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BMP signaling coordinates gene expression and cell migration during precardiac mesoderm development

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ABSTRACT

Gene regulatory networks control the progressive specification of cell types and govern morphogenetic events during development. However, how morphogenetic events reciprocally affect gene expression remains poorly understood. Here, we analyzed the function of BMP signaling and expression of downstream target genes during cell migration of the precardiac mesoderm (trunk ventral cells, TVCs) in ascidian embryos. Our results indicate that migrating TVCs experience increasing BMP signaling as they migrate towards the ventral trunk epidermis, which expresses sustained levels of *Bmp2/4*. This increasing signaling intensity allows the successive activation of *GATAa*, *Tolloid*, *Bmp2/4* and *NK4*. Initial activation of *GATAa*, *Tolloid* and *Bmp2/4* contribute to a positive feedback loop involving cell migration, chordin inhibition and BMP ligand production. Sustained levels of BMP signaling become sufficient to activate *NK4* expression, which in turn contributes to a negative feedback loop inhibiting *Bmp2/4* and *Tolloid* expression. In addition, NK4 appears to inhibit cell migration thus providing a "transcriptional brake" to stop TVC migration. Our observations led us to propose a model for the coordination of cell migration and gene expression based on the temporal unfolding of a gene regulatory sub-network in a relevant developmental context.

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Introduction

The development control genes identified by genetic screens in Drosophila comprised numerous regulators of gene expression, including DNA binding transcription factors and signaling molecules (Desplan et al., 1985; Hoey and Levine, 1988; McGinnis et al., 1984; Nusslein-Volhard and Wieschaus, 1980). Decades of detailed studies complemented by system-level analyses have led to the description of gene regulatory networks (GRNs), which coordinate tissue- and cell-specific fate determination and morphogenetic events during development (Davidson and Levine, 2008; Davidson et al., 2002; Levine and Davidson, 2005; Stathopoulos and Levine, 2005). More recent studies have begun to identify the effector, or realizator, genes that execute the morphogenetic program by directly influencing cellular processes such as adhesion, actin dynamics or vesicle trafficking (Chanut-Delalande et al., 2006; Lovegrove et al., 2006; Martin et al., 2009). In parallel, mounting evidence indicate that the cellular processes, such as endocytosis, impinge on the activity of specific signaling pathways, thus potentially influencing their transcriptional outcomes (Chen, 2009; Fortini and Bilder, 2009; Furthauer and Gonzalez-Gaitan, 2009; Wiley and Burke, 2001). Finally, as development proceeds, morphogenetic movements and progressive fate specification modifies the environment of embryonic cells as well as their specific competence state. Therefore, it is intuitive to hypothesize that the genomic hard-wiring of GRNs must integrate the specific constraints of the developmental context in which they are going to unfold.

A natural consequence is that GRNs and morphogenetic patterns must have co-evolved during diversification of animal body plans. Yet, the core components of the most conserved GRNs have proven extremely stable during evolution, leading to the identification of tissue-specific GRN kernels supporting the development of conserved tissues and organs such as the visceral/precardiac mesoderm and the heart (Davidson and Erwin, 2006; Olson, 2006). For instance, in *Drosophila* and vertebrate embryos, tinman/Nkx2.5 and Pannier/GATA transcription factors are essential components of the heart kernel whose activation requires the Dpp/BMP signaling pathway, even though the morphogenetic patterns of early heart development differ substantially between Drosophila and vertebrates.

The ascidian embryo has emerged as an appealing model system to study the interface between GRNs and cell-specific morphogenetic events, due to its amenability to functional molecular studies and cellular simplicity permitting high resolution live microscopy (Christiaen et al., 2008; Imai et al., 2006; Rhee et al., 2005; Tassy et al., 2006). In addition, ascidians belong to the Tunicate phylum, which contains the closest living relatives to vertebrates that display a simplified, yet typical, chordate body plan at embryonic and larval stages. Ascidians are also characterized by invariant early cleavage

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patterns and lineage studies have demonstrated that the adult heart originates from a single bilateral pair of blastomeres in 110 cell pre-gastrula stage embryos, the B7.5 cells, which also give birth to anterior tail muscles, atrial siphon muscle and longitudinal muscle precursors in the ascidians *Halocynthia roretzi* (Hirano and Nishida, 1997) and *Ciona intestinalis* ((Satou et al., 2004); L.C. and A.S., unpublished observations). The B7.5 blastomeres are specified in response to the maternal effects of macho-1 and β-catenin, which are mediated by the transcription factors Tbx6 and Lhx3, respectively and activate the B7.5-specific expression of *Mesp*, a bHLH transcription factor required for subsequent heart development (Christiaen et al., 2009; Satou et al., 2004). Following gastrulation and two rounds of cell divisions, each B7.5 blastomere gives rise to four cells located at the anterior tip of developing tail: two small anterior and two large posterior B7.5 granddaughter cells (Davidson et al., 2006). The second division is asymmetric and sets the stage for induction of the anterior B7.5 granddaughter cells by an FGF signal, transduced by the MAPK pathway, presumably leading to the phosphorylation of the Ets1/2 transcription effector (Davidson et al., 2006). In response to this induction, the anterior B7.5 granddaughter cells express the forkhead transcription factor *FoxF* and migrate towards the ventral side of the trunk epidermis at the tailbud stage (Beh et al., 2007). These migrating cells are referred to as trunk ventral cells (TVCs), while their sister cells remain in the tail and differentiate into anterior tail muscles (ATMs). Both the Ets1/2-mediated FGF signal and FoxF transcriptional inputs are required for TVC migration (Beh et al., 2007; Davidson et al., 2006).

B7.5 lineage-specific whole genome transcription profiling has been used to analyze the transcriptional output of FGF/MAPK/Ets and FoxF activities in TVCs (Christiaen et al., 2008). This study and previous comprehensive *in situ* hybridization screens have identified numerous additional candidate regulators of TVC development (Imai et al., 2004). Notably, migrating TVCs express the *C. intestinalis* orthologs of tinman/Nkx2.5, pannier/GATA4/5/6, Dpp/Bmp2/4 and Tolloid/Bmp1 (Davidson and Levine, 2003; Satou et al., 2004), which encodes an extracellular metalloprotease known to potentiate BMP signaling through proteolytic cleavage of the secreted BMP inhibitor chordin (Hopkins et al., 2007). Here, we investigated the function of BMP signaling in regulating cell migration and the expression of heart kernel genes during TVC development. Evidence is presented that TVC migration towards a source of BMP2/4 ligand is required for the deployment of the precardiac gene regulatory network kernel via activation of *GATAa* and *NK4*.

Materials and methods

Adult animals, embryo manipulations and migration analysis

Gravid *C. intestinalis* adults were collected at the Pillar Point harbor (Half Moon Bay, CA) or obtained from M-Rep (San Diego, CA). Ripe oocytes and sperm were collected surgically and kept separate until in vitro fertilization. Fertilized eggs were dechorionated as described (Mita-Miyazawa et al., 1985). Electroporations were performed as described (Corbo et al., 1997) with 50-100 μg of plasmid DNA per construct. Each experiment was performed twice or more. The migration scoring system used in this study has been described in detailed elsewhere (Christiaen et al., 2008). Briefly, each class of migration phenotype is defined by (1) the number of cells that have migrated into the trunk (zero to four, two in wild-type embryos, classified as "3." Zero is the most severe migration defect, classified as "5") and (2) whether the cells that have migrated into the trunk remain attached (A) or not (D) to their sister cells that remained in the tail. Using this system, the wild type is described as 3D, the most severe migration defects are 4A and 5 (cells are necessarily attached in class "5").

Constructs design and molecular cloning

The Mesp *cis*-regulatory DNA lacking the endogenous start codon was described previously (Christiaen et al., 2008). The following primer sequences and design strategies were used for recombinant constructs cloning (underlined sequences indicate restriction sites, brackets indicate start codon and squared brackets indicate STOP codons):

The *C. intestinalis* type I BMP receptor, referred to as TGFb receptor-Ib in the Ghost database (http://ghost.zool.kyoto-u.ac.jp/ST2005.html; (Satou et al., 2005; Satou and Satoh, 2005)), was identified by virtue of a conserved peptide motif in the L45 loop, which determines the specificity of interaction with pathway-specific regulated Smad proteins (R-Smad; (Chen et al., 1998)). The dominant-negative truncated version of Ci-BMPR-Ib (tBMPR) was amplified using BRIb_F: AAGCGGCCGCAACC(ATG)CTGGACAATGGAC-TACCTCGTATG and dnBRIb_R: AACAATTGTTATAAAGCGTAGTCTGG-CACGTCGTATGGGTATTCCATCATCTCATTATGTCCTTCCA primers. The reverse primer encodes the sequence for C-terminal addition of an HA tag (YPYDVPDYAL*) following amino-acid 183, thus removing the C-terminal kinase domain (Namiki et al., 1997). The constitutively active Ci-BMPR-Ib (caBMPR) mutant was created by overlapping PCR to introduce a Q202D mutation in the regulatory GS domain of the full length receptor (Akiyama et al., 1997; Shi and Massague, 2003). PCR amplification of the 5′cDNA product used BRIb_F, and caBRIb_R: AAGAATTTCGATGTCTCGTGATATCGTTCTCTGTACAAGTAGAGGCATCC, primers; the 3′cDNA product was amplified with caBRIb_F: CAGA-GAACGATATCACGAGACATCGAAATTCTTCACGAAATTGGAAAAGG and BRIb_R: AACAATTGTTATAAAGCGTAGTCTGGCACGTCGTATGGGTAG-CAACTTGAATGCTCCTCTTCTTTAACA. 5′ and 3′cDNA products were then gel-purified, mixed and re-amplified using BRIb_F and BRIb_R primers to obtain the full length coding sequence for the constitutively active Ci-BMPR-Ib mutant.

Dominant-negative Ci-Smad1/5/8 was produced using PCR strategy by amplifying a truncated fragment of the coding sequence (truncation after amino-acid G432). The C-terminus was replaced by the yellow fluorescent protein Venus and an HA tag was added to the N-terminus (L.C. and Jean-Stéphane Joly, unpublished constructs).

The coding sequence for Ci-noggin was obtained by PCR on genomic DNA using nogginCDS-F: AAGCGGCCGCAACC(ATG) AACTTTGCAACTTGTTTTGCTACTTTAATGACGTGGA and nogginCDS-R: AAGAATTCGGGTGCAGTCGGTTCGAGTT primers.

The coding sequences used in this study were amplified from cDNA libraries made from total RNA using the SMART RACE kit (Clontech), Sensiscript or Omniscript reverse-transcriptase systems (Qiagen). All PCR products were cloned downstream of the *Mesp cis*-regulatory DNA using standard procedures.

In situ hybridization and β-galactosidase detection

Double fluorescent *in situ* hybridization and β-galactosidase immunostaining was performed essentially as described (Beh et al., 2007; Christiaen et al., 2008). The digoxigenin-labeled antisense RNA probes for *Bmp2/4*, *Tolloid*, *GATAa* and *NK4* were synthesized by in vitro transcription from linearized plasmid DNA. The *Bmp2/4* probe template was obtained from the *C. intestinalis* Gene Collection release 1 (clone: *cicl060n01*). *Tolloid*, *GATAa* and *NK4* probes were synthesized from 3′RACE fragments amplified using nested PCR and the following forward primers: Tolloid3RF1: AGCGTGGATTTTCTGCCTCGCATAC, Tolloid3RF2: CCCACAGTACGGCGACACCATCTAC, GATA3RF1: GCGCTAACACTACAGCCGGAAGCAT, GATA3RF2: GATCCTGAGAACCCG-GAAGTGTTCG, NK43RF1: ATCCGGATCGCCAAACAAACAACAT, NK43RF2: GGCCAGTATTGGACCTCCACGAAGA and the Universal Primer Mix from the SMART cDNA kit (Clontech) and cloned into the pCRII-TOPO vector (Invitrogen).

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Results

BMP signaling influences TVC migration

Comprehensive *in situ* hybridization surveys and cell-specific whole genome transcription profiling indicated that the TVCs express both the *Bmp2/4* and *Tolloid* genes as they migrate towards the ventral part of the trunk, at the mid-tailbud stage (Christiaen et al., 2008; Imai et al., 2004). We confirmed these previous observations by double fluorescent *in situ* hybridization and β-galactosidase immunostaining of embryos electroporated with a Mesp>lacZ transgene, which marks the B7.5 lineage, including the TVCs and anterior tail muscles (Figs. 1A, B). Examination of late neurula stage embryos indicated that the B7.5 lineage cells are located dorsally and adjacent to the ventral epidermis, which expresses high levels of the *Bmp2/4* gene (Fig. 1C). At this stage, the anterior B7.5 granddaughter cells have been induced by an FGF signal but have not migrated yet (Davidson et al., 2006). Notably, they subsequently migrate towards the ventral part of the trunk as a polarized pair of cells constituted of a leader (L) and a trailer (T) (Christiaen et al., 2008). Therefore, we reasoned that the ventral source of BMP2/4 ligand might provide a localized cue contributing to the polarization and guidance of the TVC pairs.

To test this possibility, we created expression constructs by fusing the *Mesp cis*-regulatory DNA with engineered versions of *C. intestinalis* orthologs of known regulators of BMP signaling (see Material and methods). We identified the sole type I BMP receptor (TGFβ receptor-Ib), based on the presence of a signature peptide known to bind the SMAD1/5/8 family of R-SMADs (Chen et al., 1998). The BMPR-Ib coding sequence was modified to remove the 3′ region coding for the C-terminal kinase domain, thus creating a dominant-negative form of BMPR-Ib, referred to as tBMPR. We also created a dominant-negative version of the SMAD1/5/8 ortholog, by removing the C-terminal part of the MH2 domain, including the phosphorylation site. These constructs were expected to function cell-autonomously. In addition, we used the wild-type form of *Ciona* noggin, which encodes a secreted inhibitor of BMP ligands binding (Pasini et al., 2006). Finally, a constitutively active version of BMPR-Ib (caBMPR) was created by introducing a point mutation known to over-ride the requirement for trans-activation of the kinase domain upon ligand binding and receptor dimerization.

These constructs were electroporated in fertilized eggs together with the Mesp>lacZ reporter construct and tailbud embryos were fixed for subsequent double fluorescent *in situ* hybridization and β-galactosidase immunostaining in all experiments described hereafter.

We first ought to analyze the effects of these manipulations on TVC migration. To this aim, we used a previously described migration scoring system (Christiaen et al., 2008), whereby each group of B7.5 lineage cells falls into a class of defective migration phenotype, detailed classes were subsequently grouped into simplified classes and the proportions of B7.5 lineage cell falling into each class displayed as stacked histograms (Fig. 1D). Cell-autonomous attenuation of BMP signaling using tBMPR and tSMAD inhibited TVC migration to variable degrees and were not fully penetrant (Fig. 1D). Noggin over-expression had more pronounced inhibitory effects on TVC migration, presumably due to non cell-autonomous interference with BMP signaling and/or more efficient inhibition (Fig. 1D). Notably, targeted expression of the constitutively active form of BMPR-Ib also inhibited TVC migration, but sometimes resolved in enhanced migration at later stages (data not shown). Taken together, these observations show that manipulation of BMP signaling affects TVC migration to variable extents, suggesting that tight control of BMP signaling is required for optimal TVC migration.

NK4 expression correlates with BMP signaling and TVC migration

To gain further insights into the function and regulation of BMP signaling during TVC development, we analyzed expression of several regulatory genes following manipulation of BMP signaling.

We first focused our attention on *NK4*, the ortholog of vertebrate *Nkx2.5* and *Drosophila tinman*, which are conserved direct targets of

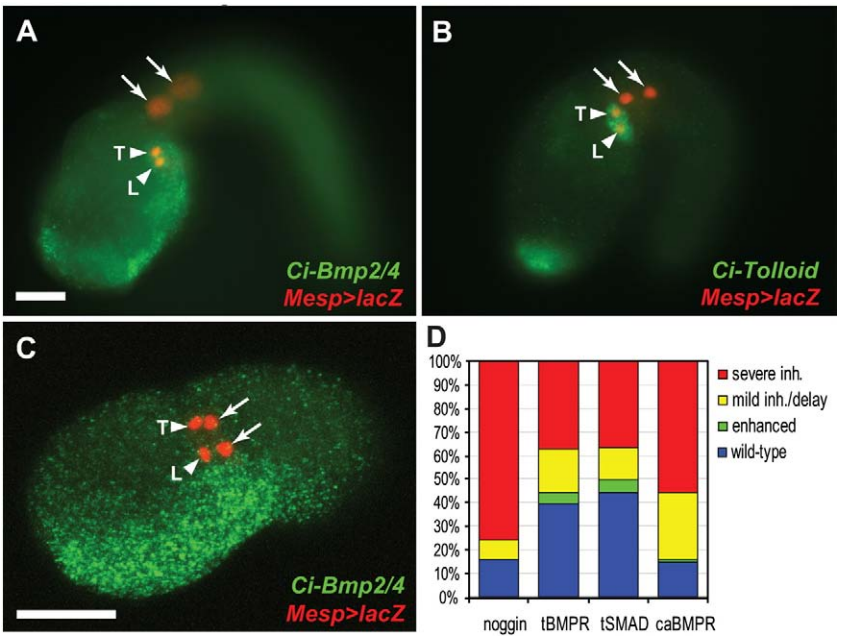

Fig. 1. BMP signaling components are expressed in TVCs. (A) Mid-tailbud embryo electroporated with Mesp>lacZ (red) and hybridized with a *Bmp2/4* antisense RNA probe (green). (B) Mid-tailbud embryo electroporated with Mesp>lacZ (red) and hybridized with a *Tolloid* antisense RNA probe (green). Note *Bmp2/4* and *Tolloid* expression in both the leader (L) and trailer (T) TVCs (arrowheads), but not in the ATMs (arrows). (C) Late neurula embryo electroporated with Mesp>lacZ (red) and hybridized with a Bmp2/4 antisense RNA probe (green). At this stage, *Bmp2/4* is not expressed in TVCs (arrowheads), but in the adjacent ventral epidermis. Scale bars ~20 μm. (D) Targeted expression of indicated constructs, under the control of the *Mesp cis*-regulatory DNA, induce variable TVC migration defects. Histograms depict the proportions of B7.5 cell progeny (typically 0 to 2 per embryo) grouped into indicated simplified classes of migration phenotypes (Christiaen et al., 2008).

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BMP signaling during precardiac development (reviewed in Furlong, 2004; Zaffran and Frasch, 2002). In wild-type mid-tailbud embryos, *NK4* is expressed in the anterior trunk endoderm, ventral epidermis and migrating TVCs, but not in their sister cells, the ATMs (Fig. 2A). Double fluorescent *in situ* hybridization and β-galactosidase immunostaining allowed us to score for gene expression and cell migration on electroporated embryos. As for cell migration, we observed that attenuation of BMP signaling using targeted expression of noggin, tBMPR or tSMAD had variable inhibitory effects on *NK4* expression in TVCs (Fig. 2C). During the course of this analysis, we observed a correlation between migration phenotypes and *NK4* expression, even in the control embryos electroporated with a Mesp>RFP transgene that showed sporadic migration defects (Fig. 2B). Therefore, we analyzed the correlation between *NK4* expression and TVC migration defects and observed that the proportions of embryos showing either no or one *NK4* positive B7.5 lineage cell increased when TVC migration was more severely inhibited (e.g. classes 4A and 5; Fig. 2D). These data indicate that *NK4* expression correlates strongly with TVC migration, suggesting that migration is required for sustained *NK4* expression in the TVCs.

However, the causal relationships between BMP signaling, TVC migration and *NK4* expression cannot be readily inferred from these correlations. To address this issue, we re-analyzed the data by scoring *NK4* expression in relation to both migration classes and experimental conditions, including targeted expression of the constitutively active caBMPR. We focused on the two classes showing the most severe inhibition of TVC migration (classes 4A and 5), in an attempt to determine whether TVC migration and *NK4* expression could be uncoupled (Fig. 2F). As mentioned above, migration inhibition altered *NK4* expression in control embryos electroporated with a Mesp>RFP transgene (Fig. 2F). Targeted expression of tBMPR or tSMAD increased the proportion of B7.5 lineage cells lacking *NK4* expression, thus indicating that attenuation of BMP signaling inhibits *NK4* expression regardless of migration (Fig. 2F). Conversely, targeted expression of caBMPR restored *NK4* expression in TVCs that had not migrated (e.g. Figs. 2E, F). Taken together, these observations suggest that BMP signaling is a primary determinant of *NK4* expression in the TVCs. Therefore, we propose that the observed correlation between *NK4* expression and TVC migration reflects the need for the TVC to migrate closer to ventral epidermal cells expressing *Bmp2/4*, in order to express sustained levels of *NK4*.

NK4, GATAa, Bmp2/4 and Tolloid have distinct requirements for BMP signaling

Following our observations on *NK4*, we analyzed expression of additional orthologs of regulatory genes known to be involved in precardiac mesoderm development and/or implicated in regulating

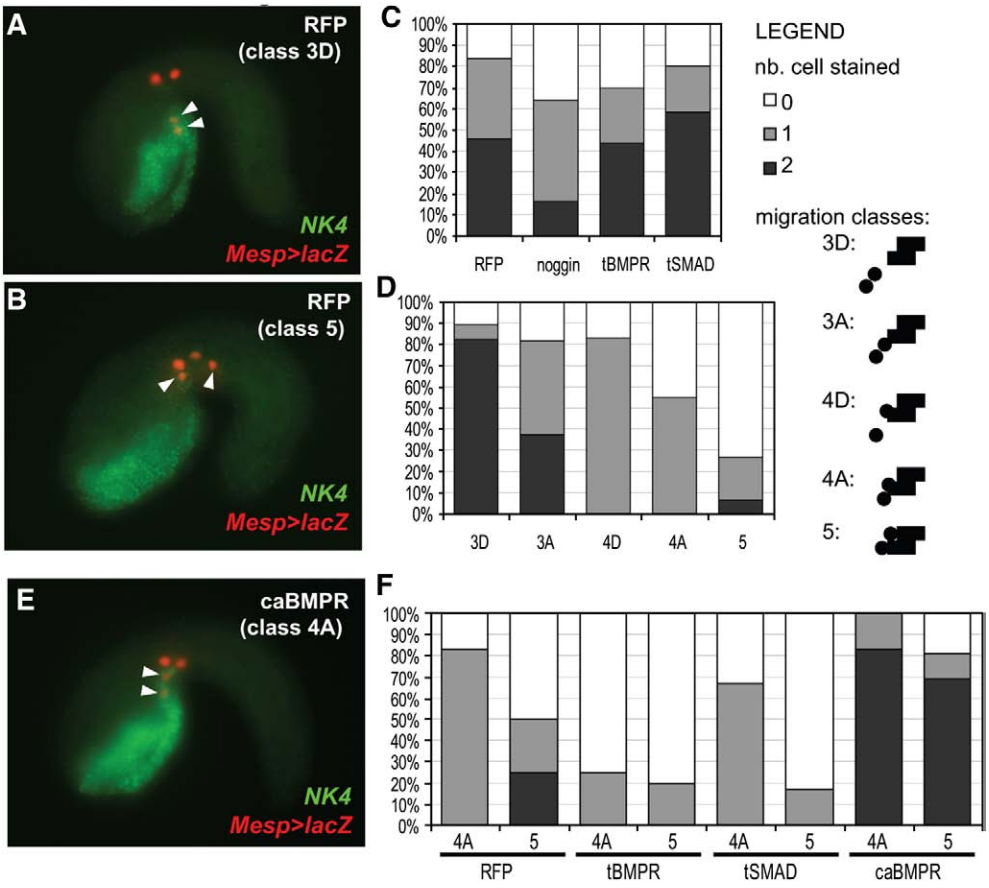

Fig. 2. *NK4* expression correlates with TVC migration and responds to BMP signaling. (A) mid-taildbud embryo electroporated with Mesp>lacZ (red) and Mesp>RFP constructs and hybridized with an *NK4* antisense probe (green). B7.5 cells show wild-type TVC migration (class 3D). Note expression in the TVCs (arrowheads). (B) Same as (A) except that TVCs have not migrated into the trunk (arrowheads) and only the anterior-most cell shows weak *NK4* expression. (C) Stacked histograms showing the proportions of B7.5 lineage groups with 0, 1 or 2 NK4-positive cells following targeted expression of indicated constructs driven by the *Mesp cis*-regulatory DNA. Note the variable decrease of NK4 expression upon attenuation of BMP signaling using noggin, tBMPR or tSMAD. (D) *NK4* expression relative to migration classes, data was pooled from the same experiments used in (C). Migration classes are depicted in the legend panel (boxes and circles represent ATMs and TVCs, respectively). (E) Mid-tailbud embryo electroporated with Mesp>lacZ (red) and Mesp>caBMPR constructs and hybridized with an *NK4* antisense probe (green). Note that TVC have not migrated normally into the trunk (class 4A, arrowheads), but show conspicuous *NK4* expression. (F) Histograms showing the proportions of B7.5 cell groups with 0 to 2 NK4-positive cells. Only classes of severe migration inhibition are showed (classes 4A and 5). Note the increased proportions of embryos showing 0 and 2 positive cells upon BMP signaling attenuation (tBMPR and tSMAD) or BMP signaling enhancement (caBMPR), respectively.

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BMP signaling, namely *GATAa*, the *Ciona* ortholog of *GATA-4/-5/-6*, *Bmp2/4* and *Tolloid*.

At the mid-tailbud stage, *GATAa* shows strong expression in the posterior trunk endoderm and TVCs (Fig. 3A); as mentioned above, *Bmp2/4* is expressed in TVCs as well as ventral epidermis and anterior palp region (Fig. 1A; see also Imai et al., 2004; Satou et al., 2004), and *Tolloid* shows expression in TVCs, notochord, palp region and parts of the central nervous system (Fig. 1B; see also Imai et al., 2004; Satou et al., 2004). Cell-autonomous attenuation of BMP signaling using the tBMPR and tSMAD constructs inhibited expression of *GATAa*, *Bmp2/4* and *Tolloid* to variable extents. As seen for *NK4*, expression of *GATAa*, *Bmp2/4* and *Tolloid* correlated positively with TVC migration (Figs. 3C, E, G). However, expression of *GATAa* and *Tolloid* appeared less sensitive to migration inhibition, as indicated, for example, by the higher proportion of B7.5 lineage halves of classes 3A and 4A showing conspicuous expression in two cells (compare Figs. 3C and G to Fig. 2D). In contrast, *Bmp2/4* expression appeared more variable and sensitive to migration (compare Fig. 3E to Fig. 2D). Added to our analysis of *NK4* expression, these observations suggest that *NK4*, *GATAa*, *Bmp2/4* and *Tolloid* are activated by distinct levels of BMP signaling in the TVCs. Namely, *GATAa* and *Tolloid* appear to be activated by even low levels of signaling, whereas *Bmp2/4* and *NK4* may need medium to high levels of signaling (Fig. 3H).

In principle, BMP-induced activation of *Tolloid* and *Bmp2/4* could contribute to a positive feedback whereby BMP signaling in migrating TVCs would increase with time (see Discussion). In this context, differential sensitivity to BMP signaling should convert in gene activation at different time points. To test this possibility, we analyzed *NK4*, *GATAa*, *Tolloid* and *Bmp2/4* expression at the early tailbud stage (Fig. 4). As expected, we observed conspicuous TVC-specific expression of *GATAa* and *Tolloid*, while *NK4* and *Bmp2/4* expression could not be detected above background levels (Fig. 4). Thus, we conclude that progressive increase in BMP signaling during TVC migration allow the successive activation of genes requiring higher levels of signaling (see discussion).

NK4 contributes to a negative feedback on BMP signaling

During the course of these analyses, we observed conflicting effects of attenuating BMP signaling on *Bmp2/4* and *Tolloid* gene expression (L.C. unpublished observations). Even though targeted

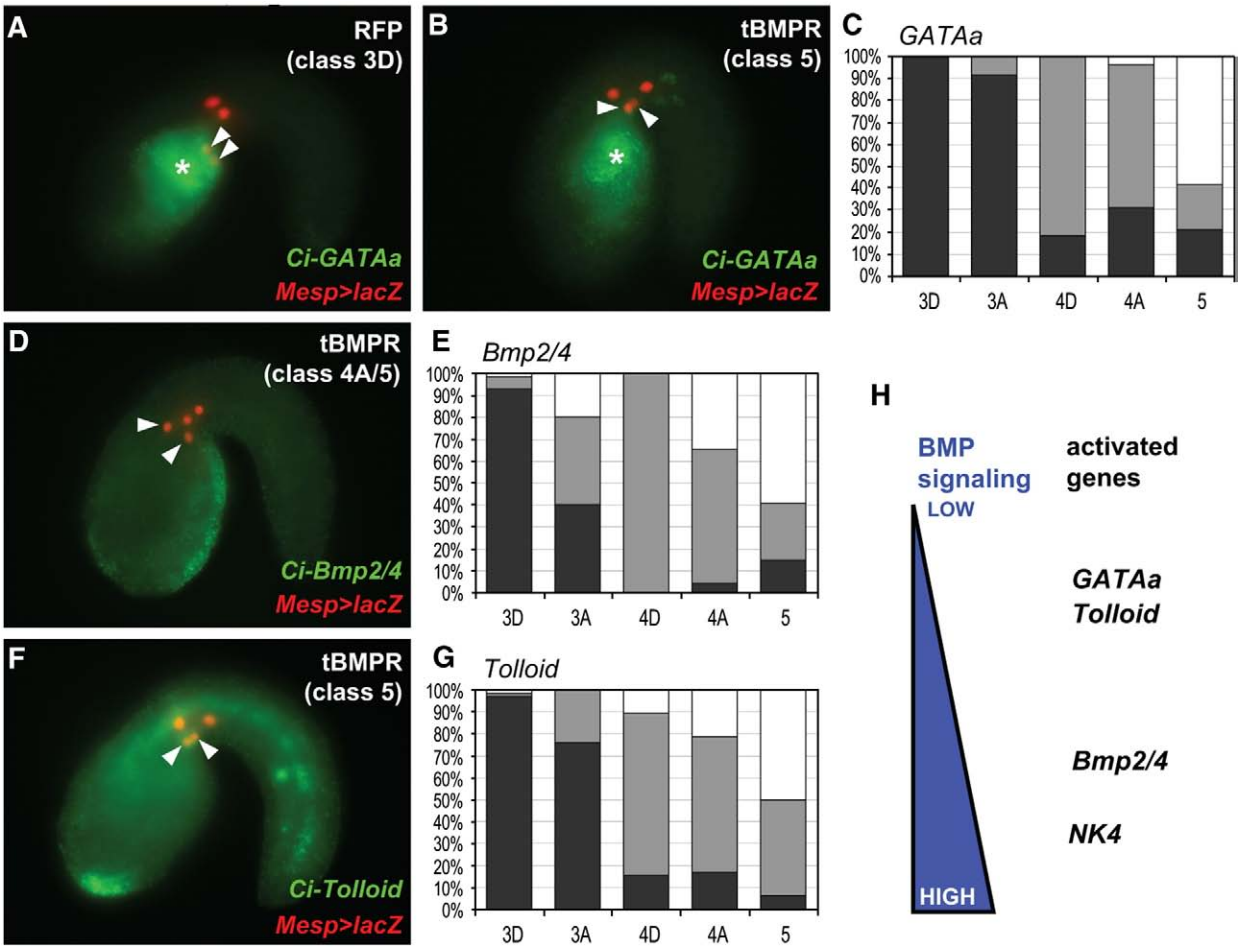

Fig. 3. *GATAa*, *Bmp2/4* and *Tolloid* expression correlate with TVC migration, but have distinct requirements for BMP signaling. (A) Mid-tailbud embryo electroporated with Mesp>lacZ (red) and Mesp>RFP constructs and hybridized with a *GATAa* antisense probe (green). B7.5 cells show wild-type TVC migration (class 3D). Note expression in the TVCs (arrowheads) and posterior trunk endoderm (*). (B) Mid-tailbud embryo electroporated with Mesp>lacZ (red) and Mesp>tBMPR constructs and hybridized with a *GATAa* antisense probe (green). B7.5 cells show inhibited migration (class 5) and reduced *GATAa* expression (arrowheads). (C) *GATAa* expression relative to migration classes, as depicted in Fig. 2. Note the decreased proportions of B7.5 cell groups showing 2 GATAa-positive cells in classes of severe migration inhibition (4D, 4A and 5). (D) Mid-taildbud embryo electroporated with Mesp>lacZ (red) and Mesp>tBMPR constructs and hybridized with a *Bmp2/4* antisense probe (green). B7.5 cells show inhibited migration (class 4A/5) and no *Bmp2/4* expression (arrowheads, compare to Fig. 1A). (E) *Bmp2/4* expression relative to migration classes. (F) mid-taildbud embryo electroporated with Mesp>lacZ (red) and Mesp>tBMPR constructs and hybridized with a *Tolloid* antisense probe (green). B7.5 cells show inhibited migration (class 5) and reduced *Tolloid* expression (arrowheads, compare to Fig. 1B). (G) *Tolloid* expression relative to migration classes. (H) Summary interpretation: *GATAa*, *Tolloid*, *Bmp2/4* and *NK4* are activated in TVCs by increasing levels of BMP signaling.

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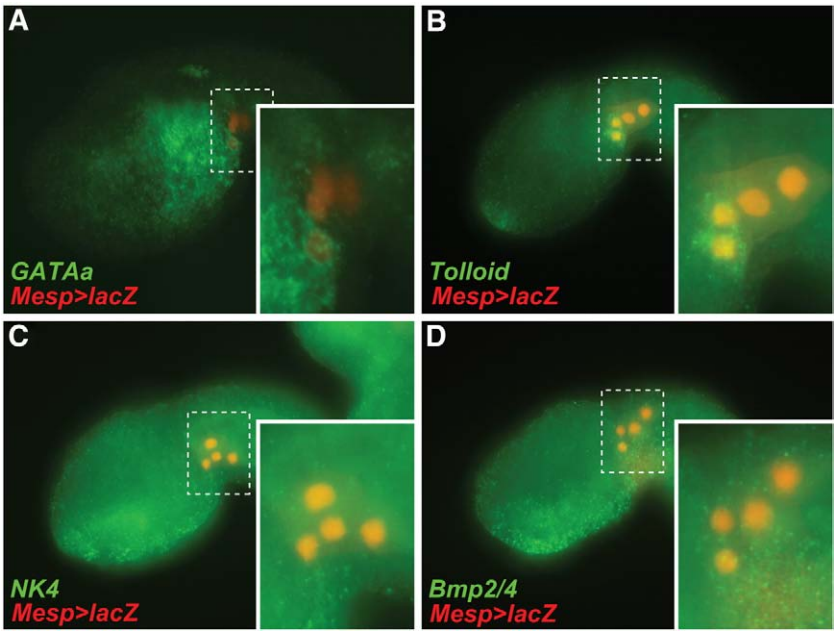

Fig. 4. *NK4*, *GATAa*, *Tolloid* and *Bmp2/4* expression in early tailbud embryos. (A–D) Early-tailbud embryos electroporated with Mesp>lacZ, detected by immunofluorescence (red), and hybridized with the indicated DIG-labeled antisense RNA probes (green).

expression of noggin, tBMPR or tSMAD mostly inhibited *Bmp2/4* and *Tolloid* expression, residual staining sometimes appeared qualitatively stronger. This sporadic incidence of upregulation upon perturbation of the BMP pathway hinted at the possibility of a negative feedback loop onto *Bmp2/4* and *Tolloid*, in response to increasing amounts of signaling beyond the initiation of gene expression. Such a combination of positive and negative feedbacks on TGFβ signaling has been described in numerous analogous systems and is thought to constitute a homeostasis mechanism ensuring the maintenance of appropriate levels of signaling (Miyazono, 2000). In addition, recent studies showed that Nkx2.5 activity is required to attenuate BMP signaling during early cardiac development in the mouse embryo (Prall et al., 2007).

Therefore, we asked whether NK4 activity could contribute to the attenuation of *Bmp2/4* and/or *Tolloid* expression downstream of BMP signaling by over-expressing NK4. To this aim, we used a TVC-specific enhancer previously isolated from the *FoxF* gene, which is one of the first genes to be activated in TVCs, prior to cell migration. Since *NK4* expression is inhibited by targeted expression of tBMPR, we reasoned that the effects of NK4 over-expression should be more conspicuous in a "tBMPR background" and analyzed *Bmp2/4* and *Tolloid* expression following targeted expression of tBMPR and/or NK4 (Fig. 5). We first observed that targeted expression of NK4 slightly inhibited TVC migration, and could not rescue the mild migration defects obtained by expression of tBMPR, suggesting that NK4 has inhibitory effects on TVC migration (Fig. 5C).

Next, we analyzed *Bmp2/4* and *Tolloid* expression by double fluorescent *in situ* hybridization and β-galactosidase immunostaining. We observed that NK4 over-expression inhibited both *Bmp2/4* and *Tolloid* expression in TVCs (Figs. 5A, B, D, E) and this effect did not seem to be a consequence of cell migration inhibition since it was also observed on cells that showed a wild-type migration pattern (3D class, Fig. 5F). Notably, the effect of NK4 over-expression on *Bmp2/4* expression was not altered by co-expression of tBMPR (Figs. 5D, F). In contrast, the negative effect of NK4 on *Tolloid* was surprisingly abolished upon co-expression with tBMPR (Figs. 5E, F), which suggests that yet unknown molecular mechanism involving sustained BMP signaling is required for NK4-mediated repression of *Tolloid* expression in the TVCs.

Discussion

A dynamic model for BMP-mediated coordination of cell migration and gene expression

Here we presented experimental evidence supporting a dynamic model for the coordination of a regulatory sub-network and cell migration in the ascidian precardiac mesoderm. The key features of the model are that (1) BMP responsive genes are activated by distinct levels of signaling. In particular, *GATAa* and *Tolloid* expression are activated by low levels of signaling presumably available before the onset of TVC migration, while *NK4* expression requires higher levels of signaling, after the cells have approached the ventral epidermis; (2) migration towards the ventral epidermis exposes the TVCs to higher concentrations of the BMP2/4 ligand, thus possibly resulting in higher levels of intra-cellular BMP/Smad signaling. This progressive increase in signaling intensity is further reinforced by a positive feedback mechanism involving *Tolloid* and *Bmp2/4* expression in the TVCs; (3) high levels of BMP signaling promotes a negative feedback mechanism, whereby NK4 function inhibits TVC migration and thus functions as a "transcriptional brake."

Given these elements, the dynamic model can be described from both regulatory and developmental perspectives as follows (Fig. 6): following induction of the TVC fate by FGF signaling, the anterior B7.5 granddaughter cells are exposed to low levels of BMP signaling originating from the ventral epidermis and counter-acted by a dorsal source of chordin (Imai et al., 2004; Pasini et al., 2006). This low level of BMP/Smad signaling is sufficient to initiate expression of *GATAa* and *Tolloid* in the only competent cells, i.e. the TVCs. Tolloid activity initiates a positive feedback loop by inhibiting chordin function. In parallel, GATAa function contributes to the initiation of TVC migration (Jeni Beh, Ph.D. thesis and personal communications). Migration towards the ventral epidermis and Tolloid-positive feedback promotes the increase in intra-cellular BMP/Smad signaling, which allows the activation of *Bmp2/4* expression. Together with continuous migration and Tolloid activity, *Bmp2/4* expression further enhances the positive feedback loop, thus promoting further increase in intra-cellular BMP/Smad signaling. By the mid- to late-tailbud stage, the

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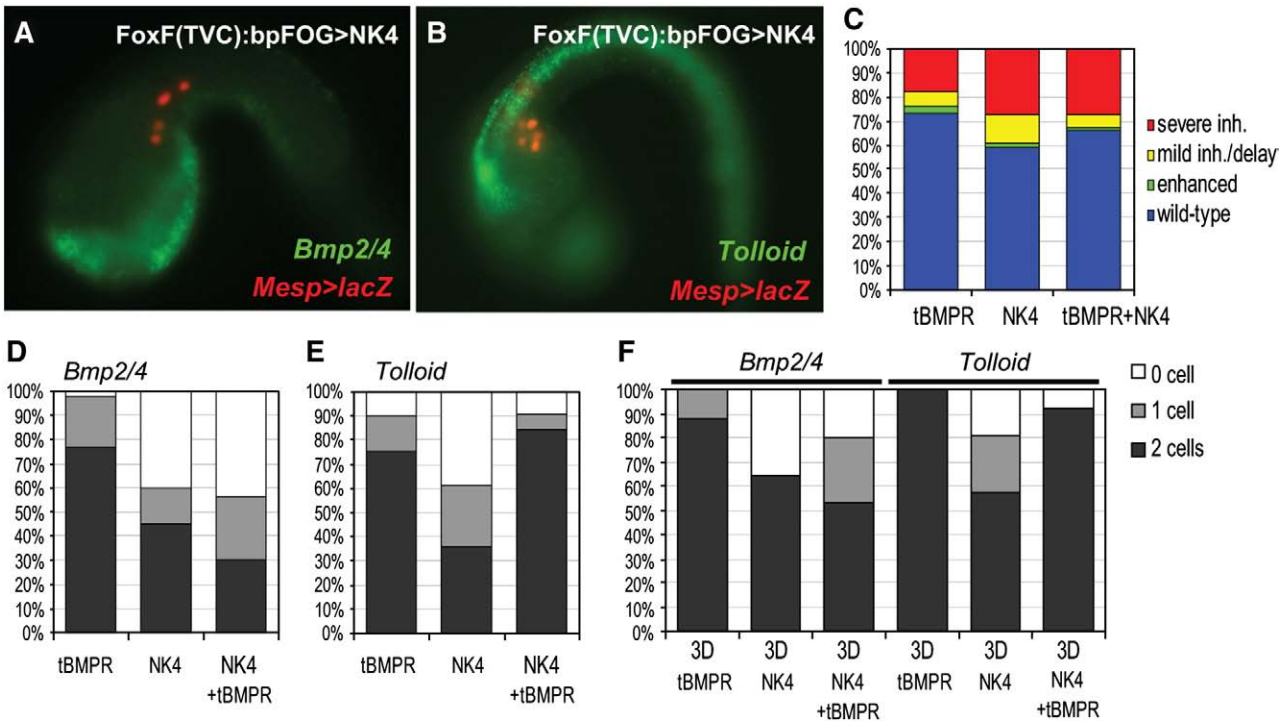

Fig. 5. NK4 contributes to a negative feedback loop on BMP signaling and TVC migration. (A) Tailbud embryos electroporated with Mesp>lacZ (red) and FoxFTVC:bpFOG>NK4 constructs and hybridized with *Bmp2/4* (A) and *Tolloid* (B) antisense RNA probes (green). In both embryos, the TVC have not migrated normally and show strongly reduced gene expression. (C) tBMPR and NK4 over-expression have mild inhibitory effects on TVC migration. (D–F) Gene expression histograms showing that NK4 inhibits *Bmp2/4* (D) and *Tolloid* (E) expression. These effects are not only indirect consequences of migration inhibition since they can be observed on 3D class B7.5 cell groups, which have normally migrated into the trunk (F).

high levels of BMP/Smad signaling activate *NK4* expression, which in turn initiates a negative feedback loop on *Bmp2/4* and *Tolloid* expression and may also inhibit expression of genes required TVC migration. The proposed model depends on the architecture of the gene regulatory network and on the developmental context brought about by cell migration.

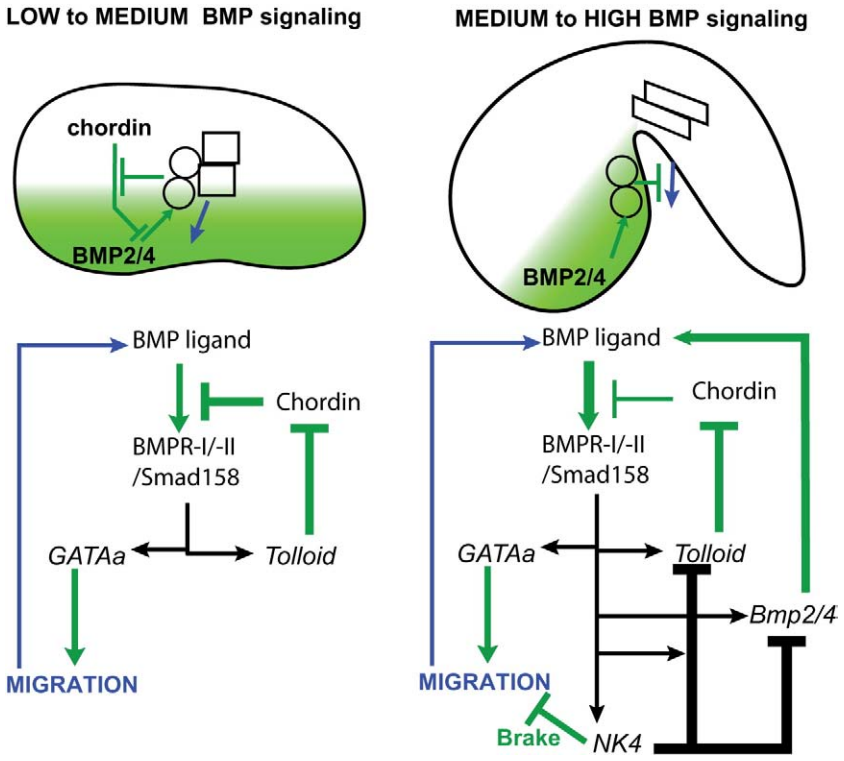

Fig. 6. Hypothetical summary model: BMP signaling coordinates TVC migration and gene expression. The two main "regulatory states" are shown. See Discussion for a step-by-step description of the model. Note that indicated steps are arbitrary delimitations between regulatory events that overlap to some extend. Green represents protein regulation, black represents regulatory interactions, -> and –| represent activation and inhibition/repression, respectively. Strong early dorsal expression of *chordin* has been showed by Imai et al. (2004) and can be visualized on the ghost database at http://ghost.zool.kyoto-u.ac.jp/cgi-bin3/photoget2.cgi?cicl016e09.

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Evolutionary considerations and future directions

Differential responses of target genes to BMP/Smad signalling is a key feature of the proposed model, which has been described in analogous systems whereby position-dependent responses are triggered by distinct concentrations of a morphogen (e.g. Gurdon and Bourillot, 2001; Simeoni and Gurdon, 2007). Here, the differential response of *GATAa*, *Tolloid*, *Bmp2/4* and *NK4* occurs in the same cells, each locus being presumably exposed to the same concentration of trans-acting factors (e.g. P-Smad1/5/8–Smad4 hetero-dimers) at a given time. Therefore, gene-specific responses must be encoded by the associated *cis*-regulatory DNAs. In fact, in vertebrate and Drosophila embryos, both *GATA4/Pnr* and *Nkx2.5/tinman* are direct transcriptional targets of BMP/Dpp signaling. In Drosophila, enhancer-mediated differential responses have been shown for target genes of the dorso-ventral Dpp gradient, whereby enhancers with a low affinity for Mad-containing transcription complexes direct expression to the region of the dorsal ectoderm with highest Dpp/Mad signaling (Ashe et al., 2000; Wharton et al., 2004). By analogy, we would expect the TVC enhancer of *NK4* to exhibit a lower affinity for Smad complexes than the TVC enhancers of *GATAa* and *Tolloid*.

Another essential feature of the proposed interpretation is the sequential activation of positive and negative feedback loops for the coordination of BMP signaling, gene expression and cell migration. Both of these regulatory loops are common features of BMP signaling, which directly activates positive and negative regulators of the core pathway in diverse tissue types (e.g. Karaulanov et al., 2004; Miyazono, 2000). Notably, the proposed model includes a tissue-specific component in the negative feedback loop, involving BMP-mediated activation of *NK4*, and a similar mechanism is probably conserved in vertebrates. Indeed, enhancer analysis showed that precardiac expression of *Nkx2.5* is directly activated by BMP/Smad signaling (Lee et al., 2004; Lien et al., 2002). In addition, analysis of double *Nkx2.5* and *Smad1* knockout mice demonstrated that Nkx2.5 function attenuates BMP signaling during subsequent cardiac development (Prall et al., 2007). It is of note that in both vertebrates and Drosophila, GATA factors contribute to the activation and/or maintenance of tinman/Nkx2.5 expression (Alvarez et al., 2003; Klinedinst and Bodmer, 2003; Lien et al., 1999; Searcy et al., 1998). Further studies will be required to determine whether this conserved regulatory connection is also present in the ascidian precardiac GRN.

Combinatorial effects of BMP signaling and tissue-specific regulators also determine the competence of anterior B7.5 granddaughter cells to migrate and activate precardiac kernel genes. For instance, previous studies demonstrated that both Mesp, FGF signaling and FoxF are required for TVC-specific gene expression including *Bmp2/4*, *Tolloid*, *GATAa* and *NK4* (Beh et al., 2007; Christiaen et al., 2008; Davidson et al., 2006; Satou et al., 2004). Although no direct link has been established between FoxF genes and heart development in vertebrates, similar regulatory interactions are at work during lateral mesoderm development in mice, whereby FoxF1 and BMP4 functions are required for *Gata4* activation (Rojas et al., 2005). In line with these observations, the *Drosophila* ortholog of *FoxF*, *biniou*, has been shown to play a critical role during visceral mesoderm development, in part by regulating the maintenance of *tinman* expression, together with Dpp signaling inputs (Zaffran et al., 2001).

In Drosophila, mesoderm precursor cells ingress ventrally during gastrulation. Subsequent differentiation and migration towards the dorsal side of the embryo require FGF signaling (Furlong, 2004; Kadam et al., 2009; Zaffran and Frasch, 2002). Therefore, early FGF signaling is required to establish the competence and specification of the precardiac mesoderm, through maintenance of *tinman* expression by the dorsal source of Dpp. This is strikingly similar to the situation observed in ascidian embryos, with the notable difference that the dorso-ventral polarity of cell migration and Dpp/Bmp sources appear to be inverted.

It is not clear that Dpp signaling contributes to dorsal mesoderm migration in Drosophila. Indeed, precocious expression of *tinman* throughout the mesoderm might inhibit the positive feedback loop discussed above. In contrast, recent studies in zebrafish have shown that a left–right asymmetric source of BMP is implicated in the cell migrations underlying heart looping in zebrafish (Smith et al., 2008). However, the effects of BMP signaling on cell migration are tissue- and stage-dependent, since it also negatively influences lateral mesodermal cell migration during gastrulation (von der Hardt et al., 2007).

Therefore, FGF-dependent cell migration of the early Drosophila mesoderm towards a dorsal ectodermal source of Dpp is most similar to the situation observed in *Ciona* embryos. In addition, this source of Dpp also determines the subsequent unfolding of the precardiac mesoderm GRN. In vertebrates however, the BMP signals contributing to precardiac mesoderm specification originate primarily from the anterior endoderm. Further studies will be required to determine the relative contribution of each tissue in *Ciona* embryos, since several candidate BMP ligands are also expressed in the posterior trunk endoderm adjacent to the TVCs (Imai et al., 2004). Nevertheless, the conserved role of Dpp/BMP signaling in precardiac mesoderm specification stands out given the diversity of signaling sources used in vertebrates, *Drosophila* and possibly *Ciona*. Therefore, we speculate that changes in the expression profiles of extracellular ligands, most probably through *cis*-regulatory DNA modifications, are required to allow conserved GRN kernels to operate in divergent morphogenetic environments.

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