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# *FoxF* is essential for FGF-induced migration of heart progenitor cells in the ascidian *Ciona intestinalis*

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Heart development requires precise coordination of morphogenetic movements with progressive cell fate specification and differentiation. In ascidian embryos, FGF/MAPK-mediated activation of the transcription factor Ets1/2 is required for heart tissue specification and cell migration. We found that *FoxF* is one of the first genes to be activated in heart precursors in response to FGF signaling. We identified the FoxF minimal heart enhancer and used a cis-trans complementation test to show that Ets1/2 can interact with the *FoxF* enhancer in vivo. Next, we found that FoxF function is required downstream and in parallel to the FGF/MAPK/Ets cascade for cell migration. In addition, we demonstrated that targeted expression of a dominant-negative form of FoxF inhibits cell migration but not heart differentiation, resulting in a striking phenotype: a beating heart at an ectopic location within the body cavity of juveniles. Taken together, our results indicate that *FoxF* is a direct target of FGF signaling and is predominantly involved in the regulation of heart cell migration.

**KEY WORDS: FGF signaling, Ascidian, Cardiac morphogenesis, Directed cell migration, Forkhead**

## INTRODUCTION

Heart development is governed by the tight coupling of morphogenesis and differentiation. In amniotes, complex heart anatomy results from a series of morphogenetic processes, including cell migration and cardiac tube looping. Heart morphogenesis is accompanied by the differentiation of several cell types, including the cardiomyocytes that confer contractile properties to the beating heart (for a review, see Garry and Olson, 2006).

Cardiogenesis is controlled by a highly conserved cassette of regulatory genes, which includes *tinman/Nkx2.5*, *pannier/Gata4/6*, *Hand* and T-box genes (Davidson, 2007; Davidson and Erwin, 2006; Olson, 2006). How this network coordinates cardiac morphogenesis and heart cell differentiation remains poorly understood. Here and elsewhere we have studied the earliest events in heart formation in an emerging model system, the ascidian *Ciona intestinalis*.

Despite their simplicity, tunicates, which include ascidians, are the closest living relatives of the vertebrates (Delsuc et al., 2006). The ascidian heart consists of a single layer of striated cardiomyocytes, surrounded by a pericardial sheath. This simple single-compartment heart beats rhythmically and can undergo reversible contractions (for details, see Davidson, 2007).

Lineage studies showed that the adult heart derives from two founder cells (the B7.5 blastomeres in the early gastrula embryo), which also form the larval anterior tail muscles (Davidson and Levine, 2003; Hirano and Nishida, 1997). By the end of neurulation, the B7.5 daughter cells constitute bilateral clusters of two small anterior and two large posterior cells occupying an anterior location in the tail. Shortly thereafter, the small anterior B7.5 lineage cells migrate to the ventral side of the trunk, hence their designation as trunk ventral cells (TVCs). The large posterior daughter cells remain in the tail, where they differentiate into muscle cells. The TVCs are the heart precursor cells in ascidian embryos. During subsequent stages of embryogenesis, they migrate into the trunk and fuse at the ventral midline in a fashion reminiscent of vertebrate heart precursor cells (Davidson and Levine, 2003).

Previous studies illuminated some aspects of the genetic regulation of early heart development in ascidians. The Mesp basic helix-loop-helix (bHLH) transcription factor is expressed exclusively in B7.5 cells from the 110-cell stage until the end of gastrulation (Imai et al., 2004; Satou et al., 2004). Morpholino knock-down showed that Mesp activity is required for both TVC migration in the embryo and cardiomyocyte differentiation during metamorphosis (Satou et al., 2004). In addition, ascidian orthologs of the conserved heart specification genes *NK-4* (*tinman/Nkx2.5*), *GATA-a* (*pannier/GATA4/5/6*), *Hand* and *Hand-like* (initially termed *NoTrlc*) (Imai et al., 2003) are expressed in the TVCs (Davidson, 2007; Davidson and Levine, 2003; Satou et al., 2004). *NK-4*, *Hand* and *Hand-like* are downregulated upon Mesp knock-down (Satou et al., 2004). Mesp is also thought to upregulate *Ets1/2* expression in the B7.5 lineage, thus conferring competence to respond to an unknown extrinsic FGF signal, possibly FGF9/16/20 (Davidson et al., 2006) (B.D., unpublished).

FGF signaling, transduced via the MAPK pathway, was recently shown to induce both heart tissue specification and TVC migration (Davidson et al., 2006). This induction event takes place only in the anterior B7.5 daughters, thus distinguishing the heart precursors from their sister tail-muscle cells. Heart specification and cell migration are both transcriptionally regulated by Ets1/2 in response to FGF signaling.

Migration and specification are thus tightly linked by common molecular determinants. However, it has been possible to uncouple TVC migration from cardiac muscle induction by targeted expression of a constitutively activated form of Mesp, the Mesp:VP16 fusion protein, in the B7.5 lineage (Davidson et al., 2005). TVCs are sometimes arrested in the anterior tail, and differentiate into disorganized aggregates of contractile cardiomyocytes at an ectopic location in the juvenile. Although the molecular basis of this uncoupling is not known, this result suggests that distinct, but interconnected, genetic pathways differentially regulate the acquisition of cardiac tissue identity and migratory behavior.

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Here, we present evidence that the forkhead/winged helix transcription factor FoxF is essential for TVC migration, but not for heart muscle specification. *FoxF* orthologs are highly conserved among diverse metazoans, and have been analyzed in vertebrates and *Drosophila* (Adell and Muller, 2004; Kaestner et al., 2000; Yagi et al., 2003). *FoxF* is one of the first genes to be activated in the anterior B7.5 lineage following FGF induction. We identified a FoxF minimal heart enhancer and used a cis-trans complementation test to show that Ets1/2 is an immediate activator of the enhancer in vivo. A dominant-negative form of FoxF inhibited cell migration but not subsequent heart differentiation, resulting in a striking phenotype: a beating heart in an ectopic position within the body cavity. These observations suggest that FoxF is a direct downstream effector of the FGF/MAPK/Ets signaling pathway and is required to induce the migratory behavior of heart precursors.

## MATERIALS AND METHODS

### Embryological techniques

*Ciona intestinalis* adults were collected at the Half Moon Bay harbor or obtained from San Diego, and maintained under constant illumination at 16-18°C. Eggs and sperm were used for in vitro fertilization following standard methods. Fertilized eggs were dechorionated and electroporated as previously described (Corbo et al., 1997). A FoxF morpholino oligonucleotide (5′-TGGTGTCCTGTCACTTCCATACTTC-3′) was purchased from Gene Tools. Morpholino injection was performed as described (~40 pl, 0.3-0.5 mM) (Imai et al., 2000). Juveniles were obtained by transferring electroporated embryos to uncoated Petri dishes filled with filtered artificial seawater with ampicillin (80 μg/ml). Water was changed 48 hours later, and every 24 hours subsequently. Heart phenotypes were scored from first water change and ongoing for the following 5 days.

### Histochemistry and in situ hybridization

X-gal staining was performed as described (Locascio et al., 1999), except that embryos were fixed in MEM-GA (0.1 M MOPS, pH 7.4, 0.5 M NaCl, 2 mM $MgSO_4$, 1 mM EGTA, pH 8.0, 0.2% glutaraldehyde, 0.05% tween-20) for 30 minutes at room temperature and staining was performed at 37°C. Stained embryos were mounted in glycerol. Double fluorescent in situ hybridizations and immunohistochemistry were performed as described by Dufour et al. (Dufour et al., 2006). β-galactosidase was detected using a mouse monoclonal antibody (dilution 1:1000, Promega, Z378A); antisense RNA probes for either *FoxF*, *Hand-like*, *GATA-a* or *NK-4* were visualized with TSA-fluorescein and the amplification kit according to the manufacturer's recommendations (PerkinElmer, NEL741). Embryos were mounted in ProLong Gold (Invitrogen, P36931) and analyzed with a LEICA TCS SP2 confocal microscope.

### Molecular cloning

The coding sequence for the FoxF DNA binding domain (FoxF-DBD) was amplified from the *Ciona* Gene Collection library clone ciel007c02. The Mesp>*FoxF*:VP16 and Mesp>*FoxF*:WRPW fusion genes were derived from the previously reported Mesp>*Mesp*:VP16 and Mesp>*Mesp*:WRPW fusion genes (Davidson et al., 2005), by replacing the Mesp bHLH domain with the FoxF-DBD fragment.

Approximately 3 kb of the *FoxF* 5′ flanking sequence was PCR-amplified from genomic DNA and cloned into the pCESA vector containing a *lacZ* reporter gene (Harafuji et al., 2002). The minimal *FoxF* TVC enhancer (–1135 to –840) was PCR amplified and cloned upstream of the *Ci-FoxAa* basal promoter included in pCESA. Small deletions and point mutations were introduced in the FoxF regulatory sequences using the QuickChange site-directed mutagenesis kit (Stratagene, 200519-5).

### Migration phenotype analysis

Both the Mesp>*GFP* and Mesp>*lacZ* reporters were used to assess migration phenotypes. Transformed embryos and larvae expressing the Mesp>*GFP* reporter gene were fixed in 4% formaldehyde overnight and mounted in glycerol or ProLong Gold (Invitrogen, P36931). Migration defects were grouped into five distinct phenotypic classes based on the relative position of the B7.5 lineage cells within the embryo (Fig. 3G). At least two independent experiments were performed for each condition. The proportions of each phenotypic class were compared between conditions using a $\chi^2$ test.

## RESULTS

A provisional circuit diagram for early *Ciona* embryogenesis was determined by systematic morpholino-mediated knock-down assays and subsequent gene expression analyses (Imai et al., 2006). Selective disruption of *Mesp* gene activity causes reduced expression of several downstream genes, including *Tolloid*, *NK-4*, *Hand-like* and *FoxF*, in the B7.5 lineage. Previous studies suggested a possible role for *FoxF1* (also known as *Foxf1a*) in mesenchyme migration during the development of the lung, gall bladder and liver in mouse embryos (Kalinichenko et al., 2001; Kalinichenko et al., 2002; Mahlapuu et al., 2001). We therefore explored the possibility that *FoxF* plays a role in heart migration during *Ciona* embryogenesis.

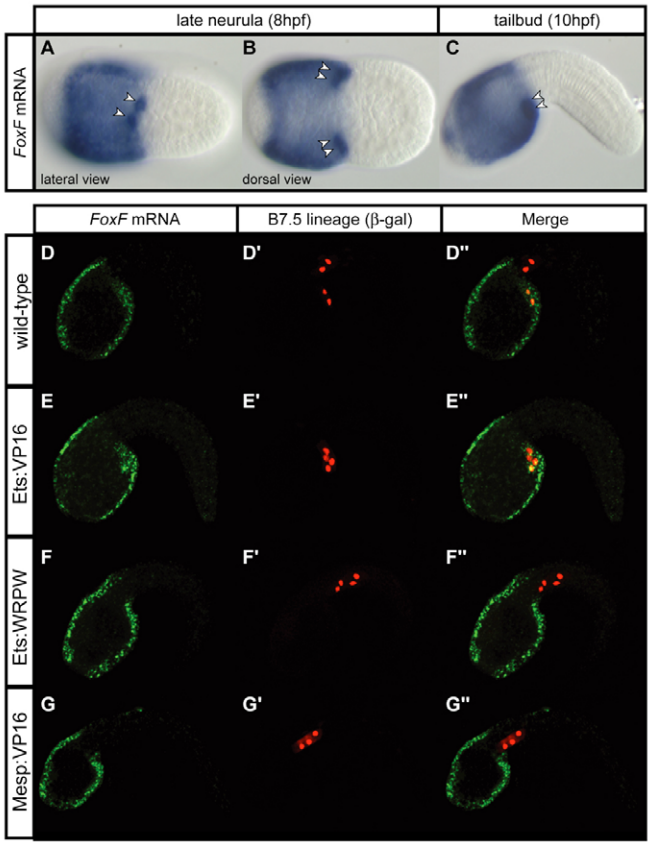


**Fig. 1. *FoxF* expression in anterior B7.5 cells requires Ets1/2 activity.** (**A-C**) In situ hybridization on neurula (A,B) and tailbud (C) embryos. Arrowheads (A,B) indicate the anterior B7.5 cells, which migrate and form the trunk ventral cells (TVCs; white arrowheads in C). Notice the *FoxF* expression in trunk epidermal cells. (**D-G″**) The B7.5 lineage cells were visualized with an anti-β-galactosidase antibody (red), and *FoxF* expression was revealed by fluorescent in situ hybridization (green). (D-D″) Embryo electroporated with the Mesp>*lacZ* control. Within the B7.5 lineage, *FoxF* is only expressed in the anterior TVCs and not in the posterior tail muscles. (E-E″) Embryo co-electroporated with Mesp>*lacZ* and Mesp>Ets:VP16. Ets:VP16 induces all four B7.5 lineage cells to migrate and express *FoxF*. (F-F″) Embryo co-electroporated with Mesp>*lacZ* and Mesp>Ets:WRPW. Ets:WRPW inhibits both the migration of B7.5 cells and *FoxF* expression. (G-G″) Embryo co-electroporated with Mesp>*lacZ* and Mesp>*Mesp*:VP16. All B7.5 cells remain in the tail; anterior cells fail to express *FoxF*.

### *FoxF* expression correlates with heart migration

The *FoxF* expression pattern was determined by in situ hybridization (Fig. 1A-C). At the late neurula stage, *FoxF* transcripts were detected in the trunk epidermis, and in the two TVCs at the anterior end of the tail (Fig. 1A,B, arrowheads). At the tailbud stage, epidermal expression persisted, while the TVCs had moved to a ventro-posterior position in the trunk (Fig. 1C, arrowheads). Hence, *FoxF* is expressed in both the trunk epidermis and TVCs.

Double-staining assays were conducted to determine the detailed *FoxF* expression pattern within the B7.5 lineage. Fertilized eggs were electroporated with a Mesp>*lacZ* fusion gene, and an anti-β-galactosidase antibody was used to visualize *lacZ* expression in the complete B7.5 lineage, i.e. the anterior heart precursor cells (TVCs) and posterior tail muscles (Fig. 1D′-G′). Endogenous *FoxF* expression was visualized by fluorescent in situ hybridization (Fig. 1D-G). Double-staining assays confirmed that *FoxF* is expressed in the anterior heart precursors, but not in the posterior cells forming tail muscles.

Previous studies demonstrated that heart induction occurs at the late neurula stage in the anterior B7.5 cells in response to an FGF signal, which induces the activity of the transcription factor Ets1/2 (Davidson et al., 2006). In an initial attempt to determine whether *FoxF* expression requires Ets1/2 activity, embryos were co-electroporated with constitutively active (Ets:VP16) and dominant-negative (Ets:WRPW) forms of Ets1/2 under the control of the *Mesp* enhancer. Co-electroporation of the Mesp>Ets:VP16 fusion gene caused the posterior B7.5 lineage to migrate into the trunk and form supernumerary heart precursors (Fig. 1E-E″) (for details, see Davidson et al., 2006). All four cells, the normal heart cells and transformed muscle cells, expressed *FoxF* upon Ets:VP16 overexpression. By contrast, *FoxF* expression was lost in embryos that were co-electroporated with a Mesp>Ets:WRPW fusion gene, which blocked heart induction and converted the entire B7.5 lineage into tail muscles (Fig. 1F-F″).

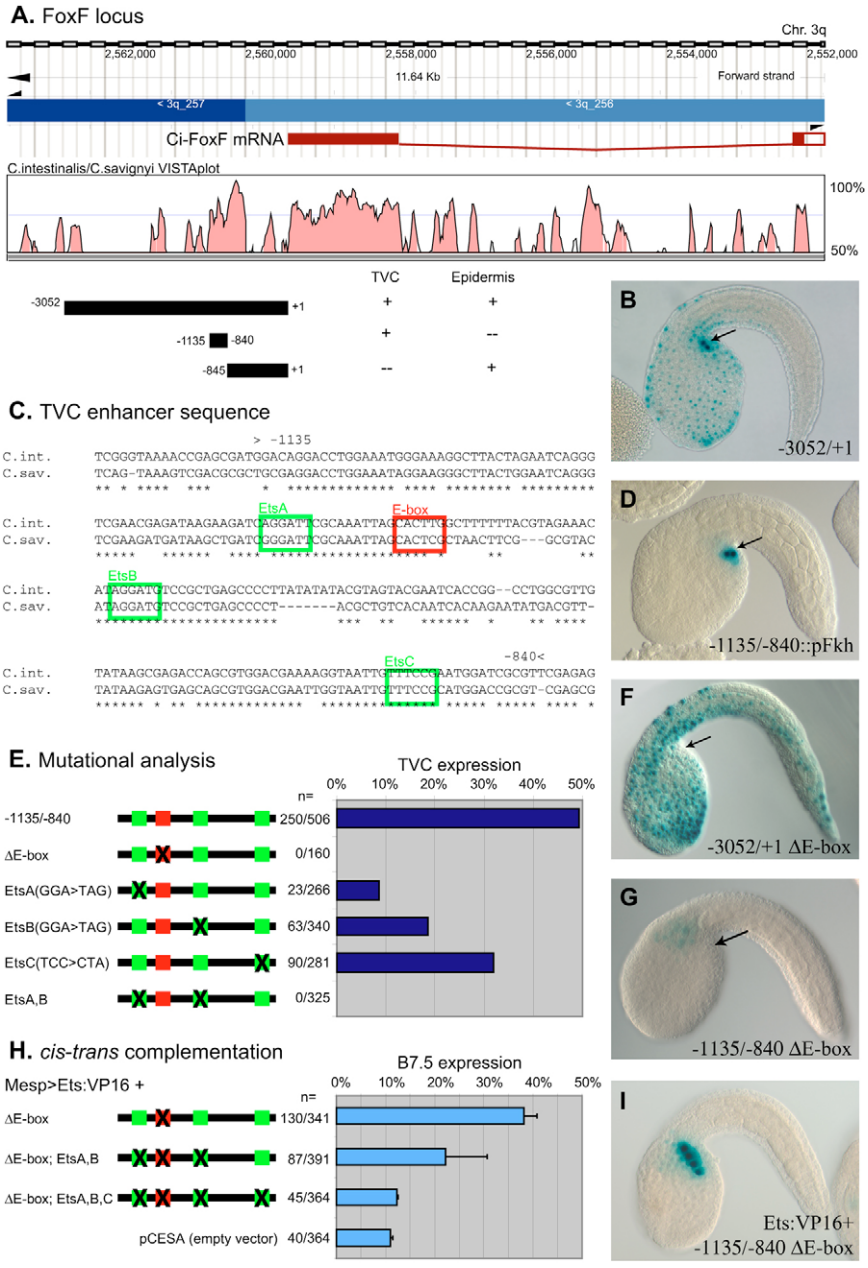


**Fig. 2. *FoxF* is an immediate target of the FGF/MAPK/Ets pathway.** (**A**, top) Map of the *FoxF* gene (red) and 5′ upstream region on chromosome arm 3q. (A, middle) VISTAplot showing sequence conservation (50-100%) between two *Ciona* species (http://genome.lbl.gov/vista/index.shtml). (A, bottom) Summary diagram of trunk ventral cell (TVC) and epidermis expression with various 5′ enhancers fused to the *lacZ* reporter. (**B**) Embryo expressing the –3052 to +1 enhancer attached to the *lacZ* reporter, showing TVC (arrow) and epidermal expression. (**C**) The *FoxF* minimal TVC enhancer sequence (–1135 to –840) is highly conserved between *C. intestinalis* and *C. savignyi*. Boxes indicate the E-box (red) and the three Ets1/2-binding sites (green). (**D**) A 295 bp (–1135 to –840 bp) genomic DNA fragment, fused to the *Forkhead* (*FoxA-a*) basal promoter, drives *lacZ* expression specifically in the TVCs (arrow). (**E**) Mutational analysis. The histogram displays the proportions of embryos showing TVC staining (*n*=total embryos). Diagrams depict the wild-type –1135 to –840 fragment and the indicated mutations. (**F**) The –3052 to +1 cis-regulatory region with deleted E-box motif drives expression in epidermis only. (**G**) The –1135 to –840 (ΔE-box) does not drive *lacZ* expression in TVCs (arrow). (**H**) Cis-trans complementation test. Ets:VP16 can restore enhancer activity of the –1135 to –840 (ΔE-box) construct. The cis-trans complementation is abolished when the three Ets1/2 sites are mutated (errors bars indicate standard deviation). (**I**) The –1135 to –840 (ΔE-box) enhancer co-electroporated with Mesp>Ets:VP16. Ets:VP16 causes all four B7.5 cells to migrate into the trunk and causes the mutated enhancer to drive *lacZ* expression in all cells. Arrows in B,D,F,G,I point to the TVCs (not stained in F and G).

The preceding analysis suggests that *FoxF* expression is downstream of Ets1/2-mediated heart specification. To determine whether *FoxF* expression correlates with either migration or tissue specification, we examined tadpoles that express the Mesp>*Mesp*:VP16 fusion gene. Targeted expression of Mesp:VP16 caused sporadic inhibition of migration, but did not prevent heart tissue differentiation in juveniles (Davidson et al., 2005). Cells that failed to migrate into the trunk upon Mesp:VP16 over-expression did not express *FoxF* (Fig. 1G-G″). By contrast, these same non-migrating B7.5 lineage cells do express *Hand-like* and undergo heart tissue differentiation in the juvenile (Davidson et al., 2005). Thus, *FoxF* and *Hand-like* expression differentially correlate with migration and tissue specification, respectively.

In summary, *FoxF* expression in anterior B7.5 lineage cells requires FGF-induced Ets1/2 activity and correlates better with cell migration than heart tissue specification.

## FoxF is an immediate target of the FGF/MAPK/Ets pathway

The preceding results raise the possibility that *FoxF* is a direct transcriptional target of the FGF/MAPK/Ets pathway in the B7.5 lineage. To test this hypothesis, we isolated and characterized *FoxF* cis-regulatory sequences. The genomic region upstream of the first *FoxF* exon is highly conserved between *Ciona intestinalis* and *Ciona savignyi* (Fig. 2A), which often points to functional non-coding DNA. We found that 3 kb of the 5′ flanking region drives *lacZ* reporter gene expression in the trunk epidermis and heart precursors, thus recapitulating the endogenous *FoxF* expression pattern in electroporated tadpoles (Fig. 2B).

A series of 14 truncated constructs were generated and analyzed in an effort to identify a minimal heart enhancer (Fig. 2A and J.B., unpublished). We mapped a 295 bp TVC-specific enhancer between 1135 and 840 bp upstream of the translation initiation codon (Fig. 2A,C). When fused to a *Ci-FoxAa* basal promoter, reporter gene expression driven by this enhancer was restricted to the heart precursor cells (Fig. 2D). *FoxF* expression in the trunk epidermis depends on separate elements that map within the proximal 845 bp of the 5′ flanking region (Fig. 2A and J.B., unpublished).

Close examination of the 295 bp TVC enhancer revealed the presence of three putative Ets1/2-binding sites matching the consensus recognition sequence MGGAWNY (Choi and Sinha, 2006) (Fig. 2C). To test whether these sites are required for enhancer activity, point mutations were introduced in the minimal *FoxF* TVC enhancer and assayed by electroporation and X-gal staining (Fig. 2E). Point mutations in each individual putative Ets1/2 site significantly reduced TVC expression of the transgene. Because no single alteration completely eliminated reporter gene expression; we combined mutations of the two sites that showed the greatest effects (Fig. 2E, EtsA and EtsB sites). Strikingly, combined mutations of the EtsA and EtsB sequences completely abolished reporter gene expression (Fig. 2E). These results show that putative Ets1/2-binding sites are required for *FoxF* minimal TVC enhancer activity.

Further evidence that Ets1/2 can directly transactivate the minimal TVC enhancer of FoxF stems from a cis-trans complementation test. This test was based on the requirement for a second sequence motif, CACTTG, which was also found to be essential for the activity of the FoxF cardiac enhancer. This motif conforms to an E-box (CANNTG consensus). Deletion of this sequence from either the full-length *FoxF>lacZ* fusion gene or the minimal TVC enhancer abolished reporter gene expression in heart precursor cells (Fig. 2E-G; ΔE-box construct). In addition, ectopic expression of the constitutively activated form of Ets1/2 induces *FoxF>lacZ* expression in both the anterior and posterior B7.5 lineages (Davidson et al., 2006). Therefore, we reasoned that, if Ets is a direct activator, the hyper-active Ets:VP16 fusion protein should be able to restore the activity of a defective *FoxF* enhancer lacking the E-box motif.

Indeed, co-electroporation of the Mesp>Ets:VP16 fusion gene with the damaged *FoxF* enhancer resulted in robust *lacZ* expression in the entire B7.5 lineage, as compared with an empty-vector control (Fig. 2H,I). To further test whether this cis-trans complementation results from direct activation by Ets:VP16, we repeated the experiment using a mutant *FoxF* enhancer lacking all three putative

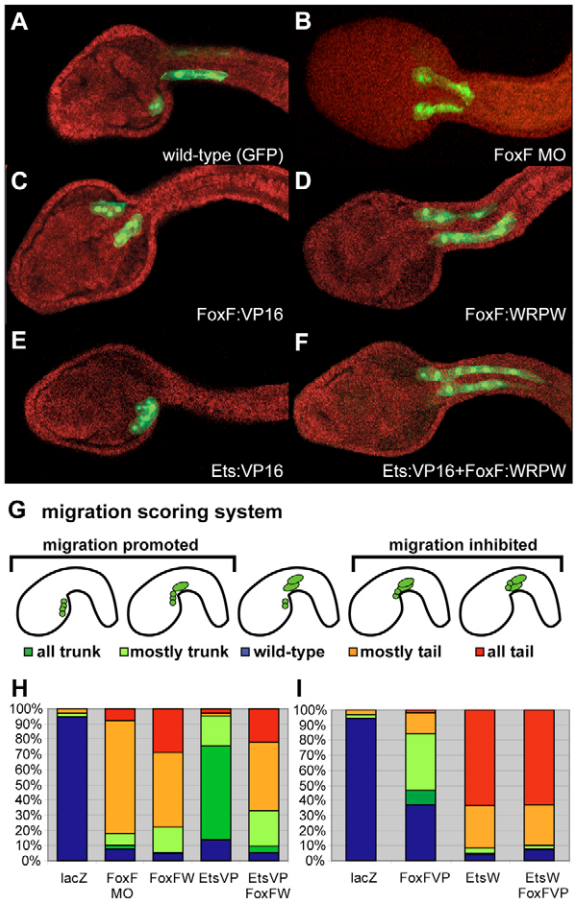


**Fig. 3. FoxF function is necessary for TVC migration.** (**A-F**) Embryos electroporated (A,C-F) or injected (B) with Mesp>*GFP* to mark the B7.5 lineage (green). The red channel detects the fluorescent background of the *Ciona* embryo. (A) Wild-type embryo with normal anterior TVC and posterior tail muscle positions (lateral view). (B) Embryo co-injected with Mesp>*GFP* and *FoxF* morpholino. Anterior B7.5 cells fail to detach from their sister muscle cells and to migrate into the trunk (ventral view). (C) Mesp>*GFP* co-electroporated with Mesp>*FoxF*:VP16. All B7.5 lineage cells have migrated into the trunk (ventral view). (D) Mesp>*GFP* co-electroporated with Mesp>*FoxF*:WRPW. All B7.5 cells remain in the tail. (E) Mesp>*GFP* co-electroporated with Mesp>Ets:VP16. All B7.5 cells migrate into the trunk (lateral view). (F) Mesp>*GFP* co-electroporated with Mesp>Ets:VP16 and Mesp>FoxF:WRPW. Inhibited TVC migration occurs that is comparable to that observed with FoxF:WRPW alone. (**G**) The five distinct classes of migration phenotypes. (**H**,**I**) Proportions of embryos distributed among the five phenotypic classes in each condition, including EtsVP/FoxFW and EtsW/FoxVP epistasis tests; color coding is as in G.

Ets1/2-binding sites as well as the E-box motif. These additional mutations abolished specific trans-activation of the enhancer by Ets:VP16.

The preceding results suggest that the FoxF cardiac enhancer is directly regulated by Ets1/2, and by an unidentified factor that binds the E-box motif.

## FoxF is essential for TVC migration

Several methods were used to interfere with FoxF function in heart precursor cells, including morpholino injection and targeted expression of a constitutive repressor form of FoxF, obtained by attaching the FoxF DNA binding domain to the *Drosophila* Hairy WRPW repressor motif (FoxF:WRPW construct). Mesp>*GFP* or Mesp>*lacZ* reporter constructs were used to visualize the B7.5 lineage cells and assess migration defects in tailbud embryos, after normal TVC migration to a ventro-lateral position in the trunk (Fig. 3A). A migration scoring scheme was developed to take into account the observed phenotypic variability (Fig. 3G).

Injection of a *FoxF* morpholino led to an inhibition of TVC migration in 80% of the examined embryos ($n$=32/40; Fig. 3B,H). However, only 37.5% of these embryos ($n$=15/40) showed normal morphology, suggesting that gross morphological defects – and possibly migration inhibition – might arise from FoxF disruption in the trunk epidermis, consistent with the dual expression of *FoxF* in both tissues (see Fig. 1A-C).

To circumvent potential indirect effects arising from disruption of the trunk epidermis via morpholino injection, we targeted expression of FoxF:WRPW in the B7.5 lineage using the *Mesp* enhancer. This allowed us to assess the cell-autonomous effects of *FoxF* gene activity in heart precursor cells. Sporadic defects were observed in embryos electroporated with the Mesp>*GFP* construct alone (5.5%, $n$=17/306; no significant difference was observed with the Mesp>*lacZ* reporter construct). By contrast, targeted expression of the constitutive repressor FoxF:WRPW fusion protein severely inhibited migration (77.6%, $n$=422/544; Fig. 3D,H). These results are consistent with the morpholino gene-disruption assays (Fig. 3H), suggesting that transcriptional activation by FoxF promotes TVC migration.

An epistasis experiment was conducted in order to establish a more definitive link between *FoxF* gene activity and heart cell migration. As shown previously, targeted expression of the constitutively active form of Ets1/2 (Ets:VP16) causes both anterior and posterior B7.5 lineage cells to migrate into the trunk and form cardiac tissues (Fig. 3E,H) (Davidson et al., 2006). Co-expression of FoxF:WRPW with Ets:VP16 appears to reverse the Ets:VP16 effect, inhibiting cell migration (Fig. 3F). Although B7.5 lineage cells migrated in some embryos, the proportion of tadpoles showing inhibited migration was indistinguishable from that observed with FoxF:WRPW alone (Fig. 3H; $\chi^2$ test, $P$=0.108). This result shows that normal FoxF function is required downstream of the FGF/MAPK/Ets cascade to promote cardiac cell migration.

Because a dominant-negative form of FoxF inhibits Ets:VP16-induced cell migration, we asked whether FoxF activity would be sufficient for the heart cells to migrate in the absence of Ets1/2 activity. To this aim, we engineered a constitutive activator form of FoxF, by fusing its *forkhead* domain to the VP16 trans-activation domain. The Mesp>*FoxF*:VP16 transgene seemed to enhance the migration of B7.5 lineage cells in 47.3% of the observed tadpoles (Fig. 3C,I). However, only 9.4% ($n$=67/712) of the observed embryos showed complete migration of the entire B7.5 lineage into the trunk (versus 61.6%, $n$=122/198 with Ets:VP16; Fig. 3H,I), and normal TVC migration was slightly inhibited in 13.8% of the

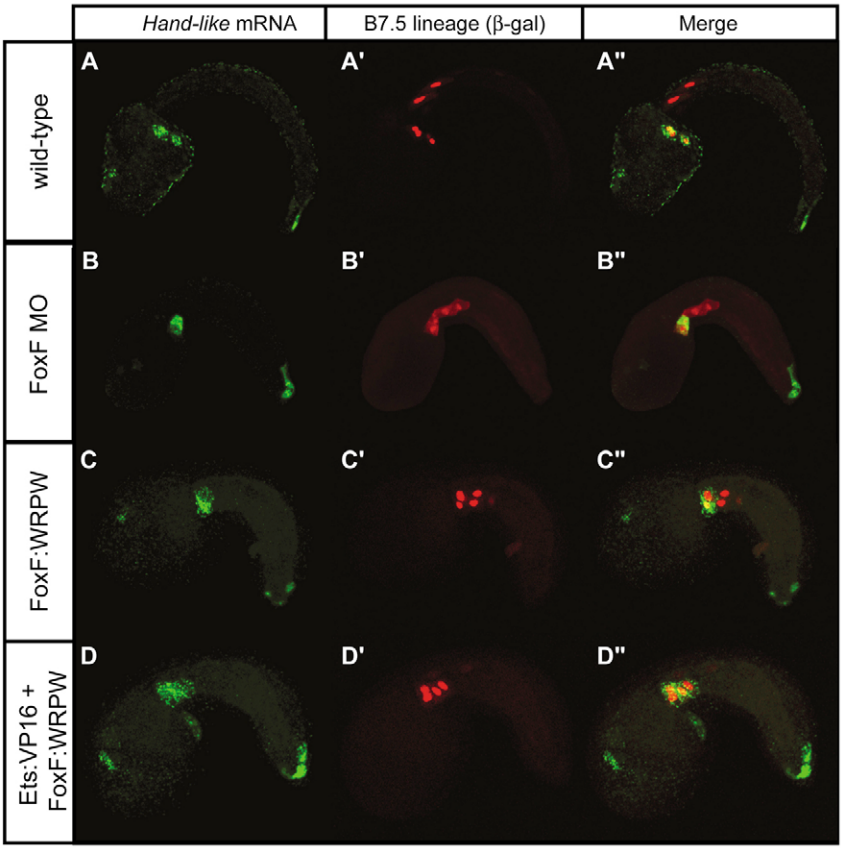


**Fig. 4. *FoxF* function is not required for *Hand-like* expression in the TVCs.** Electroporated *Ciona* embryos hybridized with the *Hand-like* probe (green) and the B7.5 lineage cells visualized with an anti-β-galactosidase antibody (red). (**A-A″**) Wild-type embryo electroporated with Mesp>*lacZ* alone. *Hand-like* is expressed in anterior trunk ventral cells (TVCs), but not in posterior tail muscles. (**B-B″**) Embryo co-injected with Mesp>*lacZ* and *FoxF* morpholino. TVC migration is inhibited, but *Hand-like* expression persists in the anterior cells. (**C-C″**) Embryo co-electroporated with Mesp>*lacZ* and Mesp>*FoxF*:WRPW. TVC migration is inhibited, but *Hand-like* exhibits normal expression in two anterior B7.5 cells. (**D-D″**) Embryo co-electroporated with Mesp>*lacZ*, Mesp>Ets:VP16 and Mesp>*FoxF*:WRPW. *Hand-like* expression is present in all B7.5 cells even though migration is inhibited by FoxF:WRPW.

embryos (Fig. 3I, 'mostly tail' class, *n*=98/712). These observations suggest that FoxF activity alone can mediate some aspects of heart cell migration. However, these results are not conclusive regarding sufficiency because Ets1/2 is potentially still active in B7.5 lineage cells.

We therefore performed a complementary epistasis assay, co-expressing the FoxF:VP16 chimera with the dominant-negative Ets:WRPW fusion protein (Davidson et al., 2006). The Mesp>Ets:WRPW fusion gene inhibited TVC migration in 91.7% (*n*=628/685) of the electroporated embryos (Fig. 3I). Co-expression of FoxF:VP16 did not rescue the migration defects induced by Ets:WRPW, which were indistinguishable from the effects of Ets:WRPW alone ($\chi^2$ test, $P$=0.258; Fig. 3I). These results confirm that FoxF is not sufficient for the migration of B7.5 lineage cells. Instead, it appears that the FGF/MAPK/Ets pathway is required in parallel with FoxF to induce cardiac cell migration.

## FoxF function is not absolutely required for early heart specification

Previous studies established that Ets1/2 activity induces both cell migration and cardiac fate specification during early heart development (Davidson et al., 2006). We therefore tested the possibility that FoxF plays an additional, cell-autonomous role in cardiac fate specification downstream of FGF signaling. To this aim, we first analyzed cardiac gene expression in embryos electroporated with the dominant-negative *FoxF* fusion genes.

As indicated previously, *Hand-like* expression correlates with heart muscle specification. Injection of the *FoxF* morpholino or expression of the dominant-negative *FoxF* transgene (Mesp>*FoxF*:WRPW) in the entire B7.5 lineage did not seem to alter the normal *Hand-like* expression pattern. Indeed, in embryos showing inhibited cell migration, *Hand-like* exhibited normal expression in the anterior, but not posterior, B7.5 lineage cells (Fig. 4B-C″).

To gain further insight into the regulatory relationship between FoxF and the heart specification cassette, we investigated *NK-4* and *GATA-a* expression in embryos electroporated with the Mesp>*FoxF*:WRPW construct. Both *GATA-a* and *NK-4* were silent in the absence of FGF signaling, but were ectopically expressed in extra migrating cells upon targeted expression of Ets:VP16 (see Fig. S1 in the supplementary material).

The dominant-negative FoxF construct had variable effects on *GATA-a* and *NK-4* expression (Fig. 5 and see Fig. S1 in the supplementary material). We focused our attention on embryos showing inhibited migration, and found that both *GATA-a* and *NK-4* expression were either unaffected (Fig. 5C,D) or lost (Fig. 5E,F) in embryos displaying conspicuous migration defects. *GATA-a* and *NK-4* expression was maintained in 35-50% of the FoxF:WRPW-expressing embryos (see Fig. S1 in the supplementary material). Taken together, these observations suggest that disruption of FoxF function has limited effects on cardiac specification, because expression of core heart differentiation genes (*Hand-like*, *GATA-a* and *NK-4*) can persist in embryos showing severely inhibited migration (see Discussion).

## Disruption of FoxF function causes a dramatic repositioning of the beating heart

Our observations raise the possibility that FoxF regulates cell migration, but not heart tissue specification. To further evaluate this possibility, we repeated the Ets:VP16/FoxF:WRPW epistasis test and assessed *Hand-like* gene expression in tailbud embryos.

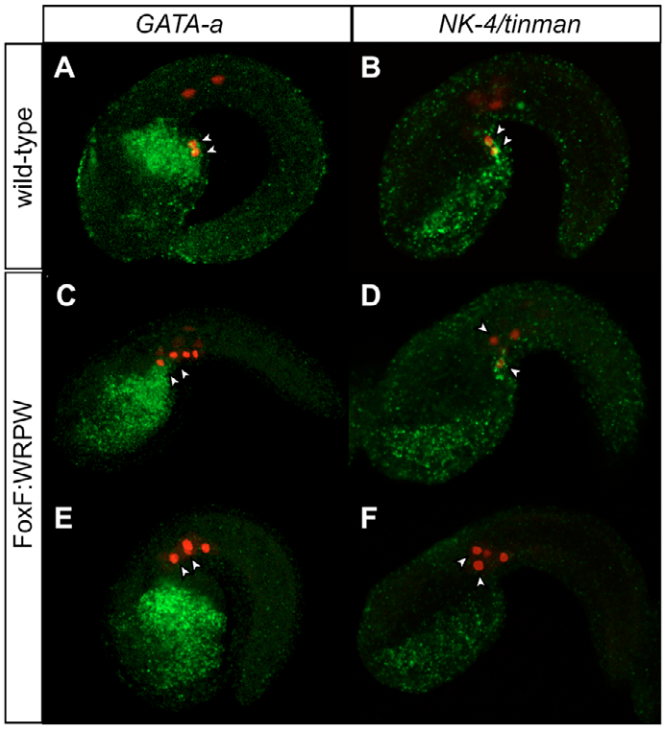


**Fig. 5. The effects of FoxF:WRPW on *GATA-a* and *NK-4* expression are variable.** Embryos electroporated with Mesp>*lacZ* and the indicated constructs (left) were stained for β-galactosidase (red) and either *GATA-a* (**A**,**C**,**E**) or *NK-4* (**B**,**D**,**F**) mRNAs (green). (A,B) Wild-type embryos. (C-F) Embryos electroporated with Mesp>*FoxF*:WRPW. *GATA-a* and *NK-4* expression persists (C,D) or is abolished (E,F) in non-migrating trunk ventral cells (TVCs). Arrowheads indicate anterior B7.5 cells.

Our previous studies showed that the Mesp>Ets:VP16 transgene induces the complete B7.5 lineage to migrate and express *Hand-like* (Davidson et al., 2006). As shown earlier, co-expression of the dominant-negative FoxF:WRPW protein inhibited migration, so that both the anterior and posterior B7.5 lineage cells remained in the tail (Fig. 3F). However, all of the cells expressed *Hand-like* (Fig. 4D-D″), suggesting that Ets:VP16 is still able to induce cardiac muscle fate in the absence of migration and FoxF function. In this experiment, heart tissue specification and cell migration were more completely uncoupled, with the entire B7.5 lineage being converted into heart muscle precursors that remained at the anterior end of the tail.

In order to determine whether these misplaced cells can form a beating heart, tadpoles expressing various combinations of the Mesp>*lacZ*, Mesp>Ets:VP16 and Mesp>*FoxF*:WRPW transgenes were grown through metamorphosis and allowed to develop into juveniles. Heart phenotypes were grouped in four distinct classes based on morphology, size and position (Table 1). In all conditions, we observed general heart defects, which might result from the dechorionation procedure, which was shown to interfere with metamorphosis (Sato and Morisawa, 1999). Most of the remaining Mesp>*lacZ*-electroporated juveniles displayed normal heart morphology and position. As shown previously, overexpression of the Ets:VP16 chimera led to an expansion of the heart tissue, sometimes resulting in the striking 'two-compartments' heart phenotype, whereas Ets:WRPW strongly

**Table 1. Heart phenotypes in juveniles**

| | | % with phenotype | | | |
|---|---|---|---|---|---|
| Experiment | *n* | Wild type | Heart tissue in tail (tail-heart) | Expanded heart | Misc. heart defects |
| *lacZ* | 46 | 69.6 | 0 | 4.3 | 26.1 |
| Ets:VP16 | 168 | 47.6 | 0 | 19.6 | 32.7 |
| Ets:WRPW | 131 | 27.5 | 1.5 | 6.1 | 64.9 |
| FoxF:VP16 | 120 | 53.3 | 0 | 13.3 | 33.3 |
| FoxF:WRPW | 191 | 51.8 | 7.9 (2.6) | 2.1 | 38.2 |
| Ets:VP16+FoxF:WRPW | 257 | 38.1 | 17.5 (8.2) | 8.6 | 35.8 |
| Ets:WRPW+FoxF:VP16 | 53 | 35.8 | 5.7 | 9.4 | 49.1 |

Results are expressed as percentage of juveniles falling into four phenotypic classes. 'Wild type' corresponds to a single heart (normal size and position). 'Heart tissue in tail' groups juveniles showing either the tail-heart phenotype, disorganized heart-like structure, or twitching tissue in the tail. 'Expanded heart' denotes an enlarged heart or the double heart described in Davidson et al. (Davidson et al., 2006). 'Miscellaneous heart defects' refer to the absence of heart or disorganized heart in a normal position. (*n*=number of juveniles scored.)

impaired heart formation (Davidson et al., 2006). In Mesp>*FoxF*:WRPW-electroporated juveniles, we found twitching heart-like tissue mis-positioned at the base of the resorbed tail in 7.8% of animals (*n*=15/191, Table 1). In 5.2% of cases (Table 1), the heart-like tissue appeared disorganized, but the other 2.6% showed the so-called 'tail-heart' phenotype (see below).

In populations co-expressing Ets:VP16 and FoxF:WRPW, mis-positioned heart-like tissue was observed in an increased proportion of juveniles (Table 1). Noticeably, approximately half of these animals (46.7%, *n*=21/45) showed the 'tail-heart' phenotype: the ectopic heart tissue formed a hollow compartment, a pericardial coelom and was beating in a rhythmic manner, although it did not effect the circulation of blood cells, because it lacked afferent and efferent blood vessels (Fig. 6B,D; see Movies 1 and 2 in the supplementary material).

These observations provide striking evidence that targeted disruption of *FoxF* function in the B7.5 lineage specifically blocks cell migration during embryogenesis, with little to no impact on subsequent cardiac tissue differentiation.

Where does the 'tail-heart' come from? Our analysis of heart differentiation genes shows that *Hand-like* expression is independent of FoxF function, because it was retained in non-migrating TVCs after *FoxF*-morpholino injection or targeted expression of the FoxF:WRPW fusion protein.

On the other hand, *GATA-a* and *NK-4* expression was reduced upon FoxF:WRPW overexpression. The cellular and molecular basis for this effect is unknown, but suggests that additional linkages might connect the core heart regulatory network to cell migration in ascidian embryos (summarized in Fig. 7).

In line with the uncoupling hypothesis, we found that *GATA-a* and *NK-4* expression persisted in 50% and 36%, respectively, of the embryos with inhibited TVC migration upon FoxF:WRPW overexpression. Hence, expression of three core heart-differentiation genes, *Hand-like*, *GATA-a* and *NK-4*, is maintained in 15-35% of embryos in which heart migration is inhibited. It seems likely that juveniles showing mis-positioned heart tissue develop from these embryos, in which the early heart-specification network appears unaffected.

## DISCUSSION

Previous studies established a role for Mesp and the FGF/MAPK/Ets signaling pathway in both heart tissue specification and cardiac cell migration in *Ciona* embryos. Here, we presented evidence that the winged helix transcription factor FoxF is required for FGF-induced cardiac cell migration, but not for heart muscle specification. Moreover, the FGF/MAPK/Ets pathway appears to directly activate *FoxF* expression in heart precursor cells. The uncoupling of heart migration and differentiation is strikingly illustrated by the development of a beating heart at the wrong location within the body cavity.

### Making a heart without moving

In ascidian embryos, heart specification in the rostral B7.5 lineage requires Mesp activity and Ets1/2 activation in response to the FGF/MAPK pathway, which induces all aspects of cardiac specification, including migration and subsequent differentiation. Previous observations suggested that heart muscle specification and cell migration could be uncoupled to some extent (Davidson et al., 2005). Here, we showed that the transcription factor FoxF is expressed in response to FGF signaling and is required for cell migration. However, FoxF function appears dispensable for heart muscle differentiation, because embryos expressing the dominant-negative FoxF:WRPW could develop into juveniles with mis-positioned heart tissue.

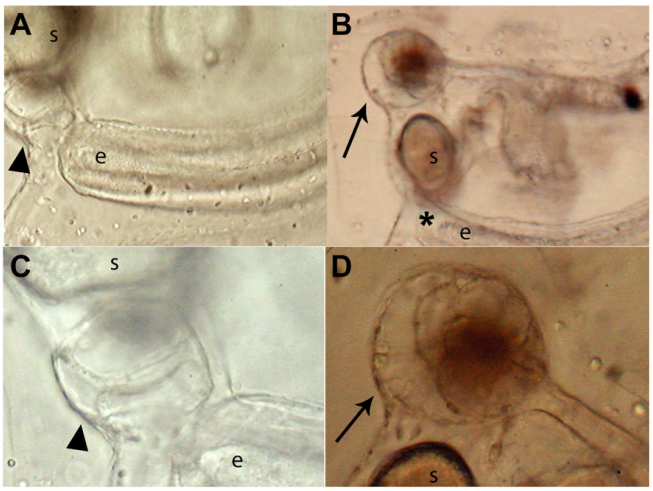


**Fig. 6. Co-expression of Ets:VP16 and FoxF:WRPW results in a mis-positioned beating heart.** (**A**,**C**) Wild-type heart (arrowhead) located between the endostyle (e) and the stomach (s). (**B**,**D**) Juvenile co-electroporated with Mesp>Ets:VP16 and Mesp>*FoxF*:WRPW. Beating heart tissue (arrow) is located where the tail is being reabsorbed above the stomach (s). Heart is missing from the wild-type location (*).

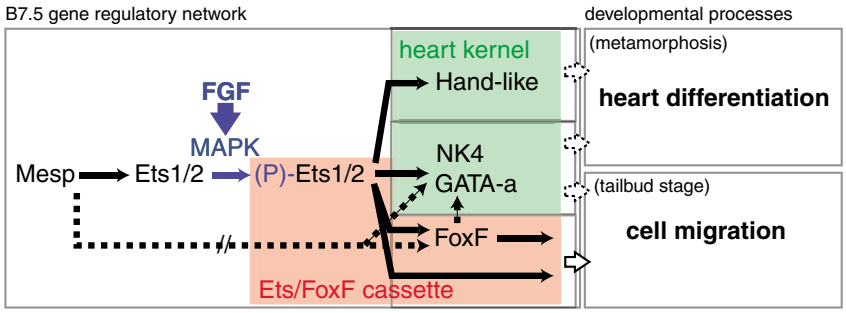


**Fig. 7. The early heart gene network regulates heart differentiation and migration.** *Mesp* expression initiates heart specification in the B7.5 lineage. Mesp is thought to upregulate *Ets1/2* expression in the whole B7.5 lineage. An FGF signal activates Ets1/2 in the anterior B7.5 cells, thus inducing both heart muscle specification and cell migration. Activated Ets1/2 activates process-specific genes for both heart differentiation (the heart-kernel genes) and cell migration (*FoxF*) in parallel pathways. Mesp presumably functions in parallel to Ets1/2 to regulate *GATA-a*, *NK-4* and *FoxF* expression. FoxF and activated Ets1/2 function in parallel to control heart cell migration.

## An essential role for Ets1/2 co-factors in heart cell migration

FGF signaling can regulate fate decision and morphogenesis via distinct intracellular pathways (e.g. Sivak et al., 2005). In *Ciona*, FGF/MAPK/Ets signaling controls cardiac fate specification predominantly via Ets1/2-mediated transcriptional activation; the constitutively active Ets:VP16 fusion protein can fully restore cardiac specification in cells expressing a dominant-negative form of the FGF receptor (Davidson et al., 2006) (B.D., unpublished).

On the other hand, the uncoupling of cardiac muscle specification and cell migration upon overexpression of FoxF:WRPW indicates that the programs governing tissue specification and cell migration diverge downstream of the FGF/MAPK/Ets cascade. How does activated Ets1/2 trigger distinct regulatory networks in the same cells?

In ascidian embryos, distinct Ets1/2 co-factors might account for tissue- or process-specific gene activation in response to FGF (e.g. Bertrand et al., 2003; Kumano et al., 2006). For instance, early Mesp activity is required for all aspects of cardiogenesis in ascidians (Satou et al., 2004). Mesp presumably upregulates *Ets1/2* expression, but might also function in parallel to the FGF/MAPK/Ets pathway. Indeed, targeted expression of the Mesp:VP16 fusion protein downregulates *FoxF*, whereas *Hand-like* expression persists in the non-migrating anterior B7.5 daughter cells (Davidson et al., 2005). By contrast, the FGF/MAPK/Ets cascade is required for TVC expression of both *FoxF* and *Hand-like*. Thus, the Mesp:VP16 chimera does not seem to interfere with FGF signal transduction through Ets1/2. Instead, we propose that Mesp:VP16 interferes with the ability of Mesp to indirectly regulate Ets1/2 co-factors upstream of *FoxF* (Fig. 7).

We found that a CACTTG motif is required for *FoxF* minimal TVC enhancer activity, which could be restored by co-expression of the hyper-active Ets:VP16 chimera. This further supports the hypothesis that an Ets1/2 co-factor is required for TVC-specific activation of *FoxF* in response to FGF signaling. As mentioned above, this motif matches the E-box consensus (CANNTG). It might therefore bind cardiac bHLH transcription factors in vivo, but alternative possibilities can be envisioned. Indeed, we found that the CACTTG motif also matches the Nkx2.5/tinman consensus sequence CAMTTR (Sandelin et al., 2004; Zaffran and Frasch, 2002). Further investigation will be required to identify Ets1/2 co-factors and determine their precise roles in the selective regulation of cell migration via *FoxF* regulation.

## An Ets/FoxF circuit specifically regulates cell migration

FGF signaling has extensively documented roles in regulating cell type specification and morphogenesis of mesoderm derivatives (reviewed in Thisse and Thisse, 2005). Our results point to an essential role of FoxF in the transcriptional control of cardiac cell migration downstream of FGF/MAPK/Ets signaling. In vertebrates and *Drosophila*, FGF signaling and *FoxF* orthologs have been implicated in a variety of morphogenetic processes involving mesenchyme cells derived from the lateral plate mesoderm, consistent with their widespread expression in these tissues (e.g. Mahlapuu et al., 2001; Malin et al., 2007; Mandal et al., 2004; Michelson et al., 1998; Zaffran et al., 2001). Here, we found that a hyper-active form of FoxF slightly enhanced migration of the B7.5 lineage cells, but failed to rescue the migration defect caused by the dominant-negative Ets1/2. These results suggest that Ets1/2 activity is also required in parallel with FoxF to regulate the full spectrum of genes required for TVC migration (summarized in Fig. 7).

Most of our internal organs arise from primordia that undergo directed migration to ensure that they are positioned in an orderly fashion within the body cavity. It seems likely that similar principles seen for migration of the ascidian heart primordium will also apply to additional organ systems. In particular, we speculate that genes such as *FoxF* will serve to connect gene regulatory cassettes controlling organogenesis to the process of directed cell migration.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/18/3297/DC1

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