NK4 Antagonizes Tbx1/10 to Promote Cardiac versus Pharyngeal Muscle Fate in the Ascidian Second Heart Field

Wei Wang, Florian Razy-Krajka, Eric Siu, Alexandra Ketcham*, Lionel Christiaen*

Department of Biology, New York University, New York, New York, United States of America

Abstract

The heart and head muscles share common developmental origins and genetic underpinnings in vertebrates, including humans. Parts of the heart and cranio-facial musculature derive from common mesodermal progenitors that express NKX2-5, ISL1, and TBX1. This ontogenetic kinship is dramatically reflected in the DiGeorge/Cardio-Velo-Facial syndrome (DGS/CVFS), where mutations of TBX1 cause malformations in the pharyngeal apparatus and cardiac outflow tract. Cardiac progenitors of the first heart field (FHF) do not require TBX1 and segregate precociously from common progenitors of the second heart field (SHF) and pharyngeal muscles. However, the cellular and molecular mechanisms that govern heart versus pharyngeal muscle specification within this lineage remain elusive. Here, we harness the simplicity of the ascidian larva to show that, following asymmetric cell division of common progenitors, NK4/NKX2-5 promotes GATAa/GATA4/5/6 expression and cardiac specification in the second heart precursors by antagonizing Tbx1/10-mediated inhibition of GATAa and activation of Collier/Olf/EBF (COE), the determinant of atrial siphon muscle (ASM) specification. Our results uncover essential regulatory connections between the conserved cardio-pharyngeal factor Tbx1/10 and muscle determinant COE, as well as a mutual antagonism between NK4 and Tbx1/10 activities upstream of GATAa and COE. The latter cross-antagonism underlies a fundamental heart versus pharyngeal muscle fate choice that occurs in a conserved lineage of cardio-pharyngeal progenitors. We propose that this basic ontogenetic motif underlies cardiac and pharyngeal muscle development and evolution in chordates.

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Introduction

The combined cardiac and craniofacial malformations observed in Cardio-Velo-Facial/Di George Syndrome patients arise from complex sets of defects in the development of the pharyngeal apparatus [1–4]. Developmental studies focused on amniote model systems have documented the ontogenetic proximity of the branchiocardio-muscular primordia to the heart and head muscles. However, the relative complexity of vertebrate embryos has hindered the identification of progenitor cells and the analysis of the precise cellular characteristics of the heart versus pharyngeal muscle fate choice.

Tunicates are the closest living relatives of the vertebrates [12] and the ascidian Ciona intestinalis recently emerged as a simple and relevant model for chordate heart development [13,14]. In ascidian embryos, each one of the bilateral B7.5 blastomeres uniquely expresses the conserved cardiac determinant Mesp and gives birth to four cells: two heart progenitors, the trunk ventral cell and two pharyngeal muscle progenitors.
**Author Summary**

Mutations in the regulatory genes encoding the transcription factors **N**KX2-5 and **T**BX1, which govern heart and head muscle development, cause prevalent congenital defects. Recent studies using vertebrate models have shown that the heart and pharyngeal head muscle cells derive from common progenitors in the early embryo. To better understand the genetic mechanisms by which these progenitors select one of the two developmental trajectories, we studied the activity of these transcription factors in a simple invertebrate chordate model, the sea squirt *Ciona intestinalis*. We show that the sea squirt homolog of **N**KX2-5 promotes early heart specification by inhibiting the formation of pharyngeal muscles. Conversely, the **T**BX1 homolog determines pharyngeal muscle fate by inhibiting **G**ATAa and thereby the heart program it instructs, as well as promoting the pharyngeal muscle program through activation of **COE** (Collier/Olf-1/EBF), a recently identified regulator of skeletal muscle differentiation. Finally, we show that the **N**KX2-5 homolog protein directly binds to the **COE** gene to repress its activity. Notably, these antagonistic interactions occur in heart and pharyngeal precursors immediately following the division of their pluripotent mother cells, thus contributing to their respective fate choice. These mechanistic insights into the process of early heart versus head muscle specification in this simple chordate provide the grounds for establishing the etiology of human congenital cardio-craniofacial defects.

Anterior tail muscles (ATMs) [15–20]. Electroporation of fertilized eggs with constructs using the Mesp or FoxF enhancers allows the visualization and molecular manipulation of B7.5 lineage cells or TVCs, respectively [16,20]. Following migration, each bilateral pair of TVCs divides asymmetrically and medio-laterally to form small medial first heart precursors (FHPs) and large lateral secondary TVCs. The latter divide again asymmetrically to form small median second heart precursors (SHPs) and large lateral atrial siphon muscles (ASMs) precursors (Figure 1A) [21]. **ASM**-specific expression of the Collier/Olf-1/EBF (**COE**) transcription factor triggers **ASM** specification, up-regulates the conserved LIM-homeobox gene *Iset*, and contributes to inhibiting the heart program specifically in the **ASM** (Figure 1A) [21]. **ASM** precursors subsequently migrate dorsally and form a ring of cells expressing myosin heavy chain 3 (**MHC3**) in the atrial siphon primordium. During metamorphosis, **ASM** precursors continue to divide and give birth to definitive **ASM** and longitudinal muscle (LoM) precursors, which migrate into the body wall [21,22]. Meanwhile, the heart precursors divide and form a compartment containing **MHC2**-cardiomyocytes (Figure 1A,B). Hence, we uncover regulatory mechanisms that distinguish the **COE**-expressing **ASM** from the SHPs following asymmetric cell divisions of the common TVC progenitors, thus providing insights into the cellular and molecular basis for heart versus pharyngeal muscle fate choice within the cardiogenic lineage.

**Results**

**NK4 Activity Promotes Heart Specification at the Expense of ASMs**

**NK4** is the sole *Ciona* homolog of the conserved homeobox cardiac determinants **NK4**/**N**kx2-5/*Coxl/miman (Figure S1) [23,24]. From the neurula to tailbud stages, **NK4** is broadly expressed in the ventral epidermis, anterior trunk endoderm, and TVCs, where its expression is regulated by Mesp, Fibroblast Growth Factor (FGF) signaling, FoxF, **G**ATAa, and Bone Morphogenetic Protein (BMP) signaling (Figure S2) [15,17,19,20,24–26]. Fluorescent in situ hybridization and immunohistochemical (FISH-IHC) assays on Mesp->**N**sC1a-**Z**-expressing larvae indicated that **NK4** expression persists at low levels until 20 hpf in both the **ASM**, FHPs, and SHPs (Figure S2A-D). Expression became undetectable in the heart region during metamorphosis and at juvenile stages (Figure S2E–F).

To investigate the TVC-specific functions of **NK4**, we generated a dominant-negative **N**448**K** DNA-binding domain mutant (dn**NK4**) [27,28] and expressed dn**NK4** or wild-type **NK4** using the FoxF minimal TVC enhancer [20,21]. We raised larvae electroporated with Mesp->**N**sC1a-**Z** and either FoxF->mCherry, -**NK4**, or -dn**NK4** to the early juvenile I stage (stage 38) [29] and performed double FISH-IHC using an anti-β-galactosidase antibody together with **MHC2** and **MHC3** antisense RNA probes to visualize the B7.5 lineage-derived cardiomyocytes and **ASM**/LoM, respectively (Figure 1A,B) [21]. In control juvenile hearts, **MHC2** marks differentiated β-Gal+/**MHC2**- cells, while **MHC3** is specifically expressed in the B7.5 lineage-derived, βGal+, **ASM**, and LoMs and also in the A7.6 lineage-derived, βGal-, oral siphon muscles (OSMs) (Figure 1A,B, [21,30]. Targeted expression of dn**NK4** reduced the volume of the heart by ~50% (903±92 μm^3^ per half versus 1,800±487 μm^3^ in controls; Table S1) and markedly inhibited **MHC2** expression in 80% of the juveniles (28/35; Figure 1C). In these animals, the **ASM**/LoM populations did not differ notably from control juveniles. Conversely, **NK4** overexpression inhibited the formation of β-Gal+/**MHC3**+ **ASM** and LoMs (25/35; Figure 1D), while increasing the volume the TVC-derived β-Gal+ heart-like tissue by ~250% (4,464±277 μm^3^ per half versus 1,800±172 μm^3^ in controls; Table S1). This indicates that sustained **NK4** activity promotes heart specification at the expense of the **ASM**/LoM fate within the cardiogenic mesoderm.

**NK4 Activity Inhibits COE Expression in the SHPs**

Since the **ASM** determinant **COE** is expressed immediately after the asymmetric division of the secondary TVCs (Figures 1A and S3) [21], we investigated the effects of **NK4** activity on early **COE** expression. The majority (82%, 28/34) of control 20 hpf larvae contained four βGal+/**COE**+ and six βGal+/**COE**– cells per electroporated half (averaging 3.8±0.01 **COE**+ and 5.7±0.17 **COE**– cells per half, n=34; Figure 2A,D). Importantly, this pattern can be interpreted clonally given that stereotyped cell divisions separate the **COE**– heart and **COE**+ **ASM** precursors (Figure 1A). Targeted expression of dn**NK4** increased the number of **COE**+ cells to 6.3±0.31, at the expense of **COE**– cells (2.8±0.25 per half, n=40; Figure 2B,D). The total number of βGal+ TVC-derived cells in dn**NK4**-expressing larvae did not significantly change by 20 hpf, indicating that **COE** was ectopically expressed in cells that do not normally activate it (Figure 2D). Conversely, **NK4** overexpression abolished **COE** expression in 24% (7/29) of electroporated larvae. Mosaic incorporation of the transgenes reduced the number of **COE**+ cells to 1–3 per half in 38% (16/29) of the larvae. The average number of **COE**+ cells was reduced to 2.2±0.4 (n=29), and the total number of βGal+, **COE**– cells increased to 6.3±0.4 (n=29, Figure 2C,D). Targeted **NK4** and dn**NK4** expression also exerted opposite negative and positive effects on **COE** expression, respectively, in 18, 24, and 28 hpf larvae (Figures 2E–G and S4A–I). Notably, since TVC-specific expression of dn**NK4** caused additional cells to express **COE** and migrate towards the atrial siphon placode in 24 and 28 hpf larvae, the absence of a clear excess of **ASM**/LoM cells in “dn**NK4**-derived” juveniles suggests that a reduction in **ASM**/LoM
proliferation counteracts the effects of an initial excess of precursors on the final ASM/LoM population. By contrast, the heart did not seem to compensate for either the excess or reduction of the initial number of precursors since both dnNK4 and NK4 affected the juvenile heart volumes. Taken together, these results indicate that sustained NK4 activity inhibits COE expression in the TVC derivatives, which could explain its negative effects on ASM/LoM development.

To test if NK4 regulates heart versus ASM fate specification by inhibiting COE expression, we performed epistasis assays by analyzing ASM ring formation and MHC3 expression in 28 hpf larvae following combined inhibitions of NK4 and COE functions (Figure S5). In control 28 hpf larvae, 7.25 ± 0.1 ASM cells per side formed typical rings containing 5.5 ± 0.2 MHC3+ cells (n = 43; Figure S5A,G,H). Note that we recently identified an unsuspected diversity among ASM precursors, whereby only a fraction of the

Figure 1. NK4 promotes heart formation at the expense of the ASMs and LoMs in Ciona intestinalis. (A) Summary of early cardio-pharyngeal development: cell divisions, lineage, and migration of the TVCs. Green, TVCs and secondary TVCs (2nd TVC); red, FHPs and SHP; blue, ASMs and LoM precursors; OSMs, Oral Siphon Muscles; ATMs, anterior tail muscles; hpf, hours postfertilization. Green and blue arrows, TVC and ASM migrations, respectively. (B–D) 72 hpf juveniles electroporated with Mesp>nls:lacZ (red) to mark B7.5 lineage cells and FoxF>mCherry (B), FoxF>dnNK4 (C), or FoxF>NK4 (D). Double FISH-IHC with MHC2 (green) and MHC3 (blue) probes mark cardiomyocytes and ASM/LoMs, respectively. White dotted lines outline whole bodies. Insets show magnified hearts. Numbers indicate juveniles showing the phenotype and total. Scale bar, 25 μm.

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cells expressed MHC3 and began to differentiate before metamorphosis (Razy-Krajka et al., under review), which is consistent with the above observation. Targeted dnNK4 expression increased the number of ASM cells to 8.5 ± 2.8, including 6.6 ± 0.4 MHC3+ cells (n = 35; Figure S5D,G,H). Co-expression of a dominant negative form of COE lacking the N-terminal DNA binding domain (COE\textsuperscript{D321}) [31] reduced the excess ASM migration and ectopic MHC3 expression induced by dnNK4 (Figure S5C,F–H). The constitutive repressor COE:WRPW blocked ASM migration and MHC3 expression regardless of NK4 activity (Figure S5B,E,G,H). These data indicate that ectopic activation of COE mediates the ASM-promoting effects of dnNK4, further supporting the notion that NK4-mediated inhibition of COE expression is required to promote heart versus ASM fate specification.

The Ciona ortholog of Islet1 (Ci-Islet) is up-regulated by COE in the ASM [21]. FISH-IHC indicated that Islet is expressed in all TVC daughter cells, but more highly in the ASMs compared to the heart precursors (Figure S3) [21]. We previously identified a COE-dependent ASM-specific Islet enhancer, which suggested that ASM-specific transcriptional inputs downstream of COE govern Islet expression [21]. Here, using intron-specific antisense RNA probes, we detected nascent Islet transcripts in all the TVC derivatives starting around 18 hpf, after the two asymmetric divisions, but exclusively in the ASM in 20 hpf larvae (Figure S3). These data show a widespread pan-TVC, but transient, activation followed by ASM-specific maintenance, thus emphasizing the complexity of Islet regulation in the cardio-pharyngeal mesoderm.

Consistent with a predominant maintenance of Islet downstream of COE in the ASM, targeted expression of dnNK4 or NK4 caused up-regulation or reduction of Islet expression, respectively (Figure S6). Thus, as it is the case in mice [32] and zebrafish [33], NK4 activity appears to negatively regulate Islet expression in the cardiogenic mesoderm, at least in part through its effects on COE.

**NK4 Activity Promotes GATAa Expression in Secondary TVC Derivatives**

To test whether NK4 overexpression converted the ASM into heart precursors, we assayed expression of the conserved early heart marker GATAa, which is highly expressed in the TVC progenitors at early tailbud stages but appears to be down-regulated by the late tailbud stage, before the first asymmetric division [17]. GATAa became undetectable in either secondary TVCs or FHPs after the first asymmetric division (Figure S7B). Expression became detectable again around 18 hpf, primarily in

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**Figure 2. NK4 represses COE and activates GATAa in TVC derivatives.** (A–C, E–G, H–J) Larvae electroporated with Mesp\textsuperscript{nls}:lacZ (red), FoxF\textsuperscript{mCherry} (A, E, H), FoxF\textsuperscript{dnNK4} (B, F, I), and FoxF\textsuperscript{NK4} (C, G, H). FISH of COE transcripts (green) at 20 hpf (A–C) and 18 hpf (E–G) and GATAa transcripts (green) at 18 hpf (H–J). White dotted lines indicate the midline. Target expression of –dnNK4 in TVC lineage induces ectopic COE expression in SHPs (white arrowheads), in addition to ASM precursors (open arrowheads), but not in the FHPs (B, F, arrows). Conversely, FoxF\textsuperscript{NK4} represses COE expression (C, G). In wild-type embryos, endogenous GATAa expression is restricted to heart precursors (arrows, H). FoxF\textsuperscript{dnNK4} does not affect GATAa expression in the FHP (arrows, I). In FoxF\textsuperscript{NK4} expressing larvae, GATAa transcripts can be detected in all TVC derivatives, including the lateral-most (open arrowheads). Scale bar, 25 μm. (D) Numbers of βGal and COE expressing cells per electroporated half at 20 hpf, in mCherry controls, dnNK4, or NK4 expressing larvae. n, number of embryo halves scored. Error bar, standard error of the mean (SEM), Student’s t test compared experimental condition to the control. **p<0.05. (K) Interpretation of TVC lineage fate re-programming by Fox\textsuperscript{dnNK4} and FoxF\textsuperscript{NK4}. FoxF\textsuperscript{dnNK4}, SHPs are converted into ASMs; FoxF\textsuperscript{NK4}, ASMs are converted into heart precursors.

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the FHPs, and appeared weaker in the SHPs (Figure S7C). We used GATAa intron-specific antisense RNA probes to detect nascent transcripts every half-hour after the end of TVC migration and further clarify the dynamics of GATAa transcription in the TVC derivatives. Two nuclear dots of nascent transcripts were detected in each one of the TVCs towards the end of their migration (Figure S7E). Following the first asymmetric cell divisions, nascent transcripts were undetectable in the FHPs and rarely in the secondary TVCs (Figure S7F,G). Notably, after the asymmetric division of secondary TVCs in 16 hpf larvae, GATAa nascent transcripts became detectable again in the FHPs but not in the SHP until 18.5 hpf, when nascent GATAa transcripts were still observed in the FHPs and became detectable in the SHPs but not in the ASM (Figure S7H–L). These observations illuminate the dynamics of GATAa transcription in the heart precursors: GATAa first shuts off following TVC migration before being reactivated successively in the FHPs and only about 2.5 h later in the SHPs.

We next examined GATAa expression following manipulations of NK4 activity. GATAa expression in the ~18 hpf FHPs did not significantly change upon targeted expression of dnNK4, while the possibility of a reduced SHP expression could hardly be evaluated due to low expression levels in control animals (Figures 2I, S4, and S7). These data suggest that reactivation of GATAa in the FHPs is independent of NK4 activity. In contrast, we observed that overexpression of NK4 up-regulated SHP expression of GATAa and caused ectopic activation in the lateral-most TVCs at 18 hpf (Figures 2J and S4). Therefore, NK4 activity appears sufficient to promote cardiac-specific GATAa expression in the secondary TVC derivatives, and it may be necessary for GATAa reactivation specifically in the SHPs.

The above observations point to different requirements for NK4 activity in the FHPs and SHPs. TVC-specific expression of dnNK4 never converted all of the derivatives into ASM/LoM (Figure 1B; Table S1), nor did we observe ectopic activation of COE in all Mesp2>naslacz positive TVC derivatives (Figures 2B,D and S4). DnNK4 caused ectopic COE expression as early as 18 hpf, in cells positioned nearest to the ASM precursors (Figures 2B,F and S4). This stereotyped cell distribution suggested that only SHPs activated COE in response to dnNK4, while the GATAa+ FHPs failed to activate COE upon mis-expression of dnNK4 (Figure 2K). Using intron-specific probes, we detected ectopic transcription of COE in the nuclei closer to the ASM, as early as ~17–17.5 hpf but not between 15.5 and 16.5 hpf (Figure S4K). We hypothesized that only the derivatives of the secondary TVCs are competent to activate COE and become ASM precursors, while an NK4-dependent mechanism blocks COE activation in the SHPs, which then reactivate GATAa expression with a ~2.5 h delay compared to the FHPs and form heart tissue.

**NK4 Activity Restricts Tbx1/10 Expression to the ASM Precursors**

In vertebrate development, the T-box DNA binding transcription factor TBX1 is an essential determinant of the pharyngeal mesoderm expressed in both the second heart field and branchiomeric muscle progenitors [3,34,35]. We previously reported that the Ciona homolog Tbx1/10 is expressed in the LoM precursors in metamorphosing juveniles [21]. Here, using FISH-IHC, we found that Tbx1/10 expression is initiated specifically in the large lateral secondary TVCs, after the first asymmetric division (Figure 3A,B). Following the second asymmetric division, high Tbx1/10 expression is maintained in the ASM precursors at 16 to 18 hpf, while gradually fading in the SHPs (Figure 3C,D). Using Tbx1/10 intron-specific probes, we confirmed the secondary TVC-restricted activation of Tbx1/10 expression and found that the SHPs cease to transcribe Tbx1/10 shortly after the second asymmetric division (Figure 3G), while nascent transcripts could still be detected in the ASM precursors (Figure 3E–H).

Double FISH-IHC showed that COE and Tbx1/10 are co-expressed in the ASMs, while the SHPs retain only low amounts of Tbx1/10 transcripts in 16 hpf larvae (Figure 3Q,T). Thus, the common progenitors of the ASM and SHPs specifically express Tbx1/10, a conserved essential regulator of pharyngeal mesoderm development, which is maintained specifically in the ASM precursors and rapidly shut off in the SHPs.

We first asked whether NK4 could be responsible for the SHP-specific termination of Tbx1/10 expression. DnNK4 overexpression in the TVC progenitors significantly increased the numbers of Tbx1/10+ cells, from 3.4±0.1 cells per side in control larvae to 4.3±0.2 (p = 0.0027, Figure 3E,F,K). We confirmed these data using intron-specific probes that showed active Tbx1/10 transcription in the SHPs of FoxG2>dnNK4-expressing, but not in control 16.5 hpf larvae (Figure 3M–P). Notably, Tbx1/10 expression did not expand to the FHPs in response to dnNK4, indicating that the restriction of Tbx1/10 activation to the secondary TVCs is independent of NK4 activity (Figure 3J,N,P). Conversely, NK4 overexpression completely suppressed TVC-specific expression of Tbx1/10 in 74% (23/31) of the larvae, thus reducing the average number of Tbx1/10+ cells to 0.3±0.1 per side (Figure 3K,L). Taken together, these data indicate that NK4 activity is required to inhibit Tbx1/10 expression in the SHPs.

Since targeted expression of dnNK4 causes ectopic activation of COE at 18 hpf (Figures 2F and S4), we performed double FISH assays to test whether Tbx1/10 and COE co-localize in control and experimental larvae. In control larvae, we observed COE transcripts in the lateral-most cells that display the highest levels of Tbx1/10 expression (Figure 3Q,T). In dnNK4-expressing larvae, ectopic COE only expanded to the medial SHP cells that maintained high Tbx1/10 expression (Figure 3R). Finally, both Tbx1/10 and COE expression were strongly inhibited by NK4 overexpression (Figure 3S). These observations open the possibility that Tbx1/10 defines the competence of the secondary TVC derivatives to activate COE and form ASM/LoM precursors.

**NK4 Antagonizes Tbx1/10-Mediated GATAa Inhibition and COE Activation**

We first used short hairpin RNA (shRNA)-mediated RNA interference (RNAi) to knock down Tbx1/10 activity and test whether it is required to promote COE and inhibit GATAa expression in the cardio-pharyngeal mesoderm. We applied a plasmid-based strategy using RNA Polymerase III–mediated shRNA expression in Ciona intestinalis microRNA miR-2213 [37,38]. We used a combination of shRNA constructs targeting two sites in the Tbx1/10 coding region and that efficiently down-regulated expression of a GFP::Tbx1/10 fusion protein (unpublished data). Electroporation of U6>shTbx1/10 constructs down-regulated the endogenous Tbx1/10 mRNA as evaluated by FISH (Figure 4A–D). Notably, U6>shTbx1/10 constructs induced a delay in secondary TVC division, which precluded the analysis of COE and GATAa expression at early time points (i.e., 16 to 18 hpf). In 19 and 20 hpf larvae, TVC divisions had resumed and double FISH-IHC assays using COE and GATAa probes showed that Tbx1/10 knock-down caused ectopic activation of GATAa in the lateral-most TVC derivative, while COE expression was markedly down-regulated (Figure 4E–L). These results indicate that Tbx1/10...
Figure 3. NK4 contributes to restricting *Tbx1/10* expression to the ASM precursors. (A–D, I–K and Q–S) *Tbx1/10* mRNAs (green); (E–H, M–P) *Tbx1/10* nascent transcripts (green) and (Q–S) COE mRNA (blue). Larvae electroporated with Mesp>nls:Gfp (red), FoxF>mCherry (I, M and O), FoxF>dnNK4 (J, N and P), and FoxF>NK4 (K). Ventral views, dotted lines indicate the midline; hpf, hours postfertilization; stage 23, 14 hpf at 16°C. Scale bar, 10 μm. Targeted expression of dnNK4 in the TVCs induces ectopic *Tbx1/10* expression in the SHP, but not in the FHPs (J arrows). FoxF>NK4 represses *Tbx1/10* expression in all TVC derivates (K). (L) Histograms showing the number of *Tbx1*+ and βGal+ cells per half. n, number of embryo.
activity is required to inhibit GATAa and promote COE expression in the ASMs.

We further tested whether the ectopic COE activation observed upon dnNK4 expression depends upon Tbx1/10 activity by co-electroporating FoxF>dnnK4 and U6>shTbx1/10 constructs and assaying COE and GATAa expression in 19 and 20 hpf larvae by double FISH-IHC (Figure 4G,H,K,L). These larvae were indistinguishable from larvae electroporated with U6>shTbx1/10 constructs alone, indicating that Tbx1/10 activity is required to mediate dnNK4-induced activation of COE in the SHPs, while NK4 activity appears dispensable to cause ectopic GATAa expression in the absence of Tbx1/10. These data also support the hypothesis that the remaining Tbx1/10 activity in the 15.5 to 17.5 hpf SHPs contributes to delaying GATAa reactivation. The latter eventually occurs probably because NK4 activity inhibits the maintenance of Tbx1/10 expression.

We reasoned that the absence of Tbx1/10 could explain why the FHPs expressed GATAa but did not activate COE and form ASMs in response to dnNK4 overexpression. We tested this possibility by mis-expressing Tbx1/10 and assaying COE expression at 20 hpf (Figure 5A–E). Tbx1/10 overexpression caused 56% (22/39) of the larvae to express COE in five or more cells per half (Figure 5B,E), resulting in a significant increase in the average number of COE+ cells from 3.3 ± 0.1 in controls (n = 29) to 5.1 ± 0.3 in FoxF>Tbx1/10-electroporated larvae (n = 39, t test p = 8.2 × 10⁻⁶; Figure 5E). This ectopic expression of COE was similar to that observed with FoxF>dnnK4 (4.3 ± 0.2, n = 52; Figures 2B,F and S4). However, upon targeted expression of either

Figure 4. RNAi-mediated loss of Tbx1/10 function inhibits COE and causes ectopic GATAa expression. Open arrowheads, ASM precursors; white arrowheads, SHPs; arrows, FHPs. Dotted lines indicate the midline. (A–C) Larva (16 hpf) electroporated with Mesp>nls: lacZ (loading control) (A), U6>shTyr (shRNA targeting the pigment cell-specific Tyrosinase, used as negative control, B), and U6>shTbx1/10 (C). TVCs are labeled with Mesp>nls: lacZ by immunostaining. U6>shTbx1/10 knocked down the endogenous Tbx1/10 transcripts and induced a delay in secondary TVC division. Double FISH detection of COE (blue) and GATAa (green) expression in larva of 19 hpf (E–G) and 20 hpf (I–K). ShRNA-mediated knock-down of Tbx1/10 caused ectopic activation of GATAa in the lateral-most TVCs, while COE expression was markedly down-regulated (F, J). Note the persistent expression of COE in an ASM that did not receive the plasmids (open arrowhead in J). The co-electroporation of FoxF>dnnK4 with U6>shTbx1/10 shows no ectopic COE expression, but a remarkable down-regulation in the lateral-most TVCs. Ectopic GATAa expression is detected in the lateral-most TVCs as well as in larvae electroporated with U6>shTbx1/10 only (G, K). Histograms showing the number of Tbx1/10+, COE+, GATAa+, and bGal+ cells per half (D, H, and L). n, number of embryo halves scored. Student’s t test compared experimental condition to the control. **p<0.05; error bar, standard error of the mean (SEM).

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Figure 5. Tbx1/10 activity promotes COE and inhibits GATAa expression in the secondary TVC derivatives. (A–D, F–I) Larva electroporated with Mesp->nlsGAL (red) and indicated combinations of FoxF->mCherry, FoxF->dnNK4, FoxF->NK4, and FoxF->Tbx1/10. FISH detection of COE mRNAs (green) at 20 hpf (A–D) and GATAa mRNAs (green) at 18 hpf (F, G) and GATAa nascent transcripts at 18.5 hpf (H, I). White dotted lines indicate the midline. ASM precursors, open arrowheads; SHPs, white arrowheads; FHPs, arrows. Scale bar, 10 μm. In control larvae (FoxF->mCherry, A), COE expression is restricted to ASM precursors; with FoxF->Tbx1/10 (B), weak COE expression expanded to a subset of heart precursors. Combined FoxF->Tbx1/10 and FoxF->dnNK4 strongly activated COE expression in all TVC derivatives (C). FoxF->Tbx1/10 and FoxF->NK4 caused sporadic ectopic activation of COE (D). (E) Histograms showing the numbers of COE+ and |Gal+ cells per electroporated half. n, number of embryo halves scored. Student’s t test compared experimental conditions to the control. **p<0.01; error bar, standard error of the mean (SEM). In the control, endogenous GATAa expression is restricted to heart precursors (F, H). FoxF->Tbx1/10 inhibits GATAa expression in all heart precursors (G, I). Histogram (J), numbers of |Gal and GATAa expressing cells per electroporated half at 18 hpf. Error bar, standard error of the mean (SEM); Student’s t test compared experimental condition to the control. **p<0.05.

Figure 6. NK4 binds to the minimal ASM enhancer of COE. (A) Combined snapshots of the ANISEED [74] and VISTA [75] browsers showing the KH2008 transcript models [76] for COE and conservation between Ciona intestinalis and Ciona savignyi. Pink peaks indicate conserved noncoding sequences (>65% identity per 80 bp). (B) Alignment of the conserved noncoding region corresponding to the minimal ASM enhancer (Figure S8). Above sequence, C. intestinalis; bottom sequence, C. savignyi. Putative NK4 and Tbx1/10 sites are highlighted in green and blue, respectively. Coordinates are expressed relative to the translation start site (ATG, where A is +1). (C) ChIP-qPCR data expressed as average fold enrichment relative to the mock control (ChIP using nonspecific IgG on the chromatin extracted from larvae electroporated with FoxF->NK4;2xFLAG), for ChIP using an anti-FLAG tag antibody on chromatin samples obtained from larvae electroporated with GFP:2xFLAG, NK4:2xFLAG, or dnNK4:2xFLAG. Error bars, standard error of the mean (SEM); calculated over biological triplicates and qPCR performed with two primer pairs for COE and two pairs for the internal loading control Brachury.

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Tbx1/10 or dnNK4, three or four β-gal+ cells remained COE– in more than 50% of the electroporated halves (average numbers of COE– cells were 3.8±0.3 and 4.2±0.2 with Tbx1/10 and dnNK4, respectively). These cells likely represent the FHPs, suggesting that Tbx1/10 or dnNK4 alone are not sufficient to promote COE expression in the FHPs.

As reported above, GATAa is reactivated in the FHPs, where its expression appeared independent of NK4 activity, while the delayed reactivation in the SHPs may require NK4 function. We reasoned that persisting, NK4-sensitive, Tbx1/10 activity could be antagonizing GATAa expression specifically in the SHPs, but not in the FHPs that do not express Tbx1/10. We tested this possibility by mis-expressing Tbx1/10 in all TVC progenitors and assayed GATAa expression at 18 hpf. Tbx1/10 mis-expression eliminated all TVC-specific expression of GATAa in 57% (28/49) of the larvae, causing the average number of GATAa+ cells to significantly decrease from 2.5±0.2 to 0.75±0.15 cells per side (t test p = 3.3×10^-11; Figure 5F–J). Intronspecific probes indicated that Tbx1/10 mis-expression inhibited GATAa transcription in 18.5 hpf larvae (Figure 5I). Thus, Tbx1/10 is sufficient to inhibit GATAa reactivation in all TVC derivatives. These data, taken together with the ectopic GATAa observed upon Tbx1/10 loss of function, indicate that remaining Tbx1/10 activity in the SHP may delay the reactivation of GATAa but that NK4 activity in the SHPs ultimately antagonizes Tbx1/10-mediated inhibition of GATAa reactivation, thus promoting heart fate specification in the SHPs.
To test whether NK4 could be inhibiting Tbx1/10-mediated activation of COE in addition to its inhibitory effect on the maintenance of Tbx1/10 expression, we co-electroporated FoxF>Tbx1/10 and FoxF>dnNK4 and assayed COE expression at 20 hpf. This combination caused 60% (40/69) of the larvae to express COE in all TVC derivatives (Figure 5C,E). The number of COE+ cells significantly increased to 7.4±0.2 (n = 59, t test p = 8.4×10⁻³¹; Figure 5C,E); at the expense of the β-gal+/COE− second heart precursors (SHPs) and FHPs (0.7±0.2 cells per half, n = 59; Figure 5C,E), while the total cell number did not change (3.1±0.1 versus 8.7±0.3 in control larvae). This shows that combining Tbx1/10 mis-expression and NK4 inhibition is sufficient to activate COE throughout the TVC progeny. Thus, in addition to its inhibitory effect upstream of Tbx1/10 expression, NK4 activity is also normally required to prevent Tbx1/10-mediated activation of COE specifically in the SHPs.

Combined overexpression of Tbx1/10 and NK4 further tested the mutual Tbx1/10 versus NK4 antagonism upstream of COE expression. NK4 overexpression completely inhibited COE in >40% of the larvae, reducing the average number of COE+ cells to 1.3±0.2 cells per half (n = 46; Figure 3E). Co-expression of Tbx1/10 rescued COE expression to an average of 4.3±0.4 cells per half (n = 32; Figure 5D,E). These data indicate that NK4 exerts its inhibitory effects on COE expression both upstream and downstream of Tbx1/10 activity.

To test whether NK4 could directly repress COE expression, we isolated a minimal ASM enhancer located ~2.5 kbp upstream of the translation start site (Figures 6 and S8). This COE minimal ASM enhancer overlaps with a noncoding sequence conserved between the sibling species Ciona intestinalis and C. savignyi (Figure 6A,B). Analysis of these sequences with the commercial MatInspector software [39] identified putative binding sites for NKX family transcription factors (Figure 6B). The algorithm did not detect putative TBX binding sites, but visual inspection identified two motifs similar to the conserved TBX half-site (Figure 6C,D). This shows that combining Tbx1/10 overexpression with NK4 inhibition can convert the TVC derivatives into ASM/LoM precursors, at the expense of the cardiac fate, in both the FHPs and SHPs.

Combined expression of Tbx1 and NK4 rescued the volume of heart-like β-gal+ tissue to 108% of controls, albeit reducing MHC2 expression and causing conspicuous ASM/LoM defects (Figure 7E, Table S1). Conversely, 3/11 animals had an enlarged MHC2+ heart, but did not entirely lack MHC2+ ASM/LoMs (Figure 7F, Table S1). Taken together, these results support the notion that NK4 and Tbx1/10 can antagonize each other’s effects upon heart versus ASM fate specification.

**Discussion**

In summary, here we presented evidence that the precise spatio-temporal deployment of an NK4-Tbx1/10 antagonism governs heart versus ASM/LoM fate specification upstream of COE and Gata4a in the ascidian cardio-pharyngeal mesoderm. We propose a model whereby the TVCs first undergo stereotyped asymmetric divisions that separate the lateral secondary TVCs and medial FHPs (Figure 7G). The lateral secondary TVCs activate Tbx1/10 before undergoing another stereotyped asymmetric medio-lateral division. The large lateral ASM precursors activate COE in response to Tbx1/10 activity. In the SHPs, NK4 promotes cardiac specification by inhibiting Tbx1/10 expression and antagonizing Tbx1/10-mediated inhibition of Gata4a reactivation, activation of COE expression, and subsequent ASM development. Our preliminary ChiP-qPCR data indicate that NK4-mediated inhibition of COE expression in the SHPs might occur through direct repression. In the absence of Tbx1/10 expression, the FHPs are the first to reactivate Gata4a expression and assume a cardiac fate, possibly independently of NK4 activity.

A key aspect of the proposed model is the deployment of cross-regulatory interactions specifically in the secondary TVCs, SHPs, and/or ASMs. Even though the prevalent use of the minimal FoxF enhancer, which is active early in the TVCs [20], can cause precocious effects, several lines of evidence suggest that the proposed regulatory interactions do occur in the secondary TVCs and/or derivatives. First, the wild-type temporal profiles obtained using intron-specific probes showed that Tbx1/10 transcription is quickly repressed specifically in the SHPs, while Gata4a is reactivated with an approximate 2.5 h delay compared to the FHPs. Second, upon expression of dnNK4, Tbx1/10 transcription was maintained specifically in the SHPs but did not expand to the FHPs, ectopic COE expression was also restricted to the SHPs, and Gata4a expression in the FHPs was unaffected. We thus reasoned that our manipulations altered essential regulatory events that occur after the separation of the FHPs from the secondary TVCs and contributed to re-program specifically the SHPs into ASMs.

Our model also raises several questions that should be addressed in future studies. First, the above argument does not formally rule out the possibility that early TVC inputs (e.g., BMP signaling, see below and [26]) contribute to later Tbx1/10, Gata4a, and/or COE expression in the secondary TVCs, FHPs, SHPs, and ASMs and could be affected by precocious manipulation of NK4 activity.
Future studies capitalizing on enhancer analyses and whole genome profiling data (Razy-Krajka et al., under review) will uncover additional regulators of ASM versus heart fate specification. Second, since NK4 transcripts were detected throughout the TVC lineage, and high levels of NK4 activity were sufficient to repress Tbx1/10 and COE expression, what prevents NK4-mediated inhibition of Tbx1/10 and COE expression in the secondary TVCs and ASM? Since differential regulatory inputs between the heart and ASM precursors occur following stereotyped oriented asymmetric divisions, we reason that differences between the SHPs and ASM arise as consequences of the asymmetric secondary TVC division. This may contribute to the observed delay in ectopic COE expression in the FoxF>dnNK4-expressing larvae, even though these cells maintained Tbx1/10 transcription. Future studies ought to identify the molecular mechanisms that link oriented asymmetrical TVC division to the secondary TVC and ASM-specific inhibition of NK4-mediated repression of Tbx1/10 and COE.

Previous studies of the ascidian heart progenitors indicated that early GATAa activity and BMP signaling control the TVC expression of NK4, which eventually feeds back negatively on two regulators of BMP activity, Bmp2/4 and Tolloid [17,26]. Regulatory interactions between BMP signaling, NK4, and GATAa orthologs are thought to be part of an ancient gene regulatory subnetwork referred to as the “heart kernel” [45,46]. Activation of pannier/GATA4/5/6 downstream of tinman/NKX2-5 is one of the essential core regulatory interactions, which ascidians seemed to have rewired since the expression of GATAa starts before and determines that of NK4 [17]. Our results indicate that, in turn, NK4 activity is required for GATAa reactivation in the SHPs but probably not in the FHPs. In addition, we propose that this interaction involves a double-negative gate network motif whereby

Figure 7. Combined Tbx1/10 mis-expression and NK4 inhibition can convert all TVC derivatives into differentiated ASMs and LoMs in 72 hpf juveniles. (A–F) Juveniles electroporated with Mesp>nls:lacZ(red) and indicated constructs were hybridized with MHC3 (blue) and MHC2 (green) probes. (A) FoxF>mCherry control, (B) FoxF>Tbx1/10 reduced heart volume and the number of MHC2+ cells in 10/20 animals; (C–D) combined FoxF>dnNK4 and FoxF>Tbx1/10 converted most (C) or all (D) TVC derivatives into differentiated ASMs and LoMs; (E–F) combined FoxF>NK4 and FoxF>Tbx1/10 showed least dramatic effects than each one alone: ASM/LoM formation defects are observed, 8/11 juveniles have reduced hearts and MHC2 expression, and 3/11 juveniles have enlarged heart and increased MHC2 expression. White dotted lines outline whole bodies. Insets show magnified hearts. Scale bar, 25 μm. (G) Summary model of the NK4 and Tbx1/10 mutual antagonism regulating cardiac versus ASM fate specification within a conserved clonal topology for progressive fate choices. The cells and approximate time windows are showed (i.e., align with the timeline above). Greyed labels indicate inactive genes and regulatory interactions. The question marks point to the unknown mechanism(s) that prevent(s) NK4-mediated inhibition of Tbx1/10 and COE expression in the secondary TVCs and ASM founder cells.

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NK4 activity prevents the maintenance of Tbx1/10 expression, which would inhibit and actually contributes to delaying GATAa reactivation in the SHFs.

In mouse models for the Cardio-Velo-Facial/Di George syndrome, defects in the development of second heart field derivatives, such as the outflow tract, arise from altered Tbx1 dosage, including overexpression [47,48]. Transcription profiling of the posterior pharyngeal mesoderm in Tbx1 mutants and transgenic mice with additional copies of the human Tbx1 indicated that Tbx1 inhibits the expression of early heart differentiation markers, including Tbx3 and GATAa [49]. Thus, the negative influence of Tbx1/10 activity on GATAa5/6 expression in the pharyngeal mesoderm is likely to be a conserved regulatory interaction in chordates. The inhibitory effects of Tbx1 on cardiac differentiation have been interpreted as a role for Tbx1 in maintaining a pluripotent progenitor state presumably required for proliferation of cardiac progenitors in the second heart field [34,50–52]. In our study, secondary TVC divisions were markedly delayed in Tbx1/10 knock-down experiments. Future studies will be required to determine how Tbx1/10 influences cell division and whether this delay indirectly contributes to the observed deregulation of COE and GATAa in addition to the direct effect of the loss of Tbx1/10 function. Our observations suggest that ancestral roles of Tbx1/10 in the cardio-pharyngeal mesoderm were to promote cell divisions and oppose cardiac specification during pharyngeal muscle development.

What is the molecular mechanism for the proposed NK4-Tbx1/10 cross-antagonism? Our ChIP-qPCR data suggest that NK4 directly binds to and represses the COE ASM enhancer. The presence of putative TBX sites in this enhancer suggests that Tbx1/10 could also directly activate COE, which remains to be tested experimentally, as is the possibility of a direct repression of Tbx1/10 expression by NK4. A strikingly similar regulatory mechanism was recently demonstrated for the mouse Fgf10 enhancer, where Tbx1 binds and directly activates expression in the anterior second heart field, while Nkx2-5 also binds but represses enhancer activity in the myocardium, in part by competing with Islet1 for the same homeodomain binding sites [53]. Such a competition with Islet could also account for NK4-mediated repression of COE in the SHP, but presumably not at the initial stage of its expression since Islet is only activated about 2 h after COE in the TVC derivatives. Future studies will be required to determine if COE and Islet maintain each other's expression in the ASM precursors.

The NK4-Tbx1/10 antagonism possibly occurring at the COE enhancer might also involve direct protein–protein interaction. For instance, co-immunoprecipitation assays using tagged proteins indicated that Tbx1 and Nkx2-5 can physically interact in transfected 293T cells [54]. Interestingly, in this study Tbx1 and Nkx2-5 appeared to directly cooperate to activate the Pit2 ASE enhancer on the left side of the SHF [54]. Nkx2-5 was also shown to form a ternary complex with Tbx2 and the Atrial natriuretic factor (Anf) atrioventricular canal (AVC)-specific enhancer to directly repress the latter in the mouse myocardium [55]. Taken together, these and our data suggest that ancient molecular interactions between cardio-pharyngeal members of TBX and NKK families of transcription factors govern a range of enhancer activities between cardio-pharyngeal members of TBX and NKK orthologs during pharyngeal muscle fate specification within an ancestral lineage of cardio-pharyngeal progenitors in tunicates and vertebrates, where this simple ontogenetic motif underlies heart and head muscles development and evolution. More specifically, it is tantalizing to speculate that prolonged Tbx1 expression in the amniote second heart field has fostered the emergence of novel heart compartments by antagonizing heart differentiation, thus maintaining a proliferative progenitor state in the SHF and expanding on the ancestral heterochrony between first and second heart fields' specification that is observed in extant chordates.

Studies in vertebrates and flies have documented a requirement for Tbx1 activity in noncardiac muscle development. The Drosophila ortholog of Tbx1/10, wg-1, contributes to myogenesis in the visceral mesoderm [56,57]. In amniotes, the branchiomiomuscles derive from the pharyngeal mesoderm and are severely affected by impaired Tbx1 function, which is required for proper activation of the myogenic factors MyoD and Myf5 [3,58–61]. A recent study identified the COE orthologs, E82 and –3, as essential determinants of MyoD expression and head muscle development in Xenopus [31]. Mis-expression of the cardio-pharyngeal determinant Mesp1 up-regulates E82 expression in mouse embryonic stem cells [62]. Taken together, these and our observations lead us to predict that Tbx1/10 genes act as conserved upstream regulators of COE orthologs during pharyngeal muscle development in vertebrates and tunicates.

Established lineage topologies within the mouse cardio-pharyngeal mesoderm show that precursors of the FHF diverge before the separation of the SHF from pharyngeal/branchiomiome muscle progenitors [0,9]. Nkx2.5 mutant mice display profound defects in the formation of SHF derivatives, which fail to proliferate, but form FHF-derived left ventricle tissue, and loss of Nkx2.5 function also up-regulates Islet1 expression in the mouse cardiogenic mesoderm [32]. In zebrafish, SHF progenitors also reside in the gata4+/+nkh2.5+ part of the anterior lateral plate mesoderm and fail to proliferate normally in nkh2.5 morphant embryos, while initial SHF specification appeared to occur normally as judged by latent TGFβ binding protein 3 (lbp3) expression [63]. However, combined inhibitions of nkh2.5 and nkh2.7 caused an increase in atrial tissue formation at the apparent expense of the ventricle [64] and opposed the specification of Islet+ SHF progenitors [33]. In Drosophila, the NK4 ortholog tinman inhibits Collier expression during muscle-type diversification [65], suggesting that a negative regulatory interaction between NK4 and COE orthologs is part of an ancient network for muscle specification. Future studies will be required to determine if SHF progenitors can also be re-specified into COE-dependent branchiomiomuscle precursors downstream of Tbx1/10 in Nkx2.5 mutants. We propose that a conserved cross-antagonism between NK4 and Tbx1/10 orthologs acts upstream of GATAa and COE orthologs for heart versus pharyngeal muscle fate specification within an ancestral lineage of cardio-pharyngeal progenitors in tunicates and vertebrates, where this simple onogenetic motif underlies heart and head muscles development and evolution. More specifically, it is tantalizing to speculate that prolonged Tbx1 expression in the amniote second heart field has fostered the emergence of novel heart compartments by antagonizing heart differentiation, thus maintaining a proliferative progenitor state in the SHF and expanding on the ancestral heterochrony between first and second heart fields' specification that is observed in extant chordates.

Materials and Methods

Embryo Preparation

Gravid Ciona intestinalis adults were purchased from M-Rep (San Diego, CA). Isolation of gametes, fertilization, dechorionation, electroporation, and development were conducted as described [66–69]. Juveniles were obtained by letting the larvae settle and metamorphose on plastic Petri dishes. Filtered artificial sea water was supplemented with antibiotics (streptomycin and penicillin, 50 µg/mL each) and changed daily. The amount of DNA for electroporation was typically 70 µg, except for Mesp>nlslacZ (50 µg) and for Mesp>Lifeact:mCherry (20 µg). Lifeact fusions appeared toxic at higher concentrations. Embryos were fixed at...
the different developmental stages for 2 h in 4% MEM-PFA and stored in 75% ethanol at −20°C [66].

Molecular Cloning and Sequence Analyses

The coding sequences (CDS) of NK4 and Tbx1/10 (Genbank Accession: KC196542) were amplified by RT-PCR from total RNA isolated from 16 hpf Ciona intestinalis larvae using the following primers: NK4_CDS_F, 5'-AAAGGCGCCAAACATGATTTCCCTAGTCCGGTTGGATCGACT-3'; NK4_CDS_R, 5'-TTGCTTCACTCAGTTCGACAGCCGAAGCTTAT-3'. The CDS were cloned downstream of the Foxf minimal TVC enhancer (Foxf-TVC) fused to the basal promoter of the Friend of Gaeta gene [bpFOG] [70]. The Tbx1/10 coding sequence was amplified using Tbx1_CDS NotI_F2, 5'-AAAGGCGCCAAACATGATTTCCCTAGTCCGGTTGGATCGACT-3'; Tbx1_CDS ClaI_R2, 5'-AAAGCGGCCGCAACATGATTTCCCTAGTCCGGTTGGATCGACT-3'; Tbx1_CDS BglII_R2, 5'-AAAGGCGCCAAACATGATTTCCCTAGTCCGGTTGGATCGACT-3'.

The N448K point mutation of NK4 was introduced by overlapping PCR strategy using the primers NK4_N448K_F, 5'-AAAGGCGCCAAACATGATTTCCCTAGTCCGGTTGGATCGACT-3'; NK4_N448K_R, 5'-AAAGGCGCCAAACATGATTTCCCTAGTCCGGTTGGATCGACT-3'.

mCherry was fused to N-terminal LifeAct peptide [71] using double-stranded DNA oligonucleotides and subcloned downstream of the Meep enhancer (Mesp>lifeact>mCherry) to visualize the cell cortices of B7.5 lineage cells. Mesp>nIslacZ was used as described previously [16].

FISH-IHC

Double FISH-IHC was performed following a protocol modified from Christian et al. [66]. Hybridized probes were revealed using the Tyramide Signal Amplification (TSA) with either Cyanine5 or Fluorescein TSA Plus Evaluation Kits (Perkin Elmer, MA). Anti-Digoxigenin-POD Fab fragment (Roche, IN) was added to detect the fluorescein-conjugated probe. Anti–Digoxigenin-POD Fab fragment (Roche, IN) was first used to detect Digoxigenin-conjugated probe. Anti–β-galactosidase monoclonal mouse antibody (Promega) was co-incubated with anti-DIG-POD for immunodetection of Mesp>nIslacZ products. A 10 min treatment with WB2 buffer (50% formamide, 2× SSC, 0.1% Tween20) at 55°C was used to denature anti-Digoxigenin-POD antibody after the first Fluorescence reaction [72]. Anti-Fluorescence-POD Fab fragment (Roche, IN) was added to detect the fluorescein-conjugated probe following and revealed by Cyanine5 TSA reaction. Goat anti-mouse secondary antibodies coupled with AlexaFluor-555 were used following the second TSA reaction to detect β-galactosidase-bound mouse antibodies.

Confocal Imaging and Heart Volume Quantification

Samples were mounted in 50% glycerol/PBS with 2% DABCO. Images were acquired with a Leica TCS SP5 or a Leica TCS SP8 X confocal microscope, using 20× or 63× objectives. Z-stacks were acquired at 1.5 μm (20×) or 1 μm (63×) z steps. Maximum projections were processed with maximum projection tools from the LEICA software LAS-AF.

The measurements of juvenile heart volume were conducted using Velocity 5 (PerkinElmer, MA). Volume of Mesp>nIslacZ labeled heart cells was detected on confocal stacks. Three filters were set for objective detection as following: (1) regions of interest were segmented by percentage intensity (lower threshold, 20%; upper threshold, 100%), (2) noise was removed from objects with a Fine Filter setting, and (3) touching objects separated with object size guide as 100 μm². Quantifications were exported into Excel for further analysis.

shRNA-Mediated RNAi Knock-Down of Tbx1/10 Function

The coding sequence of Ct-Tbx1/10 was used to identify shRNA target sites with the DSIR [73] and Public TRC Portal (http://www.broadinstitute.org/rnai/public/seq_search) algorithms. Candidate 21-mer target sites were trimmed to 20 mer, and those that begin with A or G and end with A were further considered. Candidate 20 mer were BLASTed against the Ciona intestinalis genome on the Ghost database and selected to correspond to the microRNA mIR-2213 [30]. A vector containing the Ciona U6 promoter [36] was used to clone complementary oligos encoding four shRNA constructs: shTbx1-A-F, GGCAAGAGTCCACCAAGATTTGTAATAATTAGGAAATTGCTCT: shTbx1-A-R, GCTGTTAATTAAGTCAGCTCTTATTGTTT; shTbx1-B-F, GCTGTTAATTAAGTCAGCTCTTATTGTTT; shTbx1-B-R, GCTGTTAATTAAGTCAGCTCTTATTGTTT.

The coding sequence of NK4-N448K was amplified by RT-PCR from total RNA isolated from 16 hpf Ciona intestinalis larvae using the following primers: NK4_N448K_F, 5'-AAAGGCGCCAAACATGATTTCCCTAGTCCGGTTGGATCGACT-3'; NK4_N448K_R, 5'-AAAGGCGCCAAACATGATTTCCCTAGTCCGGTTGGATCGACT-3'.

Ciona Brachyury [44] and provide an off-target negative/loading control for qPCR. The primers used in qPCR were as follows: En_CiCOE_F1, 5'-CTGGTCCTATTCCCAATTCCAGATATTTCTCCATCGATTTACTGGAG-3'; En_CiCOE_R1, 5'-GAACCCTAAGGAAGATGTTTCTTTGAAGTTTCTTTAATGCGG-3'; En_CiCOE_R2, 5'-TTATGGCTATTCTTTACCACTTTAATTCTTTAATGCGG-3'; En_CiCOE_R3, 5'-TAATTCCCTGAGTCCCAATTCCAGATATTTCTCCATCGATTTACTGGAG-3'; En_CiCOE_R4, 5'-TTATGGCTATTCTTTACCACTTTAATTCTTTAATGCGG-3'. The data showed used 30 μg each of U6>shTbx1/10-A and -B per electroporation.

Chromatin Immunoprecipitation (ChiP)

A 2xFLAG tag was cloned to the C terminus of coding sequences to make the following constructs: Foxf>GFP:2xFLAG, Foxf>dNk4:2xFLAG, Foxf>CtTbx1/10:2xFLAG. We used 50 μg of plasmid for each electroporation. The larvae from three electroporations were combined as one ChiP sample. All samples were harvested at 16 hpf and cross-linked at 22°C for 15 min by adding formaldehyde to the final concentration of 1%. Chromatin extracted from larvae was sheared into 400 bp fragments by S220 Focused-ultrasonicator (Covaris, MA). The cross-linked enhancer fragments were incubated with DYKDDDDK tag antibody (Cat. No: 2368, Cell Signaling Technology, MA) overnight and then captured by Dynabeads M-280 sheep anti-γ-galactosidase monoclonal mouse antibody (Promega) was co-incubated with anti-DIG-POD for immunodetection of Mesp>nIslacZ products. A 10 min treatment with WB2 buffer (50% formamide, 2× SSC, 0.1% Tween20) at 55°C was used to denature anti-Digoxigenin-POD antibody after the first Fluorescence reaction [72]. Anti-Fluorescence-POD Fab fragment (Roche, IN) was added to detect the fluorescein-conjugated probe following and revealed by Cyanine5 TSA reaction. Goat antimouse secondary antibodies coupled with AlexaFluor-555 were used following the second TSA reaction to detect β-galactosidase-bound mouse antibodies.

Quantitative PCR (qPCR)

Each ChiP sample was obtained in three independent biological replicates. Two pairs of primers were designed within the minimal COE enhancer. Two pairs of primers were also used to amplify the enhancer region of Ciona Brachyury [44] and provide an off-target negative/loading control for qPCR. The primers used in qPCR were as follows: En_CiCOE_F1, 5'-CTGGTCCTATTCCCAATTCCAGATATTTCTCCATCGATTTACTGGAG-3'; En_CiCOE_R1, 5'-GAACCCTAAGGAAGATGTTTCTTTGAAGTTTCTTTAATGCGG-3'; En_CiCOE_R2, 5'-TTATGGCTATTCTTTACCACTTTAATTCTTTAATGCGG-3'; En_CiCOE_R3, 5'-TAATTCCCTGAGTCCCAATTCCAGATATTTCTCCATCGATTTACTGGAG-3'; En_CiCOE_R4, 5'-TTATGGCTATTCTTTACCACTTTAATTCTTTAATGCGG-3'.

Chromatin Immunoprecipitation (ChiP)

Quantitative PCR (qPCR)

Each ChiP sample was obtained in three independent biological replicates. Two pairs of primers were designed within the minimal COE enhancer. Two pairs of primers were also used to amplify the enhancer region of Ciona Brachyury [44] and provide an off-target negative/loading control for qPCR. The primers used in qPCR were as follows: En_CiCOE_F1, 5'-CTGGTCCTATTCCCAATTCCAGATATTTCTCCATCGATTTACTGGAG-3'; En_CiCOE_R1, 5'-GAACCCTAAGGAAGATGTTTCTTTGAAGTTTCTTTAATGCGG-3'; En_CiCOE_R2, 5'-TTATGGCTATTCTTTACCACTTTAATTCTTTAATGCGG-3'; En_CiCOE_R3, 5'-TAATTCCCTGAGTCCCAATTCCAGATATTTCTCCATCGATTTACTGGAG-3'; En_CiCOE_R4, 5'-TTATGGCTATTCTTTACCACTTTAATTCTTTAATGCGG-3'.
5′ CACGCAAGACAAATGGGAAG 3′; En_CiBra_R2 5′ GGTGGCAGCTCTATGTTTACT 3′. Quantitative PCR was performed using the SYBR Green method on a LightCycler 480 system (Roche Diagnostics, IN). Each qPCR was performed in technical triplicates, and the average values were used for subsequent calculations. Fold enrichments were calculated using the ΔΔCt method: For each ChIP sample and corresponding input, the ΔCt for En_COE_1 and En_COE_2 versus the average Brachyury was calculated first. Then ChIP versus input ΔΔCt was calculated for each En_COE primer pair and each sample. Finally, fold enrichment over mock control was calculated for each replicate. Average fold enrichments relative to mock controls are shown in Figure S6. Similar values were obtained relatively to the GFP:2xFLAG negative control (Table S2).

Supporting Information

Figure S1 Conserved features of NKX2-5 homedomain proteins across species. (A) Alignment of NK4 orthologues across species using ClustalX 2.1 (http://www.clustal.org/clustal2/); CiNKX4, Ciona intestinalis, NP_001080190, 299 aa [79]; MmNKX2-5, Mus musculus, NP_001158401, 268 aa[83]; HsNKX2-5, Homo sapiens, NP_524433, 416 aa [84]. Tinman, Drosophila melanogaster, NP_571496, 314 aa [82]; GATAa, Danio rerio, NP_004378, 244 aa [81]; VnxKX2-5, Xenopus laevis, NP_001080190, 299 aa [79]; MnNKX2-5, Mus musculus, NP_032726, 318 aa [80]; HaNKX2-5, Homo sapiens, NP_004378, 324 aa [81]; DlNKX2-5, Danio rerio, NP_571496, 314 aa [82]; VNXKX2-3′5′, Saccoglossus kowalevskii, NP_001158401, 268 aa [83]; Tinnman, Drosophila melanogaster, NP_524433, 416 aa [84]. Tinman domain, Homeobox, NK2-SD domain, and Tyrosine-Rich domain [85] are highlighted in boxes. CiNKX4 lacks the Nks2-5 box and GIRARW motif. (B) Domain structure of CiNKX4. Asparagine (N) at amino acid residue position 448, which is critical for DNA binding capability of the homeobox [28], is mutated to Lysine (K) to generate the dominant negative version of NK4 (dnNK4).

Figure S2 Endogenous expression of NK4. (A–D) NK4 endogenous expression pattern during TVC lineage specification. Mesp>LifeAct:mCherry (blue) and Mesp>nlslacZ (red) were detected by immunostaining, indicating cell boundary and nucleus, respectively; NK4 mRNAs (green) were detected by FISH. NK4 transcripts are present throughout the TVC lineage and endoderm from 12 to 20 hpf at 18°C. Scale bar, 25 μm. (A–D) FISH detection of NK4 transcripts (green) at 48 hpf (E), 72 hpf (F), Red, Mesp>nlslacZ positive B7.5 lineage cells; the heart areas (dotted line square) were rescanned at higher magnification (63x objective; E′, F′). NK4 transcripts were not detected in the juvenile heart. Scale bar, 10 μm. (TIF)

Figure S3 COE expression initiates prior to that of Islet. (A) Proportions of embryos and larva showing COE (blue) or Islet (red) expression at indicated stages of TVC development (the diagrams correspond to the stages described in Figure 1A). COE expression starts in the ASM precursors immediately after the second asymmetric division. Islet expression starts after COE throughout the lineage first, and quickly becomes stronger in the ASMs. (B) Initiation of COE expression visualized in early ASMs by two nuclear dots, indicating that this cDNA probe labels nascent transcripts. Ventral view of larva with TVC lineage progenitors in early class 2 pattern. Larva is electroporated with Mesp>nlslacZ to mark the TVC lineage (red). DAPI channel to visualize the nucleus (blue). COE transcripts are detected by FISH (green). TVC lineage cells (squared) are magnified. (C–E) Islet nascent transcripts detected by Islet intron-specific probe (green). TVC lineage is marked with Mesp>nlslacZ (red). Islet transcription is activated in all the TVC derivatives around 18 hpf, but exclusively in the ASM precursors (open arrowheads) at 20 hpf. Scale bar, 25 μm. (TIF)

Figure S4 NK4 represses COE in TVC derivatives. (A–C, E–G) FISH of COE (green) in 28 hpf (A–C) and 24 hpf (E–G) larvae; FoxF>dnNK4 induces ectopic COE expression and results in extra-numerary TVC lineage cells to contribute to the formation of the ASM ring (B). Conversely, FoxF>DNK4 represses COE and inhibits ASM ring formation (C). Mesp>nlslacZ (red) labels B7.5 lineage cells; COE transcripts (green) detected by FISH. White dotted lines indicate the midline. Scale bar, 25 μm. Counts of COE+/− (D, H, I) and GATAa+/− (J) cells following manipulations of NK4 activity at 28 hpf (D), 24 hpf (H), and 18 hpf (I, J). Student’s t tests compare each experimental condition to the control. **p<0.05 and *p<0.01. The significant increase in GATAa− cell numbers observed in (J) is due to a slight increase in the total number of cells per half in these larvae, which could be caused by premature divisions in the dnNK4 condition (see text for details). (K) FISH-IHC with COE intronic probes at indicated stages of larval development with indicated constructs. Note that no ectopic COE expression was detected before 17–17.5 hpf. (TIF)

Figure S5 NK4 regulates heart and ASM specification via COE. (A–F) Lateral views of 28 hpf larvae, showing the ventral heart precursors and dorso-lateral ASM rings. Mesp>nlslacZ (red immunostaining) marks B7.5 lineage cells. Larvae hybridized with digoxigenin-conjugated MHC3 probe (green). (A) In control larvae, four to six of the ASM ring cells express MHC3. (B) FoxF>COE:WRPW abolishes ASM migration, ring formation, and MHC3 expression in TVC derivatives. (C′–C′) FoxF>COE:Δ321 has mild or no significant effects on second migration, causes ASMs to cluster instead of forming a ring in 31% (23/74) (the cluster phenotype is scored independently) of the larvae (C′), and inhibits MHC3 expression. (D) FoxF>NK4 increases the number of migrating and MHC3+ cells. (E) FoxF>COE:WRPW inhibits the effects of FoxF>dnNK4; neither second migration nor MHC3 expression are observed. (F) FoxF>COE:Δ321 also inhibits the effects of FoxF>dnNK4; fewer cells migrate to the siphon placode and fewer MHC3+ cells are observed in the ASM ring. (G) Numbers of MHC3+/− cells per half in indicates conditions. (H) Numbers of cells that migrates to the Atrial Siphon Primordium per half. Student’s t tests compare each experimental condition to the control. **p<0.05. (TIF)

Figure S6 NK4 represses Islet in TVC lineage cells. FISH of Islet in 20 (A–C), 24 (D–F), and 28 hpf (G–I) larvae. In control larvae, Islet is expressed at higher levels in ASM precursors than in heart precursors. Targeted expression of dnNK4 causes up-regulation of Islet in heart precursors, while overexpression of NK4 inhibits Islet in all TVC derivatives. Asterisk, Islet expression in endodermal cells; dotted line, ventral midline. Scale bar, 25 μm. (TIF)

Figure S7 Endogenous expression pattern of GATAa during TVC lineage specification. GATAa mature (green) (A–D) and nascent (E–L) (green) transcripts were detected by FISH. Mesp>nlslacZ (red) were detected by immunostaining to...
Fertilized eggs were electroporated with Mesp using FISH-IHC. The total volume of s:lacZ and indicated constructs driven by FoxF(TVC)bpFOG. The contribution of Islet1-expressing splanchnic mesoderm cells to distinct heart field derivatives in the mouse embryo. Development 137: 3209–3279.

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