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Zebrafish nephrogenesis involves dynamic spatiotemporal expression changes in renal progenitors and essential signals from retinoic acid and *irx3b*

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Abstract

Kidney nephrons are comprised of proximal and distal tubule segments that perform unique roles in excretion. The developmental pathways that establish nephron segment identities from renal progenitors are poorly understood. Here, we used the zebrafish pronephros to study nephron segmentation. We found that zebrafish nephron progenitors undergo elaborate spatiotemporal expression changes of many genes before adopting a segment fate. Initially, two domains of nephron progenitors are established, then are subdivided and demarcate individual nephron segments. Using genetic and chemical genetic models of retinoic acid (RA) deficiency, we discovered that RA modulates rostral progenitor formation. To delineate downstream pathways, we knocked down the *irx3b* transcription factor and found it regulates proximal tubule segment size and distal segment differentiation. Our results suggest a model whereby RA patterns the early field of nephron progenitors, with subsequent factors like *irx3b* acting to refine later progenitor subdomains and ensure activation of segment-specific gene programs.

Keywords

kidney organogenesis; nephron; segmentation; zebrafish

INTRODUCTION

Vertebrate kidney development is characterized by the progressive formation of several kidney types. For example, mammals form three kidney structures of increasing complexity: the pronephros, the mesonephros, and finally the metanephros, which serves as the adult organ (Dressler, 2006). Other vertebrates, like fish and amphibians, initially utilize a pronephros and later form a mesonephros that functions during larval and adult stages (Dressler, 2006). Despite the serial changes in organ complexity, the nephron is the universal structural and functional unit of all vertebrate kidneys. In most cases, nephrons consist of three parts that have discrete activities: (1) a sieve (the glomerulus) comprised of an intricate arrangement of fenestrated capillaries surrounded by a capsule of podocytes that filters the blood, (2) an epithelial tubule that receives the filtrate and modifies it by

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reabsorbing and secreting metabolites, and (3) a duct that performs limited solute exchange while transporting the waste for excretion (Dressler, 2006). The tubule epithelium is patterned along its length into functionally specialized segments that express unique gene expression profiles of solute transporters (El-Dahr, et al., 2008; Kopan et al., 2007; Schedl, 2007). Proximal tubule segments perform the bulk of metabolite recovery for amino acids, salts, and sugars, while distal tubule segments are specialized for fine-tuning salt levels (Hebert et al., 2001). Interestingly, vertebrates that reside in diverse habitats ranging from the terrestrial to the aquatic possess a largely similar proximodistal complement of nephron tubule segments. Recent molecular analyses have demonstrated that zebrafish, frog, mouse, and human nephrons contain functionally comparable proximal and distal tubule segments and express many of the same solute transporter and transcription factor genes (Wingert and Davidson, 2008). Differences in segment gene expression likely reflect physiological contrasts between species.

Nephron segmentation is the process through which segment identities are produced during nephrogenesis. Several key signals have been identified that are essential for the formation of certain nephron segments, but a comprehensive understanding of segment patterning in any one model organism has not yet been achieved (Schedl, 2007; Wingert and Davidson, 2008). For example, metanephric nephron segmentation in mammals requires Notch signaling for proximal tubule formation and *Pou3f3/Brn1*, a POU-domain containing transcription factor, for distal tubule development (Nakai, et al., 2003; Cheng, et al., 2007). Notch ligands and receptors are regionally expressed in the nephron anlagen (Cheng and Al-Awqati, 2005), and conditional inactivation of *Notch2* results in a loss of proximal tubule segments (Cheng, et al., 2007). Conversely, genetic inactivation of *Pou3f3/Brn1* leads to metanephric nephrons that display a dramatic reduction in distal segments (Nakai, et al., 2003). Recent studies of pronephric nephron formation in lower vertebrates have identified roles for retinoid signaling and the Iroquois homeobox transcription factor *Irx3*. During zebrafish pronephros formation, retinoic acid (RA) is necessary for specifying proximal tubule fates, and the abrogation of RA synthesis leads to an expansion of the distal tubule and duct segments (Wingert, et al., 2007). In *Xenopus*, knockdown of *Irx3* eliminates the formation of at least one distal segment, and *Irx3* has been proposed to act as a master regulator in the specification of this segment (Alarcon, et al., 2008; Reggiani, et al., 2007). While it remains unclear if the signals that pattern vertebrate nephrons can be integrated into one universal model of nephrogenesis, trends in the regional expression of *Notch* genes, *Pou3f3/Brn1*, and *Irx3* support the enticing suggestion that nephron patterning is conserved (Wingert and Davidson, 2008).

The zebrafish pronephros is an excellent model for nephron segment patterning studies. Zebrafish embryos form an anatomically simple pronephros: it is comprised of two nephrons that derive from the intermediate mesoderm that can be visualized throughout their ontogeny (Drummond, et al., 1998; Drummond, 2003). Nephron segment lengths and boundaries can be quantified at a high resolution relative to the adjacent somites, which provide 'landmarks' along the body axis (Wingert, et al., 2007). Zebrafish pronephric nephrons possess at least eight discrete cell populations in common with mammals: the podocytes (P) which contribute to the glomerulus, a neck segment (N) that connects the glomerulus and tubule, two proximal segments (the proximal convoluted tubule (PCT) and proximal straight tubule (PST)), two distal segments (the distal early (DE) and distal late (DL)), and a pronephric duct (PD) that empties into the cloaca (C) (Wingert, et al., 2007). The parallels between fish and mammalian nephron components suggest that the zebrafish studies can generate insights into the conserved mechanisms of nephrogenesis.

In this study, we have examined the domains of gene expression in zebrafish embryo renal progenitors prior to the appearance of proximal and distal tubule segments. We discovered

that an intricate, nested pattern of transcription factor domains precedes the appearance of mature tubule segments in the nephron, and that this pattern undergoes a series of complex spatiotemporal alterations. Using *lib*, a newly isolated genetic model of RA deficiency, we found that retinoid signals influence the earliest spatial patterns of transcription factor domains. We discovered that a loss of the paraxial mesoderm disrupts the expression of the RA-biosynthesis gene *aldh1a2* and induces nephron segment defects similar to *lib*, thus demonstrating that the paraxial mesoderm is a key source of RA for nephron patterning. In addition, we found that *irx3b* is required at relatively late stages of nephron patterning for the differentiation of the first distal segment. These findings suggest a stepwise model whereby the sequential actions of RA and *irx3b* orchestrate segmentation of the zebrafish pronephros.

RESULTS

Expression domains are dynamic within the nephron progenitor territory

To study the origins of nephron segments, we analyzed the expression of transcription factors and signaling molecules between the time when the intermediate mesoderm is first detected around the 3 somite stage to the emergence of mature nephron segments around 24 hours post fertilization (hpf; equivalent to the 28 somite stage) (Wingert, et al., 2007). For each kidney gene, we determined the precise gene expression domain relative to the somites by performing double whole mount *in situ* hybridization with an age-appropriate somite marker (*myogenic differentiation 1 (myoD)* for embryos <15 somites and *myosin heavy chain (mhc)* for embryos >15 somites). Consistent with our previously published observations, we found that nephron progenitors displayed uniform expression of several transcription factors including *pax2a* and *pax8* until approximately the 5 somite stage (data not shown; Wingert, et al., 2007). Between the 6 and 8 somite stages, the nephron territory was subdivided into two molecularly distinct adjacent regions that showed subtle dynamic alterations. At the 6 somite stage, we observed a rostral domain, located adjacent to somites 2–5 and marked by expression of the Notch ligand genes *delta-c (dlc)* and *jagged-2 (jag2)*, as well as a caudal domain marked by expression of the zinc finger transcription factor *mecom* (also known as *evi1*) that began adjacent to somite 6 (data not shown; Wingert, et al., 2007). At the 8 somite stage, the *dlc* and *mecom* domains remained mutually exclusive, however the *jag2* domain expanded such that it overlapped with *mecom* at somites 6–7 (Fig. 1A, 1C, 3). Thus, a set of overlapping rostral and caudal identities is established among nephron progenitors during early somitogenesis, and likely represents the influences of early proximo-distal patterning signals occurring at this time.

By the 15 somite stage, we found a complex, nested arrangement of transcription factor domains within the intermediate mesoderm. A number of gene transcripts were expressed by all nephron progenitors, such as *hnf1ba*, *pax2a*, and *pax8* (Fig. 1B; data not shown). Genes with restricted expression domains broadly marked either the rostral, central, and/or caudal regions of the nephron field, though the precise extent of each gene varied within these partially overlapping areas. Expression of *hnf4a* was found rostrally, whereas *hnf1bb* transcripts were found throughout both the rostral and central areas (Fig. 1B, 1D). We found that *irx3b* transcripts marked the central area, and that several genes marked portions of the central as well as the entire caudal area, namely *pou3f3a*, *pou3f3b*, *mecom*, *emx1*, and *gata3* (Fig. 1B, 1D; data not shown). Interestingly, we noted that some genes at this time-point have an expression boundary that is coincident with the mature segment boundaries that appear at the 28 somite stage. For example, the anterior limit of the *mecom* expression domain (somite 9) and the posterior limit of *hnf4a* (somite 11) coincide with the eventual PST segment boundaries (somites 9–11) (Fig. 1D, 3). In addition, the posterior limits of *hnf1bb* and *irx3b* coincide with the posterior boundary of the DE segment (at somite 13)

(Fig. 3). Other gene expression domains though, like the anterior limits of *irx3b*, *pou3f3a*, and *pou3f3b*, were not coincident with subsequent segment boundaries and instead spanned the eventual boundary of the adjacent PCT and PST segments (Fig. 3). These data suggest that the boundaries of some segments can be predicted as early as the 15 somite stage, while others are defined at later stages.

The nested arrangement of transcription factor domains in nephron progenitors persisted at subsequent stages, and was still evident at the 28 somite stage, the time-point when segment-specific solute transporters mark the differentiated PCT, PST, DE, and DL tubule segments (Fig. 3). However, we found that spatiotemporal changes in gene expression occurred between the 20 and 28 somite stages. Interestingly, there was a common trend whereby genes that mark the rostral region showed no change after 15 somites, while genes that mark the central and caudal regions underwent dynamic alterations at subsequent time-points. For example, the domain of the rostral region gene *hnf4a* remained constant from the 15 somite stage onward (Fig. 1B, 2, 3). In contrast, the domains of the central and caudal region genes *etv5*, *irx3b*, *pou3f3a*, *pou3f3b*, *mecom*, *emx1*, *gata3*, and *grhl2a* showed one or more alterations at different times between 15 and 28 somites (Fig. 1B, 2, 3). In general, these changes can be summarized as posterior shifts in the anterior expression boundary of each gene, although some genes also exhibited contractions at their posterior boundary. For example, the genes *irx3b*, *pou3f3a*, and *pou3f3b* initially shared a common anterior boundary adjacent to somite 7 until the 20 somite stage. This boundary retracted to somite 9 for *pou3f3a* and *pou3f3b* at the 22 somite stage, followed by a similar retraction for *irx3b* slightly later, at the 24 somite stage. The expression domain of *pou3f3a/3b* underwent further refinement by the 28 somite stage, with a down-regulation of transcripts posteriorly. The net result of these changes is that *irx3b*, *pou3f3a* and *pou3f3b* become restricted to the PST and DE (somites 9–13) at 28 somites (Fig. 2, 3).

We found a striking correlation between the pattern of transcription factor expression domains and the location of nephron segment boundaries at the 28 somite stage (Fig. 2, 3). Surprisingly, we found only one transcription factor, *etv5*, restricted to a single tubule segment (the PST), while the others marked two or more nephron segments. The transcription factors formed an overlapping, nested fashion such that each tubule segment possessed a unique combination of transcripts. For example, the PST contained *hnf1ba*, *hnf4a*, *hnf1bb*, *etv5*, *irx3b*, *pou3f3a* and *pou3f3b* transcripts, while the DE expressed a subset of this medley that excluded *hnf4a* and *etv5* (Fig. 2, 3).

Cell proliferation is not essential for nephron segmentation during somitogenesis

One possible explanation for the dynamic spatiotemporal alterations in gene expression domains is that regional changes in cell proliferation generate shifts in the physical arrangement of nephron tubule progenitors. To explore this possibility, we treated embryos from the 15, 20, 22, or 24 somites stages with camptothecin or nocodazole to prevent cell division, and then examined the final location of each tubule segment at 24 hpf (Fig. S1). We found no evidence that an inhibition of cell proliferation altered the normal positioning of the proximal and distal segments. Treatment with proliferation-blocking compounds at younger stages was prohibitive to embryo development and precluded the ability for segmentation markers to be assessed (data not shown). Based on these experiments, we conclude that the emergence of nephron tubule segment identities occurs independently of growth after 15 somites.

Mutation of the *lightbulb* gene disrupts proximo-distal patterning of nephron segments

We next sought to gain insight into the genetic pathways that establish nephron segment fates. We isolated the *lightbulb* (*lib*) mutant in a forward genetic screen of ENU-

mutagenized zebrafish for recessive embryonic kidney defects. *lib* embryos have a truncated cervical region at 24 hpf based on the abnormally close proximity of the otic vesicle to the first somite compared to wildtypes (Fig. 4A). At 48 hpf, *lib* embryos exhibited a kink in the cervical region, pericardial edema, lacked pectoral fins, and had a pronounced trunk curvature along with a distorted lightbulb-shaped yolk (Fig. 4A). These morphological characteristics of *lib* are very similar to the hallmarks of RA deficiency in zebrafish embryos, leading us to hypothesize that *lib* had a deficit in RA bioavailability (Alexa, et al., 2009; Begemann, et al., 2001; Begemann, et al., 2004; Grandel, et al., 2002).

In the developing embryo, RA bioavailability is controlled by enzymatic production and degradation (Duester, 2008). RA is produced by the sequential action of retinol dehydrogenases (RDHs) and aldehyde dehydrogenase (Aldh or Raldh) synthesizing enzymes, and degraded by the action of cytochrome P450 enzymes belonging to the Cyp26 family (Duester, 2008). We compared the phenotype of *lib* mutant embryos to *neckless* (*nls*), which have a point mutation in the catalytic domain of *aldh1a2*, and wildtype embryos treated with diaminobenzaldehyde (DEAB), a reversible chemical inhibitor of Aldh enzymes. *nls* embryos are thought to be Aldh1a2-deficient but have less severe tissue defects compared to DEAB treatment, perhaps due to maternal Aldh1a2 activity present in the yolk (Alexa, et al., 2009; Begemann, et al., 2004). At 48 hpf, *nls* embryos had a less severe cervical kink, pericardial edema, and body curvature compared to *lib*, while DEAB-treated embryos and *lib* were more similar (Fig. 4A). Next, nephron segmentation in *lib*, *nls*, and DEAB-treated embryos was evaluated using double whole mount *in situ* hybridization with segment-specific solute transporters and *mhc* to mark the somites. Compared to wildtypes, 24 hpf *lib* embryos had reduced expression of the podocyte markers, *wt1b*, *wt1a*, and *mafb*, and the podocytes were located more rostrally, suggesting that podocyte development was partially affected (Fig. 5A; data not shown). At 48 hpf, *lib* embryos had reduced expression of both *wt1b* and *nephrin*, suggesting that podocyte numbers were not restored as development progressed (data not shown). *lib* embryos also formed shorter proximal segments, as shown by the PCT and PST markers *slc20a1a* and *trpm7* (Fig. 5A). In addition, *lib* formed expanded distal segments, as shown by the DE, DL and PD markers *slc12a1*, *slc12a3*, and *gata3*, respectively (Fig. 5B). *nls* embryos had a milder nephron segment phenotype than *lib* mutants, with reduced podocytes located at the correct location along the trunk, a slightly reduced PCT segment, and a slightly expanded DE segment (Fig. 5). DEAB treated embryos had nephrons comprised entirely of the distal segments (DE, DL, and PD) that were each expanded in length (Fig. 5). These data indicate that *lib* mutants have a nephron patterning defect more severe than *nls*, but less severe than DEAB treatment.

***lib* is a new model of Aldh1a2 deficiency**

We next undertook a positional cloning strategy to determine the genetic defect in *lib*. Using bulk segregant analysis, the *lib* gene was mapped to zebrafish chromosome 7 (Fig. 4B). Linkage analysis of 2698 meioses placed the *lib* locus between the SSLP markers z8693 and z11894, a region that includes the *aldh1a2* gene. This was surprising as the kidney phenotype of *lib* mutants is more severe than *nls* mutants, and because *nls* and its phenotypically similar alleles *no-fin* and *aldh1a2^{um22}* are all considered to represent null alleles of *aldh1a2* in the zebrafish (Alexa, et al., 2009; Begemann, et al., 2001; Begemann, et al., 2004; Grandel, et al., 2002). Nevertheless, based on the mapping and phenotype, we hypothesized that *lib* was a novel, more severe mutant allele of *aldh1a2*.

To investigate if *lib* was an *aldh1a2* mutation, we mated *lib* and *nls* adult heterozygotes and found that the mutations failed to complement one another (Fig. 4A). Compound *nls/lib* heterozygote embryos had morphological characteristics most similar to *nls* mutant embryos, with a kink in the cervical region, pericardial edema, and absent pectoral fins (Fig.

4A). In addition, compound *nls/lib* heterozygote embryos had a nephron segment pattern of intermediate severity between the *nls* and *lib* phenotypes. Compared to *nls*, compound *nls/lib* heterozygotes had a slightly shorter PST and slightly larger DE, but had no change in DL size or podocyte location as seen in *lib* (Fig. 5). Next, RT-PCR was used to isolate *aldh1a2* cDNA transcripts from *lib* mutants. Sequence analysis identified a C to A transversion at nucleotide 174, predicting a Tyr → Stop nonsense mutation at amino acid 58 (Fig. 4C). The wildtype zebrafish *aldh1a2* peptide is 518 amino acids in length, and includes a series of nucleotide binding domains, tetramerization domains, and catalytic domain (Alexa, et al., 2009). The *lib* mutation is predicted to truncate the first nucleotide binding domain and eliminate all subsequent domains. Interestingly, examination of *aldh1a2* expression in *lib* revealed that transcripts were markedly reduced both in *lib* homozygous and heterozygote embryos, consistent with nonsense-mediated decay (Fig. 4D).

To provide further evidence that the *lib* phenotype is caused by a RA synthesis defect, we investigated whether elevating RA levels could rescue *lib*. Embryos from *lib* heterozygous incrosses were injected at the 1-cell stage with wildtype *aldh1a2* cRNA or treated with exogenous RA from 60% epiboly to the 28 somite stage, then scored for morphology and pectoral fin development at 48 hpf (Fig. S2A). Overexpression of *aldh1a2* was sufficient to rescue *lib* morphology (Figs. S2A, B). Interestingly, exposure to a high dose of RA (10^{-8} M) completely rescued *lib* mutant embryos, but a lower RA dose (10^{-9} M) was insufficient to rescue *lib* (Fig. S2B). In contrast, the lower RA dose was able to fully rescue *nls* and *nls/lib* compound heterozygote embryos (Fig. S2B). These results support the notion that *lib* quantitatively possesses the most severe RA deficiency among these genotypes and that RA dosage is responsible for the graded phenotypes in *lib*, *nls/lib*, and *nls* mutants.

Next, we determined if exogenous RA application from 60% epiboly to the 28 somite stage was sufficient to rescue proximal nephron segments in *lib*. Nephron segmentation was evaluated at 24 hpf using double whole mount *in situ* hybridization with segment-specific solute transporters and the somite marker *mhc*. RA-treated *lib* mutants developed expanded proximal segments (PCT, PST) and reduced distal segments (DE, DL, PD) (Fig. S2C, D). In addition, RA treatment rescued podocyte number and positioning in *lib* embryos (Fig. S2C, D). These results demonstrate that nephron progenitors in *lib* mutants are competent to respond to an RA signal(s), and suggest that a paucity of retinoid ligands underlies the *lib* phenotype. Taken together, our analysis of the *lib* and *nls* mutants highlights that RA production by *Aldh1a2* is essential for proximo-distal patterning of the pronephros.

***lib* mutants have altered renal progenitor domains during early somitogenesis**

To investigate how RA deficiency in *lib* mutants influenced nephron segment patterning, we examined the spatiotemporal expression of renal genes between the 8–28 somite changes. At the 8 somite stage, *lib* mutant embryos had slightly shortened domains of *dlc* and *jag2* expression that were shifted anteriorly compared to wild-types (Fig. 1A, 1C). In addition, *lib* embryos had a *mecom* expression domain that was slightly expanded anteriorly compared to wildtypes (Fig. 1A). Overall, the combination of the *dlc*, *jag2*, and *mecom* domains in *lib* mutants still formed an overlapping rostral-caudal pattern similar to wildtypes, but shifted anteriorly (Fig. 1A, 1C). Since nephron progenitor patterning is altered in *lib* mutants at the earliest stages that regional differences can be detected, it suggests that RA regulates the initial proximodistal patterning of nephron progenitors, consistent with our previous observations (Wingert, et al., 2007).

More significant alterations in nephron progenitor patterning were detected in *lib* mutants at the 15 somite stage. Notable changes included anterior shifts in the rostral (*hnf4a*), central (*irx3b*), and caudal (*mecom*) expression domains (Fig. 1B, D). These shifts were not

equivalent for each domain, with *hnf4a* shifting anteriorly by 1 somite, *irx3b* by 3 somites, and *mecom* by 2 somites. The posterior boundaries of the *hnf4a* and *irx3b* domains were similarly shifted anteriorly, and also by unequal extents (3 somites for *hnf4a* and 1 somite for *irx3b*; Fig. 1B, D). The net effect of these changes was a reduction in the rostral domain and expansion in both the central and caudal domains (Fig. 1D). Despite these patterning changes, the correlations between the rostral-central-caudal domains at 15 somites and the positions of the final nephron segments that we noted in wildtype embryos were likewise the case in *lib* mutants. For instance, in both wildtypes and *lib* mutants, the boundaries of the PST segment correlate to the region of overlap between the rostral (*hnf4a*) and caudal (*mecom*) domains, despite this region being smaller and anteriorly shifted in *lib* mutants. These results provide further support for the notion that renal transcription factor domains during early pronephros development are predictive of nephron segment fates at later stages.

Between the 20–28 somite stages, *lib* embryos exhibited similar dynamic spatiotemporal shifts in central and caudal domain markers that we observed in wildtypes (Fig. S4, S5). For example, the anterior expression boundary of the central domain gene *irx3b* and caudal domain marker *mecom* retracted in *lib* and wildtypes (Fig. S4, S5). However, there was one exception: the caudal markers *pou3f3a* and *pou3f3b* failed to retract their anterior expression boundaries and remained ectopically expressed in the PCT and PST segments. Taken together, these results provide genetic evidence that RA has an early role in establishing the rostral and caudal domains within the intermediate mesoderm, and that RA is also required at later time-points to refine the expression patterns of genes such as *pou3f3a* and *pou3f3b*.

Paraxial mesoderm is necessary for proximo-distal nephron patterning

Based on our previous work showing that *aldh1a2* was highly expressed in developing somites, we hypothesized that the paraxial mesoderm was a major source of RA responsible for establishing the proximodistal pattern of the pronephros (Wingert, et al., 2007). To test this, we knocked down *tbx16/spadetail*, which encodes a T-box transcription factor required for paraxial mesoderm formation (Amacher, et al., 2002; Morley, et al., 2009), and then examined the segmentation pattern of the pronephros at 24 hpf. A deficiency in *tbx16* was associated with reduced podocytes and proximal tubule segments (Fig. 6A). Conversely, *tbx16* morphants formed an expanded DE segment, as shown by the marker *slc12a1*, while the DL (*slc12a3+*) and PD (*gata3+*) segments were not significantly altered (Fig. 6A). These patterning changes were associated with corresponding alterations in the sizes and/or positioning of the rostral, central, and caudal domains. For instance, the rostral domain marker *hnf4a* was reduced, while the central domain marker *irx3b* and the caudal domain marker *mecom* were expanded anteriorly (Fig. 6B). Precise mapping of the segment size differences relative to somite number was not possible in *tbx16* morphants due to defective somitogenesis (Fig. 6C). Analysis of *aldh1a2* expression in *tbx16* morphants at the 5 and 28 somite stages showed a significant reduction in transcript levels, consistent with the loss of paraxial mesoderm that characterizes the *tbx16* mutant phenotype (Amacher, et al., 2002). The persistent presence of *aldh1a2* transcripts in *tbx16* morphants likely represents expression in the lateral plate mesoderm along with a small number of muscle precursors that form in *tbx16* mutants (Amacher, et al., 2002). These data reveal that *tbx16* deficiency induces similar proximodistal nephron patterning defects to *lib/aldh1a2* mutants, thus supporting our hypothesis that the paraxial mesoderm provides a major source of the RA that patterns the pronephros.

RA receptor activity is also essential for nephron segment patterning

Retinoid ligands transduce their effects via heterodimeric complexes of nuclear receptors that consist of a retinoic acid receptor (RAR) and retinoid X receptor (RXR) (Duester, 2008). To explore whether modulations in retinoid receptor activity can alter nephron

segmentation, we tested the effects of retinoid receptor antagonists and agonists in wildtype zebrafish embryos. Wildtype embryos treated with an RAR- α antagonist from 60% epiboly to the 28 somite stage formed shortened proximal segments (PCT, PST) and expanded distal segments (DE, DL, PD) (Fig. S3). Conversely, treatment with an RAR- α agonist from 60% epiboly to the 28 somite stage had the opposite effect, and led to expanded proximal segments along with absent or dramatically reduced distal segments (Fig. S3). These findings provide independent evidence that RA signaling is essential for nephron segmentation, and suggest that RAR- α may be a key functional component in nephron patterning.

***irx3b* acts downstream of RA to specify the DE segment in the zebrafish pronephros**

A striking feature of *lib* mutants is the significantly enlarged DE segment. Previous studies in *Xenopus* have demonstrated that *Irx3* is needed to specify the distal segments during frog pronephros nephrogenesis (Alarcon, et al., 2008; Reggiani, et al., 2007). While the function of the zebrafish orthologue *irx3b* has not been assessed, our analysis of early transcription factor expression domains traced *irx3b* expression in medial nephron progenitors and showed a shift of this expression in *lib* mutants. To explore the functional importance of *irx3b* for specifying DE segment fate downstream of RA, we used morpholinos to knockdown this factor and examined nephron segmentation compared to mismatch control-injected animals. We observed pericardial edema in *irx3b* morphants at 72 hpf, suggestive of renal failure or salt imbalance (data not shown), and analyzed nephron segmentation in *irx3b* morphants by whole mount *in situ* hybridization. *irx3b*-deficient embryos lacked a mature DE segment, as revealed by the absence of *slc12a1* and *romk* expression at 24 hpf (Fig. 7; data not shown). In place of the DE segment, the proximal segments were slightly expanded, shown by lengthened domains of the PCT marker *slc20a1a* and PST marker *trpm7*, such that the boundary between these segments was shifted caudally along the trunk (Fig. 7A, 7C). In addition, the corpuscle of Stannius (CS), a gland derived from the tubular epithelium between the DE and DL segments and marked by *gata3*, was dramatically expanded (Fig. 7A, 7C). These results indicate that *irx3b* is essential for the expression of DE-specific markers and suggest that *irx3b* regulates the PCT/PST boundary, as well as restrict CS fate.

Consistent with the onset of *irx3b* expression at 15 somites, *irx3b*-deficient embryos showed no changes in renal gene expression until late somitogenesis. At 28 somites, we found changes in transcription factor domains that correlated with the alterations in the mature nephron segmentation pattern at 28 somites (Fig. S6). For example, *irx3b* morphants possessed an expanded domain of *hnf4a* that coincided with the expansion of PCT and PST segments (Fig. S6B). In addition, the domains of *pou3f3a* and *pou3f3b* were shifted posteriorly, such that they were located next to somite 10 compared to somite 9 in wildtype embryos, also corresponding with the shifted position of the PST (Fig. S6). Interestingly, the posterior boundary of *pou3f3a* was located at somite 14, coincident with the location of the expanded CS population observed in *irx3b* morphants, and possibly representing ectopic expression in these cells. Lastly, the domains of *mecom*, *emx1*, and *gata3* were unchanged in *irx3b* morphants, consistent with the normal location and size of the DL and PD segments (Fig. S6). The finding that transcription factor domains were not altered until approximately the 28 somite stage suggests that *irx3b* function is required late, likely between the 24 and 28 somite stages, to establish a DE-specific gene program and restrict the expression of proximal tubule markers.

The observation that the PCT/PST boundary was posteriorly shifted in *irx3b* morphants led us to test whether *irx3b* knockdown could rescue the shortened proximal segment(s) at the expense of the expanded DE in *lib* mutants. *lib* heterozygous incrosses were injected with either an *irx3b* MO or mismatch control, and then nephron segmentation

was examined at the 28 somite stage. Knockdown of *irx3b* in *lib* abrogated the DE segment, and significantly expanded the length of both the PCT and PST segments (Fig. 7A, 7C). In particular, the PST segment was doubled in length in *lib/irx3b* doubly-deficient animals, occupying the portion of the nephron that would otherwise have formed the DE in *lib* mutants. These findings indicated that while RA signaling is needed to establish the initial PCT and PST progenitor domains, the ultimate size of these segments is not irrevocably set during early nephron patterning and can be altered by modulating the activity of the later-acting gene *irx3b*.

Recent work in *Xenopus* has shown that overexpression of *Irx3* induces ectopic expression of mature distal tubule markers (Alarcon, et al., 2008; Reggiani, et al., 2007). However, it is not clear whether *Irx3* plays an early patterning role during nephron development to specify the DE domain or whether it acts later within this segment to upregulate or restrict gene expression. This issue is challenging to address in the zebrafish as early markers specific to DE progenitors have not been identified and *irx3b* transcripts are found in both PST and DE segments. To resolve this, we made use of DEAB to create embryos with nephrons comprised of DE and DL tubule segments only (Wingert, et al., 2007) (Fig. 7A). Because the PST segment is absent in DEAB-treated embryos (Wingert, et al., 2007), expression of *irx3b* in these animals will specifically mark the DE progenitors. Consistent with this, we found *irx3b* transcripts in the same region of the nephron as the DE marker *slc12a1* at the 28 somite stage (Fig. 7B). Next we investigated whether *irx3b*+ DE progenitors were still present in DEAB-treated embryos that were also injected with the *irx3b* morpholino. Expression of *irx3b* was found in DEAB-treated/*irx3b*-deficient embryos in a region equivalent to where the DE segment would normally form, despite this region now expressing the DL marker *slc12a3* (Fig. 7A, B). These results suggest that two distal domains are patterned in DEAB-treated/*irx3b*-deficient embryos, but that in the absence of *irx3b* activity the DE region fails to express DE-specific solute transporters and instead expresses DL markers. Thus, we conclude that *irx3b* in zebrafish is unlikely to be involved in the early patterning of the DE segment but rather plays a role in its terminal differentiation by regulating the expression DE-specific genes and/or repressing the gene programs of neighboring segments.

DISCUSSION

There are many gaps in our knowledge about the mechanisms that accomplish nephron patterning during kidney development. Here, we show that the nephron segments in the zebrafish pronephros arise following an elaborate series of spatiotemporal gene expression changes in the intermediate mesoderm. We have constructed a detailed transcription factor map that describes this process and is predictive of the mature segmentation pattern of the pronephros. Spatiotemporal expression domains in nephron progenitors during mid- to late-somitogenesis were unaffected by blocking cell proliferation, suggesting that cellular turnover does not significantly account for the dynamic alterations that we detected. Furthermore, our genetic evidence demonstrates that RA is essential for delineating the earliest rostrocaudal patterning events in the intermediate mesoderm, and suggest that RA dosage influences the size and positioning of subsequent transcription factor domains and thereby the mature nephron segmentation pattern. We also show that *irx3b*, acting at relatively late stages of nephron development, is involved in determining the position of the PST/DE boundary and modulating gene expression in the DE segment. Together, our study provides a new molecular map that can be utilized to guide future interrogations into the workings of nephrogenesis, and adds additional insights to previous reports about the ways that RA and *irx3b* influences nephron formation.

RA signals demarcate an early subdivision of pronephros progenitors

Several lines of evidence now indicate that retinoid signaling is crucial for pronephros patterning, and that the location of RA, time of production, and dosage are all crucial. We recently demonstrated that the *caudal* homeobox genes regulate the locale of RA by establishing the expression domains of the *aldh1a2* and *cyp26a1* genes (Wingert, et al., 2007). Axial shifts in the expression of these enzymes were associated with shifts in pronephros position along the embryo trunk, suggesting that the position of RA synthesis had important inductive consequences for nephron patterning (Wingert, et al., 2007). *aldh1a2* expression in the paraxial mesoderm has been suggested to serve as the source of retinoids that pattern the pronephros along with other nearby tissues, like the hindbrain, and our analysis of *tbx16* morphants provides further support for this hypothesis. It should be noted, however, that the disruption of the formation of the paraxial mesoderm via *tbx16* knockdown alters both *aldh1a2* expression and leads to other broad defects, thus we cannot eliminate the possibility that the change in nephron patterning in *tbx16* morphants is affected by disruptions in global patterning. Further studies are needed to delineate between RA-specific and other changes. Given the outcomes of genetic or chemical alterations in RA signaling, we favor the hypothesis that RA produced by paraxial mesoderm acts to pattern nephron progenitors. Proximal tubule segment identities rely on RA for their specification, and dramatic alterations in segment fates ensue when RA levels are greatly elevated or repressed: zebrafish embryos treated with exogenous RA develop nephrons made entirely of proximal segments, while the abrogation of RA production with DEAB leads to nephrons composed entirely of distal segments (Wingert, et al., 2007). Interestingly, segmentation changes only occur if RA bioavailability was altered from the onset of gastrulation through to early somitogenesis stages, establishing an early role for retinoid signaling in pronephros development.

In this study, our analysis of the *lib* (presumptive null allele) and *nls* (hypomorph allele) mutants provides genetic evidence that modulating RA levels produces graded effects on the nephron segmentation pattern. By morphological criteria, *lib* mutants exhibit a more severe RA-deficiency phenotype than *nls*, and form a slightly shorter PST segment concomitant with an expanded DE segment. The rescue of the *lib* mutant phenotype accordingly requires treatment with higher levels of exogenous RA than the *nls* mutant. Taken together, these lines of evidence suggest that RA dosage determines the size of the proximal tubule domain. Intriguingly, partial effects of RA were also observed when embryos were treated with an inhibitor that targets RAR- α activity. Interference with RAR- α led to reduced proximal segments and expanded distal tubule segments, but these changes were not nearly as dramatic as DEAB exposure. This finding could reflect the involvement of other RA receptors during nephron progenitor patterning, though such an interpretation may be complicated by partial drug efficacy in zebrafish cells as compared to other organisms for this particular compound. Future studies, such as RA receptor knockdown experiments, are needed to tease out the specific roles of RA receptors during nephrogenesis, and to address fully whether RA receptor activity is necessary in nephron progenitors themselves.

Our studies raise several intriguing questions about how RA actually affects the nephron progenitors. Cell-autonomy has yet to be assessed regarding RA activity in the nephron progenitors. Another pressing question is whether RA and its receptors cooperate to directly or indirectly modulate the expression of renal genes. Interestingly, a recent study provided evidence that RA participates actively to trigger gene expression of the kidney podocyte lineage. Podocyte precursors express the *Wilm's tumor 1a* (*wt1a*) gene, and *wt1a* promoter analysis discovered the presence of functional RARE elements that were shown to mediate responsiveness of the promoter to RA *in vivo* (Bollig, et al., 2009). Abrogation of RA synthesis in the *lib* mutant (this study) and with chemical inhibitors to *aldh1* enzymes

(Wingert, et al., 2007) blocks or reduces podocyte formation, respectively. These findings illuminate the possibility that RA may act directly, by activating genes required for proximal tubule fate and also by repressing distal (caudal domain) genes. However, these data do not rule out the notion that indirect mechanisms are also operative.

Interestingly, changes in RA synthesis are associated with alterations in the earliest regionalization of the intermediate mesoderm. Nephron progenitors display at least two regional identities between the 6–8 somite stages, which we have termed rostral and caudal. We found a fascinating, partial overlap of these domains at the 8 somite stage, perhaps suggestive of a more complex arrangement of molecular identities that will be better understood as more early renal markers are discovered. Nevertheless, in the *lib* setting of decreased RA production, the rostral nephron progenitor population is reduced while the caudal population is expanded, and we noted previously that DEAB treatment abrogates the rostral domain entirely (Wingert, et al., 2007). These data support the notion that RA ultimately works to induce a rostral progenitor identity among nephron progenitors. While the genes involved in nephron patterning downstream of RA are still unclear, the notch ligand genes *dlc* and *jag2* are excellent candidates, given the role of the Notch pathway in promoting proximal tubule fates during mammalian kidney development (Cheng, et al., 2005; Cheng, et al., 2007).

Conserved and unique elements of *Irx3* function during zebrafish and frog nephrogenesis

Irx genes encode homeodomain transcription factors, and members of this gene family have functions in the development of many tissues. In diverse settings ranging from imaginal discs in flies to the vertebrate neural plate, *Irx* genes act to pattern regional differences amongst cell territories and can both to activate and repress target gene expression (Cavodeassi, et al., 2001). *Irx3* has recently been identified as a requisite player in two aspects of *Xenopus* pronephros development: first, it maintains the pronephric territory at early stages, and second, it is required to form *slc12a1*-expressing distal tubule cells (Alarcon, et al., 2008; Reggiani, et al., 2007).

In this work, we show a conserved requirement for *irx3b* in distal tubule differentiation in the zebrafish pronephros. Knockdown of *irx3b* results in an absence of DE marker expression and concomitant expansions in the lengths of the PCT and PST segments. This phenotype does not appear to be caused by a failure to correctly pattern the nephron into a discrete DE domain as *irx3b* transcripts, which mark presumptive DE progenitors, are still localized to a DE-sized subdomain in DEAB-treated/*irx3b*-deficient embryos. Instead, we propose that in the absence of *Irx3b*, the DE segment adopts either a PST expression profile, in the case of *irx3b* knockdown in wildtype embryos, or a DL expression profile, in the case of DEAB-treated/*irx3b*-deficient embryos. Consistent with a late role for *irx3b* during nephron development, alterations in gene expression domains were only detectable in *irx3b* morphants at time-points that immediately preceded the onset of the segment-specific solute transporter genes. Thus, the transcriptional targets of *Irx3b* may include these transporters (acting as an activator of DE-specific transporters and/or repressor of PST and DL transporters) as well as equivalent transcription factors acting in neighboring segments. In support of this, there is precedence in other tissues for such diverse targets of *Irx* factors; regional patterning in the developing vertebrate brain utilizes a system of transcription factor antagonism between *Irx3* and *Six3*, while *Irx4* and *Irx5* in myocardial cells alters potassium ion channel gene expression (Constantini, et al., 2005; He, et al., 2009; Kobayashi, et al., 2002). We have identified putative Iroquois binding sites upstream of zebrafish *slc12a1*, consistent with the possibility that *irx3b* regulates the expression of this DE-specific transporter (Wingert and Davidson, unpublished).

In contrast, the role of *Irx3* in the maintenance of the pronephros territory does not appear to be conserved in zebrafish, highlighting one functional difference between the frog and zebrafish *Irx3* genes. In zebrafish, at least four *irx* orthologues are expressed in the zebrafish pronephros, *irx2a*, *irx3b*, *irx4a*, and *irx5a*, which may explain the difference between these species (Lecaudey, et al., 2005). Further, *Irx3* expression in *Xenopus* embryos appears to be positively regulated by retinoids, as fewer transcripts are detected in the absence of retinoids while exogenous RA is associated with elevated transcript numbers (Alarcon, et al., 2008). These alterations appear linked to early changes in the mesoderm destined to form the pronephros rather than later proximodistal patterning events (Alarcon, et al., 2008). While RA similarly appears to function upstream of *irx3b* in the zebrafish pronephros, changes in the *irx3b* domain correlate with altered nephron patterning.

Insights into conserved nephron segmentation mechanisms among vertebrates

This study has identified critical roles for RA and *irx3b* during zebrafish pronephros segmentation, but other vital signals have yet to be determined. Our characterization of transcription factor gene expression in the intermediate mesoderm has revealed a complex molecular profile that is spatially and temporally dynamic, and future functional analysis of these genes will reveal key determinants of segment identity and cross-regulatory interactions. A similar level of transcriptional complexity is beginning to be realized during mammalian nephrogenesis, largely in part due to the efforts of the GenitoUrinary Development Molecular Anatomy Project (GUDMAP), which has mapped the expression of mouse genes to different compartments of the developing metanephric kidney (Brunskill, et al, 2008; Georgas, et al., 2009; McMahon, et al., 2008). Findings from the GUDMAP, as well as previous studies, have documented a set of genes with regionalized domains within murine nephrons. For example, *Pou3f3* is restricted to prospective distal regions of the nephron, similar to our observations of the zebrafish orthologues *pou3f3a* and *pou3f3b* (Nakai, et al., 2003). It is likely that many of the same transcriptional pathways underlie proximodistal nephron patterning in all vertebrates. Therefore our work, establishing a detailed molecular description of gene expression during nephrogenesis, should provide a rich source of data for generating hypothesis-driven experiments in other model organisms such as the mouse. A greater knowledge of the molecular basis of vertebrate nephron development will help elucidate the causes of congenital kidney defects, which are often poorly understood, as well as further our understanding of the regulatory networks that maintain nephron function in the adult.

EXPERIMENTAL PROCEDURES

Zebrafish husbandry, genetic strains and *lib* mutant mapping

Zebrafish were maintained and staged as described (Kimmel, et al., 1995). Tübingen strain embryos were used for all experiments, and collected from natural matings. *lib* was recovered in a diploid ENU (N-ethyl-N-nitrosurea) screen for mutations affecting kidney organogenesis, performed as described (Solnica-Krezel, et al., 1994). Families were screened to isolate mutants with kidney development defects, as manifested by edema between 24 and 72 hpf. *lib* was cloned using meiotic mapping. *lib* mapping strains were generated by mating *lib* mutant carriers, generated and maintained on the Tübingen strain, were to the polymorphic WIK strain. Embryos were collected from pairwise matings of *lib* Tü/WIK heterozygotes, and scored at 72 hpf for edema. A panel of 239 CA simple sequence length polymorphisms (SSLP) markers were used to scan chromosomes and establish linkage as described (Wingert, et al., 2005). *aldh1a2* transcripts were isolated from wild-types and *lib* by reverse transcription PCR, using the following primers: forward ATGACCTCCAGTG-AAGTTGAACTG and reverse, TTAAGACGTCTTGCCTGACATCTT, and subcloned into pGEMT-easy (Promega) for

sequence analysis. For *lib* rescues (and *aldh1a2* expression studies, see below), full-length wild-type *aldh1a2* was subcloned into the pCS2 expression vector using the EcoRI and XhoI sites. WISH *aldh1a2* probe was generated by linearizing the plasmid with EcoRI and performing antisense RNA synthesis with T7 RNA polymerase (Roche).

Embryo chemical treatments, morpholinos and *in situ* hybridization analysis

Reported expression patterns show representative results as gathered from analysis of between 10 and 15 animals per each timepoint and genotype (mutant studies and knockdown). Gene expression domains as reported by somite boundaries were based on counts of at least 5 separate samples to ensure accurate documentation.

For chemical treatments, wild-type embryos were incubated with camptothecin and nocodazole, as described (Murphey, et al., 2006). Briefly, cell cycle inhibitors (Tocris) were dissolved to make stock concentrations of 10 mg/mL dissolved in 100% dimethyl sulfoxide (DMSO), and embryos treated at the established effective dose (Murphey, et al., 2006). For *lib* rescue studies, embryos from *lib* or *nls* heterozygous incrosses were incubated in RA treatment, as described (Wingert, et al., 2007). Briefly, all-trans retinoic acid (Sigma) was dissolved in 100% DMSO to make a 1 M stock, aliquots stored at -80°C , and embryos treated with $1 \times 10^{-8}\text{M}$ or $1 \times 10^{-9}\text{M}$ RA/DMSO, which were made by performing serial dilution of the 1M stock in E3 embryo water. For RA agonist and antagonist studies, RO 41-5232 and AM-580 (Tocris) were similarly dissolved in 100% DMSO to make a 1 M stock, aliquots stored at -80°C , and wildtype embryos were incubated in $1 \times 10^{-8}\text{M}$ RO 41-5232/DMSO or $1 \times 10^{-7}\text{M}$ AM-580/DMSO diluted in E3 embryo water. All chemical treatments were fully penetrant and produced consistent results at the doses and treatment windows that were examined.

For *Aldh1a2* rescue studies in *lib*, the full-length open reading frame of wildtype and *lib aldh1a2* alleles were subcloned into the EcoRI/XhoI sites of the pCS2 expression vector. Plasmids were linearized with NotI, and synthetic capped RNA was synthesized using SP6 mMessage machine (Ambion). Embryos were injected with 100 pg of cRNA at the 1-cell stage.

For all morpholino experiments, morpholinos were solubilized as recommended by Gene-Tools at and stored at a 4mM concentration. Dilution of morpholino stocks were injected into 1-cell stage wild-type embryos. The *tbx16* morpholino (AAGACAAGTACTCACCTCTGATAGC) targets the exon 1 donor splicing site and produces consistent results at the injected dose (Burns, et al., 2009). *irx3b* morpholinos were designed to target the 5' untranslated region and start site morpholinos, and gave similar results upon injection of ~500 pl of a 0.15 mM working dose and 0.2 mM dose, respectively: (ACCGGGAGGACTGCGGGGAACTCG) and (ATAGCCTAGCTGCGGGAGAGACATG), respectively. A 5 base-pair mismatch start site morpholino (ATTGCCTACCTGGGGGACAGAGATG) was injected for a control comparison. Whole mount *in situ* hybridization for *cdh17*, *clck*, *dlc*, *gata3*, *jag2*, *mecom*, *mhc*, *myoD*, *nbc1*, *pax2a*, *pax8*, *raldh2*, *wt1a* and *wt1b* were performed as described (Wingert, et al., 2007). Expression of *emx1*, *etv5*, *grhl2a*, *hnf1ba*, *hnf1bb*, *hnf4a*, *irx3b*, *pou3f3a*, *pou3f3b* were previously reported (Hauptmann and Gerster, 2000; Kawahara and Dawid, 2002; Lecaudey, et al., 2005; Thisse, et al., 2001).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Zebrafish nephron segments were abbreviated as follows	C, cloaca
CS	corpuscle of Stannius
DE	distal early
DL	distal late
G	glomerulus
N	neck
P	podocytes
PCT	proximal convoluted tubule, PST, proximal straight tubule

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Figure 1. Pronephros progenitors are delineated into a series of molecularly distinct regions during early somitogenesis that are RA-dependent

Gene expression patterns in the nephron territory in wildtype embryos and *lib* mutants at the (A) 8 somite stage (B) 15 somite stage and schematized respectively (C–D). Embryos were flat-mounted to remove the yolk and are shown in dorsal views with anterior to the left.

Whole mount *in situ* hybridization was used to mark kidney gene expression (purple) and the somites with *myoD* (red). Black lines indicate areas of kidney gene expression and numbers correspond to the somite position. (A) In 8 somite wildtypes, *pax2a* transcripts marked all nephron progenitors while *dlc* and *jag2* expression was restricted proximally and *mecom* expression was restricted distally; a short stretch of overlap between the *jag2* and *mecom* domains was evident. *lib* embryos had reduced *dlc* and *jag2* domains, and an expanded *mecom* domain. (B) In 15 somite wildtype embryos, *hnf1ba* marked all nephron progenitors, and the other transcription factors were restricted to subsets of cells in the rostral (*hnf4a*, *hnf1bb*), central (*irx3b*), or caudal (*pou3f3b*, *mecom*, *emx1*, *gata3*) areas of the nephron territory. *lib* had a shortened rostral *hnf4a* area while the central and caudal were expanded. (C–D) Schematic depictions of nephron progenitor domains: (top) labeled boxes indicate somite number, and (below) summary of rostral (purple), central (blue) and caudal (green) areas of the nephron progenitors. At 8 somites, the overlap between rostral and caudal areas is depicted as dark purple-colored subset of the rostral area. At 15 somites, subsets of the caudal area are depicted as dark green and light green; gray indicates divergence of non-tubule populations (future podocytes and neck segment cells) at the most

anterior regions of the renal progenitors. Asterisk marks the posterior limit of the rostral area, which shifted anteriorly in *lib* at the 8 and 15 somite stages.

Figure 2. Dynamic spatiotemporal expression changes during late somitogenesis precede the appearance of segment identities in the pronephros

Gene expression patterns of transcription factors in nephron progenitors in wildtype embryos at the 20, 22, 24, and 28 somite stage. Embryos are shown in lateral views with anterior to the left, and have been stained by whole mount *in situ* hybridization to mark kidney gene expression (purple) and the somites with *mhc* (red). Black lines indicate areas of high gene expression, and numbers correspond to the somite position. The spatial distributions of *hnf1ba*, *hnf4a*, *hnf1bb*, *emx1*, and *gata3* transcripts were unaltered between 20–28 somites of development, though note that the proximal boundary of the *emx1* and *gata3* domains at 20 somites changed since the 15 somite stage (compare to Figure 1B). The

expression domains of *pou3f3a* and *pou3f3b* were altered at 22 and 28 somites, the *irx3b* and *grhl2a* expression domains changed at 24 somites, and the *etv5* domain was restricted at the 28 somite stage. The expression domain of *mecom* exhibited multiple alterations between 20–28 somites.

Figure 3. Analysis of gene expression domain dynamics in the pronephros progenitors reveals that progressive refinements ultimately correlate with specific segment fates
(Foreground) Gene expression domains (represented by black bars) are schematized in the pronephros territory between 8 and 28 somite stages. (Background) Nephron segment identities form adjacent to particular somites (demarcated by numbered columns, top), and each segment region is uniquely color-coded to envisage a comparison between gene expression domains at each timepoint with the eventual nephron segment which arises at any given physical location in the pronephros territory. Segments and color codes are N (neck)-green, PCT (proximal convoluted tubule)-orange, PST (proximal straight tubule)-yellow, DE (distal early)-light blue, DL (distal late)-dark blue, PD (pronephric duct)-dark gray, and C (cloaca)-light gray.

Figure 4. *lib* is the most severe genetic model of *aldh1a2*-deficiency in the zebrafish

(A) Lateral views of living 24 and 48 hpf wildtype embryos, *nls* homozygotes, *nls/lib* compound heterozygotes, *lib* homozygotes, and DEAB-treated wildtypes. *aldh1a2* mutations are associated with a truncation of the cervical region evident at 24 hpf (arrows indicate the caudal boundary of the otic vesicle (OV) and the rostral boundary of the first somite (S1)). DEAB-treated embryos lack this region, with the OV and S1 located adjacent to each other (indicated by single arrow). At 48hpf, *aldh1a2* mutations are associated with a kink at the head-trunk boundary (black arrow) and pericardial edema (*). Also at 48 hpf, *lib* and DEAB-treated embryos show pronounced body curvature and develop a lightbulb-shaped yolk compared to wildtypes and other *aldh1a2* mutants. (B) Meiotic mapping placed *lib* in a genetic interval on linkage group (LG) 7 between z8693 and z11894 that includes *aldh1a2*. (C) RT-PCR and sequence analysis of *aldh1a2* transcripts from *lib* mutants detected a C → A transversion at nucleotide 174 that is predicted to introduce a Stop codon in lieu of a Tyrosine residue at amino acid position 58 in the 519 amino acid raldh2 enzyme. (D) Whole mount *in situ* hybridization analysis of *aldh1a2* expression (purple) and *krox20* expression (red) shows decreased *aldh1a2* transcripts both in *lib* mutants and *lib* heterozygote embryos, consistent with nonsense mediated decay.

Figure 5. Genetic and chemical models of RA biosynthesis deficiency exhibit a similar nephron segment phenotype of reduced proximal fates and expanded distal fates, and *lib* is the most severely affected *aldh1a2* mutant

Whole mount *in situ* hybridization analysis for nephron segment markers (purple) and *mhc* (red) at the 28 somite stage in *aldh1a2* mutants and DEAB-treated wildtype embryos. Embryos are shown in lateral views with anterior to the left, with the exception of dorsal views to show podocytes. Black brackets and arrows indicated expression domains, and numbers correspond to somite position. (A) Proximal fates of podocytes, the PCT and PST, were all reduced in the absence of RA biosynthesis, while (B) distal fates of the DE, DL, CS, and PD were expanded. (C) Summary of nephron segmentation with respect to embryo somite number. Each color represents a different epithelial population: podocytes (green), PCT (orange), PST (yellow), DE (light blue), CS (red lettering indicates location among nephron tubule cells), DL (dark blue) and PD (black), and the overlap between DL and PD-

expressed genes is indicated in white. The nephron segment phenotypes among RA-deficient zebrafish embryos showed a graded severity such that: *nls* homozygote < *nls/lib* compound heterozygotes < *lib* homozygotes < DEAB-treated wildtype embryos.

Figure 6. *tbx16*-dependent paraxial mesoderm formation is essential for proximo-distal nephron patterning

Whole mount *in situ* hybridization analysis for nephron segment markers (purple) and *mhc* (red) at the 28 somite stage in wildtype embryos and *tbx16* MO-injected embryos. Embryos are shown in lateral views with anterior to the left. Black brackets and arrows indicate expression domains, and numbers correspond to somite position. (A) *tbx16* knockdown in wildtypes was associated with formation of reduced podocyte numbers, shown by reduced *wt1b* expression, and shorter proximal segments, shown by smaller *nbc1*, *slc10a1a* and *trpm7* gene expression domains; conversely, distal segments were expanded, shown by longer *clck*, *slc12a1*, and *slc12a3* expression domains. The DL (*gata3*-expressing segment) was unaffected. (B) *tbx16* morpholino injected animals have a reduced rostral domain (*hnf4a*), and shifts in the central (*irx3b*), and caudal (*mecom*) domains at 28 somites that are consistent with shorter proximal and expanded distal segments. (C–D) *tbx16* morpholino injected animals have dramatically reduced expression of *aldh1a2* at 28 somites (C) and 5 somites (D).

Figure 7. *irx3b* activity is requisite for DE segment identity, and the absence of RA and *irx3b* activity generates nephrons with a DL-only tubule identity

(A) Whole mount *in situ* hybridization analysis for nephron segment markers (purple) and *mhc* (red) at the 28 somite stage in wildtype embryos, *irx3b* MO-injected embryos, *lib* homozygous mutants, *lib* homozygous mutants injected with *irx3b* MO, DEAB-treated embryos, and DEAB-treated embryos injected with *irx3b* MO. Embryos are shown in lateral views with anterior to the left. Black brackets and arrows indicated expression domains, and numbers correspond to somite position. *irx3b* knockdown in wildtypes and *lib* mutants was associated with DE segment abrogation, expansion of the PCT and PST segments as well as the CS population. In DEAB-treated embryos, which normally only form DE and DL tubule segments, *irx3b* knockdown was associated with expansion of the DL while the PD is unchanged. (B) In DEAB-treated embryos, *irx3b* expression marked the DE domain that becomes defined by expression of *slc12a1*. DEAB-treated embryos injected with *irx3b* MO showed expression of *irx3b* in the same region of the pronephros, suggesting that DE regionalization had been established, but that in the absence of *irx3b* DE-specific solute transporter expression does not ensue. (C) Summary of nephron segmentation with respect to embryo somite number. Asterisks in the proximal DL of DEAB-treated wildtypes injected with *irx3b* MO indicate partial regionalization that is blocked/prevented in these animals.

Figure 8. Model of how RA and *irx3b* signals sequentially pattern the pronephros territory to generate discrete nephron segments

Nephron progenitors, derived from the intermediate mesoderm (IM) are situated adjacent to the paraxial mesoderm (PM). The rostral PM (indicated by asterisks, *) provides an early local source of RA, which is thought to diffuse to nearby tissues. Subsequent to RA signaling, nephron progenitors are broadly regionalized into rostral and caudal domains by the 6–8 somite stages. At the 15 somite stage, a nested pattern of transcription factor domains suggests the continued presence of broad rostral and caudal domains as well as an overlapping medial domain, defined by *irx3b* expression. Activity of *irx3b* sets the DE identity given its dynamic spatiotemporal shifts in this vicinity during later somitogenesis stages. Segment epithelia have unique solute transporter expression patterns at the 28 somite stage, concomitant with an end to many spatiotemporal transcription factor domain shifts in the nephron progenitors.