BMP and retinoic acid regulate anterior–posterior patterning of the non-axial mesoderm across the dorsal–ventral axis

Richard W. Naylor1, Lauren Brilli Skvarca2,3, Christine Thisse4, Bernard Thisse4, Neil A. Hukriede2,3 & Alan J. Davidson1

Despite the fundamental importance of patterning along the dorsal–ventral (DV) and anterior–posterior (AP) axes during embryogenesis, uncertainty exists in the orientation of these axes for the mesoderm. Here we examine the origin and formation of the zebrafish kidney, a ventrolateral mesoderm derivative, and show that AP patterning of the non-axial mesoderm occurs across the classic gastrula stage DV axis while DV patterning aligns along the animal–vegetal pole. We find that BMP signalling acts early to establish broad anterior and posterior territories in the non-axial mesoderm while retinoic acid (RA) functions later, but also across the classic DV axis. Our data support a model in which RA on the dorsal side of the embryo induces anterior kidney fates while posterior kidney progenitors are protected ventrally by the RA-catabolizing enzyme Cyp26a1. This work clarifies our understanding of vertebrate axis orientation and establishes a new paradigm for how the kidney and other mesodermal derivatives arise during embryogenesis.
The patterning of different cell types along the dorsal–ventral (DV), anterior–posterior (AP) and animal–vegetal axes determines the organization of the embryonic body plan. In zebrafish, the mesoderm arises from an equatorial marginal zone on the yolk cell. The formation of the shield on one side breaks the radial symmetry of the marginal zone and acts as a major signalling centre for patterning. Classically, the shield marks the dorsal side of the embryo with the DV axis running equatorially to the opposite (ventral) side. Pregastrula fate mapping has shown that while cells of the shield do indeed give rise to the axial mesoderm (prechordal plate and notochord), consistent with being dorsal derivatives, the arrangement of precursors in the remaining ventrolateral mesoderm is more consistent with being aligned with the AP, and not DV, axis of the post-gastrula embryo1–3. For instance, ventrolateral mesoderm generates much of the posterior tissues including the posterior lateral plate mesoderm, pronephros, blood, posterior somites and tail. While mesoderm towards the shield region gives rise to anterior tissues such as the head mesoderm, heart and anterior trunk somites3–5. As a result, there has been uncertainty as to whether the classic early gastrula DV axis corresponds to the actual DV axis or to the AP axis with regards to the mesoderm layout in post-gastrula embryos.

The zebrafish embryonic kidney (pronephros), comprised of two tubules that are segmented along the AP axis6,7, provides a useful tissue to help resolve the alignment of ventrolateral fates and axes. The pronephric tubules are subdivided into two anterior segments, the proximal convoluted tubule (PCT) and the proximal straight tube (PST), and two posterior segments, the distal early (DE) and distal late (DL) segments8. The pronephros arises from the intermediate mesoderm, which together with the posterior lateral plate mesoderm, forms the majority of the mesodermal tissue lateral to the trunk somites (a.k.a. the posterior lateral mesoderm (PLM)). All of this mesoderm ultimately descends from the ventrolateral mesoderm of the early gastrula5,5. Given this, it is likely that a better understanding of how the pronephros is patterned along the AP axis will give insights into how the non-axial mesoderm is aligned with respect to the classic gastrula DV axis.

Bone morphogenetic proteins (BMPs) are thought to be responsible for inducing and patterning mesodermal fates along the DV axis. Traditional models propose that a morphogen gradient of BMPs, established in part by the secretion of BMP antagonists from the shield, extends across the mesoderm with a gradient of BMPs, established in part by the secretion of BMP antagonists from the shield, extends across the mesoderm with a gradient of BMPs, established in part by the secretion of BMP antagonists from the shield. We demonstrate that AP patterning of the pronephros occurs in sequential phases, with BMPs first acting to establish broad anterior and posterior non-axial mesodermal territories. RA then acts downstream of BMPs, but RA is synthesized from vitamin A by the activities of alcohol dehydrogenases followed by aldehyde dehydrogenases, such as Aldh1a2, and is degraded by the Cyp26 family of p450 enzymes9. Together, Aldh1a2 and the Cyp26 enzymes are major regulators of RA availability10,11. In zebrafish, expression of aldhlta2 is initially found around the margin of the late blastula embryo (excluding the shield) but by late gastrulation, transcripts become highly expressed dorsally. It is around this stage that an RA gradient can first be detected emanating from the dorsal side of the embryo12. However, in the absence of a late gastrula fate map, the location of kidney progenitors relative to this RA source has been uncertain. Zebrafish embryos deficient in RA, either due to a mutation in aldhlta2 or by treating gastrula-stage embryos with diethylaminobenzaldehyde (DEAB), an inhibitor of aldehyde dehydrogenases, display a ‘posteriorized’ pronephros in which the distal segments are expanded at the expense of proximal fates7. Conversely, exogenous RA treatment dose-dependently ‘anteriorizes’ the pronephros. This effect is opposite to the established role of RA during neuroectoderm patterning where RA signalling aligned with the animal–vegetal pole promotes posterior identities12–15. Together, these findings suggest a model in which kidney progenitors arising from the mesoderm during gastrulation are patterned into different anterior and posterior fates in response to RA.

In this study, we analyse the patterning of the zebrafish pronephros and perform late gastrula fate-mapping experiments to show that the classic gastrula DV axis corresponds to the AP axis with regard to the non-axial mesoderm. By examining various mesoderm markers we show that the actual DV axis of the non-axial mesoderm during gastrulation is largely aligned with the animal–vegetal pole. We demonstrate that AP patterning of the pronephros occurs in sequential phases, with BMPs first acting to establish broad anterior and posterior non-axial mesodermal territories. RA then acts downstream of BMPs, but also across the classic gastrula DV axis, such that anterior kidney fates are induced ‘dorsally’ while posterior kidney progenitors are protected ‘ventrally’ by Cyp26a1.

### Results

RA induces anterior kidney fates during gastrulation. Prior studies showed that DEAB treatment from early gastrulation onwards results in a loss of proximal tubule formation (PCT and PST segments) and a concomitant increase in the size of the distal segments (DE and DL). We have since found that an even earlier onset of DEAB treatment, from the blastula stage (dome stage) onwards, results in a more severe phenotype with treated embryos lacking the PCT and PST segments (marked by slc4a4 expression), as well as the DE segment (marked by slc12a1 expression) and showing a greater expansion of the slc12a3+ DL segment (Fig. 1). By contrast, addition of DEAB or the RA receptor-β selective antagonist BMS189453 from the end of gastrulation (bud stage) onwards results in only mild AP-patterning defects (Fig. 1). On the basis of these findings we conclude that the formation of PCT, PST and DE segments are largely dependent on RA made during a pre-gastrula to late gastrula developmental window.

### Expression analysis defines different mesodermal territories

One of the challenges of studying how morphogens pattern the mesoderm has been a lack of markers that can distinguish the different mesodermal subpopulations that make up the late gastrula embryo. To help solve this, we identified the gene zulu (zebrafish-specific uncharacterized LOC100537766 of unknown function), discovered from an expression screen, as a late gastrula pan-mesodermal marker26 (Supplementary Fig. 1). At 85% epiboly, zulu is highly expressed dorsally. It is around this stage that an RA gradient can first be detected emanating from the dorsal side of the embryo27. In zebrafish, expression of aldhlta2 is initially found around the margin of the late blastula embryo (excluding the shield) but by late gastrulation, transcripts become highly expressed dorsally. It is around this stage that an RA gradient can first be detected emanating from the dorsal side of the embryo21. However, in the absence of a late gastrula fate map, the location of kidney progenitors relative to this RA source has
patterns of the markers *tbx6, tbx16, ntlα, pax2a, pax8, lbx2, twist1α* and *fsta* we found that the mesoderm can be subdivided into different domains at 85% epiboly (Fig. 2b). Dorsally, the non-axial mesoderm can be divided into an upper domain (herein referred to as the anterior lateral mesoderm) and a lower non-axial mesoderm can be divided into an upper domain into different domains at 85% epiboly (Fig. 2b). Dorsally, the anterior trunk paraxial mesoderm29. The upper domain was found to express *pax2a* and *twist1α* marking the upper PLM and *lbx2* transcripts on the ventral side are in the hypoblast layer (Fig. 2a). The expression patterns of *twist1α* and *lbx2* suggest that the PLM is further divided into upper (animal) and lower (vegetal) domains, with *twist1α* marking the upper PLM and *pax2a* and *lbx2* transcripts being restricted to the lower PLM (Fig. 2b). We found that *twist1α* and *lbx2* transcripts in the posterior of the embryo label non-overlapping domains at early somitogenesis stages, most likely corresponding to the posterior lateral plate mesoderm (*twist1α*) and the intermediate mesoderm (*lbx2*+) (Supplementary Fig. 2).

Within the PLM, the *cyp26a1* expression domain does not extend as laterally as the other markers expressed in this mesodermal subdivision (*zulu, pax2a* and *pax8*) but instead shows a more ventrally restricted pattern (Fig. 2a). Double *in situ* hybridizations revealed that expression of *cyp26a1* in the PLM included the lower *lbx2*+ domain (presumptive intermediate mesoderm; Supplementary Fig. 4A) while sagittal sections confirmed that *cyp26a1* transcripts on the ventral side are in the hypoblast layer (Supplementary Fig. 4B).

**Lineage labelling of RA-sensitive fates.** To more precisely determine the location of the kidney progenitors within the PLM, we performed lineage labelling by photo-activation of caged fluorescein dextran in 85% epiboly-stage embryos. To ensure the targeting was reproducible, we projected a 306-point Cartesian

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**Figure 1 | Inhibition of RA signalling affects pronephros segmentation during gastrulation.** Whole-mount double *in situ* hybridization analysis at 24 hours post fertilisation (h.p.f.) for nephron segment markers *slc4a4* (red), *slc12a1* and *slc12a3* (purple) treated with BMS189453, DEAB or DMSO (vehicle control). Embryos are shown as dorsal views with anterior to the left. White arrows indicate posterior end of *slc4a4*+ domain and black arrows indicate the boundary between the DE and DL segments. Scale bar, 100 μm.
grid onto each embryo (each point being 40 μm apart). We found that embryo size at this stage is largely constant (mean animal–vegetal pole distance was 664 μm, s.d. = 5.9 μm; n = 20). As the PLM is not morphologically discernible, we used the expression pattern of zulu to guide our targeting. We determined that the border between the upper zulu high PLM and the lower paraxial mesoderm domains is coincident with a horizontal axis running midway between the animal pole (defined by the anterior extent of the prechordal plate) and the margin, which corresponds closely to the y7 and y8 axes on our fate-mapping grid.

Groups of 10–20 cells were uncaged along the y7 and y8 axes at regulator coordinates across the dorsal to ventral sides of the embryo (Fig. 4a). A cluster of cells located at the (x6, y8) co-ordinate were found to give rise to anterior kidney progenitors (proximal tubule), based on co-labelling of the tracer with pax2a transcripts (n = 9; Fig. 4a), while (x3, y7) labelled more posterior kidney progenitors (distal tubule; n = 7; Fig. 4a, for transverse sections of these embryos see Supplementary Fig. 5). We also determined that gata1þ blood progenitors, tbx5aþ pectoral fin progenitors and nkx2.5þ heart progenitors, could be labelled at co-ordinates (x3, y5), (x8, y6) and (x10, y10), respectively (Fig. 4a). In addition, we confirmed that cells in the posterior trunk paraxial mesoderm domain (x5, y7), APM (x10, y5) and anterior lateral mesoderm (x11, y12) contributed to the posterior paraxial mesoderm, anterior somites and head mesoderm, respectively (Supplementary Fig. 6).

To relate the positions of the kidney progenitors to the mesodermal domains, we double-stained the embryos immediately after photo-activation for uncaged dextran and transcripts for aldh1a2, cyp26a1 and zulu (Fig. 4b). Both anterior and posterior kidney progenitors were localized within the zulu high domain. Anterior kidney progenitors localize closest to the aldh1a2 high dorsal domain, near the dorsalmost limit of the PLM, while the posterior kidney progenitors are located more ventrally within the subregion of the PLM that expresses cyp26a1 (Fig. 4b). Both anterior and posterior progenitors express low levels of aldh1a2, and consistent with this, double in situ hybridizations showed that pax2a transcripts initiate within the weaker, more lateral, expression domain of aldh1a2 (Fig. 4c). This orientation with anterior kidney progenitors located dorsally and posterior kidney progenitors located ventrally is consistent with the AP axis of the PLM running laterally from the ventral to dorsal sides of the embryo. These data support a model in which the concentration of RA is highest in the dorsalmost PLM, where it induces anterior kidney fates, and lower in the ventral PLM where posterior kidney fates arise.
A similar analysis performed for the pectoral fin progenitors showed that these cells are positioned more dorsally, outside of the PLM, but still adjacent to the \( \text{aldh1a2} \) high domain. Heart progenitors were found to lie close to the pectoral fin progenitors but further towards the animal pole and therefore relatively distant to the RA source. Blood progenitors were not examined, however a subset were mapped to position \((x, y)\), slightly more vegetal to posterior kidney progenitors \((x, y)\), placing them in the \( \text{cyp26a1} \)-expressing ventral subregion of the PLM. Overall the fate-mapping data show that progenitors requiring RA for their establishment (anterior kidney and pectoral fin) are localized around the perimeter of the \( \text{aldh1a2} \) high domain while progenitors inhibited by RA (posterior kidney, blood and heart) are found in more distal positions.

Cyp26a1 restricts the effects of RA. In certain contexts, Cyp26 enzymes are proposed to shape the RA gradient by acting as a degradative ‘sink’\(^{24,35,36}\). In doing so, Cyp26 enzymes may create a boundary that limits RA diffusion. To explore the role of cyp26a1 in the context of kidney patterning, embryos deficient in Cyp26a1 activity were generated by morpholino-mediated knockdown and by treatment with R115866, a pan-Cyp26 inhibitor, from the late blastula stage onwards. Both methods resulted in similar ‘anteriorized’ kidney phenotypes, characterized by an expansion in the anterior segments \((\text{slc4a4}^+, \text{PCT and PST segments})\), a posteriorly displaced \( \text{slc12a1}^+ \) DE segment and a concomitant reduction in the \( \text{slc12a3}^+ \) DL segment (arrows, Fig. 5). Treatment of embryos with R115866 from the end of gastrulation (tailbud stage) onwards had only mild effects on renal patterning, indicating that kidney-associated Cyp26 activity is mostly required during gastrulation (Fig. 5). We conclude from these observations that Cyp26a1 is needed in the PLM during gastrulation to restrict the anteriorizing effects of RA.

BMP regulates the \( \text{aldh1a2} \) and \( \text{cyp26a1} \) expression domains. We next examined the effects of modulating BMP signalling, given its critical role in the formation of ventrolateral fates. To do this, embryos were treated from the late blastula stage onwards with the BMP receptor inhibitor dorsomorphin (DM) to induce a moderately ‘dorsalized’ phenotype (Fig. 6). To assess PLM formation in these embryos we examined \( \text{zulu} \) expression at 85% epiboly and found the \( \text{zulu} \) high PLM domain to be smaller and more ventrally restricted (Fig. 6). These results indicate that BMP signalling is needed to establish the extent of the PLM across the ventral to dorsal sides of the embryo.

To assess how modulating the BMP pathway influences RA signalling, we investigated the expression of \( \text{aldh1a2} \) and \( \text{cyp26a1} \) in DM-treated embryos. The \( \text{aldh1a2}^\text{high} \) dorsal domain was found to be expanded towards the ventral midline in DM-treated late gastrula embryos, whereas the ventral expression of \( \text{cyp26a1} \) is absent in these embryos (Fig. 6). These observations suggest that BMP inhibition leads to increased RA signalling in more ventral regions of the embryo by increasing \( \text{aldh1a2} \) expression and reducing \( \text{cyp26a1} \) expression. In agreement with this, DM-treated embryos display an ‘anteriorized’ kidney phenotype with a severely reduced DL segment (Fig. 7a). To confirm that this phenotype was mediated downstream of BMP signalling via an increase in RA synthesis, embryos were double-treated with...
Figure 4 | Fate-mapping analysis of late gastrula embryos show proximal tubule and pectoral fin progenitors are juxtaposed to the aldhl2high domain.

(a) Cells on the left-hand side of embryos at the 85% epiboly stage were lineage-labelled at various co-ordinates on the Cartesian grid shown (embryos are orientated with animal pole towards the top and dorsal side to the right). At the 10-somites stage, co-localization of uncaged cells and kidney (pax2a+), blood (gata1+), pectoral fin (tbx5a+) and heart (nkx2.5+) tissues was determined by whole-mount double in situ hybridization. Labelled cells on the right-hand side of the embryo (white asterisk) are the result of the laser passing through the embryo and uncaging cells on the contralateral side. Embryos are flat-mounted with anterior to the left (note: tbx5a is stained purple due to being a weaker probe). (b) Top schematic shows a flat-mounted embryo with the highlighted region corresponding to the panels below and the positions that label progenitors of the proximal (PT) and distal (DT) tubules, pectoral fin (PF) and heart (H). Bottom panels show whole-mount double in situ hybridization of these uncaged populations (red) relative to the expression domains of aldhl2, cyp26a1 and zulu (purple) at 85% epiboly. (c) Lateral views of 70% (left) and 80% (centre) epiboly-stage embryos double stained for aldhl2 (purple/black) and pax2a (red) transcripts. Higher magnified view of the indicated region is shown in the right-hand panel. A, animal pole; V, vegetal pole. Schematic at the bottom represents an outline of PLM. IM, intermediate mesoderm; LPM, lateral plate mesoderm. Scale bar in a, 100 μm in top panels and 200 μm in lower panels. Scale bars in b and c, 100 μm.
DM and DEAB resulting in a phenotype switch to a 'posteriorized' (slc12a3-only) kidney (Fig. 7a).

To examine the effects of increased BMP signalling, embryos were injected with bmp2b mRNA, resulting in a mix of moderately to severely ventralized embryos. In moderately ventralized embryos, the zulu high domain was increased laterally towards the dorsal midline, whereas in severely ventralized embryos, this domain extends around the entire circumference of embryo, indicative of radial ventralization (Fig. 6). In mildly ventralized embryos, the aldh1a2high dorsal domain is reduced, whereas it is absent in severely ventralized embryos. Conversely, the ventral expression domain of cyp26a1 is expanded laterally in ventralized embryos (Fig. 6). These results, the reciprocal of those obtained from BMP inhibition, are consistent with increased BMP signalling leading to an enlarged cyp26a1+ PLM territory and a reduced or absent aldh1a2high dorsal domain. Concomitant

**Figure 5 | Knock down of Cyp26a1 anteriorizes the kidney.** Whole-mount double in situ hybridization analysis at 24 hours post fertilisation (h.p.f.) for nephron segment markers slc4a4 (orange), slc12a1 and slc12a3 (purple) in embryos injected with cyp26a1 morpholino or treated with R115866 or DMSO (vehicle control). Embryos are shown as lateral views with anterior to the left. Arrowhead indicates junction between the DE and DL segments. Scale bar, 100 μm.

**Figure 6 | BMP signalling regulates the size of aldh1a2 and cyp26a1 and zulu expression domains.** Whole-mount in situ hybridization analysis for aldh1a2, cyp26a1 and zulu at the 85% epiboly stage in embryos treated with DM dihydrochloride, DMSO (vehicle control) or injected with bmp2b mRNA. Embryos are shown flat-mounted and orientated with the animal pole at the top and the vegetal pole at the bottom. Scale bar, 200 μm.
with these changes, embryos with a moderately ventralized phenotype develop abnormally long, but relatively well-patterned, kidneys, whereas severely ventralized embryos display a completely 'posteriorized' \((\text{slc12a3}^-\text{-only})\) kidney (Fig. 7a). Taken together, these data support a model in which BMP signalling influences kidney patterning by regulating the size of the mesodermal domains that express \(\text{aldh1a2}\) and \(\text{cyp26a1}\).

One corollary of this model is that the effects of RA on the kidney must be occurring downstream of the mesoderm-patterning effects of BMP during gastrulation. To test this, embryos were first ventralized by \(\text{bmp2b}^-\text{RNA}\) injection, as well as treated with DEAB from the dome stage to the end of gastrulation (bud stage) to generate 'BMP-high/RA-low' embryos. As expected, these embryos develop 'posteriorized' kidneys (Fig. 7b). When exogenous RA was added to 'BMP-high/RA-low' embryos at the end of gastrulation, the kidney phenotype switched to being 'anteriorized' \((\text{slc4a4}^-\text{-only};\) Fig. 7b). Similarly, in embryos without altered BMP levels, the posteriorizing effect of DEAB treatment from dome to bud stage can be reversed by the addition of RA from late gastrulation (Fig. 7b). These results indicate that RA can act as late as the end of gastrulation to influence kidney fates and supports the notion

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**Figure 7 | RA acts downstream of the BMP signalling pathway during kidney patterning.** (a) Whole-mount double in situ hybridization analysis for nephron segment markers \(\text{slc4a4}\) (red) and \(\text{slc12a3}\) (purple) in embryos at the 15-somites stage injected with \(\text{bmp2b}^-\text{mRNA}\) or treated with DM dihydrochloride, DEAB and DMSO (vehicle control) at the indicated stages. Embryos are shown as dorsal views with anterior to the left. (b) Embryos were treated as shown and stopped at the 15-somites stage for double in situ hybridization staining for the anterior pronephros marker \(\text{slc4a4}\) and the distal pronephros marker \(\text{slc12a3}\). All panels are dorsal views of flatmounted embryos. Scale bar, 200 \(\mu\)m.
that RA functions sequentially to BMP during non-axial mesoderm patterning.

Effects of BMP and RA on cell movements. In addition to altering cell fates, the BMP gradient is proposed to link the DV and AP axes by regulating dorsal convergence and extension movements.37 These movements involve the migration of ventrolateral cells towards the dorsal side of the embryo together with their intercalation, which drives elongation of the body axis.38,39 The highest level of BMP signalling (encompassing 20°–30° of the ventral margin at the shield stage) creates a ‘no convergence no extension zone’ (NCEZ) resulting in the ventralmost cells moving vegetally, but not dorsally, and contributing to the tailbud and other posterior fates. To determine how the positions of anterior and posterior kidney progenitors relate to the NCEZ, we uncaged tracer in a 20° wedge of the ventral margin and then subsequently labelled the kidney progenitors at the 85% epiboly stage. By imaging live embryos, we found that the kidney progenitors are located well outside of the NCEZ (Supplementary Fig. 7). In addition, over a 1-h time period, we observed that the anterior (x6, y6) kidney progenitors migrate dorsally while the posterior (x8, y8) kidney progenitors move both dorsally and vegetally (n = 3, Fig. 8a). Inhibiting BMP signalling with DM causes both populations of cells to move predominately vegetally, most likely due to the loss of directional cues conferred by the BMP gradient (Fig. 8a). Together, these findings are consistent with the notion that a BMP gradient across the ventral to dorsal sides of the embryo directs dorsal migration and that the kidney progenitors, being outside of the NCEZ, undergo differential convergence movements corresponding to their position along this gradient.

We next determined how blocking BMP signalling affects the fates of cells at 85% epiboly that would normally give rise to the anterior (x6, y8) and posterior (x8, y8) kidney by repeating our fate-mapping experiments. We found that DM treatment causes cells at position (x6, y8) to contribute to mid-trunk tissues, rather than the kidney, while cells at position (x8, y8) give rise to the anteriormost part of the pax2a + intermediate mesoderm (Fig. 8b, see Supplementary Fig. 8a for lateral and oblique views). These results are in line with our observation that the PLM territory is ventrally restricted when BMP signalling is inhibited. As a result, cells that would normally form the posterior kidney end up being the dorsalmost cells of the PLM in DM-treated embryos and therefore populate the anterior intermediate mesoderm. Furthermore, the inhibitory effects of DM on dorsal convergence and extension provide an explanation for why the intermediate mesoderm stripes remain so posteriorly restricted in DM-treated embryos.

Significant effects of DM on the fates and positions of cells that normally contribute to pectoral fin (x6, y8) and heart (x10, y10) were also found, with both positions giving rise to upper trunk tissues, most likely anterior paraxial mesoderm, while heart progenitors formed aberrantly in lateral and ventral regions of the embryo (Fig. 8b and Supplementary Fig. 8b).

To assess whether RA also dually regulates cell movement and patterning like BMP signalling, we examined live cell movements and repeated the fate-mapping experiments in the presence of DEAB. Live imaging of uncaged cells in DEAB-treated embryos showed that the kidney progenitors undergo similar dorsal cell convergence movements as control embryos (Fig. 8a). Fate mapping of cells at positions (x6, y8) and (x8, y8) in RA-deficient embryos showed that they end up in relatively normal anterior and posterior domains of the intermediate mesoderm, respectively (Fig. 8b). Given that the kidney is ‘posteriorized’ (slc12a3+ only) in these embryos (Fig. 7a), we conclude that, in the absence of RA, anterior kidney progenitors migrate to their normal position within the intermediate mesoderm but adopt a posterior fate. In addition, we found that cells at positions (x6, y8) and (x10, y10), which normally label pectoral fin and heart progenitors, respectively, both give rise to heart progenitors in DEAB-treated embryos, consistent with previous reports (Fig. 8c). Taken together, these findings further support our model that RA signalling influences the fine patterning of the non-axial mesoderm, downstream of BMPS, without having major effects on dorsal convergence movements.

Discussion

With the advent of better fate maps in Xenopus it was proposed that the classic gastrula-stage DV axis may represent the AP axis, with more dorsally located cells contributing to anterior tissues. While the zebrafish fate map for mesoderm has not been revisited at the same level of resolution, the fate map for the ectoderm is generally consistent with the traditional view that the AP axis is coincident with the animal–vegetal axis and not the classic gastrula-stage DV axis. As a result, there has been some confusion and uncertainty in the field regarding the spatial arrangement of the mesoderm along the zebrafish DV axis. In this study we make use of the kidney, with its defined segmentation pattern along the AP axis, to demonstrate that, with regard to the non-axial mesoderm, the classic DV axis of the gastrula corresponds to the AP axis while the DV axis aligns with the animal–vegetal pole. These results agree with the latest Xenopus fate map and with grafting experiments in zebrafish, which demonstrated that ventral grafts formed posterior mesodermal tissues while more dorsal grafts formed anterior mesodermal structures. As this arrangement is the opposite to that found for the ectoderm it indicates that the AP and DV axes of the non-axial mesoderm and ectoderm are initially spatially distinct. As a result, it is impossible to consider the early embryo in terms of single AP and DV axes, which are aligned for all germ layers. Instead, the germ layer axes only become entrained following gastrulation movements.

Our data indicate that BMP signalling plays a role in the initial subdivision of the non-axial mesoderm into broad territories along the AP axis. Our marker analysis identified an anterior territory (head and upper trunk mesoderm) and a posterior territory (lower trunk mesoderm and tail). Although not examined in our study, we expect the posterior territory to be further subdivided into lower trunk mesoderm and tail subdomains, as there is evidence that tail precursors are set aside during early gastrulation but patterned later (Fig. 9). We found that the respective sizes of the non-axial mesoderm territories is set by the level of BMP signalling, consistent with a large body of evidence showing that loss of BMP signalling causes a loss of posterior structures. Inducible BMP inhibition experiments have shown that BMP signalling acts during critical temporal intervals to pattern DV fates in a rostral to caudal progression but has little involvement in AP patterning. While the mesoderm was not extensively studied, using wta1a, pax2a and gata1 expression, as read-outs of increasingly more ‘ventral’ (posterior) fates, it was revealed that BMP signalling also influences the patterning of these mesodermal tissues in a temporal fashion. As our data indicate that the AP and DV axes of the ectoderm and mesoderm are initially oriented at right angles to each other, we interpret these findings as indicating that BMP signalling in the non-axial mesoderm temporally regulates AP, but not DV, patterning. As a result, the BMP signalling gradient can be considered to function progressively over time to pattern AP identities in the non-axial mesoderm and DV tissues in the ectoderm, thus revealing common, but germ layer-specific, effects of BMPS on each axis.
The Progressive Critical Intervals model proposes that the temporal aspect of BMP signalling provides a mechanism to co-ordinate patterning such that AP and DV fates are acquired simultaneously but progressively along the embryo. While this model works well for the ectoderm, a better understanding of the temporal aspects of mesoderm patterning is needed to fully understand how this model translates to the mesoderm.

Given that twist1a expression in posterior lateral plate mesoderm precursors initiates between 50 and 60% epiboly while transcripts for lbx2 (ref. 61) and pax2a in the intermediate mesoderm appear at 70% epiboly, we speculate that DV fates may be progressively acquired along the animal–vegetal pole.

While the BMP gradient is capable of inducing specific identities, such as epidermis and neural crest, our experiments...
suggest that the fine AP patterning of kidney fates occurs later in gastrulation in response to RA. Notably, our observation that RA treatment at the end of gastrulation switches the kidney from being anteriorized to posteriorized in a ‘BMP-high/RA-low’ embryo strongly supports the role of RA acting later in development, after the temporal effects of BMP signalling have occurred. Given that we observed little effect on dorsal convergence movements when RA synthesis was inhibited, we do not believe the patterning effects of RA on the kidney are the result of abnormal cell positioning during gastrulation. Instead, our data suggest a model in which a pool of RA is established across the PLM, with high levels on the dorsal side acting to induce anterior kidney fates while more ventrally located kidney progenitors are protected from RA by Cyp26a1. This model is in keeping with the established function of RA as an AP-patterning molecule but rather than acting along the animal–vegetal axis, as it does for hindbrain patterning, it acts laterally, across the dorsal to ventral sides of the gastrula (Fig. 9).

Furthermore, this role for RA does not appear specific to zebrafish, as in Xenopus the expression domains of aldh1a2 and cyp26a1 relative to the kidney anlage show a similar spatial arrangement at the end of gastrulation (Supplementary Fig. 9).

How RA signalling distinguishes the different anterior fates of the kidney and whether this occurs as a result of a morphogenic gradient remains unclear. In the hindbrain it has been proposed that Cyp26 enzymes control the shape of an RA gradient by regulating localized RA degradation. The ‘read-out’ of this gradient is then postulated to induce different rhombomere identities within the neuroectoderm. In our studies, knockdown of Cyp26a1 activity results in the kidney being mildly anteriorized. This observation suggests that Cyp26a1 has a role in restricting RA activity but is not likely required to form the gradient. We envision that Cyp26a1 acts in a boundary-forming capacity that protects ‘ventral’ PLM progenitors from the anteriorizing effects of RA and thereby fine-tunes the proportion of anterior fates (PCT, PST and DE) induced relative to the DL fate. Because the entire kidney adopts a DL fate when RA signalling is blocked, we consider the DL segment to represent the default differentiation state of the pronephros.

In addition to kidney, the formation of erythroid cells, pectoral fin and heart is also sensitive to RA levels. With regard to red blood cells, their formation is inhibited by RA, which is consistent with our fate mapping that places their progenitors in the cyp26a1þ PLM. When embryos are treated with DEAB to block RA signalling, the anterior limit of the gata1þ blood stripes shifts from being level with somite 6 to being level with somite 1 or 2 (ref. 17). This observation suggests that the more ‘dorsal’ (cyp26a1−) PLM is competent to form blood but this fate is inhibited due to high RA levels. RA signalling also inhibits cardiac specification while it plays a permissive role for pectoral fin progenitors. Blocking RA causes the size of the cardiac progenitor field to expand into the region where the pectoral fin develops. Our fate mapping supports this interaction as both progenitor types are localized in close proximity within the anterior lateral mesoderm domain with cardiac progenitors located further away from the aldh1a2high dorsal domain than pectoral fin progenitors. The effects of RA on the patterning of multiple tissues (kidney, blood, heart, pectoral fin and hindbrain), as well as endodermally derived tissues such as the pancreas, raises the possibility that the aldh1a2high dorsal domain acts as a
One-cell embryos were injected with 1–5 nl of 12NATURE COMMUNICATIONS | DOI: 10.1038/ncomms12197 | www.nature.com/naturecommunications

... was purchased from Gene Tools LLC and resuspended in 1

... mesodermal AP and DV axes arise during development and are aligned across the classic gastrula stage DV axis, while actual DV patterning of the mesoderm occurs along the animal–vegetal pole. Together, these results clarify our understanding of how the mesodermal AP and DV axes arise during development and provide a new paradigm for how non-axial mesoderm derivatives, such as the kidney, form during embryogenesis.

Methods
Zebrafish husbandry. Zebrafish were maintained and staged according to established procedures and in accordance to the University of Auckland’s Animal Ethics Committee (protocol 001343). Embryos were collected from paired matings of wild-type Tübingen (Tg) adults for all lineage labelling and drug treatment experiments.

Embryo preparation for lineage labelling. A unit of 2μg ml−1 of dextran DMN-Caged fluorescein (Molecular Probes LLC) was injected into embryos at the one-cell stage. Injected embryos were grown to ~ 85% epiboly, dechorionated in 0.5 mg ml−1 pronase from Streptomyces griseus (Roche Diagnostics), washed three times in E3 embryo medium, then individually placed into wells on a Petri dish containing a bed of 1% agarose. Embryos were orientated into wells on a Petri dish containing a bed of 1% agarose. Embryos were orientated and left for 1 h to allow the agarose to harden before being used for further experimentation. Only the uncaged cells on the left-hand side of the embryo were used for the fate mapping analysis.

Morpholinos and mRNA synthesis. A previously described morpholino oligonucleotide to cyp26a1 (5′-CGCGAACATGACGCGAAAAGCAAAGAAA-3′) was purchased from Tools for Genes (http://www.tfgene.com). The 10–15 nmol aliquots; embryos were dechorionated and incubated in 50–100 M DEAB. RA (Sigma-Aldrich) was dissolved to 10 mM in DMSO and stored at −20 °C in 50–100 μl aliquots; embryos were treated with 0.3 μM RA. DM dihydrochloride (Sigma-Aldrich) was dissolved to 10 mM with water and stored at −80 °C in 100–500 μl aliquots; embryos were dechorionated and incubated in 10 mM DM (we found chorionated embryos had greatly reduced dorsalized phenotypes when treated with DM). R115866 (Active Biochem) was dissolved to 10 mM with DMSO and stored at −20 °C in 50–100 μl aliquots; embryos were treated with 20 μM R115866. BMS189453 (Tocris) was dissolved to 10 mM with DMSO and stored at −20 °C in 100–500 μl aliquots; embryos were treated with 10 μM BMS189453. All control embryos were incubated in an equivalent DMSO concentration to the highest concentration of drug used.

Data availability. The authors declare that the data supporting the findings in this study are available within the article or from the corresponding author on request.

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**Author contributions**

R.W.N., L.B.S. and A.J.D. carried out the experiments; R.W.N. and A.J.D. designed the experiments; A.J.D. wrote the manuscript.

**Additional information**

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