The *tbx2a/b* transcription factors direct pronephros segmentation and corpuscle of Stannius formation in zebrafish

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**ABSTRACT**

The simplified and genetically conserved zebrafish pronephros is an excellent model to examine the intricate processes of cell fate decisions during the development of nephron segments as well as the origins of associated endocrine cells that comprise the corpuscles of Stannius (CS). Using whole mount *in situ* hybridization, we found that transcripts of the zebrafish genes *t-box 2a (tbx2a)* and *t-box 2b (tbx2b)*, which belong to the T-box family of transcription factors, were expressed in the caudal intermediate mesoderm progenitors that give rise to the distal pronephros and CS. Deficiency of *tbx2a, tbx2b* or both *tbx2a/b* reduced the size of the distal late (DL) segment, which was accompanied by a proximal convoluted segment (PCT) expansion. Further, *tbx2a/b* deficiency led to significantly larger CS clusters. These phenotypes were also observed in embryos with the *from beyond* (*fby*) mutation, which encodes a premature stop codon in the *tbx2b* T-box sequence. Conversely, overexpression of *tbx2a* and *tbx2b* in wild-type embryos expanded the DL segment where cells were concomitant with the adjacent DE, and also decreased CS cell number, but notably did not alter PCT development—providing independent evidence that *tbx2a* and *tbx2b* are each necessary and sufficient to promote DL fate and suppress CS genesis. Epistasis studies indicated that *tbx2a* acts upstream of *tbx2b* to regulate the DL and CS fates, and likely has other targets as well. Retinoic acid (RA) addition and inhibition studies revealed that *tbx2a* and *tbx2b* are negatively regulated by RA signaling. Interestingly, the CS cell expansion that typifies *tbx2a/b* deficiency also occurred when blocking Notch signaling with the chemical DAPT (*N*-[3,5-diethylaminobenzylidene]-S-phenylglycine t-butyl ester). Ectopic activation of Notch in *Tg(hsp70::Gal4; UAS::NICD (NICD))* embryos led to a reduced CS post heat-shock induction. To further examine the link between the *tbx2a/b* genes and Notch during CS formation, DAPT treatment was used to block Notch activity in *tbx2a/b* deficient embryos, and *tbx2a/b* knockdown was performed in NICD transgenic embryos. Both manipulations caused similar CS expansions, indicating that Notch functions upstream of the *tbx2a/b* genes to suppress CS ontogeny. Taken together, these data reveal for the first time that *tbx2a/b* mitigate pronephros segmentation downstream of RA, and that interplay between Notch signaling and *tbx2a/b* regulate CS formation, thus providing several novel insights into the genetic regulatory networks that influence these lineages.

**1. Introduction**

Kidneys are excretory organs with an intricate architecture and a rich diversity of cell types. Typically, renal cells are arranged into functional units known as nephrons, while other cells constitute interstitial supporting populations. Vertebrate kidney development progresses through a series of two or three stages, depending on the species, in which a pronephros, mesonephros, and a metanephros are formed (Saxen, 1987). Each kidney organ is composed of nephrons, though their numbers increase and their arrangements are progressively more complex with each successive stage (Dressler, 2006). For example, in mammals the pronephros and mesonephros contain up to 50 million nephrons each, while the metanephros contains only about 1 million nephrons. The simplification and genetic conservation of the zebrafish pronephros has made it an excellent model to examine the intricate processes of cell fate decisions during the development of nephron segments as well as the origins of associated endocrine cells that comprise the corpuscles of Stannius (CS).

The corpuscle of Stannius formation in zebra
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several dozen nephrons that are situated in parallel arrays, while the ensuing adult metanephric kidney has thousands or even millions of nephrons that are organized in elaborate arborized configurations (Takasato and Little, 2015).

At present, one major aspect of vertebrate kidney development that remains largely enigmatic is how nephron tubular epithelial cells acquire segment-specific fates during nephrogenesis. The meager understanding of nephron segmentation is due in part to the complexity of mammalian kidney anatomy and limited models to study nephrogenesis in vitro (Costantini and Kopan, 2010). However, nephron structure is broadly conserved among vertebrates (Romagnani et al., 2013). Furthermore, in recent years there has been an increasing appreciation of the usefulness of the zebrafish pronephros as a simplified, genetically tractable experimental system for nephrogenesis studies in the context of organ development and regeneration (Drummond and Wingert, 2016).

The embryonic zebrafish kidney is a functional pronephros composed of two nephrons that form rapidly, becoming segmented into phenotypically distinct regions by 24 hours post fertilization (hpf) (Gerlach and Wingert, 2013). Each segment plays discrete and essential roles in renal physiology including, but not limited to, the absorption and secretion of particular metabolites and electrolytes, which is mediated by the expression of solute transporters (Ebarasi et al., 2011). It has been shown that the solute transporter genes that are expressed in each pronephric segment correspond with genes that are expressed in similar segments in nephrons of the mature mammalian kidney, thereby establishing the relevance for segmentation research using zebrafish (Wingert et al., 2007; Wingert and Davidson, 2008). These segments include the podocytes (P), neck (N), proximal convoluted and straight tubule (PCT, PST), distal early and late (DE, DL) tubule, and a pronephric duct (PD) (Wingert et al., 2007; Wingert and Davidson, 2008). In zebrafish, nephron segment patterning is known to be reliant on retinoic acid (RA), produced largely from the paraxial mesoderm, which divides the renal progenitor field (derived from the intermediate mesoderm) into rostral and caudal domains that are further induced to form the series of tubule segments (Wingert et al., 2007; Wingert and Davidson, 2011). The ongoing application of the zebrafish pronephros model has begun to further elucidate the cast of key transcription factors and signaling pathways that are expressed by developing nephron segments, and defined a growing number of their functional roles, such as Notch signaling in governing tubule epithelial fate choices (Ma and Jiang, 2007; Liu et al., 2007; O’Brien et al., 2011; Naylor et al., 2013; Kroeger and Wingert, 2014; Li et al., 2014; Gerlach and Wingert, 2014; McKee et al., 2014; Marra and Wingert, 2014, 2016; Miceli et al., 2014; Cheng and Wingert, 2015).

In addition to the pronephros, the intermediate mesoderm field gives rise to groups of endocrine cells called the corpuscles of Stannius (CS) that are initially located in the vicinity of the DE and DL segment precursors and then later coalesce into a pair of clustered organs that are situated dorsal to the pronephros (Elizondo et al., 2005; Wingert et al., 2007). The CS are endocrine glands found in bony fish (Garrett, 1942; Krishnamurthy, 1976). The CS are responsible for the synthesis and secretion of stanniocalcin 1 (STC1), a glycoprotein hormone that regulates calcium and phosphate homeostasis in fishes through its actions on the gills and kidneys (Elizondo et al., 2005; Krishnamurthy, 1976; Kaneko et al., 1992). As such, the CS are thought to be important regulators of calcium uptake from the aquatic environment (Elizondo et al., 2005). Although the CS and the proteins they secrete were previously considered to be an endocrine system that is unique to fishes, intriguing evidence has implicated the existence of STC-like proteins in humans and other higher vertebrates (Wagner et al., 1995; Chang et al., 1995). To date, however, not much is known about the genetic factors that induce the CS lineage or regulate its differentiation. CS fate is reliant on RA signaling within the zebrafish embryo, where elevated RA levels block CS formation and RA biosynthesis abrogation expands the CS (Wingert et al., 2007). More recently, the single-minded family bHLH transcription factor 1a (sim1a) was discovered to be both necessary and sufficient for CS formation, and shown to act downstream of RA in promoting stc1-expressing cell fate (Cheng and Wingert, 2015).

In searching for other candidate nephron segmentation modulators, we noticed that expression of the t-box 2b (tbx2b) transcription factor was annotated within the distal pronephros at 24 hpf during zebrafish embryogenesis, similar to its paralog t-box 2a (tbx2a) (Dheen et al., 1999; Thiese and Thisse, 2004; Slanchev et al., 2011; Thu et al., 2013), in an area that is now known to correspond to the site of the distal tubule, duct, and CS (Wingert et al., 2007). Interestingly, in situ hybridization showed Tbx2 expression in the mesonephros and also in the E12.5 metanephric tubules of the developing murine kidney (Chapman et al., 1996). More recently, Tbx2 expression was also reported in the ureretic bud tips (GUDMAP: 10896), which give rise to the collecting duct system (Little and McMahon, 2012). Previous research in Xenopus has shown that Tbx2 loss of function results in an enlarged pronephros, whereas ectopic activation of Tbx2 inhibits nephric mesoderm differentiation in embryos (Cho et al., 2011). Further, Tbx2 repressed expression of the Notch factor Hey1 to control pronephric morphogenesis, suggesting a link between Tbx2 and Notch during nephrogenesis in Xenopus (Cho et al., 2011). Until the present study, however, no one had examined the roles of tbx2a/b in nephron segment formation.

Here, we identified tbx2a and tbx2b as essential regulators of pronephros and CS development. Gene knockdown studies and analysis of the tbx2b mutant from beyond (fbzf4144) showed that loss of one or both tbx2a and tbx2b led to a short DL segment and significantly larger CS clusters. Further, overexpression of tbx2a or tbx2b was sufficient to expand the DL and significantly reduce CS cell number. Epistasis experiments revealed that tbx2a acts upstream of tbx2b, and suggested that tbx2a mitigates the DL and CS lineages by regulating an additional target(s) as well. In testing the relationship of tbx2a/b genes with previously known nephron patterning pathways, we determined that RA signaling negatively regulates their spatiotemporal expression. Finally, we found that Notch signaling is essential to restrict CS fate, and that the tbx2a/b transcription factors act downstream of Notch to repress CS formation. In sum, this research has identified new roles for tbx2a/b in nephron segmentation, and revealed for the first time that Notch signaling and tbx2a/b modulate CS genesis.

2. Materials and methods

2.1. Zebrafish husbandry and ethics statement

Zebrafish were housed in the Center for Zebrafish Research at the University of Notre Dame Freimann Life Science Center. The Institutional Animal Care and Use Committee (IACUC) approved studies all under protocols 16–025 and 16-07-3245. Wild-type Tübingen, fbzf4144, and hspa70::Gal4; UAS::Nicd adults and embryos were maintained and staged as previously described (Westerfield, 1993; Kimmel et al., 1995). Embryos were raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) at 28 °C (Westerfield, 1993).

2.2. Whole mount and fluorescence in situ hybridization

Whole mount in situ hybridization was performed as described (Cheng et al., 2014). Antisense riboprobes were generated for smyc1, slc12a1, slc12a3, slc20a1a, trpm7, stc1, and sim1a, as previously reported (Wingert et al., 2007; Cheng and Wingert, 2015). The DNA template for the tbx2a riboprobe was generated from IMAGE clone 6964146 using the PCR primers (5'- ATGCGTATTATCCCTTTTCACGCGCACAGGCCGGCCG-3' and 5'-ATTAACCTCTAATAGGTCCTTCCAGAGTGTGTTCCGAAGTGAGTGCTGCCG-3').

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The DNA template for tbx2b was generated from IMAGE clone 5914621 with PCR primers (5'-ATGAGAGATCCAGTTTTTAC-3', 5'-AATTAATCATCCACTAAGGGTACATTGGTGA-3'). PCR products were purified using the Qiagen PCR purification kit and riboprobes generated using t3 RNA polymerase. Detection was performed using anti-digoxigenin and anti-fluorescin antibody (Roche) and NBT/BCIP/INT staining reagents. Fluorescent whole mount in situ hybridization was performed as described (Brend and Holley, 2009).

2.3. Morpholinos

All morpholino oligonucleotides were obtained from Gene Tools, LLC. tbx2a splice morpholino was designed to target the splice acceptor site of exon 1 (5' -GGAAACATTCTCTATGACGAAAG-3') (Thu et al., 2013). tbx2b splice morpholino was designed to target the splice donor site of exon 3 (5'-AAAATATGGGTACATACCTTGTCG-3') (Ribiero et al., 2007); tbx2b ATG morpholino (5' -CCCTAGAACTGATCTGCTATCGG-3') (Gross and Dowling, 2005); tbx2ab ATG morpholino (5' -AAAACTGATCTCTCATCGGTAC-3') (Sedletcaia and Evans, 2011). Embryos at the 1-cell stage were injected with approximately 2 nl of morpholino from a 0.25 mM dilution. Injected embryos were incubated at 28 °C until fixation in 4% paraformaldehyde/PBST at the 28 ss. Embryo undergoing gene expression analysis were stored in methanol at −20 °C.

2.4. RT-PCR

RNA was extracted from 24 hpf tbx2a and tbx2b splice morphants using Trizol (Invitrogen). For tbx2a morphant and control cDNA, primers were used to amplify exons 1–3 of tbx2a (5’ -GGCTGACCTCTGTGATTACAT-3’; 5’ -GTCGTTGGCTCTAACGATATGAAACCTGGGTTGGTATTTG-3’; 5’ -TGCATTGAATTTA-3’). Regions within exon 2 were amplified using RT-PCR (5’ -AAAAATATGGGTACATACCTTGTCG-3’). Regions within exon 1 were amplified using 5’ -GGAAACATTCTCTATGACGAAAG-3’. Regions within exon 3 were amplified using 5’ -AAAATATGGGTACATACCTTGTCG-3’. Regions within exon 2 were amplified using 5’ -GTCGTTGGCTCTAACGATATGAAACCTGGGTTGGTATTTG-3’ (Ribeiro et al., 2007).

2.5. Overexpression studies

The open reading frames of zebrafish tbx2a and tbx2b were subcloned into the pCS2 vector. To prepare for in vitro transcription reactions, the tbx2a template was linearized using NotI restriction enzyme, and the tbx2b template was linearized with ApaI I. cDNA for each gene was produced using the mMessage mMachine Sp6 kit (Ambion). cRNAs were injected into 1 cell stage embryos and fixed in PFA at 24 hpf.

2.6. Chemical treatments

1 M stocks of RA and DEAB were created by dissolving compounds (Sigma-Aldrich) in 100% DMSO aliquots and stored at −80 °C (Wingert et al., 2007; Lengerke et al., 2011). For RA treatments, embryos at the 60% epiboly stage were incubated in RA/DMSO/E3 solutions until 24 hpf. Three RA treatment doses were tested; 1×10^{-6} M, 1×10^{-7} M, and 1×10^{-8} M. For DEAB treatments, 1.6×10^{-7} M DEAB/DMSO was administered to embryos at either 50% epiboly or 90% epiboly, washed off at 24 hpf, and embryos were fixed as previously described.

2.7. Genotyping and heat shock experiments

Heterozygous mutants were identified using fin clip DNA and genotyping assays as detailed in previous studies: fshb144 (Snelson et al., 2008) and hsp70::Gal4; UAS::NICD (Scheer and Campos-Ortega, 1999; Scheer et al., 2002). NICD heat shock experiments were conducted by incubating embryos at the 15 somite stage in 37 °C for 1 h. Embryos were then incubated at 28 °C until the 28 ss and processed as described earlier.

2.8. Cell counts and statistics

At least 15 embryos were examined for each morpholino and drug treatment experiment, which were performed in triplicate. A minimum of three genotype-confirmed mutant embryos were acquired for each condition represented. CS cell counts were taken in each of the two nephrons in the embryos examined. CS counts were averaged across three replicates for each condition and tested for significance using a non-paired t-test.

3. Results

3.1. Expression of tbx2a and tbx2b during zebrafish intermediate mesoderm development

Due to the teleost genome duplication event, zebrafish possess two tbx2 genes, designated tbx2a and tbx2b, which share approximately 80% peptide identity (Sedletcaia and Evans, 2011). Previous studies have shown that tbx2b is strongly expressed in the distal pronephros at 24 hpf (Dheen et al., 1999; Slanchev et al., 2011). In comparison, slightly discrepant patterns of tbx2a have been reported in the distal pronephros, ranging from weak to strong expression in the area (Dheen et al., 1999; Thisse and Thisse, 2004; Thu et al., 2013). Despite the variations, these data suggest that tbx2a and tbx2b might be involved in nephrogenesis, which occurs during the first day of zebrafish embryo development when each bilateral stripe of intermediate mesoderm precursors are assembled into nephron segments or a closely associated CS gland (Fig. 1A; Cheng and Wingert, 2014).

To explore this, we first performed additional expression studies to clarify the spatiotemporal domains of tbx2a and tbx2b in the intermediate mesoderm. Whole mount in situ hybridization with RNA antisense riboprobes specific for tbx2a or tbx2b transcripts, as well as corresponding sense controls, was conducted on wild-type embryos at numerous developmental stages during the time frame when renal progenitors undergo segmental patterning (Fig. 1B, Supplementary Fig. 1). tbx2a and tbx2b transcripts were present in the caudal intermediate mesoderm as early as the 8 somite stage (ss), and were maintained in the developing pronephros through the 28 ss (Fig. 1B, Supplementary Fig. 1). Notably, at the 14 ss, tbx2a was expressed in a larger domain of the intermediate mesoderm compared to tbx2b, which occupied a shorter domain and was expressed weakly in that area (Fig. 1B, C). By the 16 ss, tbx2a and tbx2b occupied analogous pronephros domains, though tbx2b expression was weaker (Fig. 1C). At the 28 ss, or approximately 24 hpf, when segmentation of the pronephros is fully established, transcripts encoding tbx2a and tbx2b were restricted to the area where the DL and the PD reside within the pronephros (Fig. 1B, Supplementary Fig. 1). As demonstrated by fluorescent in situ hybridization at the 28 ss, tbx2a and tbx2b were co-localized in this distal pronephros area, though the tbx2b renal expression domain was slightly broader, and tbx2a was more robustly expressed in the posterior duct region (Fig. 1D). These findings suggested that tbx2a/b are localized to renal progenitors during nephrogenesis, and specifically implicated them as possible candidates for regulating distal intermediate mesoderm fates such as the DL segment.

Due to the spatial distribution of tbx2a and tbx2b expression at the
28 ss, we next determined if the transcripts were localized to a particular emergent lineage(s) in the pronephros and/or the CS glands. To assess their presence in the DL, we performed double fluorescent in situ hybridization with \( slc12a3 \). Transcripts encoding \( tbx2a \) and \( tbx2b \) were co-localized to \( slc12a3 \)-expressing cells (Fig. 2A). The CS lineage is situated at the anterior aspect of the DL at this time, being a small group of cells that emerge from the intermediate mesoderm in a location next to the 15th somite (Elizondo et al., 2005; Wingert et al., 2007), which co-express \( sim1a \) and \( stc1 \) (Fig. 2B). Given this close proximity of the CS and the DL, we assessed expression of \( stc1 \) along with \( tbx2a \) or \( tbx2b \) (Fig. 2B). Interestingly, \( tbx2a \) transcripts were not detectable in \( stc1+ \) cells while all \( stc1 \)-expressing cells were \( tbx2b + \) (Fig. 2B). These data confirmed our hypothesis that \( tbx2a \) and \( tbx2b \) are expressed in the DL at the 28 ss, and revealed that \( tbx2b \) is also expressed by the CS lineage at this stage.

### 3.2. \( tbx2a/b \) promote the formation of the DL segment

To investigate the possibilities that \( tbx2a \) and/or \( tbx2b \) participate in nephron patterning, we next undertook loss of function studies. We utilized gene knockdowns and a \( tbx2b \) deficient mutant, \( fgy^{cht} \), which encodes a T-to-A transversion that introduces a premature stop codon.
within the T-box DNA-binding domain (Snelson et al., 2008). For the knockdowns, previously reported morpholinos (MOs) for tbx2a, tbx2b and tbx2a/b were used that either targeted the start site of the gene or interfered with pre-mRNA splicing (Thu et al., 2013; Gross and Dowling, 2005; Ribeiro et al., 2007; Sedletcaia and Evans, 2011). We used RT-PCR to validate that the use of tbx2a and tbx2b splice-blocking MOs interrupted splicing activity, which in each case led to some products that are predicted to encode truncated proteins (Supplementary Fig. 2). Although wild-type transcripts were still produced when implementing each splice-blocking MO, the expression of transcripts encoding Tbx2 peptide truncations that remove the 3’-terminal region has been reported to produce dominant-negative effects (Stott et al., 1993; Rao, 1994; Dheen et al., 1999). Therefore, to next assess pronephros development, wild-type embryos were injected at the 1-cell stage, and then morphants and uninjected siblings were fixed at the 28 ss to examine nephron segmentation using double whole mount in situ hybridization. To help define alterations in nephron segment pattern formation, we detected transcripts encoding slow myosin heavy chain 1 (smyhc1) to mark the somites.

Interestingly, deficiency of tbx2a, tbx2b, or combined tbx2a/b loss of function was associated with a reduced distal tubule domain along with an expanded proximal region (Fig. 3). The PCT expresses the gene solute carrier family 20, member 1a (slc20a1a), in a domain that was situated adjacent to somites 5–8 in wild-type embryos, while tbx2a, tbx2b, and tbx2a/b morphants exhibited an expansion of this segment, such that the PCT was situated adjacent to somites 5–9 (Fig. 3).
Comparison, the PST segment, which can be detected using a riboprobe corresponding to the transient receptor potential cation channel, subfamily M, member 7 (trpm7), was unaffected in size within tbx2a, tbx2b, and tbx2a/b morphants, but displayed an axial location shift that correlated with the expanded PCT segment domain (Fig. 3). The DE domain was marked by solute carrier family 12, member 1 (slc12a1), which encodes a Na/K/Cl cotransporter, and had a similar length in wild-types and tbx2a, tbx2b, and tbx2a/b morphants but experienced a one somite posterior shift in tbx2a/b deficient embryos compared to wild-type embryos due to changes in the domain size of the PCT and DL segments. 

To further assess these segment alterations, the absolute lengths of the PCT, PST, DE and DL domains were measured in triplicate cohorts of wild-type controls in addition to tbx2a, tbx2b, and tbx2a/b deficient embryos (Supplemental Fig. 3A). Overall, these phenotypes were penetrant and consistent in the majority of morphant embryos (Supplementary Fig. 3B–G). Of note, we have a robust ability to score the boundaries of the PCT, DE and the DL due to the nature of these respective riboprobes, which are not prone to background staining. By comparison, the trpm7 riboprobe used to label PST can produce some background. For this reason, PST phenotypes were scored more conservatively than the other segments, but we nevertheless observed 50–60% of tbx2a/b deficient animals with a very clear PST shift. Statistical analysis of these segment lengths confirmed that there was not a significant change in either the PST or DE segments due to single or double tbx2 knockdown (Supplemental Fig. 3A). In contrast, we found that there was a statistically significant decrease in DL segment length in single and double tbx2 deficient embryos compared to wild-type controls, as well as an increase in the PCT length associated with tbx2 loss of function (Supplemental Fig. 3A). We also explored whether double tbx2 knockdown altered mesodermal patterning. Analysis of several intermediate, paraxial, and lateral plate mesoderm markers suggested that these divisions were normally inculcated by early somitogenesis stages (Supplemental Fig. 4; data not shown).

Next, segment changes were assessed in both the tbx2b deficient embryo...
line fbyc144 and in fbyc144 injected with the tbx2a splicing MO. In both of these experimental contexts, we found that the loss of tbx2b or the combined loss of tbx2a/b induced a similar reduction in the DL segment domain, along with an expansion in the PCT segment domain; further, there were corresponding distal shifts in the location of the PST and DE (Fig. 3). Interestingly, approximately 20% of fbyc144+/− mutants displayed a normal segment pattern, consistent with partial penetrance of this particular allele—though 50% of fbyc144+/− also displayed pronephric and CS phenotypes, which might reflect a dominant negative effect or possibly haploinsufficiency (data not shown).

We conclude from these findings that tbx2a and tbx2b are required for normal nephron segmentation in the absence of earlier mesoderm patterning events. In light of the expression domain of tbx2a and tbx2b in the distal IM, we hypothesized that these factors specifically promote DL fate, and that the alterations in PCT formation might be a secondary, i.e. indirect phenotype. Further, the observation that dual loss of tbx2a and tbx2b resulted in a similar phenotype to either knockdown alone suggested the possibility that they act in the same pathway to influence normal nephron segmentation.

3.3. tbx2a/b act to inhibit the formation of the CS during nephrogenesis

As mentioned previously, the CS, to date found only in bony fishes, are sac-like bodies located adjacent to the kidney. Based on the distal IM expression domains of tbx2a and tbx2b during early somitogenesis, as well as the specific expression of tbx2b in the CS at 28 ss (Fig. 2B), we hypothesized that CS development might also be regulated by one or both of the tbx2 genes.

To explore this possibility, we performed whole mount in situ hybridization on wild-type controls and tbx2-deficient embryos to examine expression of the CS marker sim1, which encodes precise and reliable quantification of CS cell number (Supplemental Fig. 5). In wild-type embryos, the CS possessed an average of 4 cells per nephron (Fig. 4A, B). By comparison, knockdown of tbx2a, tbx2b, or both tbx2a/b induced an apparent expansion in the size of the CS (Fig. 4A). Likewise, fbyc144 mutant embryos exhibited an increased CS (Fig. 4A). To determine if these changes were due to elevated cell numbers, we quantified CS cell number in tbx2-deficient embryos (Fig. 4B). While tbx2a knockdown induced a small but significant increase in CS cell number, both tbx2b deficient and tbx2a/b doubly deficient embryos exhibited a dramatic, approximately three-fold increase in the number of stcl+ cells (Fig. 4B).

In a previous study, the transcription factor sim1a was shown to be necessary and sufficient for the formation of the stcl-expressing CS lineage (Cheng and Wingert, 2015). Notably, sim1a expression precedes that of stcl, as it is expressed in CS precursors at the 24 ss, while stcl is expressed in CS cells beginning at the 28 ss (Cheng and Wingert, 2015). Therefore, we next investigated whether tbx2-deficiency affected CS formation by influencing the expression of sim1a. To do this, whole mount in situ hybridization was utilized to assess the spatiotemporal expression domain of sim1a in the context of tbx2a, tbx2b and dual tbx2a/b deficiency. Here, we found that the number of sim1a+ CS cells was significantly increased in tbx2a, tbx2b, and tbx2a/b deficient animals (Fig. 4C, D). Taken together, this data suggests that tbx2a/b are factors that inhibit CS formation, most likely by limiting the expression of sim1a and consequently affecting the number of stcl+ cells that develop to form the CS lineage.

3.4. tbx2a/b are necessary and sufficient to promote DL formation and inhibit the CS

To determine whether increased levels of tbx2a and/or tbx2b transcripts were sufficient to induce changes in nephron cell identity, we injected wild-type embryos with tbx2a or tbx2b capped RNA (cRNA). Overexpression of either tbx2a or tbx2b resulted in a somite expansion in the slc12a3-expressing DL segment at a minimum cRNA dose of 50 pg and a maximum dose of 750 pg (Fig. 5A). There was no observed change in the PCT, PST, or DE segments upon injection of 500 pg/nL of tbx2a or tbx2b cRNA (Supplementary Fig. 6). Testing at additional doses of 50, 100, 250, or 1000 pg/nL tbx2a or tbx2b cRNA also failed to initiate changes in these segments (data not shown).

To further assess segment alterations after tbx2a or tbx2b overexpression, the absolute lengths of the PCT, PST, and DE domains were measured (Fig. 5D; Supplementary Fig. 6B). Statistical analysis of these segment lengths confirmed that there was not a significant change in the PCT, PST or DE due to alterations in tbx2a/b (Supplemental Fig. 6A). To assess how the DL expansion aligned with the adjacent DE segment, we performed double fluorescent in situ hybridization with the markers slc12a3 and slc12a1 (Fig. 5B). This revealed that there was co-mingling of distinct slc12a1+ and slc12a3+ cells in the pronephros region situated adjacent to somite 13 (Fig. 5B). These data indicated that the expansion of slc12a3+ cells populating this area was actually mosaic after tbx2a or tbx2b overexpression, and thus resolve how an expansion of the DL domain occurred while the DE domain was unchanged.

Next, we performed rescue studies using cRNA paired with tbx2a or tbx2b splicing targeting MOs to verify that the observed DL segment changes seen in our morphants were specifically due to loss of tbx2a or tbx2b, respectively, and to test if tbx2b could specifically rescue fbyc144 mutant embryos. We observed a normal DL in ~40% of tbx2a MO + tbx2a cRNA and ~50% of tbx2b MO + tbx2b cRNA embryos, as well as fbyc144 mutants + tbx2b cRNA, compared to the 2–3% wild-type phenotype observed in embryos injected with either cRNA or MO alone, which was based on measurements of absolute length of the DL (Fig. 5C, D, Supplementary Fig. 7A).

We also examined CS development using whole mount in situ hybridization to detect slc1 transcripts following overexpression of either tbx2a or tbx2b (Fig. 6). Addition of either tbx2a cRNA or tbx2b cRNA resulted in a significantly lower number of CS cells (Fig. 6A, B). This difference further suggests that both tbx2 factors have potent downstream consequences that can restrict CS development. With these morpholino knockdown studies, we also assessed the ability of tbx2a or tbx2b overexpression to specifically rescue the expansion of the CS lineage, and the ability of tbx2b to rescue CS ontology in fbyc144. Approximately 60% of tbx2a/b MO + tbx2a/b cRNA injected embryos had a wild-type CS with between 3 and 7 cells associated with each nephron (Fig. 6A, B, Supplementary Fig 7B). Quantification of the CS cells revealed that there were no significant differences in cell number between tbx2a rescues and wild-type, tbx2b rescues and wild-type, or tbx2a rescues and tbx2b rescues (Fig. 6B). Taken together, the compilation of our various loss of function, overexpression and rescue studies demonstrate that tbx2a/b are necessary and sufficient to promote the formation of the DL and suppress the formation of the CS.

3.5. tbx2a is epistatic to tbx2b during DL and CS development, and likely has other targets

Given the intriguing observation that the expression of tbx2a transcripts in the distal IM slightly precede the expression of tbx2b in this region, and the finding that the combined loss of tbx2a/b function led to identical outcomes as either factor alone, we hypothesized that they may occupy a shared pathway in which tbx2a does indeed predicate tbx2b. To explore this, we examined the expression domains of tbx2a and tbx2b transcripts in tbx2a and tbx2a morphants, respectively (Fig. 7A, B). The knockdown of tbx2b was not associated with any alteration in the tbx2a pronephros expression domain at the 28 ss compared to wild-type control embryos (Fig. 7A). In contrast, tbx2a knockdown was linked to a reduced tbx2b expression domain in the distal pronephros at the 28 ss compared to wild-type embryos.
Measurements of the absolute lengths of these \textit{tbx2a} and \textit{tbx2b} domains, followed by statistical analysis, confirmed that the nephron expression domain of \textit{tbx2b} was significantly reduced in \textit{tbx2a} morphants compared to wild-types, and that there was no difference in \textit{tbx2a} expression between \textit{tbx2b} morphants and wild-type embryos (Fig. 7C). The restriction of the \textit{tbx2b} expression domain in \textit{tbx2a} morphant embryos at the 28 ss suggested that \textit{tbx2b} transcripts were lost in the developing CS (Fig. 7B).

We performed double fluorescent \textit{in situ} hybridization studies in \textit{tbx2a} morphant embryos to examine the spatial localization of both \textit{tbx2b} and \textit{stc1} transcripts (Fig. 7D). We found that \textit{stc1+} cells lacked \textit{tbx2b} transcripts (Fig. 7D) in \textit{tbx2a} deficient embryos, consistent with the model that \textit{tbx2a} knockdown leads in part to the loss of \textit{tbx2b} expression in the CS lineage.

Next, we performed rescue studies to determine whether overexpression of \textit{tbx2b} or \textit{tbx2a} was sufficient to ameliorate the CS and DL

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**Fig. 4.** \textit{tbx2a/b} deficiency leads to an increase in the number of cells in the CS. (A) The CS was labeled by whole mount \textit{in situ} hybridization to detect transcripts encoding \textit{stc1} at the 28 ss stage. (B) In wild-type embryos, each CS is typically comprised of about 4–6 \textit{stc1+} cells per nephron. However, \textit{tbx2a/b} deficiency in morphants and \textit{fby^{c144}} mutants induced a significant increase of the \textit{stc1+} cell number in the CS. (C) Labeling the CS with \textit{sim1a} revealed a similar phenotype consistent with an inhibitory role of \textit{tbx2a/b} in CS development. (D) Quantification of \textit{sim1a+} CS cell number revealed that \textit{tbx2a}, \textit{tbx2b} and \textit{tbx2a/b} morphants and \textit{fby^{c144}} mutants all led to a significantly greater number of CS cells than wild-type embryos. For each experiment, at least 15 morphant embryos and 3 genotypically confirmed \textit{fby^{c144}} mutant embryos were examined. (*) *p* < 0.05, **(*p* < 0.001. Abbreviations: CS (corpuscle of Stannius).
phenotypes caused by *tbx2a* or *tbx2b* loss of function, respectfully (Fig. 7E–H; Supplemental Fig. 7). The overexpression of *tbx2b* rescued the CS expansion and DL reduction in close to or exceeding a majority of *tbx2a* morphants, where approximately 40% of embryos had a wild-type DL and approximately 70% a wild-type CS (Fig. 7E, Supplemental Fig. 7). This data is consistent with the conclusion that *tbx2b* acts downstream of *tbx2a*.

Surprisingly, however, overexpression of *tbx2a* also rescued the CS expansion and DL reduction in some *tbx2b* morphants, though the frequency of the rescue was comparatively less, with approximately 30% of embryos showing a wild-type DL and 20% a wild-type CS (Fig. 7G, Supplemental Fig. 7). Given the multiple lines of evidence that suggest *tbx2b* acts downstream of *tbx2a*, our interpretation of these findings is that *tbx2a* promotes the DL and inhibits the CS through one or more alternative targets as well.

3.6. RA regulates both *tbx2a* and *tbx2b* during zebrafish nephrogenesis

RA signaling has previously been established as both a critical activator and suppressor of segment patterning in the zebrafish pronephros (Wingert et al., 2007; Wingert and Davidson, 2011; Li et al., 2014; Cheng and Wingert, 2015; Marra and Wingert, 2016). In wild-type embryos, RA acts as a morphogen to promote the formation of proximal segments at the expense of distal segments. When RA concentrations are low or absent, only the formation of distal segments occurs. Thus, the early-acting RA chemical gradient creates the diversity of segments seen in the zebrafish nephron. In previous studies, *sim1a* was placed downstream of RA signaling, as its domain was altered by chemical treatments with exogenous RA or the RA biosynthesis inhibitor, 4-diethylaminobenzaldehyde (DEAB) (Cheng and Wingert, 2015). Since *tbx2a/b* also alter the *sim1a* domain, we aimed to investigate the relationship between RA and *tbx2a/b*. To determine this, wild-type embryos were treated with exogenous RA concentrations between 1×10^{-6} M and 1×10^{-8} M from 90% epiboly to the 28 ss, or with DEAB at a concentration of 1.6×10^{-5} M starting at either 50% or 90% epiboly until the 28 ss.

After performing whole mount *in situ* hybridization on chemically treated embryos and controls using probes for *tbx2a* and *tbx2b*, we found that RA and DEAB treatments do alter the *tbx2a/b* expression domains (Fig. 8). The minimum RA concentration for effects appeared to be 1×10^{-8} M. At 1×10^{-7} M, the pronephric expression domains of
10 representative embryos from each condition. The number of CS cells in embryos injected with contrast, the number of CS cells in rescue study embryos was not signi

3.7. Notch signaling and tbx2a/b cooperate to repress CS development in the zebrafish

Several studies have demonstrated essential roles of the Notch signaling pathway, downstream of RA signaling, in mediation of the renal progenitor fate decisions during nephron pattern formation (Cheng and Kopan, 2005; Cheng et al., 2007; Georgas et al., 2009). For example, there is evidence that RA acts upstream of Notch to mediate cell fate choice within the pronephros (Li et al., 2014; Marra and Wingert, 2016), which led us to hypothesize that the activities of tbx2a and tbx2b tracks with the DL and the CS, as the expression of these genes correlates with the previous observations of reduction and expansion of the DL segment and the CS during ectopic RA addition and RA inhibition by DEAB, respectively (Wingert et al., 2007; Wingert and Davidson, 2011). While these observations are consistent with the interpretation that tbx2a and tbx2b are each negatively regulated by RA signaling during nephrogenesis, these experiments do not establish whether the interaction is direct or indirect.

Fig. 6. tbx2a/b are necessary and sufficient to inhibit CS cellular expansion. (A) Dorsal images of wild-type 28 ss embryos injected with tbx2a and tbx2b cRNA show that tbx2a/b overexpression results in a decrease in the number of stc1-expressing CS cells. Rescue studies were conducted by co-injecting wild-type with either tbx2a cRNA and tbx2b MO or tbx2b cRNA and tbx2b MO and fby−144 mutants with tbx2b cRNA. In all three conditions, the CS was successfully rescued. (B) The number of CS cells in each nephron was counted for at least 10 representative embryos from each condition. The number of CS cells in embryos injected with tbx2a cRNA and tbx2b cRNA were significantly lower than wild-type embryos. In contrast, the number of CS cells in rescue study embryos was not significantly different than wild-type embryos. (*p < 0.05, **p < 0.001, N.S.=not significant). Abbreviations: cRNA (capped RNA), CS (corpuscle of Stannius).

both tbx2a and tbx2b was reduced to just the duct/cloaca region, and at 1×10^{-6} M RA, pronephric expression of tbx2a and tbx2b was abolished (Fig. 8). Reciprocally, the domains of both tbx2a and tbx2b expression were expanded in embryos that had been treated with DEAB at both 50% and 90% epiboly (Fig. 8). The phenotypes observed in these drug-treated embryos were highly penetrant and consistent across replicates (Supplementary Fig. 8).

These results are consistent with the notion that the expression of tbx2a and tbx2b tracks with the DL and the CS, as the expression of these genes correlates with the previous observations of reduction and expansion of the DL segment and the CS during ectopic RA addition and RA inhibition by DEAB, respectively (Wingert et al., 2007; Wingert and Davidson, 2011). While these observations are consistent with the interpretation that tbx2a and tbx2b are each negatively regulated by RA signaling during nephrogenesis, these experiments do not establish whether the interaction is direct or indirect.

To do this, we utilized the double transgenic line Tg(hsp70::GAL4, UAS::NICD) to ectopically activate Notch signaling through heat-shock induction at the 15 ss. Conversely, to block the Notch signaling pathway, wild-type embryos were treated with 100 µM of the gamma-secretase inhibitor DAPT at 90% epiboly through the 28 ss. Surprisingly, ectopic activation of Notch resulted in a striking reduction in CS formation in the transgenic embryos, while wild-type siblings underwent normal CS development (Fig. 9A). In contrast, DAPT-treated wild-type embryos had a significantly expanded CS (Fig. 9A). Again, alterations in the size of the CS were due to changes in the number of stc1+ cells, as shown by cell number quantification (Fig. 9B). These data suggest that Notch also acts to inhibit CS formation during zebrafish nephrogenesis in a pattern similar to loss and gain of tbx2a/b.

To investigate whether tbx2a/b cooperates with Notch in regulation of the CS, we performed MO knockdown of tbx2a/b in NICD embryos and then performed heat-shock induction at 15 ss. Interestingly, whereas tbx2a/b morphant siblings showed an increased number of cells which expressed markers of the CS lineage, activated NICD-tbx2a/b morphants failed to show a reduction in CS formation that is characteristic of uninjected activated NICD mutants (Fig. 9). In addition, tbx2a/b morphants as well as fby−144 mutant embryos were subjected to DAPT chemical treatments to block Notch signaling. While a CS expansion was evident, the effects of blocking both Notch and tbx2a/b were not significantly different than inhibiting either of these components alone (Fig. 9). The inability of tbx2a/b absence to rescue the diminished CS of heat-shocked NICD embryos or additively increase the CS clusters of DAPT-treated embryos suggests that tbx2a/b function downstream of Notch signaling in a common pathway to suppress CS differentiation.

4. Discussion

Knowledge of the complex genetic requirements for successful nephron segment formation in zebrafish has a number of applications in developmental biology and regenerative medicine. Here, our work has illuminated the requirement for two previously unexplored genetic participants during the process of nephron segmentation: tbx2a and tbx2b. Additionally, we have found a new role for these genes, along with the Notch signaling pathway, during the specification of the CS.

Our observations that the expression of tbx2a and tbx2b transcripts is restricted to the caudal renal progenitors led us to hypothesize that these genes play a part in segment patterning of the distal pronephros, and possibly formation of the CS. Indeed, loss of function studies resulted in a decrease in the DL segment, and overexpression of either tbx2a or tbx2b was sufficient to expand the DL, which lead us to conclude that the actions of both factors promote the formation of the
**Fig. 7.** tbx2a functions upstream of tbx2b during pronephros segment and CS development. (A, B) Whole mount in situ hybridization was performed with tbx2a probe on 28 ss wild-type embryos and embryos injected with tbx2b MO. The tbx2a domain remained the same somite length despite the loss of tbx2b. In contrast, loss of tbx2a caused a notable decrease in the tbx2b domain. (C) The length of tbx2a and tbx2b expression domains of five representative embryos was measured (μm). While tbx2a expression is not significantly different wild-type and tbx2b morphants, the size of the tbx2b domain is significantly smaller than their wild-type siblings. (D) Fluorescent in situ hybridization was used to examine the distal region of the pronephros of tbx2a morphant embryos at the 28 ss, which revealed that stc1+ CS cells lacked tbx2b transcripts. DAPI (blue) was used to label nuclei. (E, F) A portion of embryos injected with tbx2b MO and tbx2a cRNA exhibited a normal number of CS cells, but the average number of CS cells was significantly larger than the wild-type controls. A more robust rescue was seen in embryos injected with tbx2a MO and tbx2b cRNA, which on average had a similar number of CS cells compared to wild-type embryos. (G, H) DL lengths were rescued in some embryos by tbx2a cRNA in tbx2b morphants, and by tbx2b cRNA in tbx2a morphants, such that the average DL lengths were not significantly different from wild-type controls. (F, H) CS cells and DL lengths were counted in each nephron in 10 individuals in each treatment. (*p < 0.05, **p < 0.001, N.S.=not significant). Abbreviations: MO (morpholino), cRNA (capped RNA), DL (distal late), CS (corpuscle of Stannius).
As tbx2a/b are not expressed proximally, it seems reasonable to surmise that the expansion of the PCT segment during tbx2 deficiency is a secondary consequence of the DL alteration. The mechanism(s) by which this occurs is(are) extremely intriguing to contemplate. Understanding the regulation of cellular dynamics in the context of nephron segment patterning, as well as elucidating if and how adjacent subtypes of renal progenitors interact, are just two avenues for investigation that are likely to provide vital clues. Next, while our epistasis experiments indicate that tbx2a is upstream of tbx2b, they also suggest tbx2a mitigates the DL and CS lineages by regulating an additional target(s). The identification of these mystery components, and determination of where they fit in the working model of nephrogenesis (Fig. 10) are very important areas for continued research. Further, our results have led us to hypothesize that part of the mechanism for how RA negatively regulates the DL during nephrogenesis occurs through its negative regulation of the tbx2a and tbx2b expression domains. However, the present study does not resolve whether RA signaling directly or indirectly regulates tbx2 genes, and as such this is another important point for subsequent work.

We also discovered that tbx2a/b and the Notch signaling pathways repress CS development, providing key formative insights into the genetic components that influence this poorly characterized lineage. Noticeably, tbx2a/b deficient embryos formed significantly larger CS clusters, as shown by the elevated number of stc1 and simil1a expressing cells. A similar CS expansion was seen when Notch signaling was blocked using DAPT. Notch overexpression also led to a depression in

**Fig. 8.** tbx2a/b are downstream of retinoic acid in the nephrogenesis pathway. Wild-type embryos were treated with exogenous RA at 3 dosages from 90% epiboly to the 28 ss. Whole mount in situ hybridization was then performed using riboprobes for tbx2a and tbx2b. Both the tbx2a and tbx2b domains decreased with all three doses of RA, with total abrogation of expression occurring at 1×10^-6 M. In contrast, DEAB treatment induced an expansion in the tbx2a/b domains at both treatment time points. This suggests that RA negatively regulates tbx2a/b expression, either directly or indirectly, in the development of the distal pronephros. At least 20 representative embryos were examined in each treatment.

**Fig. 9.** Notch signaling also inhibits CS differentiation, but in an upstream or independent way than tbx2a/b. NICD embryos were heat-shocked at 15 ss to ectopically activate Notch signaling and fixed at 24 hpf. Post heat-shock induction, NICD embryos exhibited a dramatic reduction of the CS, while siblings showed normal CS clusters. To block Notch signaling, wild-type embryos were incubated in 100 µm DAPT/E3 solution from 90% epiboly to the 24 hpf stage. DAPT treatment resulted in a significant elevation in the number of CS cells. Heat-shocked NICD tbx2a/b morphants exhibited a significant expansion in the CS that was in contrast to the reduced CS observed in uninjected heat-shocked NICD siblings. The number of cells in the these CS clusters was not significantly different to the expansion seen in wild-type embryos treated with tbx2a/b MO. DAPT-treated tbx2a/b morphants and fbyc144 mutants exhibited an expansion in the CS that was not significantly different than wild-type embryos treated with DAPT alone. Together, these results suggest that tbx2a/b are acting in the same pathway, downstream of Notch to suppress the CS. Representative images were based on thorough examination of at least 15 embryos per treatment. (*p < 0.05, **p < 0.001, N.S.=not significant).
CS formation. As knockdown of tbx2a/b in NICD transgenic embryos failed to repress CS formation post heat-shock induction, and DAPT-treated tbx2a/b morphants exhibited no additional CS expansion, we conclude that tbx2a/b act downstream of Notch to inhibit CS development (Fig. 10). More studies are needed to decipher the relationship between tbx2a/b with each other, as well as how these factors interact with particular Notch pathway components, during segment patterning and CS formation.

4.1. The roles of Tbx2 in development and tbx2a/b functions in patterning the pronephros

The vertebrate T-box gene family, which arose from a single primeval gene in a common metazoan ancestor, consists of over 17 transcription factors that are now appreciated to play many roles in animal development (Abrahams et al., 2010). Tbx2 belongs to the Tbx2 subfamily (which includes Tbx3, Tbx4, and Tbx5) and is expressed in numerous tissues during embryogenesis, including endoderm and mesoderm derivatives such as the lung and kidney, respectively (Bollag et al., 1994; Chapman et al., 1996). Tbx2 is a potent suppressor of transcription, though an activation domain has also been identified, suggesting that the protein may have versatile roles (Paxton et al., 2002; Abrahams et al., 2010). Indeed, Tbx2 is involved in a multitude of events. For example, elegant genetic studies have established that mammalian Tbx2 has distinct roles during heart development (Harrelson et al., 2004; Cai et al., 2005), eye development (Behesti et al., 2009), lung growth (Ludtke et al., 2013), limb field specification and posterior digit identity (Isaac et al., 1998; Logan et al., 1998; Suzuki et al., 2004; Nissim et al., 2007), and mammary gland development (Douglas and Papaioannou, 2013)—just to name a few.

Consequent to the teleost genome duplication, zebrafish have retained two tbx2 genes, which share 79.5% peptide identity (Sedletcaia and Evans, 2011). To date, both redundant and unique roles have been attributed to the zebrafish tbx2a and tbx2b paralogs: tbx2a has a distinct role in regulating morphogenesis of the pharyngeal arches (Thu et al., 2013), while tbx2b is required for development of the pineal organ (Snelson et al., 2008), and both tbx2a/b are functionally redundant for regulating heart chamber development (Sedletcaia and Evans, 2011).

With respect to vertebrate kidney development, Tbx2 was detected in total RNA from the adult murine kidney (Bollag et al., 1994). The spatiotemporal expression of Tbx2 during renal development of the mouse has been described previously in the mesonephros and metanephric tubules at E12.5 (Chapman et al., 1996). Additionally, Tbx2 transcripts have been detected in the Wolfian (mesonephric) duct of developing mouse embryos, as well as in the ureteric bud tips of the collecting duct system during branching morphogenesis of the metanephric kidney (GUDMAP: 10896). In light of this and the present findings, exploring the functional role of Tbx2 in mammalian kidney will be important in future studies. Tbx2 null murine embryos die due to their cardiovascular defects, which has precluded analysis of its function in renal development (Harrelson et al., 2004), though a conditional null allele has been created that could be invaluable for further analyses (Wakker et al., 2010). As we have observed that the zebrafish tbx2 homologs, tbx2a and tbx2b, are both expressed in a region of the distal pronephros which consists of both DL and a purported partially overlapping PD segment, an interesting hypothesis for prospective studies is that tbx2a/b have conserved roles in PD/collecting duct development as well. At present, exploration of this possibility is currently limited by a paucity of molecular markers of the zebrafish PD, but advances might be achieved by targeted expression profiling of these cells or through the assessment of other genes expressed in the ureteric bud tips.

Despite the conservation in tbx2 expression in the renal duct networks among various organisms, the molecular mechanisms underlying how tbx2 impacts kidney development continue to be unresolved. In tadpoles, overexpressing Tbx2 inhibited nephric mesoderm formation and Tbx2 transcripts were localized to the non-nephric mesenchyme of the IM that surrounds the nephric anlage (Cho et al., 2011). In contrast, Tbx2 inactivation expanded the boundary of the pronephric nephron, resulting in an enlarged pronephros (Cho et al., 2011). These results implied that Tbx2 plays a role in determining the size of the pronephros territory (Cho et al., 2011). Unlike these findings, alterations of tbx2a/b levels during zebrafish pronephric development did not alter pronephros size but led to changes in nephron segment pattern.

While the role of Tbx2 in mammalian nephrogenesis remains to be determined, the observed conservation in renal expression across vertebrates suggests the possibility that Tbx2 participates in mammalian kidney organogenesis in a way similar to zebrafish by promoting distal segment and/or collecting duct formation. Whether alterations in Tbx2 are linked to human renal congenital abnormalities or other diseases may be a valuable area for future study. Additional work to understand the molecular consequences of alterations in tbx2a/b using the zebrafish may provide a useful model to explore these aspects, as has been the case for a number of other genetic alterations (Pourreetezadi and Wingert, 2016).

4.2. tbx2a/b and induction of the CS in teleosts

The CS has been characterized for its secretion of Stanniocalcin (STC), a homodimeric glycoprotein hormone with an integral role in calcium and phosphate homeostasis in fishes (McCudden et al., 2001). STC in fish acts to prevent hyperkalemia and is released into the bloodstream in response to serum Ca$^{2+}$ elevations, whereas in the kidney it stimulates phosphate reabsorption in order to balance excess Ca$^{2+}$ in the serum (Lu et al., 1994). Interestingly, the mammalian counterparts of this protein, STC1 and STC2, are found in low levels in blood serum but their subcellular functions remain to be resolved (Yeung et al., 2011). Despite relatively intensive studies on the CS gland and STC, not much is known about how the CS is formed and what molecular mechanisms regulate its differentiation.

Recent findings by our lab have identified the transcription factor...
**sim1a** as a critical modulator of CS differentiation, as disrupting **sim1a** function completely abrogated the formation of stc1-expressing cells of the CS in the zebrafish pronephros (Cheng and Wingert, 2015). Our data has added further knowledge on the regulation of CS development during zebrafish nephrogenesis. Due to our observations that stc1 and sim1a are co-localized in CS cells at the 28 ss, we postulate that **sim1a** is another viable marker to examine the CS at this time point. In addition, loss of tbx2a/b resulted in a significant increase in the number of CS cells, which was detected by an increase in cells expressing stc1 and sim1a. This places tbx2a/b as an upstream repressor of **sim1a**, which leads to suppression of CS development. This model is further strengthened by our data that overexpression of tbx2a/b caused a drop in the number of CS cells. Interestingly, as a patterning factor, **sim1a** is also involved in negotiating the PCT/PST boundary (Cheng and Wingert, 2015). As our studies demonstrate that tbx2a/b negatively regulate **sim1a**, this could provide an explanation for the expansion of the PCT upon loss of tbx2a/b which could be tested experimentally in future studies.

### 4.3. Notch signaling pathway is also involved in CS regulation during zebrafish nephrogenesis

Previous studies in mammalian models demonstrated the crucial role of the Notch signaling pathway in effecting cell fate decisions in the mammalian nephron (Cheng et al., 2003, 2007; Cheng and Kopan, 2005). In the zebrafish pronephros, studies have shown that Notch signaling inhibits the formation of multiciliated cells (MCCs) to advance transporter cell fates such as the PST (Liu et al., 2007; Ma and Jiang, 2007; Marra et al., 2016). While the cellular lineages of the CS have not yet been extensively characterized, we hypothesized that Notch could be involved in regulating CS formation. Chemically inhibiting Notch signaling with DAPT resulted in an increase in the numbers of CS cells in embryos. Conversely, ectopically activating Notch via our NICD heat-shock transgenic line resulted in an almost complete loss of CS cells. We have therefore identified Notch signaling as a suppressor of CS development in the zebrafish pronephros. This novel role for Notch signaling is important for continuing to comprehend how this critical developmental pathway functions in the zebrafish pronephros and its associated tissues.

In addition to reporting tbx2a/b and Notch signaling as repressors of CS development, we also provide baseline information about how these factors are positioned within the network of CS regulatory genes. Loss of tbx2a/b paired with loss and gain of Notch lead to CS cell number increases that was comparable to loss of tbx2a/b alone. This indicates that tbx2a/b act downstream of Notch signaling to suppress the CS. Whether this relationship involves direct activation by Notch or a cascade of intermediates is currently unknown, thus future studies will focus on resolving the mechanistic details of this interaction.

### 4.4. The links between nephrogenesis and kidney disease

In this study, we identified the T-box transcription factors tbx2a and tbx2b as crucial regulators of nephron patterning, where they may also possibly act to regulate renal progenitor differentiation during zebrafish nephrogenesis. The specification and patterning of the nephron progenitor is a complex process that requires integrative regionalization of each progenitor population, as highly specialized nephron epithelial cells made in each domain carry out distinct physiological functions. Whereas applications of zebrafish and other models such as amphibians continue to advance the knowledge of nephrogenesis (Desgrange and Cereghini, 2015), a large gap nevertheless remains regarding the broad picture of the molecular control behind renal progenitor specification. Understanding the genetic, molecular, and cellular mechanisms of nephrogenesis has far reaching implications for kidney diseases ranging from congenital anomalies of the kidney and urinary tract (CAKUT) to acquired renal injuries because a common aspect that unifies kidney diseases with diverse pathologies is the loss of function or dysfunction of nephrons. The rising incidence of kidney disease and limitations for treatment pose ongoing medical challenges (Saran et al., 2015). Discoveries of the key modulators during nephron patterning and the molecular mechanisms underlying renal progenitor commitment are needed to better understand the processes of nephrogenesis and kidney ontogeny to help create new, innovative regenerative therapeutics. As researchers continue to endeavor to cultivate renal precursors, through emergent techniques like organoid culture (Chambers et al., 2016), the information collected from the analysis of simplified nephron models such as the vertebrate pronephros can continue to assist in illuminating lineage relationships and gene regulatory networks.

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**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2016.10.019.

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