

# Wnt3a-mediated chemorepulsion controls movement patterns of cardiac progenitors and requires RhoA function

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The heart is the first organ to function during vertebrate development and cardiac progenitors are among the first cell lineages to be established. In the chick, cardiac progenitors have been mapped in the epiblast of pre-streak embryos, and in the early gastrula they are located in the mid-primitive streak, from which they enter the mesoderm bilaterally. Signals controlling the specification of cardiac cells have been well documented; however, migration routes of cardiac progenitors have not been directly observed within the embryo and the factor(s) controlling their movement are not known. In addition, it is not clear how cell movement is coordinated with cell specification in the early embryo. Here we use live imaging to show that cardiac progenitors migrate in highly directed trajectories, which can be controlled by Wnt3a. Ectopic Wnt3a altered movement trajectories and caused cardia bifida. This was rescued by electroporation of dominant-negative DN-Wnt3a into prospective cardiac cells. Explant essays and mutant analysis showed that cellular guidance involved repulsion in response to Wnt3a and required RhoA function. It has been shown that Wnt3a inhibits cardiogenic cell specification through a  $\beta$ -catenin-dependent pathway. On the basis of our results, we propose that Wnt3a concomitantly guides the movement of cardiac progenitors by a novel mechanism involving RhoA-dependent chemorepulsion.

**KEY WORDS:** Wnt signalling, Cell migration, Chemorepulsion, Cardiac progenitors, Chick

## INTRODUCTION

The heart is the first organ to function during vertebrate development and the cells that will become the heart, mesoderm-derived cardiac progenitors, are among the first cells to ingress through the primitive streak during gastrulation. In avian embryos, future cardiac cells have been identified in the epiblast of pre-streak embryos (Hatada and Stern, 1994) and in the mid-primitive streak at Hamburger Hamilton (HH) stage 3 (Garcia-Martinez and Schoenwolf, 1993; Hamburger and Hamilton, 1951; Rosenquist, 1970). In the chick, a simple, contractile heart tube forms by HH stage 10, which is after approximately 48 hours of development.

The specification of prospective cardiac cells occurs concomitantly with cell migration and morphogenetic movements during early embryogenesis (Brand, 2003; Yutzey and Kirby, 2002). How these important processes are coordinated is, at present, not understood. Multiple extracellular cues have been implicated in the specification of cardiac cell fate and early heart formation, and studies in vertebrate embryos have uncovered a crucial role for bone morphogenetic proteins (Bmps), fibroblast growth factors (Fgfs) and Wnt signalling (Eisenberg and Eisenberg, 1999; Lickert et al., 2002; Marvin et al., 2001; Schneider and Mercola, 2001; Schultheiss et al., 1997; Tzahor and Lassar, 2001).

Migration routes of cells from the primitive streak towards the bilateral heart fields have been inferred from mapping experiments (Yutzey and Kirby, 2002) but the molecular signals controlling cardiac progenitor cell movements are not understood. Recently, it has become possible to observe cell movements in real time in whole embryos. This has led to an increased understanding of cell movement patterns, in particular during

streak formation and gastrulation, and the cellular and molecular mechanisms are beginning to be elucidated (Chuai et al., 2006; Cui et al., 2005; Leslie et al., 2007; Yang et al., 2002; Zamir et al., 2006). Avian embryos are particularly suited to these approaches, as they develop outside the mother, they can be cultured (Chapman et al., 2001), and they are accessible for the electroporation of expression plasmids (Itasaki et al., 1999; Momose et al., 1999) and the analysis of explants (Münsterberg et al., 1995; Yang et al., 2002).

We previously established long-term time-lapse video microscopy followed by image analysis and showed that Fgf-mediated chemotaxis is involved in guiding cell movements of prospective paraxial and lateral plate mesoderm cells during gastrulation (Yang et al., 2002). Here, we have used this approach to examine the cell movement patterns of cardiac progenitors as they migrate from the primitive streak at HH3. In particular, we investigated the effects of Wnt signalling and found that prospective cardiac cells alter their movement pattern in response to Wnt3a. Analysis of HH3 primitive streak cell explants showed that cardiogenic cells are repelled by a source of Wnt3a and migrate away from it. This behaviour was dependent on RhoA function and was abrogated by the electroporation of dominant-negative forms of Wnt3a or RhoA. The perturbation of cardiac progenitor cell movement patterns in vivo resulted in cardia bifida, and the frequency of this phenotype was reduced significantly by the electroporation of dominant-negative Wnt3a. We propose that, in addition to its effects on cardiac cell-fate specification (Marvin et al., 2001; Schneider and Mercola, 2001), Wnt3a acts as a chemorepellent signal to guide the movement of these cells, and that this involves a non-canonical, RhoA-dependent pathway.

## MATERIALS AND METHODS

### Embryo manipulations

Plasmid DNA was microinjected into the primitive streak of HH2-HH3 embryos in EC culture (Chapman et al., 2001) with their ventral side facing up. This was followed by electroporation providing 10 pulses of 12 V for 50 mseconds. Embryos were briefly incubated at 37°C (30

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minutes to 2 hours) and were then incubated at 28°C overnight. Small grafts of GFP-labelled primitive streak were dissected from HH3 donor embryos and transferred to an unlabelled, stage-matched host embryo cultured in a six-well dish. Alternatively, primitive streak explants were cultured on the area opaca of an HH3 embryo as described (Yang et al., 2002). To generate cell pellets RatB1a fibroblasts were seeded on 3-cm bacterial Petri dishes. Cells formed aggregates overnight, which were transferred to host embryos using a Gilson pipette and implanted by pushing them into a small slit created by fine tweezers. Most embryos had normal morphology after these experimental manipulations and any abnormal embryos were excluded from the analysis.

### Microscopy and image processing

Embryos in six-well dishes were mounted on an inverted wide-field microscope (Axiovert, Zeiss) and bright-field and fluorescent images were collected every 5–6 minutes over a period of 20–24 hours using Axiovision software. At the end of imaging the data was exported as TIFF files. Images were processed using Optimas VI imaging software to reveal cell movement tracks (Yang et al., 2002).

### Expression constructs, probes and whole-mount in situ hybridisation

Plasmid constructs were engineered in the pCAB $\beta$  vector containing the chicken beta-actin promoter and an IRES-GFP (Alvares et al., 2003). The chicken genome sequence (Hillier et al., 2004) was used to design primers for the amplification of complete gene coding sequences. Forward and reverse primers included restriction sites, and reverse primers contained sequences encoding an HA-tag. RhoA-V14 and RhoA-N19 mutants (Ridley and Hall, 1992) were generated by site directed mutagenesis following standard protocols. Primer sequences are as follows.

cWnt-11F+XbaI: TCTAGAATGAAGCCGAGCCCGCAATTTTTC;  
cWnt-11R+NotI-HA: GCGGCCGCTCAAGCGTAATCTGGAACATCG-TATGGGTATTTGCAGACGTATCTCTCGAC;

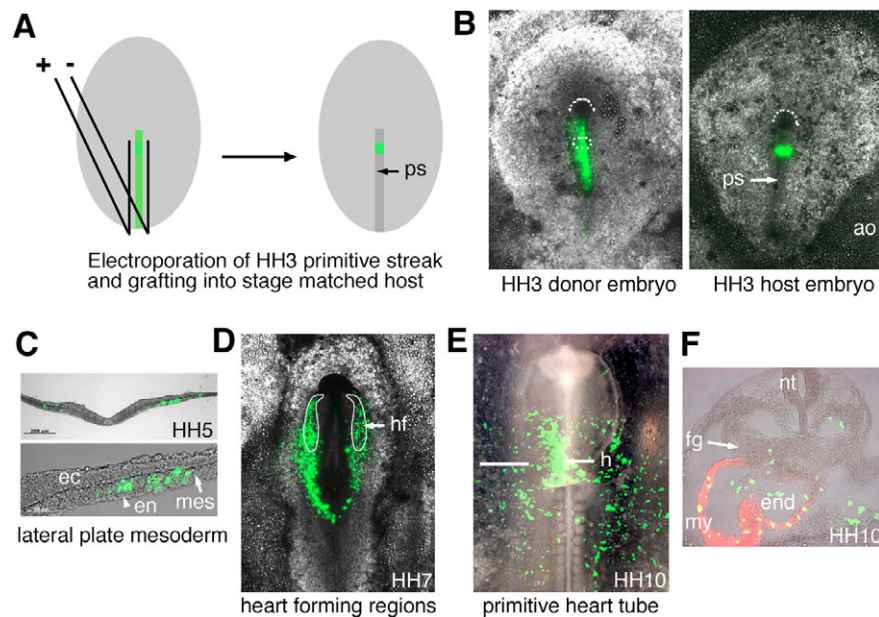
cWnt-3aF+XbaI: TCTAGAATGAAGTCGTTCTGCAGCGAAG;  
cWnt-3aR+SmaI-HA: CCCGGGTCAAGCGTAATCTGGAACATCGT-ATGGGTATTTGCACGTGTGGACGTCGTAG;  
DN-cWnt3aR+NotI-HA: GCGGCCGCTCAAGCGTAATCTGGAACAT-CGTATGGGTAGCAAAAAGTTGGGTGAGTTC.  
RhoA-V14: GTCATAGTGGGCGACGTTGCCTGCGGGAAGACC;  
RhoA-V14R: GGTCTTCCCGCAGGCAACGTCGCCCACTATGAC;  
RhoA-N19: GGTGCCTGCGGGAAGAAGTGTCTGCTGATTGTG;  
RhoA-N19R: CACAATCAGCAGACAGTTCTTCCCGCAGGCACC.

PCR products were purified, cloned into pGEM-T (Promega) and sub-cloned into pCAB $\beta$ . Prior to electroporation, all plasmids were transfected into HEK293 cells and the expression of proteins was confirmed by western blot and GFP fluorescence. The generation and functional characterisation of  $\Delta$ -Fgfr1-YFP in pEYFP (Clontech) has been described before (Yang et al., 2002).

For in situ hybridisation, embryos were harvested following imaging and fixed in 4% paraformaldehyde (PFA) at room temperature. Some embryos were allowed to develop until they reached HH10 before fixation. In situ hybridisation was essentially as described before (Schmidt et al., 2000) following standard protocols. We amplified probes for Wnt3a, Wnt11 and RhoA by PCR. Cheryl Tickle provided Fgf8 (Niswander et al., 1994) and Thomas Schultheiss provided vMHC and Nkx2.5 probes (Niswander et al., 1994; Schultheiss et al., 1997).

### Sections and immunohistochemistry

For wax and cryosections, embryos were fixed and embedded in OCT (Tissuetek) or in paraffin, following standard protocols. After sectioning, GFP was detected using a polyclonal anti-GFP antibody (Abcam, 1:500); myosin heavy chain was detected using MF20 (1:1000, Developmental Studies Hybridoma Bank). Secondary antibodies used were anti-rabbit-Alexa488 (green) and anti-mouse-Alexa568 (red), both at 1:1000 (Molecular Probes).



**Fig. 1. GFP labelling of cardiac progenitors in HH3 primitive streak.** (A) Schematic illustration of the electroporation and grafting procedure; embryos were placed in EC culture with their ventral side up. (B) HH3 donor embryo (left) after electroporation of primitive streak with pCS2<sup>+</sup>-GFP, and HH3 host embryo (right). Green fluorescence identifies labelled cells; small grafts were dissected and transplanted into a stage-matched host. The white dotted semi-circles show the anterior margin of the primitive streak; the white dotted oval indicates the graft. (C) Transverse cryosection (15  $\mu$ m) through an HH5 embryo shows GFP-positive cells in the mesoderm of the lateral plate. (D) HH7 embryo with GFP-positive cells in the area of the bilateral heart fields, indicated by a white line (Stalsberg and DeHaan, 1969). (E) GFP-positive cells in the heart tube at HH10. (F) Transverse cryosection (30  $\mu$ m) of a similar embryo, stained with anti-GFP (green) and MF20 (red) antibodies, showing that GFP-positive cells were present in endocardium and myocardium. White line in E indicates the approximate level of the section shown in F. ao, area opaca; ec, ectoderm; en, endoderm; end, endocardium; fg, foregut; h, heart; hf, heart field; my, myocardium; nt, neural tube; ps, primitive streak.



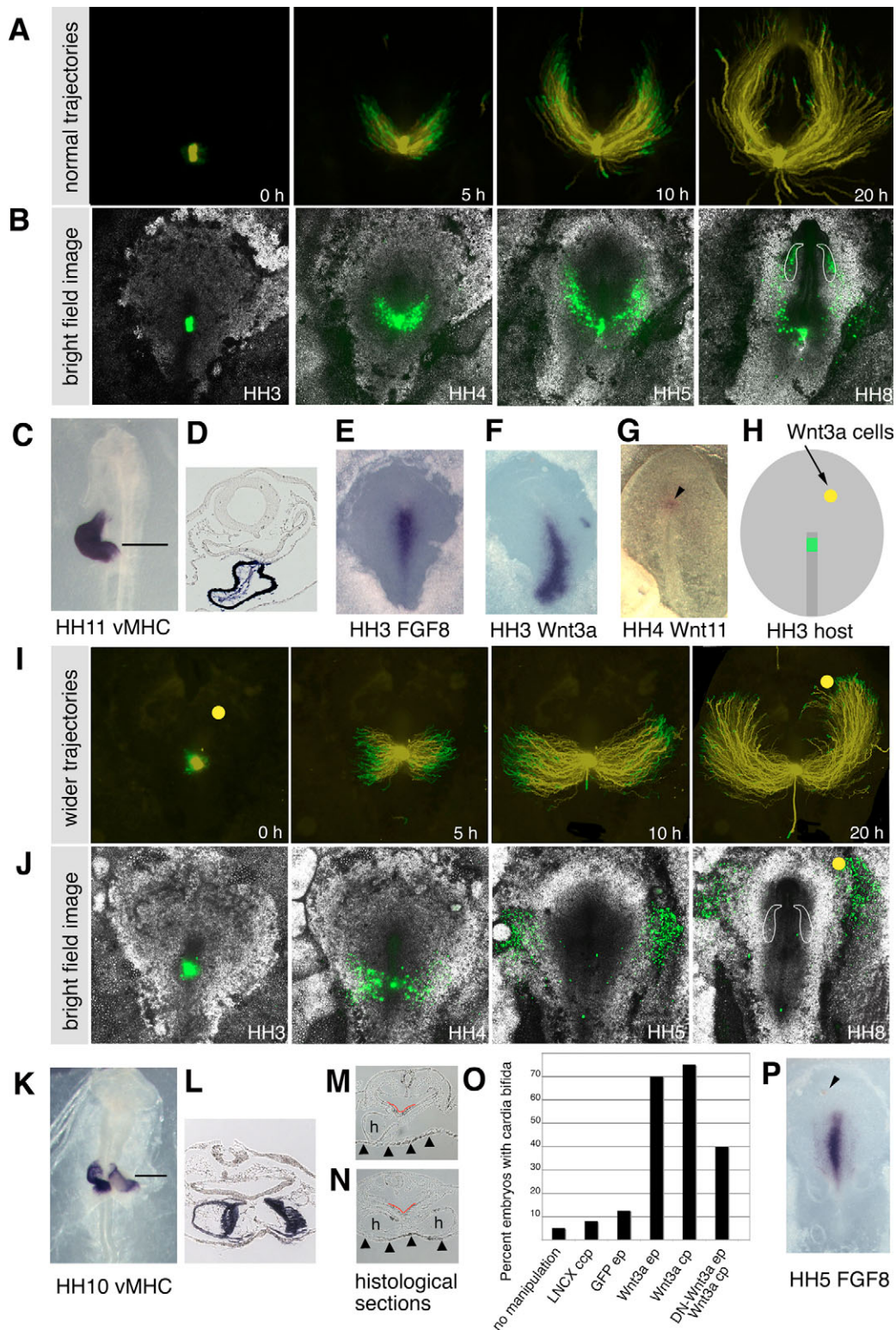


Fig. 2. See next page for legend.

## RESULTS

### GFP-labelling allows the tracking of cardiac progenitors in real time

We electroporated the primitive streak of Hamburger Hamilton (HH) stage 3 embryos in EC culture with expression plasmids encoding GFP. When GFP expression became visible, small grafts

of labelled primitive streak cells were dissected and transplanted into unlabelled host embryos (Yang et al., 2002) (Fig. 1A,B). This enabled us to follow the movement of individual cells emerging from the primitive streak. Movement tracks were monitored from HH3 until embryos reached the 6-10 somite stage (after 20-24 hours). GFP-positive cells were found in lateral plate mesoderm at

**Fig. 2. Cardiac progenitors move on directed trajectories, which are controlled by Wnt3a.** (A) Long-term video microscopy followed by image processing revealed movement trajectories; still images at the time points indicated in hours (h) are shown. (B) Corresponding bright-field images. The last panel shows the location of GFP cells in the bilateral heart fields, indicated by white outline (for the corresponding movie, see Movie 1 in the supplementary material). (C) vMHC in situ hybridisation of an HH11 embryo after live imaging shows normal heart morphogenesis and cardiac specific gene expression. (D) Paraffin transverse section of the embryo shown in C. (E,F) In situ hybridisation shows (E) Fgf8 and (F) Wnt3a transcripts are expressed in the HH3 primitive streak. (G) A weak signal for Wnt11 was detected in the node of an HH4 embryo (arrowhead). The contrast was enhanced and saturation of the magenta colour was increased to visualise this signal. (H) Illustration of Wnt3a-expressing cells (yellow dot) grafted into HH3 embryos to challenge the movement of GFP-labelled cardiac progenitors. (I) Long-term video microscopy followed by image processing revealed the movement trajectories of GFP-labelled cardiac progenitors in the presence of Wnt3a-expressing cells (yellow dot). Still images at the time points indicated show a wider trajectory. (J) Corresponding bright-field images show that most cells failed to reach the midline and remained in the area opaca and lateral mesoderm (see Movie 2 in the supplementary material). (K,L) Many embryos developed cardia bifida as shown by vMHC in situ hybridisation: whole mount (K) and section (L) of an embryo after imaging. (M,N) Paraffin sections of an HH10 embryo implanted with a control cell pellet (M) or with a Wnt3a cell pellet to induce cardia bifida (N). Black arrowheads indicate the endoderm and red lines indicate the foregut pocket; h, heart. (O) Graph showing the percentage of embryos with cardia bifida under different conditions. The embryo treatment is indicated below each bar. LNCX ccp, control cell pellet; ep, electroporation; cp, cell pellet. (P) HH5 embryo with Wnt3a-expressing cells (arrowhead) implanted at HH3 (as shown in H) and hybridised with a probe detecting Fgf8 transcripts.

HH5 and in the bilateral heart-forming regions at HH7 (Fig. 1C,D), before contributing to a single heart tube by HH10 (Brand, 2003; Yutzey and Kirby, 2002) (Fig. 1E,F).

Real-time imaging demonstrated that prospective heart cells migrated on highly directed trajectories (Fig. 2A,B; see also Movie 1 in the supplementary material). They exited the primitive streak and migrated in an anterior-lateral trajectory away from the streak towards the periphery of the embryo. Cells then moved back towards the midline and a single heart tube formed by HH10, undergoing normal morphogenesis and expressing vMHC (Fig. 2C,D).

### Wnt3a-mediated chemorepulsion guides cardiac progenitor-cell movement

To investigate whether cell movement patterns are governed by signals from the environment or whether cell behaviour is intrinsically/cell autonomously regulated, we performed heterochronic grafts. GFP-labelled cells were isolated from the anterior primitive streak of HH3 embryos and grafted into the anterior streak of a HH4 host. Prospective cardiac cells grafted into the HH4 anterior primitive streak behaved appropriately for their new environment, they displayed a narrow migration trajectory and gave rise to paraxial mesoderm, as described previously (Yang et al., 2002) ( $n=11/11$ ). Conversely, HH4 paraxial mesoderm progenitors grafted into the anterior streak of HH3 host embryos displayed movement trajectories typical for cardiac progenitors and indistinguishable from the HH3 homotypic grafts shown (Fig. 1C-F, Fig. 2A,B;  $n=11/11$ , not shown). These experiments demonstrated that cell movement patterns are established by extrinsic cues.

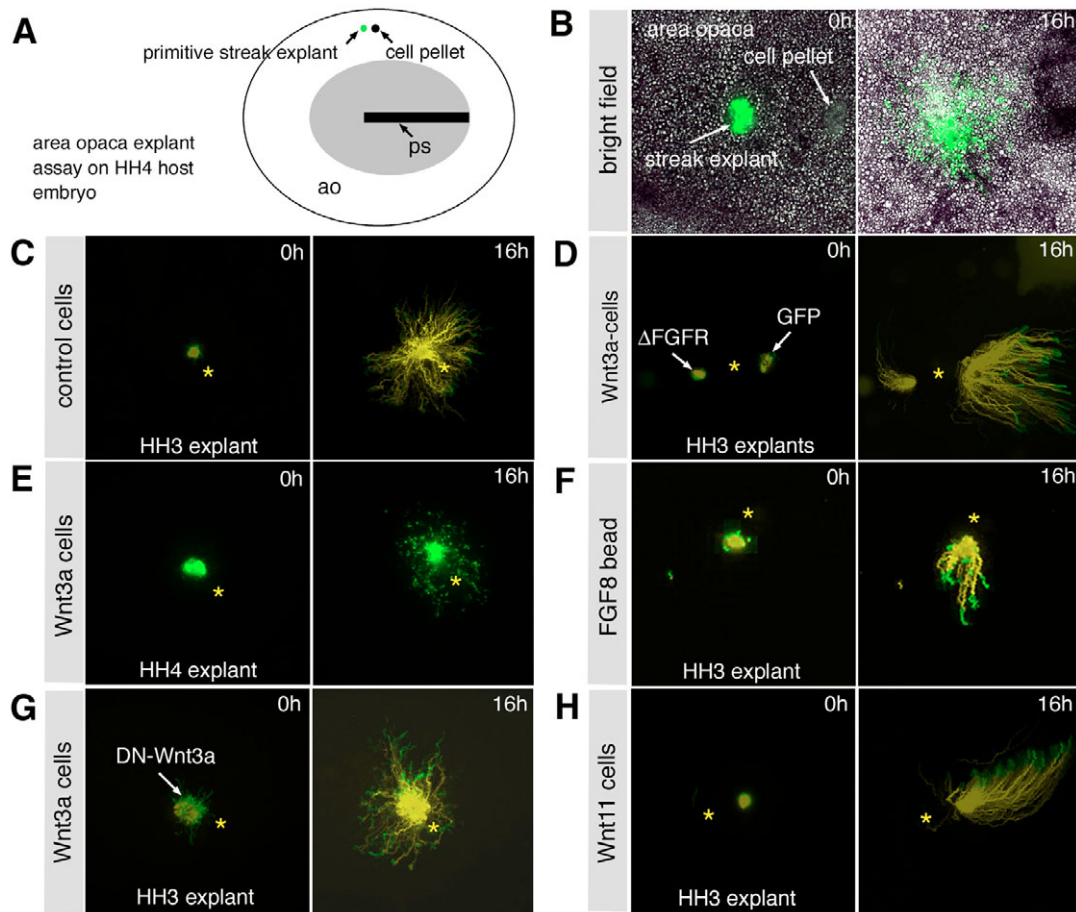
Wnt3a transcripts are highly expressed in the primitive streak of the chick gastrula (Fig. 2F) (Marvin et al., 2001). To investigate the potential role of Wnt3a in guiding the movement of cells emerging from the HH3 primitive streak, we implanted pellets of RatB1a fibroblasts expressing Wnt3a (Münsterberg et al., 1995) into the migration path of cardiogenic cells (Fig. 2H). This dramatically changed their movement trajectories and, in the majority of embryos, cardiac progenitors on both sides took a significantly wider path with initially more pronounced lateral migration before moving towards the midline (Fig. 2I; see also Movie 2 in the supplementary material). Many of the GFP-labelled cells remained in extraembryonic mesoderm or anterior lateral mesoderm and only a few contributed to the heart (Fig. 2J). In many cases, the two heart fields failed to merge and cardia bifida was observed at high frequency (75%, Fig. 2K,L,N). Similar results were obtained after the electroporation of an expression plasmid for Wnt3a (pCA $\beta$ -Wnt3a-IRES-GFP; Fig. 2O, data not shown), suggesting that Wnt3a is directly involved.

We previously showed that HH4 primitive streak cells are responsive to Fgf8, and proposed a model whereby movement trajectories of prospective paraxial and lateral plate mesoderm cells are governed by Fgf8-mediated repulsion (Yang et al., 2002). Fgf8 is expressed at high levels in HH3 primitive streak (Fig. 2E). As Wnt3a pellets affected the movement of cardiac progenitors bilaterally and at long range, we investigated the possibility that Wnt3a caused a global re-patterning of embryos and ectopic upregulation of Fgf8 transcripts; however, this was not the case (Fig. 2P).

Next, we examined whether prospective cardiac cells respond directly to Wnt3a. Small grafts from the anterior primitive streak of HH3 embryos were cultured on the area opaca (Yang et al., 2002) and exposed to different Wnt-expressing cells (Wnt1, Wnt3a, Wnt5a, Wnt7, Wnt11) or to the parental RatB1a-LNCX fibroblasts (Münsterberg et al., 1995). In the presence of control cells, GFP-labelled prospective cardiac cells were highly motile and migrated away from the graft in all directions (Fig. 3B,C). However, when challenged with Wnt3a-expressing cells, HH3 primitive streak cells migrated away from the source of Wnt3a in a highly directed manner (Fig. 3D, right explant; see also Movie 3 in the supplementary material). Mesoderm progenitors from HH4 primitive streak did not respond to Wnt3a pellets (Fig. 3E) but were repelled by Fgf8, both in vivo and if cultured on the area opaca (Yang et al., 2002). HH3 prospective cardiac cells were also able to respond to Fgf8 and were repelled by it (Fig. 3F). We previously showed that the response of HH4 primitive streak cells to Fgf8 is almost completely inhibited by misexpressing a dominant-negative Fgf-receptor-EYFP fusion protein, pFgfr1 $\Delta$ C-EYFP (Yang et al., 2002). However, expression of pFgfr1 $\Delta$ C-EYFP in HH3 cardiac progenitors did not abrogate their response to Wnt3a on the area opaca, indicating that Fgf receptor activity was not required (Fig. 3D, left explant). By contrast, HH3 primitive streak cells expressing a dominant-negative mutant of Wnt3a (Hoppler et al., 1996), DN-Wnt3a-IRES-GFP, no longer responded to Wnt3a-expressing cells (Fig. 3G). However, they were still motile, possibly due to other signals present within the explant, such as Fgf8, and behaved like unchallenged controls migrating away from the graft in all directions.

In addition to Wnt3a, Wnt11-expressing cells repelled prospective cardiac cells in this ex vivo system (Fig. 3H). However, based on the embryonic expression patterns of these ligands (Fig. 2F,G) (Eisenberg et al., 1997; Skromne and Stern, 2001) Wnt3a is more likely to provide the endogenous guidance





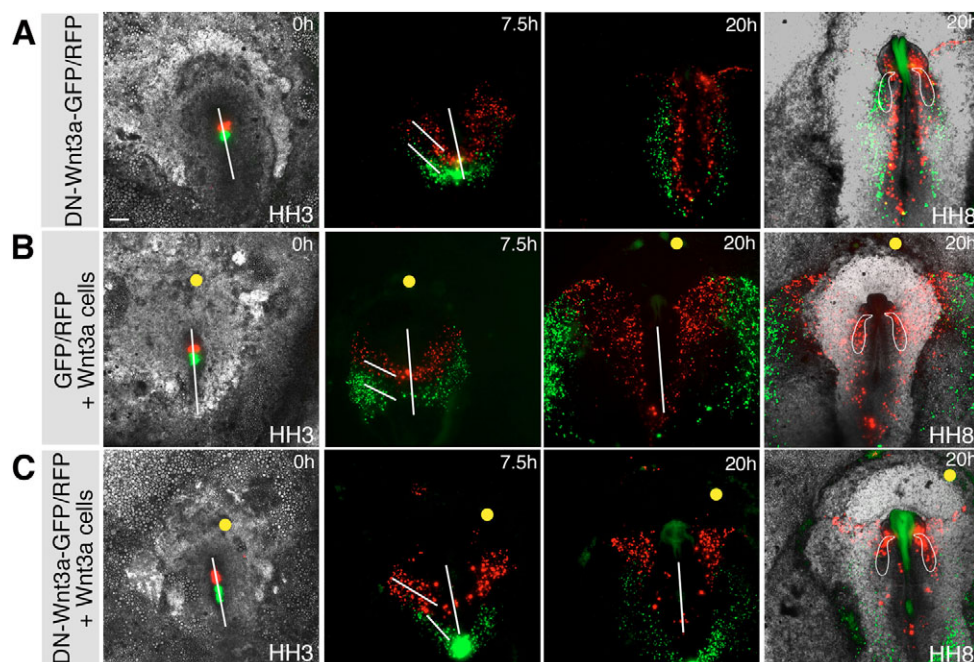
**Fig. 3. Wnt3a-expressing cells repel cardiac progenitors on the area opaca.** (A) Illustration of GFP-positive primitive streak explants cultured in the presence of a cell pellet on the area opaca of an HH4 host embryo. (B) Merged bright-field and fluorescent images of a representative streak explant and cell pellet on the area opaca. Still images from the start (0h) and end points (16h) of the experiments are shown. (C) Image tracks of movies showed that HH3 primitive streak cells migrated in all directions away from a graft cultured on the area opaca and ignored the presence of a control cell pellet (yellow asterisk). (D) HH3 primitive streak cells electroporated with GFP (on the right of the cell pellet) or with a dominant-negative form of Fgf receptor 1,  $\Delta$ -Fgfr1-YFP (on the left of the cell pellet) migrated away from Wnt3a-expressing cells (see Movie 3 in the supplementary material). (E) HH4 primitive streak cells did not respond to Wnt3a-expressing cells. (F) HH3 cardiac progenitors were repelled by a bead soaked in Fgf8. (G) HH3 cardiac progenitors electroporated with DN-Wnt3a-GFP no longer responded to Wnt3a-expressing cells. (H) Wnt11-expressing cells repelled HH3 prospective cardiac cells. Yellow asterisks indicate the position of cell pellets or beads. 0h, start of imaging; 16h, end of imaging; ao, area opaca; ps, primitive streak.

cue. Together, these experiments raise the possibility that high levels of Wnt3a in the primitive streak control the movement of cardiac progenitors.

### A dominant-negative form of Wnt3a restores movement trajectories and reduces the incidence of cardia bifida in response to Wnt3a

To determine whether DN-Wnt3a-IRES-GFP expression in HH3 primitive streak could alter the movement patterns of prospective cardiac cells *in vivo*, we compared the behavior of cells expressing DN-Wnt3a or not in single and double implants (Fig. 4). Cells labelled with RFP or DN-Wnt3a-IRES-GFP were grafted into the HH3 primitive streak at different anterior-posterior positions. In the absence of an ectopic source of Wnt3a, RFP and DN-Wnt3a-IRES-GFP labelled cells displayed identical movement patterns and migrated away from the streak in an anterior-lateral direction (Fig. 4A), which was indistinguishable from the movement pattern in single grafts (Fig. 2A,B). At HH8, the cells were found in the bilateral heart forming regions (Fig. 4A). Next, we investigated

whether DN-Wnt3a expression would compromise the ability of cells to respond to an ectopic source of Wnt3a in the embryo. As before, we challenged the movement of prospective cardiac cells and exposed them to a pellet of Wnt3a-expressing cells. Double implants confirmed that cardiac progenitors responded to Wnt3a at long distances (Fig. 4B). Both GFP- and RFP-labelled cell populations displayed wider movement trajectories in the presence of a Wnt3a cell pellet (Fig. 4B), when compared with movement trajectories in the absence of a Wnt3a cell pellet (Fig. 4A). This was consistent with the observations made using single GFP grafts, where cardiac progenitors migrated away from the streak in a more pronounced lateral trajectory (Fig. 2I,J). By HH8, some RFP-positive cells contributed to the heart-forming regions; however, the majority of RFP- and GFP positive cells were found in extra-embryonic regions and anterior lateral mesoderm (Fig. 4B, Fig. 2J). Electroporation of DN-Wnt3a-IRES-GFP rescued the movement patterns in the presence of a Wnt3a cell pellet (Fig. 4C): DN-Wnt3a-IRES-GFP-expressing primitive streak cells displayed almost normal trajectories and contributed to the heart-forming regions by HH8. It



**Fig. 4. DN-Wnt3a can rescue the effects of Wnt3a on cell movement patterns in vivo.** (A) DN-Wnt3a-GFP- (green) and RFP- (red) expressing HH3 streak cells grafted into a stage-matched host displayed normal migration patterns in control embryos. (B) A graft of Wnt3a-expressing cells affected GFP- and RFP-labelled cells at different anterior-posterior levels and caused cells to take a wider path. (C) The movement trajectories of cells expressing DN-Wnt3a-GFP were restored to normal in the presence of Wnt3a-expressing cells but RFP-transfected cells still displayed wider migration trajectories. (A-C) Panels on the left show GFP and RFP fluorescence with bright field at the start point of imaging. The two central panels show dark-field images at the time points indicated. Panels on the right show fluorescence with bright field at the end of imaging. Vertical white lines indicate the axial midline; angled white lines indicate the main trajectory of cells leaving the streak. Heart-forming regions at HH8 [based on Stalsberg and DeHaan (Stalsberg and DeHaan, 1969)] are outlined in white. Scale bar in A: ~500  $\mu$ m.

is not clear how dominant-negative Wnts act; however, it has been proposed that they act extracellularly and inhibit the receptor, and/or form inactive heterodimers with wild-type Wnt ligands (Hoppler et al., 1996). Surprisingly, neighbouring RFP-labelled cells, not expressing DN-Wnt3a-IRES-GFP, were only partially rescued (Fig. 4C). These cells still moved on a wider trajectory in the presence of ectopic Wnt3a and ended up in more lateral positions than normal (Fig. 4A,C). This suggests that DN-Wnt3a acts cell-non-autonomously but may have a limited range or activity. Embryos expressing DN-Wnt3a-IRES-GFP in the primitive streak had a significantly reduced frequency of cardia bifida in the presence of ectopic Wnt3a (40%) compared with GFP-expressing embryos in the presence of ectopic Wnt3a (75%). However, when compared with unchallenged controls (12.5%) or embryos with a control cell pellet (8.3%), the frequency of cardia bifida was still increased, consistent with a partial rescue of cardiac progenitor cell movements by DN-Wnt3a-IRES-GFP (Fig. 2O).

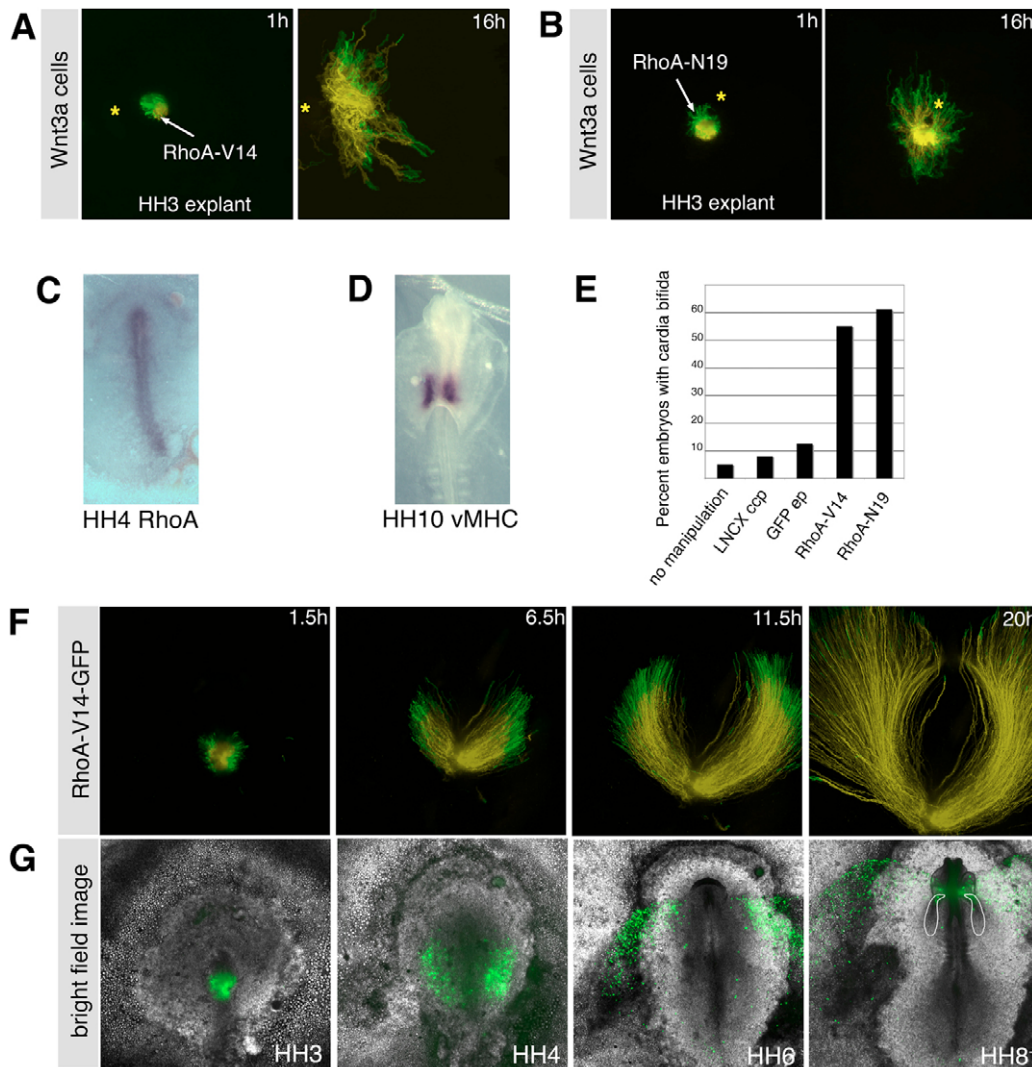
#### RhoA is required for Wnt3a-mediated repulsion and alters movement patterns in vivo

We found that RhoA transcripts are enriched in the primitive streak (Fig. 5C). To determine whether RhoA mediates the Wnt3a signal, HH3 streak cells were electroporated with a constitutively active form of RhoA, RhoA-V14 (Ridley and Hall, 1992), and challenged in an ex vivo assay. These cells responded to Wnt3a and were repelled by it (Fig. 5A). By contrast, electroporation of a dominant-negative mutant form, RhoA-N19 (Ridley and Hall, 1992), resulted in motile cells that were no longer able to respond to Wnt3a. These cells moved away from the explants in all directions and ignored the Wnt3a cell

pellet (Fig. 5B). In vivo, RhoA-N19-IRES-GFP-expressing cells migrated from the primitive streak and displayed normal trajectories (data not shown); however, they moved more slowly than controls and 60% of embryos developed cardia bifida (Fig. 5D,E). Movement trajectories of cardiac progenitors expressing RhoA-V14-IRES-GFP were initially normal. However, during the later phase of migration a proportion of the cells took a wider path, failed to migrate towards the midline and ended up either in extra-embryonic regions or remained in the lateral mesoderm (Fig. 5F,G). Many of these embryos developed cardia bifida (Fig. 5E).

#### DISCUSSION

Long-term video microscopy combined with GFP labelling enabled us to observe the movement patterns of cardiac progenitor cells in real time. Image processing revealed the migration trajectories of prospective cardiac cells in whole embryos. Embryo manipulations and the analysis of explants suggested that Wnt3a plays a crucial role in guiding these cells through a RhoA-dependent mechanism involving negative chemotaxis. Chemotaxis is an important mechanism controlling directional cell migration over either short or long distances. Diffusible chemo-attractants are detected by responding cells, and the graded activation of receptors along the length of the cell results in polarisation of the actin cytoskeleton and directed movement (Dormann and Weijer, 2003). Wnt signalling has been shown to be involved in axon guidance in the chick optic tectum and spinal cord, and in *Drosophila* nervous system development (Lyuksytova et al., 2003; Schmitt et al., 2006; Yoshikawa et al., 2003). However, this is the first report implicating Wnts in the chemotactic movement of individual cells in vertebrate embryos.



**Fig. 5. Wnt3a-mediated repulsion requires RhoA function, and dominant-negative or constitutively active forms of RhoA cause cardia bifida.** (A) HH3 primitive streak cells electroporated with a constitutively active form of RhoA, RhoA-V14, were repelled by Wnt3a-expressing cells (yellow asterisk) in area opaca explants. (B) HH3 primitive streak cells electroporated with a dominant-negative form of RhoA, RhoA-N19, ignored the source of Wnt3a and behaved like unchallenged explants or explants transfected with DN-Wnt3a (see Fig. 3C,G), migrating in all directions away from the graft. (C) RhoA transcripts were expressed in the primitive streak at HH4. (D) vMHC in situ hybridisation showed that embryos electroporated with RhoA-V14-IRES-GFP developed cardia bifida. (E) Graph showing the percentage of embryos with cardia bifida. Embryo treatment is indicated below each bar. ccp, control cell pellet; ep, electroporation. (F) Long-term video microscopy followed by image processing revealed the movement trajectories of RhoA-V14-IRES-GFP labelled cardiac progenitors. Still images at the time points indicated are shown. (G) Corresponding bright-field images show the location of GFP cells within the embryo. The bilateral heart fields are indicated by a white outline in HH8 embryos.

We found that cardiogenic cells are repelled by an ectopic source of Wnt3a in explants cultured on the area opaca (Fig. 3D). In vivo, this resulted in aberrant movement trajectories, which led to cardia bifida (Fig. 2I-L). The response of cells to Wnt3a was abrogated by the electroporation of DN-Wnt3a or RhoA-N19 in explants (Fig. 3G, Fig. 5B), suggesting that RhoA is an important effector for directional cell movement in response to Wnt3a. Wnt3a is typically thought to act through  $\beta$ -catenin-dependent mechanisms to affect cell fate decisions and is implicated in inhibiting cardiogenesis through this 'canonical' pathway (Marvin et al., 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001), components of which are expressed in the primitive streak (Schmidt et al., 2004). However, our observations are consistent with a report looking at CHO cells in culture, where it was shown that Wnt3a-dependent cell motility

involves non-canonical signalling through RhoA (Endo et al., 2005). Moreover, RhoA has been implicated in midline convergence of organ primordia in zebrafish (Matsui et al., 2005), and is upregulated during early heart development in chick (Kaarbo et al., 2003; Wei et al., 2001). Electroporation of DN-Wnt3a restored the movement pattern of cardiac progenitors in vivo, in the presence of an ectopic source of Wnt3a (Fig. 4C), and significantly reduced the incidence of cardia bifida (Fig. 2O). Interestingly, RhoA-V14, a constitutively active form of RhoA (Ridley and Hall, 1992), did not have a significant effect on the early phases of cardiac progenitor cell migration, whereas at later stages some cells took a wider path (Fig. 5F,G). We speculate that this delayed effect may be due to the fact that at high levels of Wnt3a close to the streak overexpression of an active form of RhoA had no discernable effect. In contrast, at lower



levels of the repulsive signal, RhoA-V14 caused a more pronounced outward migration, possibly by sensitising the cells. Alternatively, it is possible that RhoA-V14 interferes with the response to another factor responsible for guiding cells back to the midline.

How cells move back towards the midline is not clear at present. We propose that this is achieved through a combination of convergent extension, which drives the narrowing of the embryonic axis, as well as active migration. In this context, it is interesting to note that cells still avoid the vicinity of the grafted Wnt3a pellet as they move back towards the midline (Fig. 2I,J); however, morphogenetic forces or, potentially, other guidance signals seem to become dominant over Wnt3a-mediated repulsion at that stage. While it is likely that additional signals are required to attract cardiogenic cells towards the ventral midline, their origin and identity remains to be investigated. A population of ventral midline endoderm cells, derived from the prechordal plate, has been proposed to function as a ventral organiser of the developing head and heart (Kirby et al., 2003). Although we cannot detect any morphological changes of the ventral foregut in histological sections (Fig. 2D,L-N), it remains possible that Wnt3a affects ventral endoderm cells, for example by inhibiting the expression of a midline attractant. It will be interesting to investigate this possibility and to characterise any molecular changes using the markers that are now becoming available (Chapman et al., 2002). Taken together, we suggest that the cardia bifida phenotype is due to a more extensive lateral migration, combined with impaired movement and/or convergence back towards the midline.

Interestingly, cardiac progenitors could also respond to Fgf8 in a manner similar to paraxial mesoderm and lateral plate mesoderm progenitors that emerge from the primitive streak at HH4 (Yang et al., 2002) (Fig. 3F). By contrast, the response to Wnt3a was specific to HH3 primitive streak cells and did not require a functional Fgf receptor (Fig. 3D,E). This indicates a dynamically changing signalling environment and responsiveness of cells, and suggests that Wnt and Fgf, and possibly other pathways, act in parallel to control the correct movement behaviour of prospective cardiac cells.

In conclusion, our data supports a model whereby high levels of Wnt3a cause the repulsion of prospective cardiac cells from the streak resulting in lateral migration. This is mediated by a pathway, which involves RhoA activity necessary for directional migration. It will be particularly interesting to unravel how the different activities of Wnt3a, in both activation (Naito et al., 2006; Nakamura et al., 2003; Ueno et al., 2007; Vijayaragavan and Bhatia, 2007) and inhibition of cardiogenesis (Marvin et al., 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001), as well as cardiac progenitor cell movements, become integrated through various downstream signalling effectors.

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/6/1029/DC1>

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