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HHS Public Access

Author manuscript

Curr Opin Genet Dev. Author manuscript; available in PMC 2016 June 01.

Published in final edited form as:

Curr Opin Genet Dev. 2015 June ; 32: 119–128. doi:10.1016/j.gde.2015.02.008.

Regulation and evolution of cardiopharyngeal cell identity and behavior: insights from simple chordates

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Abstract

The vertebrate heart arises from distinct first and second heart fields. The latter also share a common origin with branchiomeric muscles in the pharyngeal mesoderm and transcription regulators, such as Nkx2–5, Tbx1 and Islet1. Despite significant progress, the complexity of vertebrate embryos has hindered the identification of multipotent cardiopharyngeal progenitors. Here, we summarize recent insights in cardiopharyngeal development gained from ascidian models, among the closest relatives to vertebrates. In a simplified cellular context, progressive fate specification of the ascidian cardiopharyngeal precursors presents striking similarities with their vertebrate counterparts. Multipotent cardiopharyngeal progenitors are primed to activate both the early cardiac and pharyngeal muscles programs, which segregate following asymmetric cells divisions as a result of regulatory cross-antagonisms involving Tbx1 and Nkx2–5 homologs. Activation of Ebf in pharyngeal muscle founder cells triggers both *Myogenic Regulatory Factor*-associated differentiation and Notch-mediated maintenance of an undifferentiated state in distinct precursors. Cross-species comparisons revealed the deep conservation of the cardiopharyngeal developmental sequence in spite of extreme genome sequence divergence, gene network rewiring and specific morphogenetic differences. Finally, analyses are beginning to uncover the influence of surrounding tissues in determining cardiopharyngeal cell identity and behavior. Thus, ascidian embryos offer a unique opportunity to study gene regulation and cell behaviors at the cellular level throughout cardiopharyngeal morphogenesis and evolution.

Introduction

The heart is a muscular organ that pumps blood through a circulatory system. In mammals, the embryonic heart tube is the first functional organ and further morphogenesis leads to a four-chambered organ. The frequency and diversity of congenital heart diseases reflects the complexity of cardiac morphogenesis. Discrete mesodermal cell populations, referred to as the first and second heart fields, are recruited sequentially to form the heart tube and

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progressively add tissue to the growing heart [1–5]. The first heart field (FHF) gives rise to the early embryonic heart tube, while the second heart field (SHF) later contributes to both arterial (*e.g.* outflow tract and right ventricle) and venous (*e.g.* right atrium) poles, (reviewed in [6]). Clonal analyses in the mouse demonstrated that cardiomyocytes of FHF and SHF origins derive from common progenitors that were initially thought to originate in the *Mesp1*+ mesoderm of the early embryo [7][8,9]. Further analyses revealed a common pharyngeal origin of the SHF and the branchiomeric/pharyngeal muscles [10–14]. Shared molecular determinants of the SHF and pharyngeal muscles have been identified and linked to the Cardio-Velo-Facial/DiGeorge syndrome, in which the 22q11.2 deletion removes the transcription factor TBX1 and is responsible for malformations of pharyngeal apparatus and cardiac outflow tract [15,16]. Developmental and genetics studies point to essential roles for the homeobox genes *Islet1/Isl1* and *Nkx2–5* alongside *Tbx1* in the cardiopharyngeal mesoderm, the source of SHF and branchiomeric muscles progenitors [13,17–21]. Mammalian cardiopharyngeal progenitors and the mechanisms underlying early heart vs. branchiomeric muscle specification remain elusive due to the complexity and relative inaccessibility of the early embryos.

The ascidian *Ciona intestinalis* has emerged as a simple chordate model to study early cardiac development with cellular resolution [22–24]. As tunicates, ascidians are marine invertebrates among the closest living relatives of the vertebrates [25–27]. Tunicates and vertebrates form the clade *Olfactores* [27]. A model tunicate, *Ciona* uniquely combines genetic and cellular simplicity, experimental amenabilities and olfactores-specific traits, which are lacking in distant genetic models including flies or nematodes [28]. The adult *Ciona* heart consists of U-shaped tube comprising two monolayers of cells: an external pericardium surrounding a contractile myocardium, with no endocardium [29–31]. The ascidian heart derives from a single pair of bilateral blastomeres in the 110-cell stage embryo [31,32]. The B7.5 blastomeres, named after Conklin, and their daughter cells, the B8.9 and B8.10 founder cells, transiently express the *Mesp/Mesogenin* sole pro-ortholog [31]. As in vertebrates, early *Mesp* function is crucial for heart development in *Ciona* [31]. The founder cells then divide asymmetrically to produce two anterior tail muscle cells (ATMs) cells and their sister cells, the trunk ventral cells (TVCs), which migrate towards the ventral side of the trunk (Figure 1; [30,31,33]). TVC specification and migration are controlled by the sequential activation of the FGF-MAPK-Ets signaling pathway and the transcription factor FoxF ([34–36]; reviewed in [22,24]). Migrating TVCs activate conserved regulators of cardiac development including *Nk4/Nkx2–5*, *Hand* and *Gata4/5/6* homologs [30,31]. The TVCs are common progenitors for the juvenile heart, atrial siphon muscles (ASM) and longitudinal body wall muscles (LoM) [32,37]. The latter muscle populations derive from *Islet*+ and *Tbx1/10*+ precursors, constituting likely homologs of the vertebrate branchiomeric muscles. Here, we review recent progress in characterizing the TVCs as multipotent cardiopharyngeal progenitors and the mechanisms underlying fate choices in their progeny. We present how the cardiopharyngeal ontogenetic motif is conserved amid specific developmental variations and gene regulatory drift in distantly related tunicates. Finally, we summarize recent insights into the crosstalk between extrinsic influences, intrinsic properties and cell behavior during TVC induction and collective migration.

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TVC are multilineage-primed multipotent cardiopharyngeal progenitors

Following migration into the trunk and association with the ventral endoderm, each TVC divides asymmetrically into a small median first heart precursor (FHP) and a large lateral secondary TVC (STVC) [33,37]. The latter divide again asymmetrically into a small medial second heart precursor (SHP) and a large atrial siphon muscle founder cell (ASMF; Figure 1; [37–39]). ASMFs immediately activate the atypical helix-loop-helix transcription factor Ebf (previously referred to as COE, for Collier/Olf/EBF; [37,40]). Gain- and loss-of-function assays, using either a repressor form or lineage-specific CRISPR/Cas9 constructs, indicated that Ebf promotes ASM specification at the expense of the heart fate [37,41]. Thus, the mechanisms controlling ASMF-specific expression of *Ebf* determine the initial heart vs. ASM fate choice in the ascidian cardiopharyngeal mesoderm.

Tissue-specific transcription profiling using fluorescence activated cell sorting (FACS) and microarrays characterized the transcriptional dynamics underlying heart vs. ASM fate choice [38]. Time-series and Ebf-perturbations datasets suggested that Ebf-inhibited genes, viewed as candidate heart-specific genes, were first expressed in the TVC prior to asymmetric cell divisions. However, fluorescent *in situ* hybridizations assays revealed that asymmetric divisions are accompanied by progressive restriction of expression of distinct TVC genes into either the heart precursors or the STVCs and then ASMFs [38]. Among the TVC genes restricted to the STVCs and ASMFs, *Hand-r* (*Hand-related*, previously *Hand-like/NoTrlc*; [31,40,42]) is necessary for *Ebf* expression. On the other hand, *Gata4/5/6* and *Hand* expressions become restricted to the heart precursors. Thus, the TVCs are transcriptionally primed for both pharyngeal and cardiac fate specification. Such multilineage transcriptional priming of multipotent progenitors is common in ascidians [43] and in vertebrate hematopoiesis but has not been documented in vertebrate cardiopharyngeal mesoderm [38]. Instead, studies using stem cell models for mammalian cardiogenesis revealed "chromatin priming", whereby cardiac enhancers are poised for future activation in mesoderm progenitors [44,45]. Future studies will determine whether late ASM- and/or heart-specific enhancers are also "primed" in multipotent cardiopharyngeal progenitors.

Regulatory cross-antagonisms segregate the early heart and ASM programs

Multilineage transcriptional priming of cardiopharyngeal progenitors begs the question as to how the segregation of heart and pharyngeal muscle programs is coordinated with asymmetric cell divisions. Among the primed transcriptional regulators, *Tbx1/10* is first expressed in the bipotent STVCs and preferentially maintained in the ASMFs, where it is necessary for *Ebf* expression [39]. Tbx1/10 function is also necessary and sufficient to inhibit *Gata4/5/6* expression and subsequent heart specification. Conversely, the *Nkx2.5* homolog *Nk4* is expressed throughout the cardiopharyngeal lineage and its repressor function is necessary to shut off *Tbx1/10* expression and prevent ectopic *Ebf* activation in the SHPs. This data indicated that STVCs' multilineage transcriptional priming is resolved through regulatory cross-antagonisms between early cardiopharyngeal regulators, which segregate the ASM and heart programs to their corresponding fate-restricted precursors [39]. Similar regulatory antagonisms involving *Nkx2.5*, *Tbx1* and *Gata4/5/6* homologs contribute

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to delaying cardiac differentiation in the mouse SHF, thus permitting proliferation and growth of the developing heart [19,21,46].

Ebf promotes differentiation and stemness in distinct pharyngeal muscle precursors

Ebf promotes subsequent ASM development including collective migration of ASM precursors (ASMP) and muscle differentiation. Ebf activates a myogenic program associated with upregulation of the sole myogenic regulatory factor Mrf [38,47]. Following division of the ASMFs, *Mrf* expression becomes restricted to the anterior- and posterior-most "outer" ASMPs, which are the only cells to activate muscle differentiation genes (Figure 1; [38]). By contrast, Notch activation in the "inner" ASMP upregulates the repressor Hes-b, which inhibits *Mrf* expression and subsequent differentiation. Instead, "inner" ASMPs proliferate and later produce the full complement of ASMs and LoMs in juveniles (Figure 1; [38]). Thus, Tbx1/10 and subsequent Ebf activities govern both Mrf-associated differentiation and the Notch-dependent maintenance of an undifferentiated, stem cell-like, progenitor state among distinct pharyngeal muscle precursors. This developmental progression is reminiscent of the vertebrate situation where Tbx1 governs branchiomeric myogenesis by controlling *MyoD* expression [48–50]. In vertebrates, producing the full complement of muscle cells also requires Notch-mediated inhibition of precocious differentiation and proliferation [51,52]. Head muscle myofibers and associated stem cells also derive from common progenitors in the early embryo [53]. Ebf homologs act upstream of *MyoD* and *Myf5* during pharyngeal and somitic myogenesis [54]. This suggests a conserved role for Ebf homologs in branchiomeric myogenesis [54–56]. Thus, ASM development in ascidians likely recapitulates key developmental transitions conserved with the branchiomeric muscles of vertebrates.

A cardiopharyngeal ontogenetic motif for chordate heart and head muscle development?

Successive gene expression profiles define transient cell identities in the cardiopharyngeal mesoderm. Together with a conserved clonal topology of the lineage, this invites a specific parallel between vertebrate and ascidian cardiopharyngeal development (Figure 1). Namely, early B-line blastomeres, TVCs, STVCs, FHPs, SHPs and ASMPs would be the ascidian counterparts of putative common *Mesp1+* mesoderm progenitors, *Nkx2.5+* pan-cardiopharyngeal progenitors, *Nkx2.5+/Tbx1+* second cardiopharyngeal progenitors (common to the SHF and branchiomeric muscles), FHF precursors, SHF precursors and branchiomeric muscle precursors, respectively (Figure 1).

Recent lineage studies challenged the existence of common *Mesp1+* pan-cardiopharyngeal progenitors in mice. Using distinct clonal analyses, both Lescroart et al. [57] and Devine et al. [58] conclude that cardiac progenitors are fate-restricted before they turn on *Mesp1*, which is first expressed in FHF and only later in the SHF, consistent with independent cardiac lineages deriving from distinct *Mesp1+* progenitors. Vertebrate *Mesp* genes function in a context-dependent manner, integrating stage of differentiation and signaling

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environment in precursors that are committed to certain fates [59]. Nevertheless, the study by Lescroart et al. supports the existence of bipotent *Mesp1+* progenitors for SHF cardiomyocytes and branchiomeric myocytes, in line with retrospective clonal analysis [12,60]. In *Ciona*, precocious expression of *Mesp* precedes cell fate diversification suggesting that gene network deployment can be evolutionarily decoupled from progressive fate restrictions. Similarly, although *Tbx1/10* is activated in the common progenitors of the SHPs and ASMPs in *Ciona*, *Tbx1* expression in vertebrates may start independently in distinct SHF and branchiomeric muscle progenitors [49,61,62].

Evolutionary changes in cardiopharyngeal development among distant ascidians

Tunicate and vertebrate embryos differ profoundly, rendering tentative comparisons between developmental processes arduous. In vertebrate development, cell-cell signaling is crucial to pattern plastic populations of thousands of progenitor cells. By contrast, the small number of progenitors in the ascidian embryo imposes hardwiring of every cell fate decision. These are thought to have fostered deep evolutionary conservation of early ascidian embryogenesis to the point that early cleavage patterns and cell lineages are perfectly conserved between the Stolfidobranchia and Phlebobranchia suborders of ascidians, despite over 500 million years of evolutionary divergence [63,64] (Figure 2A).

This divergence is reflected in the genome sequences of three Stolidobranch species of the genus *Molgula* and the two *Ciona* species [65]. Yet, early *Ciona* and *Molgula* embryos are virtually indistinguishable. The successive gene expression, cell divisions and progressive fate specification events in the *M. occidentalis* B7.5 lineage are nearly identical to that in *Ciona* (Figure 2 B and C; [65]). These homologous developmental sequences demonstrate the ancestral origin of the ascidian cardiopharyngeal ontogenetic motif and allow to study how profoundly divergent genomes encode extremely conserved embryonic processes [64,66].

Functional B7.5- and TVC-specific enhancers for *Molgula Mesp, FoxF* and *Hand-r* were identified. These enhancer constructs fail to drive reporter gene expression when transfected in *Ciona* embryo, interpreted as an acute form of developmental system drift. The homologous *Ciona* enhancers showed variable degree of activity in *Molgula* embryos, revealing widespread interspecific unintelligibility of regulatory mechanisms controlling otherwise identical patterns of gene expression.

Interspecific differences in cell behavior were also apparent. In *M. occidentalis*, TVCs migrate into the trunk following a more lateral migration path than observed in *Ciona*. This causes *M. occidentalis* heart precursors to coalesce only later, during metamorphosis, and transiently display two disjointed clusters of cardioblasts in the trunk (Figure 2C). This condition has only been induced in *Ciona* by endoderm-specific disruption of *Gata4/5/6* function [67]. Although midline convergence followed by asymmetric and oriented TVC divisions is a stereotyped cell behavior sequence in *Ciona*, its evolutionary remodeling in *Molgula* suggests that the ascidian cardiopharyngeal development is a modular assembly of specific morphogenetic events.

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Another morphogenetic difference concerns the ASMPs. In *Ciona* larvae, bilateral 4-cell ASMP clusters migrate towards atrial siphon placodes (ASP), where they divide and form conspicuous 8-cell rings (Figure 2C; [37]). Only during metamorphosis do bilateral anlagen fuse to form a single atrial siphon [68]. By contrast, ASM ring formation is not observed in *Molgula* larvae, where the single ectodermal ASP is not yet specified. This presumably causes *Molgula* ASMP to migrate dorsally into a cluster without forming a ring, as observed in *Hox1* mutant *Ciona* larvae lacking ASP [69]. These differences point the plasticity of cell-cell interactions that may have fostered diversification of cardiopharyngeal forms, while preserving ancestral patterns.

Intrinsic vs. extrinsic control of cardiopharyngeal cell fate and behavior

Ascidians offer a unique opportunity for high-resolution studies of chordate cardiopharyngeal development [22–24]. Transcriptional inputs from Mesp and FGF-MAPK-Ets govern TVC induction and their collective migratory behavior, in part through regulating *FoxF* [31,33,34,36]. Studies in vertebrates identified key roles for *MESP1*, *ETS2*, *FoxF1* and *FoxF2* in controlling specific aspects of mammalian heart specification and morphogenesis [70,71]. Whole genome studies of FACS-purified TVC cells identify distinct waves of transcriptional activation of genes in the TVCs prior to their divisions [35,72]. “Primary” TVC genes, such as *FoxF* and *Hand-r* are likely direct transcriptional targets of Ets.b as soon as the TVCs are born, while transcription of “secondary” TVC genes, such as the small GTPase-encoding *RhoD/F*, starts later in response to feed-forward inputs from MAPK-activated Ets.b and primary targets like FoxF [35,72].

Gene regulatory networks governing progressive cell fate specification also determine transient cell behaviors. Previous analyses identified numerous TVC-specific candidate migration effector genes involved in basic cellular processes, including actin dynamics, cell polarity and cell-matrix adhesion [35]. The abundance of genes encoding *trans*-membrane proteins and signaling molecules in the TVC transcriptome stresses the importance of the extracellular environment for progressive cardiopharyngeal fate specification and morphogenesis. Recent studies advanced our understanding of the roles of surrounding tissues in ascidian cardiopharyngeal development.

Asymmetric cell-matrix adhesion polarize MAPK activity and TVC induction

High-resolution analyses uncovered an essential role for cell-matrix adhesion in polarizing the signaling events controlling TVC fate specification [22,73,74]. Following asymmetric division of the B8.9 and B8.10 founder cells, FGF-MAPK signaling induces the TVCs [36]. MAPK activation triggers Ets-dependent transcription only in the two ventral/anterior-born cells from the asymmetric division (i.e. the prospective TVCs), even though founder cells seem to be uniformly exposed to an FGF9/16/20 ligand [74]. This differential induction of TVC fate is achieved by polarized matrix adhesion of the founder cells to the underlying epidermis [73,74]. A GFP-tagged talin actin-binding domain, reporting actin accumulation at adhesive foci, was enriched at the ventral founder cell membrane, re-distributed to the ventral edge throughout mitosis, and inherited by newborn TVCs after cytokinesis [73]. Cell-matrix adhesion requires Rap GTPase-dependent integrin β2 activation, which appeared necessary and sufficient to promote MAPK activation and TVC induction (Figure 3; [73]).

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This work parallels documented roles for integrin-mediated cell-matrix interactions during vertebrate cardiac development [75,76].

Surrounding tissues canalize cardiac progenitor migration

Following induction, TVCs collectively polarize and migrate in response to coordinated transcriptional inputs, extracellular cues and signaling events. Previous analyses revealed that *Gata4/5/6* plays cell autonomous and non-cell autonomous roles during *Ciona* heart development [67], as is the case in vertebrates [77–83]. In *Ciona* TVCs, Gata4/5/6 function is required for *FoxF*, *Nk4*, and *Bmp2/4* expression and migration [67]. *Gata4/5/6* is also required in the adjacent endoderm for bilateral pairs of migrating TVCs to converge and meet at the midline [67]. The resulting 'split heart' phenotype is reminiscent of the vertebrate *cardia bifida* condition [79–83]. Thus, directional heart progenitor migration in vertebrates and ascidians requires interactions with the endoderm, among other surrounding tissues, for proper positioning.

Systematic tissue-specific disruption of the secretory pathway and quantitative analyses probed the influence of surrounding tissues on collective TVC migration [84]. Quantification of the contact between TVC and surrounding tissues showed that the TVC are born touching each other, the mesenchyme, the epidermis, and their sister cells, the anterior tail muscle (Figure 4). The leader TVC usually arises for the anterior-most founder cell and is initially the only one to contact the trunk endoderm, but each TVC is capable of migrating individually, albeit imperfectly. The secretory pathway was inhibited in each TVC-surrounding tissue by expressing a dominant negative form of the small GTPase Sar1 to block endoplasmic reticulum to Golgi transport. Analyses revealed (1) a role for the mesenchyme in robust specification of the trailer TVC; (2) a role for the notochord in the timely initiation of TVC migration, possibly through chemorepulsion; (3) a role for the endoderm in the establishment and maintenance of leader-trailer polarity and (4) a role for the epidermis in TVC guidance and cell-cell adhesion. These data suggested that a combination of influences from surrounding tissues canalizes intrinsically motile TVCs towards stereotyped collective polarity and directed migration. Future studies will determine the signal and transduction pathways of TVC-specific interpretation of external cues.

Concluding remarks and future directions

Recent studies have furthered our understanding of conserved features of heart and pharyngeal muscle development between vertebrates and ascidians. Despite variations in cell topology due to larger cell populations in vertebrates, little doubt remains about the homology of vertebrate and ascidian cardiopharyngeal mesoderm [85]. Deployment of a core cardiopharyngeal gene regulatory network follows similar spatio-temporal sequences in both groups, from early mesoderm with *Tbx6* homologs acting upstream of *Mesp/Mesogenin* genes [86–88], to muscle specification and differentiation controlled by *Ebf*, *Mrf* and *Delta/Notch* homologs.

Ebf function in pharyngeal myogenesis, first studied in *Ciona*, illustrates the relevance of ascidian models, building on their amenability for high-resolution analyses, to uncover the conserved molecular basis of cardiopharyngeal development in chordates. Outstanding

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questions remain about the polarized cell-cell signaling in asymmetrical fate choices; the chromatin dynamics underlying multilineage priming, early fates' segregation and commitment to either a cardiac or pharyngeal muscle identity; the molecular underpinnings of collective polarity and directed migration and the genetic changes underlying developmental system drift.

Computational approaches using whole genome data from an increasing number of ascidian species will empower gene regulatory network comparisons across ascidians and uncover fundamental rules governing cardiopharyngeal mesoderm evolution.

Future studies will dissect the intricate relationships between TVCs intrinsic transcriptional inputs and signaling pathways that interpret behavioral cues from the environment. They will benefit from the recent development of reliable methods for gene loss-of-function using electroporation, including short hairpin RNA-mediated RNAi [38,39,89] and tissue-specific targeted genome editing using TALEN and/or CRISPR/Cas9 technologies [41,90,91].

Acknowledgments

We are grateful to Christiaen lab members for discussions. N.K. was supported by the NIH 5 T32 HD007520-14 training program in developmental genetics. Our work is supported by NIH/NIGMS grant R01GM096032 and NIH/NHLBI grant R01HL108643 to L.C.

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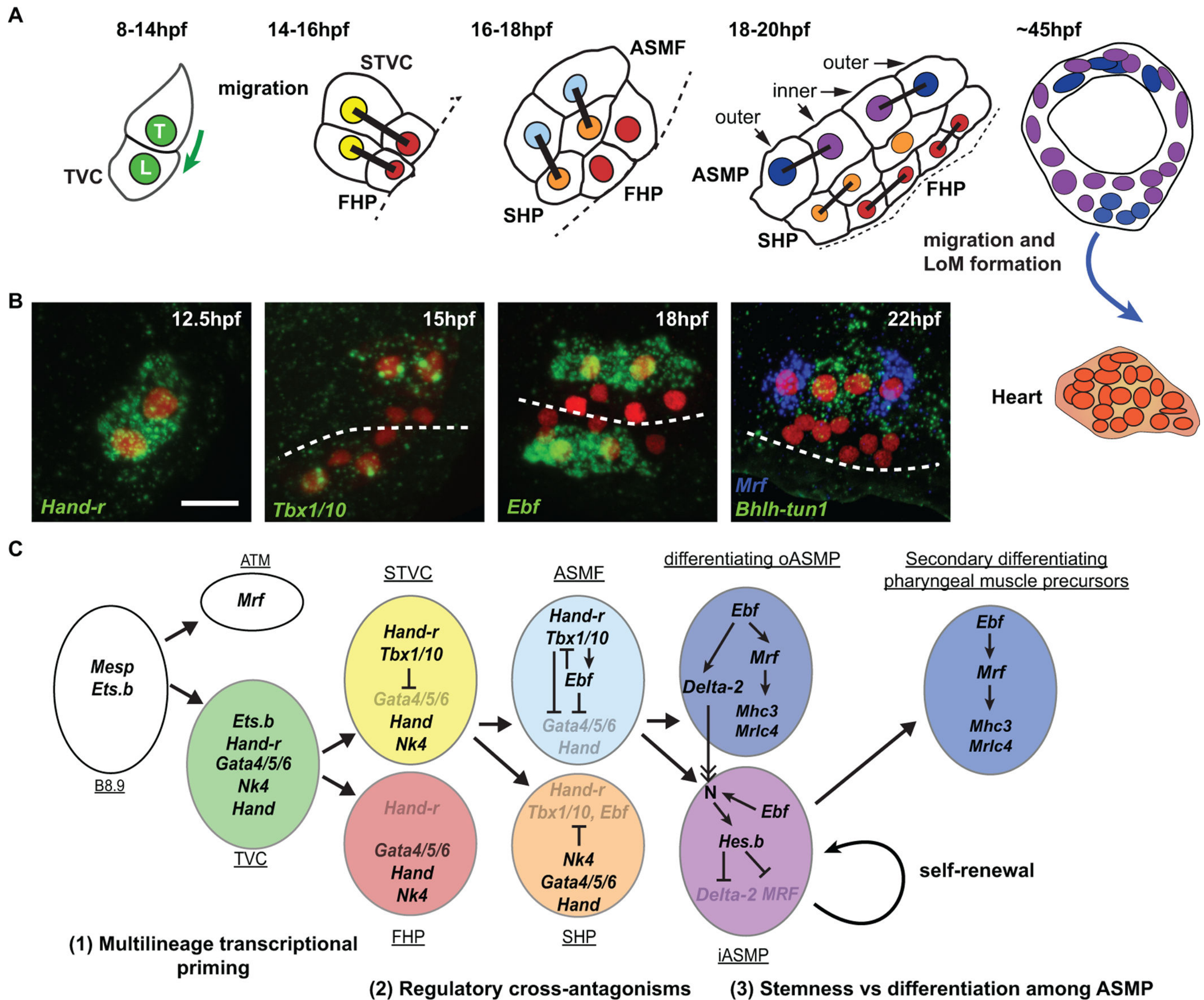

Figure 1. Schematic representation of cardio-pharyngeal development in *Ciona intestinalis*

(A) Schematic showing the trunk ventral cell progeny with divisions and migrations (green and blue arrows), from initial tail bud stage (8 hpf) to metamorphosing juvenile (45 hpf). Trunk ventral cells (TVC, green), STVC (secondary TVC, yellow), first heart precursors (FHP, red), atrial siphon muscle founder cells (ASMF, light blue), second heart precursors (SHP, orange), inner and outer ASM precursors (ASMPs, dark blue and violet, respectively), heart (red/orange). The first longitudinal muscle (LoM) derives from the ASM ring. The left side only is presented, linked nuclei indicate sister cells, dashed lines represent the midline. **(B)** Fluorescent *in situ* hybridization of key markers of the TVC progeny from 12.5 hpf to 22 hpf. The B7.5 lineage (red) is marked with Mesp>NLS::lacZ revealed by anti β-galactosidase immunostaining. Scale bar, 10 μm. **(C)** Summary of the ontogenetic interactions of the B7.5 cardiopharyngeal lineage. The TVC are transcriptionally primed pluripotent progenitors (1). Heart (*e.g. Gata4/5/6*, *Hand*) and ASM (*e.g. Hand-r*, *Tbx1/10*) transcriptional regulatory programs are segregated through cross-antagonisms coupled to

asymmetric divisions (2). A myogenic program associated with *Mrf* is deployed downstream of *Ebf*, which also promotes Notch-mediated lateral inhibition of *Mrf* and maintenance of a pool of stem cell-like muscle progenitors (3). Same color code as above.

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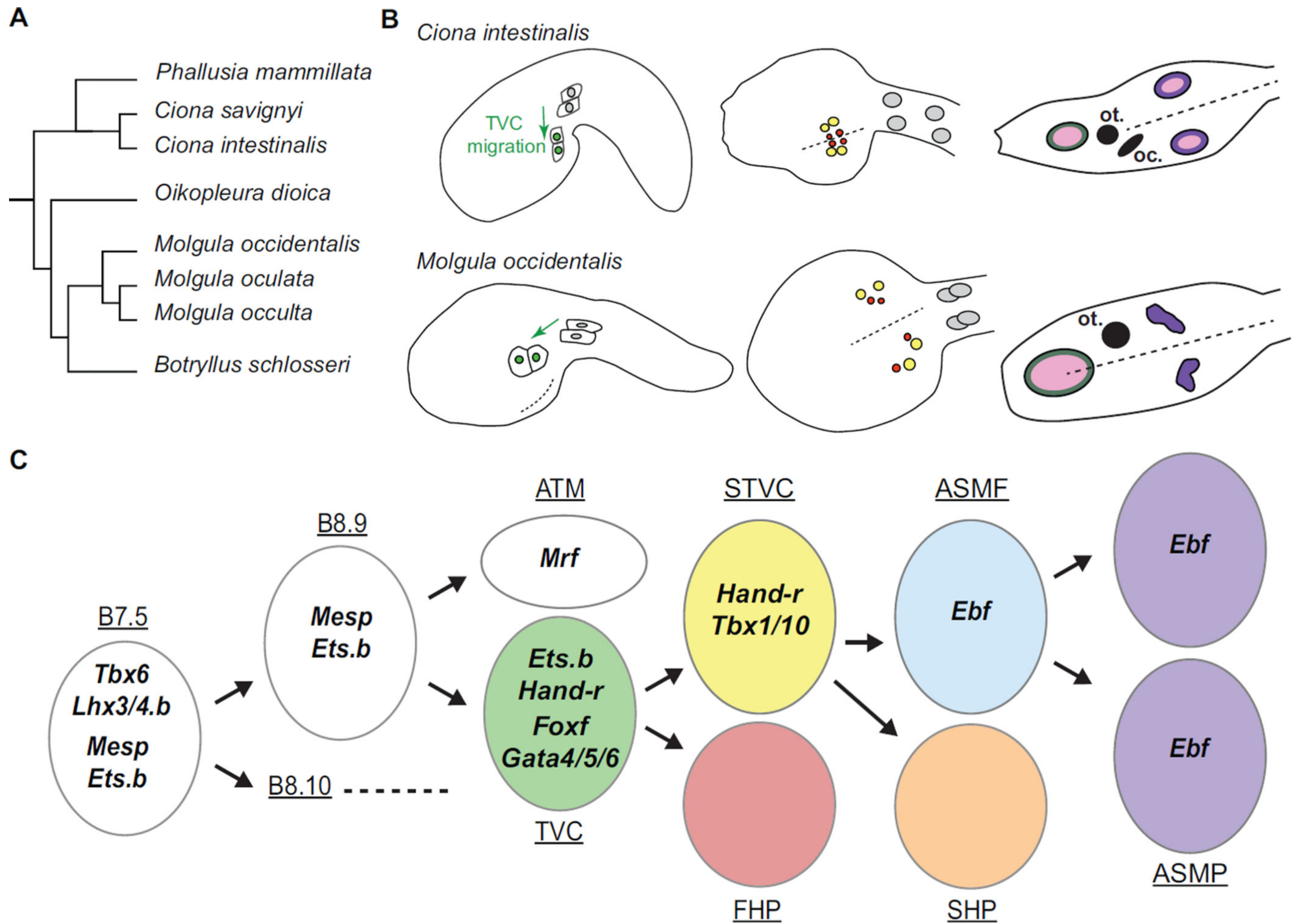

Figure 2. Variations on a conserved ontogenetic motif: comparison of the cardiopharyngeal development in *Ciona intestinalis* and *Molgula occidentalis*

(A) Simplified evolutionary tree of Tunicates based on 18s phylogeny, adapted from [92]. The *Ciona* and *Molgula* genera are positioned on the two most divergent branches within Tunicates. **(B)** Comparative schematic showing the B7.5 lineage in *Ciona intestinalis* and *Molgula occidentalis*. In both species, the TVCs (green) migrate away from the ATMs. In *Ciona intestinalis*, the TVCs converge at the midline of the ventral trunk before they divide into STVCs (yellow) and FHPs (red). In *Molgula occidentalis*, the TVCs divide more laterally into STVCs and FHPs, resulting in distinct bilateral clusters of heart precursors. In both species, the oral siphon muscle precursors (dark green) derive from a different cell lineage and form a ring surrounding a single oral placode (pink). In *Ciona intestinalis*, 4 ASMPs migrate towards the atrial placode (pink), divide and from a rings of 8 cells (violet) on either side. In *Molgula occidentalis*, the absence bilateral atrial placodes causes the ASMPs to remain as two bilateral clusters of cells. **(C)** Summary of the ontogeny of the B7.5 cardiopharyngeal lineage of *Molgula occidentalis*. Conservation of the cell division patterns is coupled to conserved spatiotemporal expression patterns of the main markers of the B7.5 lineage. Same color code as above

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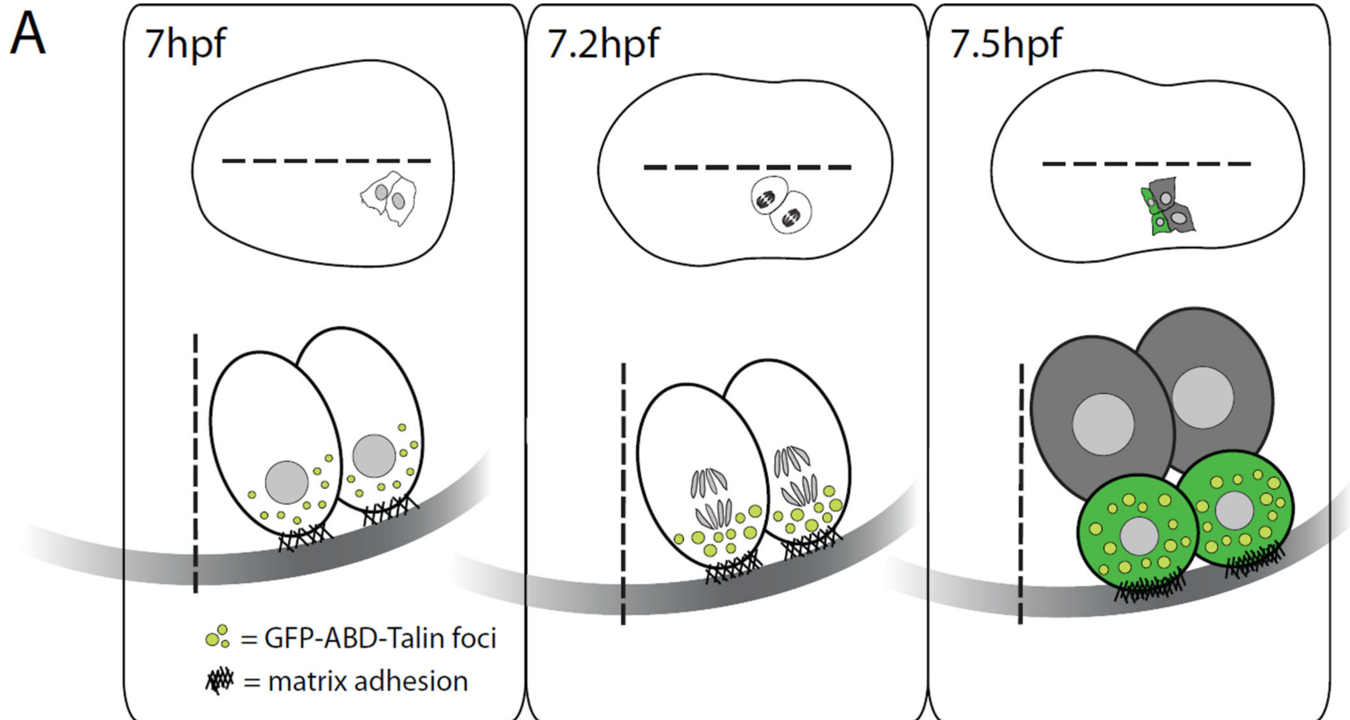

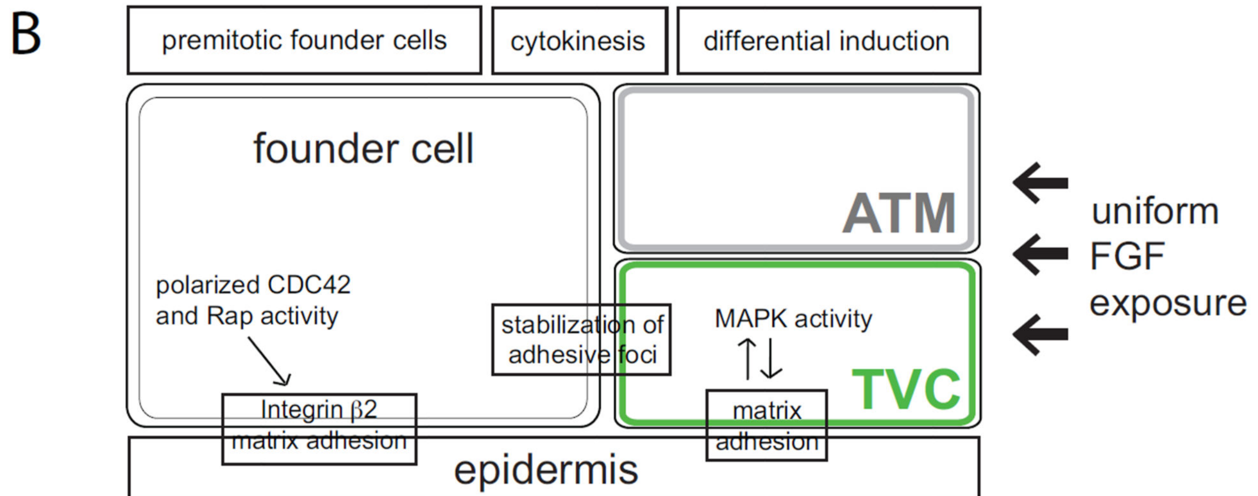

Figure 3. Model showing asymmetric induction of TVC fate by regional matrix adhesion

(A) Dorsal (top) and lateral (bottom) views of schematic B8.9 and B8.10 founder cells at neurula stage (~7hpf). GFP-tagged actin binding domain of Talin labels actin at adhesive foci (light green), which localize ventrally in founder cells before they divide; black hashes represent matrix adhesion to the underlying epidermis at the ventral border of founder cells. During mitosis, GFP-ABD-Talin foci increase in size and intensity, which correlates with stronger adhesion to the underlying ECM. After cytokinesis (~7.5hpf), two different fates are specified: ATM (gray), and TVC (green). GFP-ABD-Talin foci are inherited by TVCs, and ventral edges of newly born TVCs appear to protrude into the underlying epidermis. **(B)** Model of regional MAPK activation and asymmetric TVC induction despite uniform FGF

exposure. Polarized Rap activity in founder cells leads to regional stabilization of adhesion during cytokinesis at the presumptive TVC membrane in founder cells. Strong differential adhesion in newly born TVCs leads to differential MAPK activity and finally to TVC specification.

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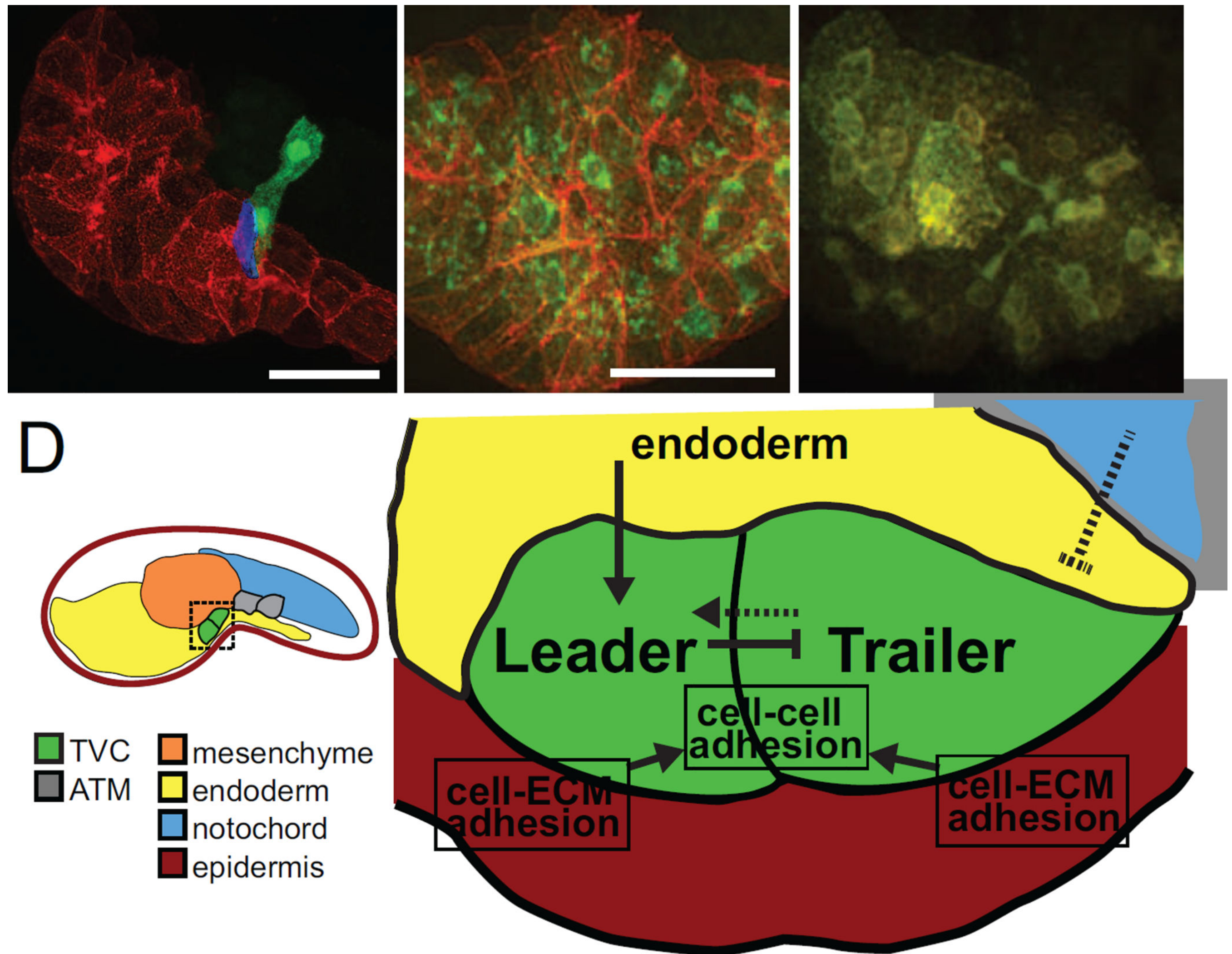

Figure 4. Surrounding tissues canalize TVCs towards directional migration

(A) Initial tailbud embryo expressing membrane-localized reporter hCD4::mCherry in the endoderm (red), and GFP in the B7.5 lineage (green). Leader and trailer TVCs are shown. Surface contact analysis between TVC and endoderm indicates that only the presumptive leader TVC contacts the endoderm (blue). Scale bar, 40 μm. **(B)** Localization of membrane localized protein hCD4::mCherry (red) at cell membranes and KDEL receptor KDELR::GFP (green) labelling the endoplasmic reticulum (ER) in control embryos. Scale bar, 40 μm. **(C)** In embryos expressing dnSar1, hCD4::mCherry accumulates in the ER and cannot be properly trafficked to the membrane, indicated by the co-localization of red and green at the ER, and the absence of red fluorescence at cell membranes. **(D)** Schematic mid-tailbud embryo showing TVCs (green) and ATMs (gray) and tissues analyzed by Gline *et al.* by tissue-specific disruption of the secretory pathway: epidermis (red), mesenchyme (orange), endoderm (yellow) and notochord (blue). Model for extrinsic tissue influences on TVCs and intercellular interactions during TVC migration. The endoderm (yellow) signals to the TVCs (green) to establish leader-trailer polarity and TVCs in turn signal to each other to maintain polarity during migration. The notochord (blue) possibly sends chemorepulsive

signals to migrating TVCs. TVCs adhere to each other during migration and also to the underlying epidermis (red) through cell-ECM interactions.

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