatively low, under the in situ hybridization detection level.

Overexpression of s1pr5 enhances Enpp4 function to induce ectopic pronephros. In order to identify whether a lipidic receptor is involved in Enpp4 phenotypes, s1pr and lpar over-expression analyses were carried out by injecting 2 ng of s1pr5, s1pr1, lpa1.1 and p2y10 mRNAs alone or in combination with 1 ng of enpp4 mRNA alone (Fig. 6a–d; Supplementary Fig. 6a–f; Supplementary Table 5) i.e half of the optimal dose to generate ectopic pronephros, see Fig. 1). At 1 ng enpp4 mRNA dose ectopic 3G8 (7%) and 4A6 (5%) staining were obtained only in rare cases (n = 94, Fig. 6d) compared to the optimized dose of enpp4 mRNA used in Fig. 1. Injection of any tested lipidic receptor mRNA alone does not induce any ectopic kidney formation with normal 3G8 and 4A6 staining in the majority of the embryos (n = 52, Fig. 6c, Fig. 5b, d, f). Only co-expression of s1pr5 and enpp4 mRNAs resulted significantly in higher ectopic 3G8 staining compared to enpp4 mRNA alone (38%, n = 89, p < 0.001, Fig. 6a, b, Supplementary Fig. 6a, c, e and Supplementary Table 5). Furthermore, the size and frequency of the ectopic pronephroi were higher than with injections of 2 ng of enpp4 alone (see Fig. 1b–e). These results indicate that, among the tested receptors, only S1pr5 enhanced Enpp4 function to generate ectopic pronephros.

The functions of Enpp4 are mediated by the lipidic S1pr5 receptor. To further confirm that S1pr5 is involved in pronephros development we performed loss of function experiments. Two s1pr5 genes are identified in X.laevis genome and s1pr5.L corresponds to our published sequence32. Despite distinct spatial expression in the adult frog, the two s1pr5 homeologs display a very similar expression profile during X.laevis embryogenesis and are both expressed in the pronephric tissues (Supplementary Fig. 7a, b). We therefore performed loss of function analyses with an anti-sense MO against Xenopus s1pr5.L and s1pr5.S (see Supplementary Figs. 6g, h and 7c, d for MOs efficiency and specificity evaluation and Supplementary Table 5 for raw data and statistical analyses). Embryos injected with 15 ng of s1pr5.L MO or s1pr5.S MO displayed significant reduced 3G8 and 4A6 staining (n = 43 and 66, respectively, Fig. 6e, f, Supplementary Fig. 7e and Supplementary Table 5) suggesting that S1pr5 receptors are required for normal pronephros formation in Xenopus.

To examine potential synergistic effects, we co-injected 7.5 ng of s1pr5.L MO with 5 ng of enpp4 MO (half of the dose used previously for single injections) and compared their phenotype to those obtained following co-injection of s1pr5 MO or enpp4 MO with control MO (Fig. 6g–i). As expected, embryos co-injected with s1pr5.L and enpp4 MOs generated the strongest phenotype and smallest pronephros, with strong reduction of 3G8 (74%) and 4A6 (81%) staining domains (n = 42, Fig. 6g, h). Enpp4 MO alone also caused strongly reduced 3G8 (65%) and 4A6 (60%) staining as previously shown (n = 40, Fig. 6i), while the s1pr5.L MO alone reduced pronephric size in both 3G8 (22%) and 4A6 (29%) domains less frequently (n = 51, Fig. 6i). Although there are no significant differences in pronephric phenotype between enpp4 MO1 + s1pr5.L MOs and enpp4 MO1 injected embryos, we concluded that co-injection of s1pr5.L and enpp4 MOs showed additive effects on the inhibition of Xenopus pronephros development based on the size of the scored pronephroi.

To further analyse the link between Enpp4 and S1pr5, we carried out injection of 2 ng of enpp4 mRNA together with 15 ng of s1pr5.L MO (Fig. 6k–l) or control MO. As expected, injection of s1pr5.L MO lowered the percentage of embryos displaying...
Enpp4 specifically interacts with the lysophospholipid phosphatidylserine. To assess if Enpp4 hydrolyses lipids and generates a ligand, which could bind to the S1pr5 receptor, phospholipid binding was tested by a protein-lipid overlay assay using commercial pre-spotted lipid membranes. Out of the 26 bioactive lipids tested, only phosphatidylserine (PS) is specifically bound by Xenopus Enpp4 (Fig. 7a, b and Supplementary Fig. 8). Moreover, this interaction is abolished when the putative catalytic site is mutated (Fig. 7c). We then tried to determine the enzymatic activity of Xenopus Enpp4. However, we could not detect any lipid derivatives, e.g. DAG, PA or LysoPS, which could be generated from the hydrolysis of PS in the membrane proteins fractions from overexpressing Enpp4 CHO cells. Taken together, these data show Enpp4 specifically interacts with PS but does not have PLA, PLC or PLD activity towards PS.

**Discussion**

This paper reports newly identified and unexpected roles of the conserved ectonucleotidase Enpp4 during vertebrate kidney development. Moreover, our findings provide a novel molecular mechanistic understanding for pronephric development and emphasizes the importance of the lipidic pathways in kidney formation (Fig. 8).

We previously showed that enpp4 is expressed in pronephric tubules16, but our present data demonstrate that low but significant levels of enpp4 can be detected at the time of proximal tubule specification.35 Weak enpp4 expression was previously detected by RT-PCR but not by ISH, in gastrula embryo16. This discrepancy is attributable to the lower sensitivity of ISH compared to RT-PCR for the detection of gene expression patterns.

Our work demonstrates that Enpp4 regulates the expression level of two of the transcription factors involved in pronephric anlagen formation, lhx1 and pax823 but not irx124. However, induction of lhx1 ectopic expression is delayed compared to pax8 one. Such a distinct expression regulation of these two pronephric genes by several signalling pathways has already been described34,35. As Lhx1 is necessary for the early patterning of the
entire kidney and subsequently growth and elongation in the development of the pronephric tubules\textsuperscript{36,37}, the reduction of \textit{lhx1} expression can explain the formation of the small pronephros in \textit{enpp4} morphants. Pax8 is necessary for the earliest steps of pronephric development and for pronephric precursors cell proliferation and can induce the formation of ectopic pronephric tubules\textsuperscript{23,38}. Therefore, the ectopic expression of \textit{pax8} in Enpp4 overexpressing embryos can explain the formation of ectopic pronephroi. We also demonstrate that Enpp4 is sufficient to generate kidney, but only from lateral mesoderm and not in ectopic non-lateral positions. This suggests that the lateral mesoderm must contain either the receptor or the substrate necessary for Enpp4 function. Furthermore, the induction of \textit{enpp4} expression in activin treated animal caps confirms the importance of mesoderm tissues for Enpp4 pronephric functions (see Supplementary Fig. 4).

The ectopic kidneys formed from \textit{enpp4} overexpression consist of tubular structures and are patterned along their proximal/distal axis. Moreover, in some rare cases, there is a complete mini-duplication of the entire pronephros. This surprising phenotype could be explained by the upregulation of the patterning of signalling pathway members. RA signalling is required during gastrulation for pronephric specification. Increased levels of RA signalling by Enpp4 overexpression could lead to \textit{pax8} expression activation and then to the formation of ectopic pronephroi\textsuperscript{23,25}. RA signalling is also required post-gastrulation for tubules morphogenesis and its downregulation in \textit{enpp4} morphants could explain tubules formation defects\textsuperscript{25}. Subsequently, RA increase could pattern these ectopic tubules, as it has been shown during zebrafish pronephric nephron segmentation patterning\textsuperscript{29,40}. However, in \textit{Xenopus} pronephros, RA signalling increases expression level of distal tubules markers\textsuperscript{41}. Moreover, RA signalling also regulates the expression of members of the Notch pathway\textsuperscript{42}, which can subsequently activate \textit{wnt4} expression\textsuperscript{28}, which then functions to pattern the proximal pronephros. Our data demonstrate that Rnfg protein is involved in mediating Enpp4 signalling, probably by its ability to modify Notch-ligand interactions\textsuperscript{43}. Therefore, we speculate that Enpp4 acts upstream or in parallel to RA signalling and upstream of Notch and Wnt pathways (see Fig. 8). As \textit{enpp4} expression was unchanged in animal caps treated with RA compared to control caps, this supports the hypothesis that Enpp4 acts upstream of the RA pathway.

A key question is how the misexpression of Enpp4, an ectonucleotidase, can alter gene expression. Phosphatidylserine translocation across the cell membrane is a well-known indicator of apoptosis but is also involved in physiological and developmental processes\textsuperscript{44,45}. Therefore, Enpp4 could bind to PS in the extracellular space during pronephrogenesis. Enpp4 enzymatic activity is essential for ectopic kidney formation, suggesting that renal alterations are due to an excess or shortage of Enpp4 generated products in the extracellular space inducing cell responses via the activation of the S1pr5 receptor. However, our data strongly suggest that the Enpp4 kidney phenotype is not linked to the biochemical lipids LPA or S1P. The fact that the observed kidney phenotype might be due to a non-catalytic effect of Enpp4 might be puzzling and unexpected, especially since Enpp6 has been suggested to play major renal physiological role through its enzymatic functions\textsuperscript{46}. However, specific functions of other Enps, such as Enpp1, Enpp2 and Enpp5, have been shown to be independent of their enzymatic activity\textsuperscript{47–50}. It is therefore possible that Enpp4 does not hydrolyse PS but its interaction with PS is necessary for the activation of Enpp4 and subsequently of S1pr5. PS binding and conformational change mechanisms have been demonstrated for protein kinase C activation in mammalian kidney cells, supporting this hypothesis\textsuperscript{51}. Although we were unable to detect any of the predicted products of PS hydrolysis, we cannot rule out that we failed to characterize Enpp4 enzymatic
activity and that Enpp4 will generate a bioactive lipid, other than S1P, which is able to bind to S1P5r, the most divergent member of the S1P4r family. Although signalling through S1P5r has been poorly studied, the activation of the S1P5r receptor has been linked to an intracellular calcium increase and inactivation of the ERK pathway, both pathways regulating proenkephalin expression via RA signalling.

We show that mouse Enpp4 can fulfill Xenopus Enpp4 functions during proenkephalinogenesis, suggesting mammalian kidney formation may be regulated by a similar mechanism demonstrated in this work. ENPP4 is highly expressed in human metanephros and kidney tumours and its expression increases in deceased donor kidney biopsies with delayed graft function after transplantation (data from human protein atlas) 56. Interestingly, ENPP4 is localized close to RUNX2 gene, whose mutations cause cleidocranial dysplasia (CCD) 57,58. Furthermore, a child with CCD and crossed renal ectopia has been reported, and given our data, we can speculate that the ectopic kidney is attributable to ENPP4 locus alterations 59.

We propose a potentially novel model of action of the lipidic pathway in kidney physiology, implicating other bioactive lipids distinct from LPA and S1P molecules or a novel non-catalytic interaction. The fact that a S1P receptor might be activated other than by S1P binding may explain the controversy regarding the beneficial actions of FTY720 in renal pathologies. Moreover, our study raises potentially fascinating possibilities regarding regenerative therapies for renal diseases. As therapies for chronic renal failure are still lacking, the identification of a novel pathway enabling the generation of ectopic kidneys may provide useful insights to therapists that enhance human renal regeneration.

Methods

Ethics statement. The work was carried out under a UK Home Office-approved animal procedures project license and approved by the University of Warwick Biological Ethics Committee.

Enpp4 cloning and site-directed mutagenesis. The Xenopus enpp4 cDNA (Accession number: BC 079717) was cloned into pcDNA3.1. Mouse Enpp4 cDNA (Accession number: BC 077249) was cloned into pCS2+ and pCDNA3.1. Site-directed mutagenesis of enpp4 was performed using a PCR-based approach. For each mutant, 2 successive rounds of PCR were carried out using the Pfx polymerase (Invitrogen) following the manufacturer’s protocol and using the primers listed below. The first round of PCR, performed using the enpp4-pRNA3 plasmid as template, allowed the amplification of two fragments of the enpp4 coding region, one upstream and containing the desired mutation (underscored in the primer sequence) and the other downstream and containing the mutation, respectively. For this, one amplification was performed using the upstream primer carrying out the mutation and the primer ORF downstream containing the stop codon and the other amplification using the downstream primer carrying out the mutation and the primer ORF upstream containing the ATG codon. The two PCR products were then mixed and a third PCR was carried out using this mixture as template using the upstream and downstream ORF primers, carrying out the BamHI and EcoRI restriction sites, respectively (in italic in the primer sequence). The final PCR product was digested by BamHI and EcoRI and inserted into the pCS2+ vector. The presence of the correct mutation was confirmed by sequencing. For each mutant the enpp4 cDNA was then extracted from the pCS2+ and cloned into the pcDNA3.1. All constructs were verified by sequencing.

mRNA synthesis and morpholino oligonucleotides. Capped mRNAs were synthesized using mMESSAGE mMACHINE Kits (Ambion) from linearized plasmids. Plasmids used were Xenopus enpp4-pRNA3 (clone BC 079717); mouse Enpp4-pCS2+; Xenopus mutant enpp4-pCS2+; Xenopus s1p5r-PC2+ (clone DGI 11014); Xenopus s1pr5-pCMV-Sport6 (clone BC 074356); Xenopus p2y10-pCMV-Sport6 (clone BC 084356) and Xenopus rnf-pCMV-Sport6 28, enpp4 MO1 (5′-gtgagattaacctgcggcaat-3′), enpp4 MO2 (5′-ggaaatgtcacacacgcagctcct-3′), enpp6 MO (5′-aaatgcggcgactgctgctgca-3′), s1pr5 MO (5′-cattctggctactgttcttttct-3′), s1pr5.5 MO (5′-cattctggctactgttcttttct-3′), rnf MO and standard control MO (5′-cctcctctcaacatcctat-3′) were designed and supplied by Genetools, LLC.

Embryo culture, dissection, microinjections and lineage staining. Xenopus embryos were staged according to Faber and Nieuwkoop 61. Kidney and pronephric anlagen dissections were performed in Barth X31. Each individual sample was injected into the lateral marginal zone of a ventral-vegetal blastomere (V2) at the 8-cell stage to target the pronephros. Experimenters were carried out to determine the enpp4 and lipidic receptors mRNAs and MOs quantities to inject, based on their abilities to alter kidney development without affecting the overall morphology of the embryos. The rnf mRNA and MO dose was used as previously published 28. The LacZ (250 pg) mRNA was used as a lineage tracer. LacZ mRNA was injected alone or in combination with standard MO as controls. Injected embryos were cultured to various developmental stages, fixed in MEME and stained for β-galactosidase activity (Red-Gal or X-Gal staining) to identify correctly targeted embryos. Only embryos that had normal pronephros formation on the uninjected side and correctly targeted β-galactosidase staining on the injected side were scored.

Analysis of molecular marker expression in embryos. Whole-mount immunohistochemistry was performed using 5G8 and 4A6 monoclonal antibodies as previously described 28. Whole-mount in situ hybridization was carried out as previously described 42. Anti-sense digoxigenin (DIG)-labelled RNA probes were synthesized from linearized template plasmids 32,63. Either BM purple (Roche Applied Science) or NBT/BCIP (Roche Applied Science) or Fast Red/Naphthol AS/MX (Sigma) was used for the colour reaction. After bleaching, embryos were photographed with a magnification of×10 for whole stage 41 embryos,×20 for whole gastrula, neurula and early organogenesis stages embryos and×32 for pronephric region.

Acrylamide embedding, cryostat sectioning and Hoechst staining. X. laevis embryos were embedded in 18 μm thickness and nuclear Hoechst staining performed 44.

RT-PCR. RT-PCR reactions were carried out on whole or dissected X. laevis embryos as described previously using the housekeeping gene as loading control. Quality of pronephric tissues dissections was assessed by amplification of the kidney markers lhx1 and pas8 and of the muscle marker myf5. Amplification conditions and primers sequences for the enpp, lpar, s1pr, lhx1 and pas8 genes have been previously published 16,25,65. Myf5 was amplified using the forward primer, 5′-actaactcacggcaccagc-3′ and the reverse primer, 5′-agatcggagattaggcggacc-3′, with the annealing temperature of 60 °C and 29 cycles. Each sample was analyzed in two independent embryo batches.

Cell culturing and transient transfection. Chinese hamster ovary (CHO) cells were cultured in HAMs F-12 (Gibco BRL) containing 10% foetal serum, NaHCO3 (1.176 g/l, 2 mM of glutamine, 5 U/ml penicillin and 37.8 U/ml streptomycin) during the 48 h prior to transfection. Cells were then transfected for 24 h using the reagent TurboFect (Fermentas) with 1 μg of the eukaryotic constructs. The transfection medium was then removed and replaced with culture medium. Approximately 48 h post transfection, cells were fixed or harvested for analyses. As a control, CHO cells were transfected with empty vector pcDNA3.1.

Anti-Enpp4 antibody production. The anti-Xl Enpp4 polyclonal antibody was raised in rabbits by direct intramuscular injection of the Xenopus laevis wild-type enpp4-pCDNA3.1 plasmid followed by electroporation (Aldevron, LLC, USA). Rabbits were immunized three times, at day 0, day 28 and 56 and terminal bleed performed at day 70.

Immunocytochemistry. Immunocytochemistry was carried out on fixed unpermeabilized cells with polyclonal antibodies anti-Xi-Enpp4 used at 1/200 or anti-mEnpp4 (CB65; see ref. 13) used at 1/400 and anti-rabbit IgG Fc ITG (Sigma) at 1/80. The staining was recorded using a Nikon Optiphot/ Digital camera system. Photographs were taken at a magnification of ×40.

Electrophoresis and western blot. Native membrane proteins were extracted from transfected cells using the ProteoExtract® Native Membrane Protein Extraction Kit (Calbiochem). Proteins from whole cells and from the membrane and soluble fractions were separated on a 12% SDS-PAGE gel. Cell or plasma membranes were solubilized in 1% Triton X-100, 1% BSA in Tris-buffered saline 0.05% Tween 20 (TBST). All subsequent washes were performed in TBST. Blots were overlaid with proteins extracts from membrane fractions of CHO cells transfected with enpp4-pCDNA3.1 or
empty vector (dilution 1/30) in blocking buffer overnight at 4 °C. Membranes were washed and incubated with pre-absorbed Enpp4 serum (dilution 1/200) for 6 h at room temperature. After several washes, the membranes were incubated with goat anti-rabbit IgG peroxidase secondary antibody for 30 min at room temperature, washed and developed using enhanced chemiluminescence. To confirm the observed binding, nitrocellulose Hybond-C extra (GE Healthcare) membranes were spotted with 0 to 200 µM of PA (Sigma P-9511) or PS (Sigma P-6641) diluted into a mix of MeOH/CHCl3/H2O (2/1/0.8, v/v). Dried membranes were then treated as described above.

Statistics and reproducibility. All experiments were repeated several times, on different batches of embryos, and pronephric phenotypes were determined in a commonly used way, blind-coded, by comparing the injected and uninjected sides. The percentages of the embryos displaying the discussed phenotypes are given in the table in bracket along with the total number of analysed embryos. All raw data and statistical analyses are presented in the Supplementary Tables (SI). Each histological analysis was numbered (see Supplementary Tables 1a, 2a, 3a and 5a) and statistically pairwise compared as indicated in the Supplementary raw data and statistical analyses are presented in the Supplementary Tables (SI).

Data availability. The authors confirm all the data supporting the findings of this study are available in the Nature Research Reporting Summary linked to this article. All the statistical analyses were performed using the R statistical software Core Team R96.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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Author contributions

K.M. and E.A.J. designed research; K.M., E.A.J., S.B. and L.M.-P. performed research; K.M., E.A.J., C.P., E.B.-G. and L.M.-P. analysed data; K.M. and E.A.J. wrote the paper with contributions from all the authors.

Competing interests

The authors declare no competing interests.

Additional information

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