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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 cryptic 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)*^{c144} 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 comingled 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-[N-(3,5-difluorophenacetyl)-L-alanyl]-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

Abbreviations: cRNA, capped RNA; CS, corpuscle of Stannius; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DEAB, 4-diethylaminobenzaldehyde; DE, distal early; DL, distal late; *fby*^{c144}, *from beyond*; hpf, hours post fertilization; IM, intermediate mesoderm; NICD, *Tg(hsp70::Gal4, UAS::NICD*; MO, morpholino; N, neck; P, podocytes; PCT, proximal convoluted tubule; PD, pronephric duct; PST, proximal straight tubule; PD, pronephric duct; RA, retinoic acid; *sim1a, single-minded family bHLH transcription factor 1a*; *slc12a1, solute carrier family 12, member 1*; *slc12a3, solute carrier family 12, member 3*; *slc20a1a, solute carrier* family 20, member 1a; *smyhc1, slow myosin heavy chain 1*; ss, somite stage; *stc1, stanniocalcin 1*; *tbx2a, t-box 2a*; *tbx2b, t-box 2b*; *trpm7, transient receptor potential cation channel, subfamily M, member 7*

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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; Thisse 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 ureteric 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 (fby^{c144})* 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, *fby^{c144}*, and *hsp70::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 $CaCl_2$, 0.33 mM $MgSO_4$) 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 *smyhc1, 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'- ATGGCTTATCACC CTTTTCACGCGCACAGGCCGGCCG-3' and 5'-ATTAACCCTCA CTAAAGGGCTCTCCCAGTAGTTGTTCGCAAGATGAGTCGTCCGA-3').