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Matrix adhesion polarizes heart progenitor induction in the invertebrate chordate *Ciona intestinalis*

Jennifer Norton¹, James Cooley¹, A. F. M. Tariqul Islam², Christina D. Cota³ and Brad Davidson^{3,*}

SUMMARY

Cell-matrix adhesion strongly influences developmental signaling. Resulting impacts on cell migration and tissue morphogenesis are well characterized. However, the *in vivo* impact of adhesion on fate induction remains ambiguous. Here, we employ the invertebrate chordate *Ciona intestinalis* to delineate an essential *in vivo* role for matrix adhesion in heart progenitor induction. In *Ciona* pre-cardiac founder cells, invasion of the underlying epidermis promotes localized induction of the heart progenitor lineage. We found that these epidermal invasions are associated with matrix adhesion along the pre-cardiac cell/epidermal boundary. Through targeted manipulations of RAP GTPase activity, we were able to manipulate pre-cardiac cell-matrix adhesion. Targeted disruption of pre-cardiac cell-matrix adhesion blocked heart progenitor induction. Conversely, increased matrix adhesion generated expanded induction. We were also able to selectively restore cell-matrix adhesion and heart progenitor induction through targeted expression of $\text{Ct-Integrin } \beta 2$. These results indicate that matrix adhesion functions as a necessary and sufficient extrinsic cue for regional heart progenitor induction. Furthermore, time-lapse imaging suggests that cytokinesis acts as an intrinsic temporal regulator of heart progenitor adhesion and induction. Our findings highlight a potentially conserved role for matrix adhesion in early steps of vertebrate heart progenitor specification.

KEY WORDS: Cell-matrix adhesion, Chordate evolution, Growth factor signaling, Heart development

INTRODUCTION

The *in vitro* characterized network linking matrix adhesion to inductive signaling is likely to influence cell fate patterning *in vivo* (Giancotti and Tarone, 2003; Streuli and Akhtar, 2009; Rozario and DeSimone, 2010). Receptor tyrosine kinase (RTK) ligands such as fibroblast growth factors (FGFs) function as pervasive inductive signals (Thisse and Thisse, 2005). Integrin-associated adhesion complexes display extensive interactions with RTKs, including FGF receptors (FGFRs), as well as downstream transduction pathways, including the MAP kinase (MAPK) cascade (Tsou and Isik, 2001; Schwartz and Ginsberg, 2002; Campos et al., 2004; Mori et al., 2008). Numerous studies have shown how RTK/integrin interactions shape embryonic cell migration and other morphogenetic cell behaviors (Ross, 2004; Ivaska and Heino, 2010; Kim et al., 2011b). By contrast, relatively few *in vivo* studies have elucidated a direct role for integrins in fate specification (Martin-Bermudo, 2000; Streuli, 2009; Rozario and DeSimone, 2010).

The current ambiguity regarding integrins in cell fate specification is exemplified by studies of vertebrate heart development. Knockdown or knockout of matrix adhesion factors severely disrupts cardiac morphogenesis but has little or no impact on early heart gene expression (Ross and Borg, 2001; Bowers and Baudino, 2010). Although these studies suggest that cell-matrix adhesion is not required for initial heart specification, further investigation is warranted. Redundancy may buffer the cell-matrix adhesion complex against the loss of a single component. Additionally, low-resolution heart marker gene analysis may not

have revealed alterations in specification. Observed perturbations in morphogenesis may partially reflect undetected disruption of earlier specification events. Furthermore, FGF signaling takes part in pre-cardiac mesoderm specification in vertebrate and *Drosophila* embryos (Beiman et al., 1996; Harvey, 2002; Kadam et al., 2009; Klingseisen et al., 2009; Nakajima et al., 2009). Although there are indications of an interplay between matrix adhesion and FGF-mediated heart progenitor specification in the *Drosophila* studies, an explicit link has not been established (McMahon et al., 2010). FGF signaling is also crucial for heart progenitor specification in the invertebrate chordate *Ciona intestinalis* (Davidson et al., 2006). However, the role of cell-matrix adhesion in *Ciona* cardiogenesis, or in any other aspect of *Ciona* development, has not been investigated.

The *Ciona* heart progenitor lineage provides an ideal model for examining the potential role of matrix adhesion in fate specification. In *Ciona* embryos, low cell numbers and rapid, stereotyped fate restriction permit high-resolution analysis of early specification events. *Ciona* heart tissue can be traced back to four B7.5 lineage founder cells. During neurulation, each pre-cardiac founder lineage cell divides asymmetrically to produce two distinct lineages (Fig. 1A-C). The smaller founder cell daughters (termed trunk ventral cells or TVCs) constitute the heart progenitor lineage, whereas the larger daughters constitute the anterior tail muscle lineage (ATM). Previous work has shown that TVC induction is directed by FGF/MAPK signaling (Davidson et al., 2006). Surprisingly, differential TVC induction occurs despite uniform exposure to FGF (Cooley et al., 2011). In a recent study, we have investigated the role of polarized protrusions in differential TVC induction (Cooley et al., 2011). This study revealed that pre-mitotic founder cells initially display uniform induction in response to ungraded FGF. FGF/MAPK signaling is gradually restricted to the presumptive TVCs as founder cells complete mitosis. Dissociation studies indicated that an extrinsic cue from the embryonic microenvironment promoted this gradual signal polarization.

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Immediately prior to division, founder cells produce localized protrusions that invade the adjacent epidermis (Fig. 1B). Perturbation studies indicate that these localized protrusions are both necessary and sufficient for polarized induction. These findings prompted us to investigate three questions: (1) What is the nature of the presumed micro-environmental cue? (2) How might invasive protrusions influence founder cell interactions with this extrinsic cue? (3) What underlies the temporal progression from uniform to localized induction during mitosis? In this study, we show that localized adhesion of founder cells to the epidermal matrix serves as an extrinsic cue for polarized induction. Our data also suggest that cooperative interactions between adhesion and protrusion promote regional inductive signaling. Additionally, our results indicate that intrinsic mitotic processes modulate spatiotemporal shifts in adhesion and signaling.

MATERIALS AND METHODS

Embryological techniques

Collection, rearing, fertilization, staging, dechoriation and electroporation were carried out as previously described (Corbo et al., 1997; Davidson et al., 2005; Hotta et al., 2007).

Antibody staining

For visualization of transgenically labeled founder cells, embryos were either fixed for *in situ* hybridization (Beh et al., 2007) and then stained with antibodies (Cooley et al., 2011), or embryos were fixed in 0.4% formaldehyde overnight at 4°C and mounted for direct visualization of fluorescence signal (Cooley et al., 2011). Antibody staining with dpERK (double-phosphorylated extracellular signal-related kinase; Sigma M9692, 1:500) was carried out (Davidson et al., 2006). Z-stacks spanning the GFP-labeled founder cells were analyzed using Imaris imaging software. Nearly every nucleus showed a low background level of staining. Therefore, nuclei were scored as positive if the level of staining was detectable above the background level (>25% pixel intensity).

Protrusion measurement

The volume of CDC42-GFP-labeled invasive protrusions were derived from 1 μ m confocal stacks using ImageJ. Staining of GFP in the CDC42-GFP assays was performed as described by Cooley et al. (Cooley et al., 2011). Only strictly ventral views were analyzed. A consistent starting position within the epidermal layer was defined by finding the center of DRAQ5-stained epidermal nuclei just above the founder cell pair being examined. The green channel (GFP) was then separated and a substack of seven or eight slices was created containing all slices ventral of the epidermal nuclei (as defined in the previous step). By consistent use of the threshold and magic wand tools, GFP-stained regions were accurately outlined in each slice. The area of the defined regions from each slice were measured and the added together to roughly approximate the GFP signal volume intruding into the epidermis for each founder cell pair. Over 16 founder cell pairs were used per trial.

Molecular cloning

All constructs were generated with PCR generated sticky end fragments using the primers listed in supplementary material Table S1.

Ex vivo adhesion assays

Dissociations were carried out as previously described (Cooley et al., 2011), using Mesp-Enscosin-3xGFP transgenic embryos, with the following changes. Out of a 400 μ l final suspension, 50 μ l was set aside for hemocytometry and 350 μ l was transferred into a Petri dish containing the coated coverslip and 3 ml of FASW+BSA. Glass coverslips (Fisher 22 \times 22 mm, #1.5) were treated with either gelatin formaldehyde (Cooley et al., 2011), fibronectin (Sigma F1141), laminin (Sigma L2020) or collagen (Sigma C7661) as follows. Fibronectin was diluted to 5 μ g/ml in 1 \times PBS and placed on a coverslip for 45 minutes at room temperature. Laminin was diluted to 20 μ g/ml and collagen was diluted to 100 μ g/ml, and both were allowed to incubate for 3 to 4 hours at room temperature. Coverslips were

then rinsed once in filtered artificial seawater (FASW) and used within 1 hour. Manufacturer-treated coverslips (fibronectin, BD 354088; laminin, BD 354087; collagen, BD 354089) produced similar results. Embryos were dissociated at stage 14 (6:30 HPF at 18°C), transferred to coated coverslips and incubated for 1.5 hours at 18°C. Cells were then fixed in 0.4% formaldehyde overnight at 4°C and mounted in 5 μ l of 70% glycerol. Coverslips were then sealed and samples scored on a compound scope. Only isolated adherent transgenically labeled B7.5 cells were used for analysis to avoid the possibility that adhesion was dependent on other cells in a cluster. The density of cells plated for each assay was determined using a hemocytometer. The raw average values \pm s.d. for each of the sample sets are given in supplementary material Table S2. In all samples, cell counts were normalized by the density of cells plated relative to controls and then a ratio was calculated comparing average adhesion in each sample to average adhesion in controls. Thus, final values accounted for differences in both cell density and levels of control adhesion.

Morpholino assay

Morpholino oligonucleotides (MOs) were purchased from Gene Tools: Antisense Talin, AGCTTCACAAGCTTACTCCATCTCT, and an Antisense Talin-control with five mis-matches, AGCTTCAGAA-CCTTAGTCGATGTCT. Dechorionated and unfertilized eggs were co-injected with MOs along with Mesp-GFP fusion reporter constructs to mark founder lineage cells. The injected eggs were fertilized and maintained at 18°C in artificial seawater containing 50 μ g/ml streptomycin sulphate.

Live imaging

Dissociated cells from 5.5 HPF (approximately stage 12) embryos were re-suspended in ice-cold 5 μ g/ml FM4-64 in calcium-free sea-water with 1% BSA (Cooley et al., 2011) from a stock solution of 100 μ g/ml H₂O (Invitrogen, T-3166) for 1-2 minutes then transferred to an FN-coated Nunc Lab-Tek Chambered #1.0 Borosilicate cover glass (Sigma, Z734640) containing 5 μ g/ml FM4-64 in filtered seawater with 1% BSA. Transgenic embryos were simply transferred to uncoated chambered cover slips at 5.5 HPF. Imaging was carried out with a Leica high-resonance scanning SP5 confocal microscope using a \times 20 C-Apochromat 1.2 W objective. Movies were created by compiling sequential z-stacks in either Imaris 6.4.2 (Bitplane) or ImageJ.

Segmentation analysis

GFP-Talin embryos were fixed according to the *in situ* fixation protocol (Beh et al., 2007) during founder cell mitosis (6:45-6:55 HPF), antibody stained (see above) and mounted in Prolong Gold Antifade Reagent with DAPI (Molecular Probes P-36931) or treated with DRAQ5 (1:5000 in PBS with 0.1% Tween, 5-minute incubation at room temperature and rinsed once before mounting, Cell Signaling 4084S). Three-dimensional projections of high-resolution confocal z-stacks (1.0 μ m section) cropped to include only one founder cell pair were analyzed using the 'Surfaces' tool in Imaris set to 'automatic creation' mode. Prior to surface detection, we applied Background Object Subtraction with a Gaussian filter (sigma = Object Diameter set at 1 μ m/2). Surfaces were then filtered by automatic thresholding at the lower limit to exclude low correlation data but with no filtering at the upper limit. Varying area sizes were created by 'region growing' from seedpoints defined by contrast at presumed borders. The Surfaces program provided comprehensive volume and average intensity measurements for each segmented GFP-Talin foci. The intensity measurements represent relative values within each founder cell pair. Thus, comparison of intensity levels between samples does not involve absolute values that would be subject to inter-sample variability.

RESULTS

Founder cell-matrix adhesion correlates with regional induction

Founder cells divide along a perpendicular axis relative to adjacent epidermis (Fig. 1C). As a result, TVCs arise with greatly increased epidermal contact in comparison with their larger sisters (Fig. 1B,C). Additionally, founder cell invasion of the epidermis is associated with differential induction (Cooley et al., 2011). These

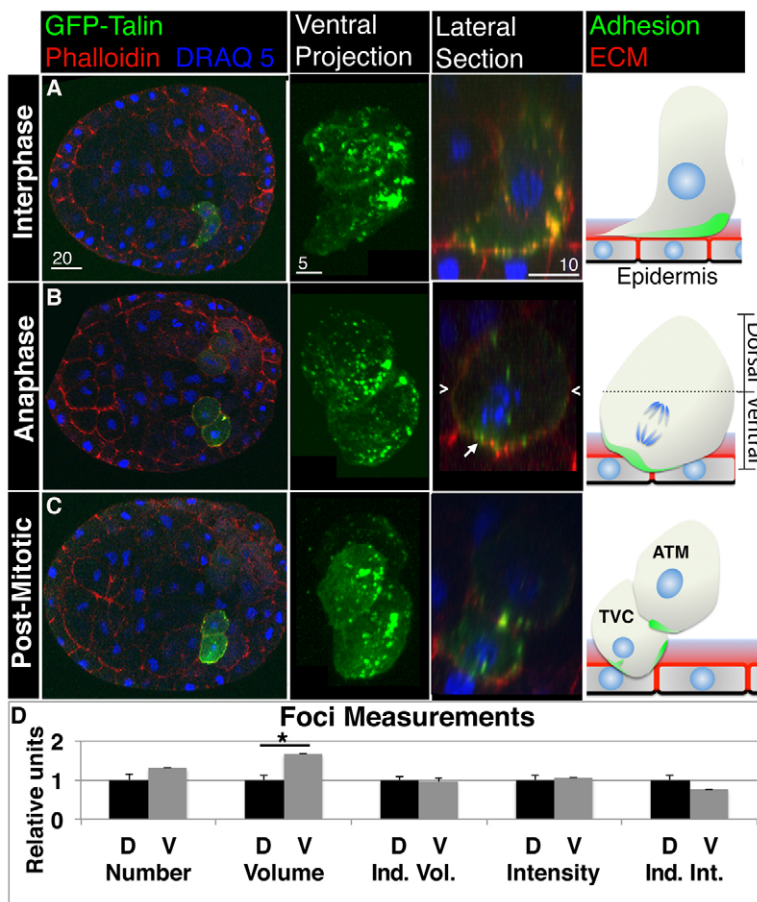


Fig. 1. Spatial correlation between founder cell adhesion and induction. (A-C) Representative micrographs and accompanying diagrams of staged Mesp-GFP-Talin founder cells, stained as indicated. In the first column, a horizontal section indicates labeled founder cell position in the embryo. Subsequent columns show sections (as indicated) of these cells. Lateral sections are taken from the more medial (top) cell of each founder cell pair. Arrow in B indicates local enrichment of GFP-Talin. (D) Segmentation analysis comparing GFP-Talin foci between the ventral (V) and dorsal (D) sides of transgenic founder cells. The demarcation used to divide founder cells into ventral and dorsal regions is illustrated in B (arrowheads). In this and all similar graphs, averages are shown for total foci number, volume and intensity in the region of interest, along with averages for individual focal volume (Ind. Vol.) and intensity (Ind. Int). * $P=0.013$, 45 founder cell pairs per experimental condition, each sample spanned four independent trials. Error bars represent s.e.m. Significance for all trials was determined using two-tailed, unpaired *t*-tests. Scale bar measurements are in μm and are indicated for each column. Embryos are oriented anterior towards the left.

observations suggest that founder cell/epidermal interactions localize inductive signaling. More specifically, we hypothesized that founder cell adhesion to either the epidermis or associated matrix proteins influences induction. To investigate these hypotheses, we visualized adhesive interactions in transgenically labeled founder cells (Fig. 1; see supplementary material Fig. S1).

We first visualized cell-cell adhesion using *Ciona* CadherinI-GFP and CadherinII-mCherry fusion constructs driven by the Mesp enhancer (Davidson et al., 2005). In the resulting transgenic embryos, Cadherin fusion proteins were enriched along the boundary between founder cell pairs (supplementary material Fig. S1). Thus, regional cell-cell adhesion does not correspond to localized induction along the ventral, epidermal boundary.

We next visualized founder cell-matrix adhesions using GFP-labeled *Ciona* Talin fragments driven by the Mesp enhancer (Mesp-GFP-Talin). These Talin fusion proteins have previously been shown to label focal adhesions in transfected mammalian cells (Singiser and McCann, 2006). In transgenic founder cells, GFP-Talin foci were strongly enriched along the ventral founder cell membrane adjacent to underlying epidermis (Fig. 1A-C). Through segmentation analysis, we quantified the number, volume and intensity of individual GFP-Talin foci (supplementary material Fig. S5). We found that total foci volume was significantly enriched on the ventral side (Fig. 1D). Ventral enrichment appeared to reflect increased numbers of GFP-Talin foci rather than an increase in the size of individual foci (Fig. 1D, Ind. Vol.).

The transition between uniform and localized induction occurs during founder cells mitosis (Cooley et al., 2011). By visualizing mitotic figures in transgenic GFP-Talin founder cells, we

determined that there is a corresponding, cell-cycle specific restriction in founder cell-matrix adhesion. Prior to mitosis, GFP-Talin foci extend along the entire ventral side of the founder cell (Fig. 1A, lateral section and diagram). During mitosis, founder cells round up and GFP-Talin foci are gradually restricted to the presumptive TVC membrane (Fig. 1B,C, lateral sections and diagrams). During anaphase, we consistently observed highly localized enrichment of GFP-Talin in the nascent TVC cortex, closely apposed to the ventral chromatin (Fig. 1B, arrow). As the founder cells complete mitosis, newly born TVCs inherit a cortex enriched with GFP-Talin (Fig. 1C).

Targeted disruption of founder cell adhesion through perturbation of Rap GTPase activity

We next began to test the hypothesized role of cell-matrix adhesion in signal polarization through functional perturbations. We reasoned that the observed correspondence between cell-matrix adhesion and induction could be purely correlative. In this case, loss of cell-matrix adhesion would have no impact on induction. Alternatively, cell-matrix adhesion might serve to spatially restrict inductive signaling. In this case, disruption of adhesion would expand inductive signaling. We also considered the possibility that cell-matrix adhesion facilitates regional activation of a uniform but weak inductive signal. In this case, loss of cell-matrix adhesion would abrogate induction.

To block founder cell-matrix adhesion, we disrupted Rap GTPase activity. Rap orthologs play a central role in integrin activation (Banno and Ginsberg, 2008; Boettner and Van Aelst, 2009; Carmona et al., 2009; McMahon et al., 2010). Rap is inactivated by

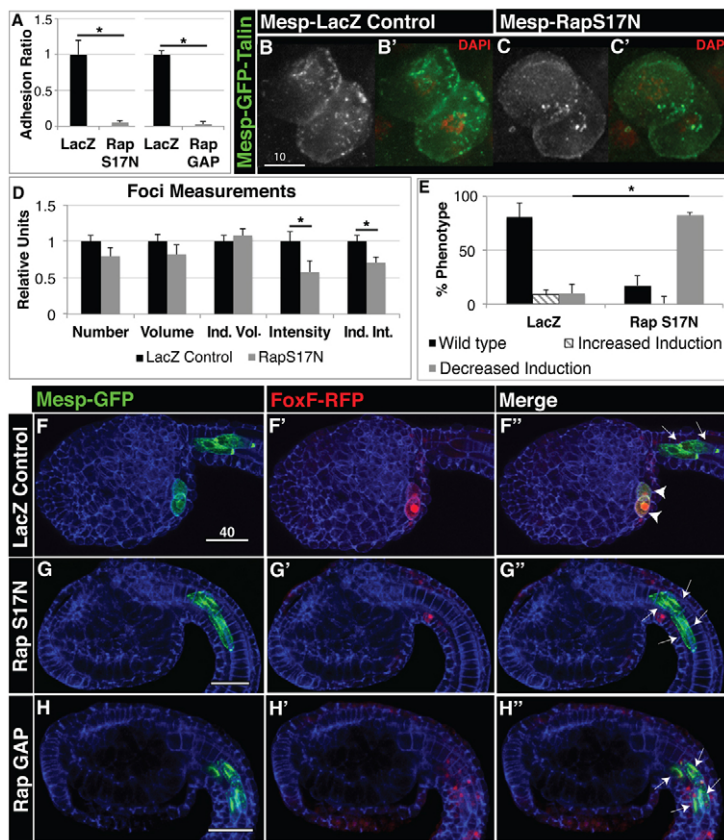


Fig. 2. Matrix adhesion is required for TVC induction.

(A) Adhesion of dissociated transgenic founder cells to fibronectin. Adhesion ratio is derived from a comparison with average number of adhesive cells in the controls. * $P=0.008$ for LacZ versus RapS17N; $P=9.93E^{-5}$ for LacZ versus RapGAP. (B-C') Ventral projections of representative GFP-Talin founder cell pairs co-transfected and stained as indicated. (D) Segmentation analysis; * $P=0.001$ for intensity and $P=0.005$ for Ind. Int.; over 35 founder cell pairs examined for each experimental condition spanning three independent trials. (E-H'') Graph (E) and representative micrographs (F-H'') showing TVC induction (FoxF-RFP) in transgenic embryos as indicated. Arrowheads in F'' indicate TVCs; arrows in F'', G'', H'' indicate uninduced founder lineage cells. Only embryos displaying normal overall morphology were scored. Bilateral founder cell clusters were scored independently. The 'decreased induction' phenotype often entailed the complete absence of FoxF-RFP-positive TVCs (G'', H''), but also included embryos in which only a single TVC was observed/cluster. (E) * $P=0.001$, three trials, $n>15$ /trial. The loss of induction phenotype represented in H was nearly uniform in all Rap GAP transgenic embryos, three trials, over 100 clones per trial. Error bars represent s.e.m. Significance for all trials was determined using two-tailed, unpaired *t*-tests. Scale bar measurements are in μm and are indicated for each row. Embryos are oriented anterior towards the left.

highly specific interactions with Rap GTPase activating proteins (Rap-GAPs). We therefore attempted to disrupt Rap activity and downstream cell-matrix adhesion through targeted expression of the sole *Ciona* RapGAP ortholog (Mesp-RapGAP). To assay the impact of this manipulation on cell-matrix adhesion, we dissociated transgenic GFP-Talin founder cells and plated them on different matrix substrates. Labeled founder cells displayed highly selective adhesion to fibronectin (FN)-coated coverslips in comparison with collagen, laminin and gelatin (supplementary material Fig. S2A). We therefore employed FN-coated coverslips for all subsequent adhesion assays. As shown in Fig. 2A, RapGAP abrogated FN adhesion in transgenic founder cells. To alleviate concerns about the specificity of RapGAP, we also attempted to disrupt adhesion through targeted expression of a dominant-negative form of *Ciona* Rap1 (Mesp-RapS17N). Transgenic RapS17N founder cells also displayed complete loss of FN adhesion in our *ex vivo* assay (Fig. 2A).

We next examined the impact of RapS17N on GFP-Talin localization. In double transgenic embryos (Mesp-GFP-Talin/Mesp-RapS17N) we often observed a dramatic decrease in the intensity of GFP-Talin foci (compare Fig. 2B with 2C). Through segmentation analysis, we found that disruption of Rap activity led to a significant decrease in the average intensity of individual GFP-Talin foci (Fig. 2D). These results confirm that targeted perturbation of Rap effectively disrupts founder cell/matrix adhesion.

Targeted disruption of Rap activity perturbs TVC induction

To assay the impact of targeted Rap perturbations on founder cell induction, we employed Mesp-GFP/FoxF-RFP double transgenic embryos (Davidson et al., 2006). The FoxF-RFP reporter provides

a rapid and reliable read-out of TVC inductive signaling within Mesp-GFP-labeled founder cells (Davidson et al., 2006). On each side of a wild-type embryo, two out of four Mesp-GFP-labeled founder lineage cells undergo TVC induction. In the resulting TVC pair, induction rapidly generates robust expression of FoxF-RFP followed by migration into the trunk region (Fig. 2F-F'', arrowheads). Co-transfection with Mesp-RapS17N or Mesp-RapGAP severely disrupted TVC induction, significantly reducing the number of FoxF-RFP-positive founder lineage cells (Fig. 2E-H). These results support the hypothesis that cell-matrix adhesion is required for regional enhancement of uniform, sub-threshold FGF signaling.

We next attempted to block cell-matrix adhesion more directly using morpholino knockdown of *Talin* (supplementary material Fig. S2). *Talin* is expressed in both notochord progenitors and heart founder cells (Christiaen et al., 2008). We therefore anticipated that *Talin* knockdown would produce tail defects, as well as potentially impacting heart progenitor specification. *Talin* morphant embryos characteristically displayed shortened tails along with defects in heart founder cell division (supplementary material Fig. S2). Although *Talin* morphants also displayed aberrant TVC induction, we could not meaningfully interpret this data owing to associated division defects.

We also attempted to block cell-matrix adhesion directly by targeted expression of truncated integrin and talin constructs. Although orthologous integrin and talin fragments have been shown to function as dominant negatives in a few defined contexts, previous data are limited and somewhat ambiguous (Retta et al., 1998; Lee et al., 2001; Lee et al., 2006; Jannuzi et al., 2002; Wegener et al., 2007; Anthis et al., 2009; Haling et al., 2011; Kim et al., 2011a). In contrast to the morpholino results, the truncated

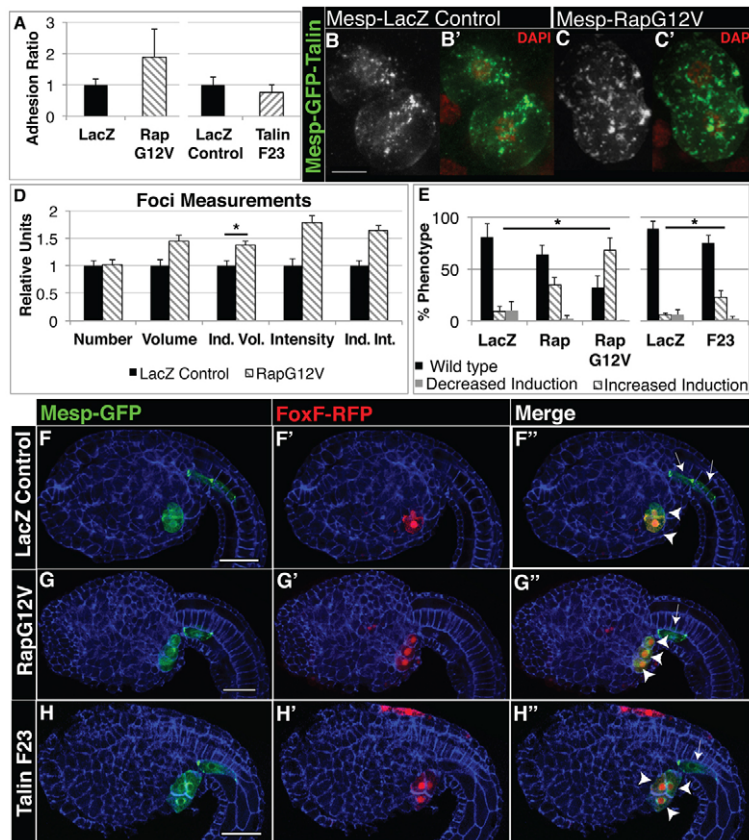


Fig. 3. Enhanced adhesion promotes increased induction. (A) Adhesion ratio of dissociated transgenic founder cells to fibronectin. (B-C') Ventral projections of representative Talin-GFP founder cell pairs stained and co-transfected as indicated. (D) Segmentation analysis; $*P=4.16E^{-6}$, over 35 founder cell pairs examined for each experimental condition. (E-H'') Graph (E) and representative micrographs (F-H'') showing TVC induction (FoxF-RFP, arrowheads; arrows indicate uninduced founder lineage cells) in transgenic embryos as indicated. (E) $*P=0.008$ for LacZ versus RapG12V and 0.009 for LacZ versus F23. RapG12V samples were conducted in association with the trials shown in Fig. 2E, three trials $n>15$ /trial. The TalinF23 data set contained over 45 clones per trial. Error bars represent s.e.m. in A,D and s.d. in F. Significance for all trials was determined using two-tailed, unpaired *t*-tests. Scale bars: 10 μ m (B); 40 μ m (F-H). Embryos are oriented anterior towards the left.

Talin construct had no impact on founder cell adhesion, division or induction, indicating a lack of dominant-negative activity (supplementary material Fig. S2). The truncated integrin constructs also had no discernible impact on *ex vivo* FN adhesion or *in vivo* TVC induction (supplementary material Fig. S2). In light of these negative results and the lack of well-established dominant-negative activity for orthologous truncations, we focused our efforts on further manipulations of Rap activity.

Targeted potentiation of Rap activity leads to expanded induction

According to our adhesion hypothesis, increased adhesion should potentiate an expansion of TVC induction. We tested this prediction through targeted expression of full-length Rap (Mesp-Rap) or constitutively active Rap (Mesp driving a GTPase insensitive Rap mutant, Mesp-RapG12V) in the founder cell lineage. We also attempted to expand cell-matrix adhesion more directly through targeted expression of a constitutively active form of Talin (Mesp driving the Talin head domain, Mesp-TalinF23) (Wegener et al., 2007). We first examined the effect of increased Rap or Talin activity on founder cell-matrix adhesion using our FN coverslip assay. We found that RapG12V led to a dramatic but variable increase in adhesion, whereas TalinF23 had no apparent impact (Fig. 3A). These results may indicate that perturbations of adhesion dynamics have variable effects on *ex vivo* matrix interactions. Alternatively, these results may simply reflect the relatively low sensitivity of our *ex vivo* adhesion assay. We also observed and quantified the impact of RapG12V on GFP-Talin foci. The observed increase in adhesion is paralleled by the appearance of relatively more intense and significantly larger Talin foci along the founder cell/epidermis boundary (Fig. 3B-

D). Using the FoxF-RFP assay, we observed a robust and significant expansion in TVC induction in Mesp-RapG12V embryos (Fig. 3E-G). Targeted expression of wild-type Rap also appeared to promote increased induction but this result was not significant (Fig. 3E). Additionally, co-transfection with Mesp-TalinF23 generated a mild but significant increase in TVC induction (Fig. 3E,H). These data indicate that increased matrix adhesion is sufficient for expanded induction.

Selective restoration of adhesion and differential induction

Rap GTPases do not function solely as modulators of adhesion (Boettner and Van Aelst, 2009). Thus, our perturbations of Rap activity may have influenced TVC induction independently of their impact on adhesion. To address this concern, we tested whether restoration of cell-matrix adhesion in the RapS17N background is sufficient to rescue induction. We attempted to restore adhesion through targeted expression of specific integrin chains. *Ciona* gene models predict 11 integrin- α and five integrin- β orthologs (Ewan et al., 2005). Expression data from sorted founder cells indicate that six α -chains ($\alpha 2$, $\alpha 3$, $\alpha 6$, $\alpha 9$, $\alpha 10$ and $\alpha 11$) and only two β -chains ($\beta 1$ and $\beta 2$) are robustly expressed in founder cells (Christiaan et al., 2008; Woznica et al., 2012). We attempted to restore adhesion through targeted expression of *Ciona* Integrin $\beta 1$ (Mesp-Int $\beta 1$), Integrin $\beta 2$ (Mesp-Int $\beta 2$) or Integrin $\alpha 11$ (Mesp-Int $\alpha 11$) in the RapS17N background. Double transgenic embryos were dissociated and the labeled founder cells (Mesp-GFP) were incubated on FN-coated coverslips. Intriguingly, we found that targeted expression of Integrin $\beta 2$ restored adhesion, whereas targeted expression of Integrin $\beta 1$ or $\alpha 11$ did not (Fig. 4A). We next examined whether the selective restoration of adhesion by Mesp-Int $\beta 2$ versus Mesp-Int $\beta 1$

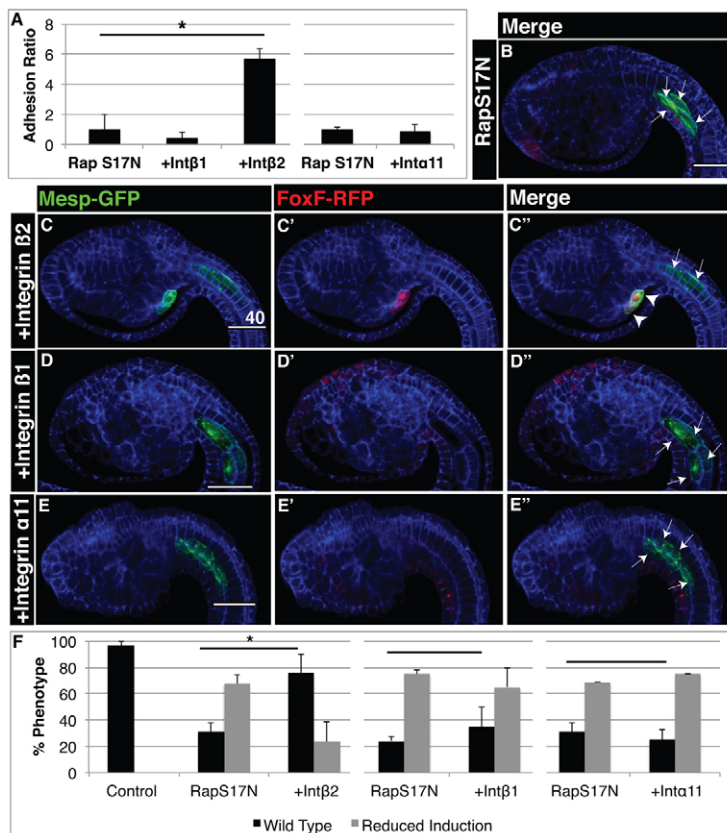


Fig. 4. Selective rescue of adhesion and induction by Integrin-β2 overexpression in RapS17N embryos.

(A) Adhesion ratios of dissociated transgenic founder lineage cells to fibronectin, $*P=0.018$ for Intβ2 co-transfection. Co-transfection with Intβ1 or Intα11 had no significant impact: $P=0.607$ and 0.575 , respectively. (B-F) Representative micrographs (B-E) and graph (F) showing TVC induction (FoxF-RFP, arrowheads) in transgenic embryos as indicated. Arrows indicate uninduced founder lineage cells. (F) $*P=1.63E^{-4}$ for percentage wild-type induction in RapS17N alone versus RapS17N + Intβ2; the percentage wild-type induction in RapS17N alone versus RapS17N + Intβ1 or + Intα11 were not significant: $P=0.246$ and 0.239 , respectively. Intβ rescue assays involved more than 33 clones per trial whereas the Intα11 assays involved more than 27 clones per trial. Data represent at least three independent trials. Error bars represent s.e.m. in A and s.d. in F. Significance for all trials was determined using two-tailed, unpaired *t*-tests. Scale bars: 40 μm. Embryos are oriented anterior towards the left.

or Mesp-Intα11 led to a corresponding, selective rescue of induction. We found that co-transfection with Mesp-Intβ2 led to a dramatic and significant restoration of differential induction in Mesp-RapS17N embryos (Fig. 4C,F). By contrast, co-transfection with Mesp-Intβ1 or Mesp-Intα11 failed to rescue induction (Fig. 4D-F). To further confirm Integrin β2 specificity in this assay, we co-transfected Mesp-RapS17N embryos with Mesp-CadherinI and Mesp-CadherinII constructs. Targeted expression of these cell adhesion proteins also failed to rescue induction (data not shown). Selective restoration of both adhesion and induction by Integrin β2 allayed concerns regarding specificity in the Rap perturbation assays.

To more closely examine the immediate impact of Integrin β2 rescue on adhesion and induction, we examined nuclear diphosphorylated extracellular-signal-regulated-kinase (dp-ERK, which is indicative of MAPK pathway activation) in stage 15 transgenic embryos. Immediately after founder cell mitosis, dp-ERK antibody staining is dramatically enriched in the nuclei of ventral daughter cells, the TVCs, in comparison with the nuclei of their more dorsal sisters, the ATMs (Fig. 5A) (Davidson et al., 2006). Consistent with the FoxF reporter data, embryos transfected with RapS17N displayed a significant reduction in the number of dpERK-positive TVCs (Fig. 5B,D). Furthermore, co-transfection with Mesp-Intβ2 significantly restored dp-ERK enrichment in ventral daughters (Fig. 5C,D). We were also able to directly observe the morphology of newly divided founder lineage cells in this assay (Fig. 5A'''-C'''). In wild-type embryos, dp-ERK positive TVCs consistently displayed a tight association with the epidermis (Fig. 5A''', white dotted line). By contrast, RapS17N transgenic founder lineage cells often lacked this tight epidermal association, were more dispersed and displayed a pronounced dorsal shift (Fig. 5B'''). Interestingly, occasional contact between RapS17N founder

lineage cells and the epidermis was accompanied by a weak level of nuclear dp-ERK staining (Fig. 5B, arrow). Strikingly, the rescue of dp-ERK in Mesp-RapS17N/Mesp-Integrinβ2 embryos was accompanied by restoration of a tight association between TVCs and the epidermis. These assays confirm that our manipulations of Rap GTPase activity directly impact founder cell adhesion and TVC induction. These assays also further validate our reliance on the established FoxF reporter assay as an accurate read-out of TVC induction. Taken together, GFP-Talin localization and Rap perturbation assays strongly support the hypothesis that matrix adhesion regionally enhances sub-threshold TVC induction. Thus, cell-matrix adhesion appears to function as the extrinsic cue delineated by our previous research.

Cooperative regulation of inductive signaling by adhesion and protrusion

We have also begun to investigate the relationship between invasive protrusions and cell matrix adhesion during TVC induction. Localized CDC42 activity and associated invasive protrusions polarize heart progenitor induction (Cooley et al., 2011). In light of our current data, we propose that protrusions polarize induction in concert with cell-matrix adhesion. Adhesion and protrusion may regulate inductive signaling through a linear, hierarchical pathway. According to this paradigm, either regional adhesion directs protrusion-mediated signaling or regional protrusions direct adhesion-mediated signaling. Alternatively, adhesion and protrusion may function cooperatively to promote polarized signaling.

To distinguish between these hypotheses, we examined the impact of Rap activity on CDC42 localization and invasive protrusions in Mesp-CDC42-GFP transgenic embryos. CDC42-GFP is highly enriched in the invasive protrusions of transgenic

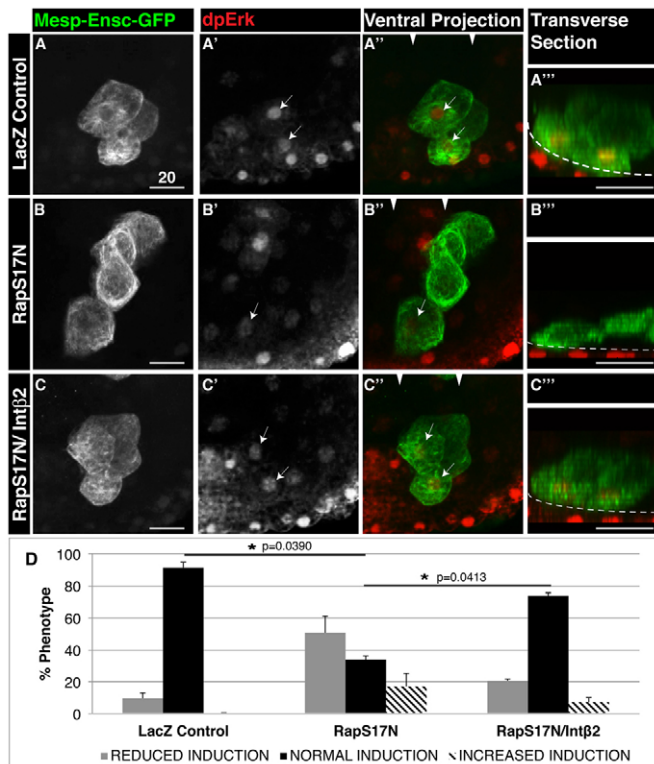


Fig. 5. Restoration of adhesion and induction by Integrin- β 2 occurs immediately after founder cell division. (A-D) Representative micrographs (A-C'') and graph (D) showing dpERK staining in transgenically labeled stage 15 founder cell nuclei (Mesp-Encs-GFP). Arrows indicate dpERK-positive nuclei. Although staining intensity was not quantified, the intensity of occasional dpErk-positive nuclei in Mesp-RapS17N embryos was low, just above background levels (B'). Additionally, co-transfection with Mesp-Int β 2 appears to only partially restore the full intensity of dp-ERK nuclear staining (C' versus A'). White triangles at the top of each ventral projection (A'', B'', C'') indicate the anterior and posterior boundaries of transverse sections in A''', B''', C'''. Broken lines indicate the epidermal surface in A''', B''', C'''. (D) Data are from two independent trials, $n \geq 5$ /trial. Error bars represent s.d. Significance for all trials was determined using two-tailed, unpaired t -tests. Scale bars: 20 μ m. Embryos are oriented anterior towards the left.

founder cells (Fig. 6A) (Cooley et al., 2011). We found that transfection with Mesp-RapS17N led to a significant reduction in the average volume of CDC42-GFP-enriched invasive protrusions (Fig. 6B,D). This result suggests that regional adhesion promotes or stabilizes CDC42 localization and invasive protrusion. By contrast, co-transfection with Mesp-RapG12V had no discernible impact on protrusion volume or phenotype (Fig. 6C,D). This result indicates that increased adhesion can enhance induction independent of any impact on protrusive activity.

We next examined how loss of adhesion impacted TVC induction in a hyperactive CDC42 background (Mesp-RapS17N/Mesp-Cdc42Q61L). Targeted hyperactivation of CDC42 (Mesp driving a GTPase defective Cdc42Q61L) delocalizes protrusive activity and generates uniform TVC induction (Fig. 6F; Cooley et al., 2011). According to strictly hierarchical models, either the hyperactive CDC42 phenotype (expanded induction, Fig. 6F) or the RapS17N phenotype (reduced induction, Fig. 6E) should predominate. In Mesp-RapS17N/Mesp-Cdc42Q61L double transgenic embryos, we

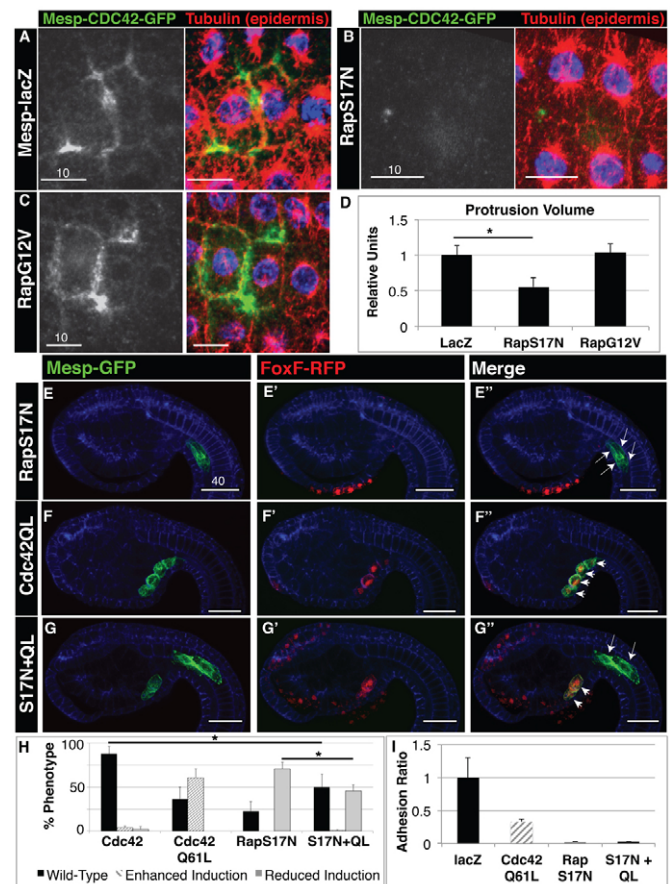


Fig. 6. Interplay between protrusion and adhesion during TVC induction. (A-C) Representative micrographs displaying CDC42-GFP-enriched founder cell membranes (GFP in white or green) invading the underlying epidermis, co-transfected and stained as indicated, DRAQ5 stained chromatin in blue. (D) Relative volumes of invasive membranes for each transgenic phenotype in relation to the control (Mesp-LacZ), $*P=0.025$, over 20 founder cell pairs examined for each experimental condition, each sample spanned three independent trials. (E-H) Representative micrographs (E-G'') and graph (H) showing TVC induction (FoxF-RFP, arrowheads) in embryos co-transfected as indicated, $*P=0.017$ for Cdc42 versus S17N and 0.011 for S17N versus QL. (I) Adhesion ratio of dissociated transgenic founder cells to fibronectin. Data were obtained from two trials, $n > 33$ /trial. Error bars represent s.e.m. in D,F and s.d. in H. Significance for all trials was determined using two-tailed, unpaired t -tests. Scale bar measurements are in μ m and are indicated for each panel. Embryos are oriented anterior towards the left.

observed a severe and significant loss of TVC induction (FoxF-RFP, Fig. 6H). Notably, the expanded induction phenotype typical of Mesp-Cdc42Q61L embryos was completely absent. These results support a linear model in which protrusions primarily impact TVC induction through modulation of cell-matrix adhesion. However, double transgenic embryos display a modest but significant decrease in the frequency of the reduced induction phenotype in comparison with single RapS17N transgenic embryos (Fig. 6H). Indeed, in a slight majority of these double transgenic embryos, TVC induction appeared normal (Fig. 6G,H). We investigated whether this partial rescue of induction might reflect a partial restoration of adhesion by CDC42Q61L. Using our *ex vivo* adhesion assay, we found that CDC42QL did not restore adhesion in the RapS17N background

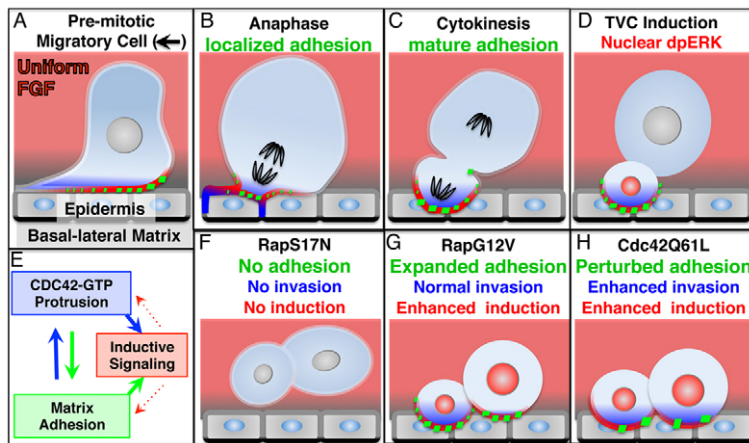


Fig. 7. Model illustrating proposed role for matrix adhesion in differential TVC induction. (A-D) Correlation between localized adhesion (green foci), protrusive actin (blue shading) and phospho-tyrosine staining (red shading, indicative of either FGF receptor or integrin activation) in wild-type founder cells. Diagrams are based on the adhesion data from this paper and data on protrusive actin and p-TYR staining from Cooley et al. (Cooley et al., 2011). (E) Proposed interactions between adhesion and protrusion and their hypothesized cooperative promotion of spatially restricted inductive signaling. The possibility that inductive signaling provides another layer of positive feedback is indicated by broken red arrows. (F-H) The characterized impact of targeted transgenic manipulations on adhesion, protrusion and induction.

(Fig. 6I). Indeed, in transgenic CDC42Q61L founder cells FN adhesion was markedly reduced.

To further explore the impact of hyperactive CDC42 on adhesion dynamics, we co-transfected Mesp-GFP-Talin embryos with either Mesp-Cdc42 or Mesp-Cdc42Q61L (supplementary material Fig. S3). Targeted expression of wild-type CDC42 (Mesp-Cdc42) had no apparent impact on GFP-Talin localization. By contrast, targeted expression of hyperactive CDC42 (Mesp-Cdc42Q61L) severely disrupted GFP-Talin localization in both pre-mitotic and mitotic founder cells. In pre-mitotic founder cells, there is a notable anterior/posterior size gradient of GFP-Talin foci along the epidermal boundary (Fig. 1A, lateral section). Using segmentation analysis, we measured a significant increase in the average intensity and volume of individual GFP-Talin foci in the posterior versus anterior region of labeled pre-mitotic founder cell pairs (supplementary material Fig. S3A). Co-transfection with Mesp-CDC42Q61L abrogates this posterior enrichment (supplementary material Fig. S3B). In mitotic founder cells, GFP-Talin is progressively restricted to the presumptive TVC membrane (Fig. 1B,C). Co-transfection with Mesp-CDC42Q61L promoted significantly enlarged GFP-Talin foci scattered along the ventral cortex (supplementary material Fig. S3C-E). The altered distribution of GFP-Talin correlates with uniform induction previously observed in Mesp-Cdc42Q61L transgenic founder lineage cells (Cooley et al., 2011).

Overall, our data do not support a simple hierarchical regulatory relationship between protrusion and adhesion. Instead, these results suggest that cooperative interactions between adhesion and protrusion may play a role in inductive signal localization.

Dynamic alterations in adhesion during founder cell mitosis

We next initiated live imaging analysis to visualize adhesion dynamics during the shift from uniform induction in pre-mitotic founder cells to differential induction in mitotic founder cells. For this analysis, we employed both dissociated and intact transgenic Mesp-3xYFP-Talin embryos. Dissociated pre-mitotic founder cells plated on FN are highly mobile and display a notable enrichment of enlarged foci clustered at the trailing edge (supplementary material Movie 1). The observed Talin gradient may reflect polarized focal adhesion maturation from the leading to trailing edge that is typically observed in migrating cells (Huttenlocher and Horwitz, 2011). The similar pattern of Talin foci observed *in vivo* (Fig. 1A) may reflect *in vivo* founder cell migration just prior to mitosis/induction (B.D., unpublished). Upon entering mitosis,

dissociated founder cells cease migration and round up, redistributing Talin foci along the adherent membrane (supplementary material Movie 2; Fig. S4). Just prior to cytokinesis, new GFP-Talin foci emerge along the reduced plane of matrix contact (supplementary material Fig. S4, bracket). These nascent foci appear to stabilize and enlarge during cytokinesis (supplementary material Movie 2; Fig. S4). We observed a similar association between cytokinesis and the maturation of adhesive foci in our *in vivo* samples (supplementary material Fig. S4; Movies 3, 4). Talin foci increase dramatically in size and intensity as founder cells complete mitosis (supplementary material Fig. S4, bracket). Intriguingly, immediately prior to cytokinesis, the presumptive TVC membrane bulges ventrally as it invades the underlying epidermis [as demarcated by the red line in supplementary material Fig. S4 and described previously by Cooley et al. (Cooley et al., 2011)] and adhesion maturation is concentrated along the invasive membrane. These observations suggest that processes associated with cytokinesis promote the localized growth/maturation of nascent focal adhesions during invasion of the adjacent epidermis.

DISCUSSION

Localized adhesion potentiates differential heart progenitor induction

Through high-resolution *ex vivo* and *in vivo* analysis, we have delineated a crucial role for matrix adhesion in *Ciona* heart progenitor induction (Fig. 7). According to our model, adhesion to the epidermal matrix directs regional enhancement of sub-threshold, uniform FGF signaling. This model is supported by the spatio-temporal correlation between gradual localization of matrix adhesion (Fig. 1; Fig. 7A-D) and the shift from uniform to differential induction during founder cell mitosis (Cooley et al., 2011). More crucially, targeted perturbations of Rap GTPase activity indicate that matrix adhesion is both necessary and sufficient for localized induction (Figs 2, 3; Fig. 7F,G). Concerns regarding Rap specificity were addressed through two complementary experiments. First, targeted enhancement of integrin activation (Mesp-TalinF23) was sufficient to expand inductive signaling (Fig. 3). Second, restoration of adhesion by targeted overexpression of a specific integrin β -chain (Integrin β 2 versus β 1 or α 11) selectively restored induction in the RapS17N background (Fig. 4). Future studies will exploit the observed differential restoration of adhesion and induction by Integrin β 2 (Fig. 4) to help dissect the precise role of this integrin β -chain and one or more associated α -chains in TVC induction. Future efforts will also include integrin knockdown studies. However, the functional properties of *Ciona*

integrins have not been previously investigated. Therefore, initial follow up studies must incorporate a fundamental characterization of *Ciona* integrin adhesion and signaling. A comprehensive exploration of the proposed matrix adhesion model must also include investigations of upstream adhesive localization, as well as the downstream impact of adhesion on inductive signaling, as described in the following sections.

Potential mechanisms for localized founder cell-matrix adhesion

The hypothesized role of localized adhesion in differential TVC induction has prompted us to consider how extrinsic and intrinsic factors may initially regionalize adhesion. One immediate hypothesis is that extrinsic matrix asymmetries in the founder cell microenvironment dictate localized adhesion. However, exploration of this hypothesis is hindered by the extremely poor characterization of *Ciona* matrix proteins (Ewan et al., 2005). The *Ciona* Fibronectin ortholog, for example, is merely a sequence-based prediction that has not been functionally characterized (Tucker and Chiquet-Ehrismann, 2009). Further progress will require extensive characterization of *Ciona* matrix proteins, including development of appropriate antibodies.

Intrinsic asymmetries in Rap activity may also contribute to localized founder cell adhesion. The impact of Rap-GAP on TVC induction indicates that Rap plays an endogenous role in establishing localized adhesion. In addition, *Ciona Rap1* is highly expressed in pre-mitotic founder cells (Christiaen et al., 2008). Rap proteins themselves are not characteristically polarized (Bivona and Philips, 2005) and we have observed uniform localization of Rap1-GFP fusion proteins in transgenic founder cells (C.C., unpublished). Therefore, future studies will focus on the potential contribution of localized Rap modulators (GAPs or GEFs).

Mitotic processes may also play an intrinsic role in adhesion localization. Through *in vivo* and *ex vivo* imaging, we have observed dramatic re-distributions of adhesive foci during founder cell mitosis (supplementary material Fig. S4). In particular, these studies point to a crucial role for cytokinesis in regional maturation of adhesive foci. It is tempting to speculate that invasion may serve to anchor a subset of adhesive foci, allowing them to persist during de-adhesion associated with mitotic rounding. Subsequently, cytokinesis may stimulate tension directed maturation/enlargement of these 'mitotic anchors' (Puklin-Faucher and Sheetz, 2009; Weber et al., 2011). Similar processes of regional anchoring during mitosis have been previously described in studies of cultured mammalian cells (Théry and Bornens, 2006).

Delineating the precise impact of adhesion on inductive signaling

Probably the most crucial gap in our model involves the precise downstream impact of adhesion on inductive signaling. To address this gap, we must first determine whether localized induction relies primarily on regional trafficking/activation of FGF receptors or regional trafficking/activation of downstream MAPK components. Previously, we attempted to visualize FGF receptor activation through the use of phospho-Tyrosine (p-Tyr) antibody staining (Cooley et al., 2011). We found that p-Tyr is enriched along the presumptive TVC membrane and that p-Tyr enrichment requires FGF signaling (Cooley et al., 2011). Furthermore, we observed a gradual spatial restriction of p-Tyr staining along the presumptive TVC membrane during mitosis. These observations suggested that p-Tyr staining accurately visualized localized FGF receptor phosphorylation underlying localized induction. However, p-Tyr staining is also strongly associated with

focal matrix adhesion (Berrier and Yamada, 2007). Thus, the characterized p-Tyr staining pattern may reflect maturation of adhesions along the epidermal matrix. Indeed, co-staining of mitotic Mesp-GFP-Talin founder cells with a p-Tyr antibody reveals a close correspondence between cortical GFP-Talin and membrane p-Tyr staining within the presumptive TVC (data not shown). New assays are required to visualize localized inductive signaling components. We are therefore developing FRET (Förster resonance energy transfer) and FRAP (fluorescence recovery after photobleaching) assays to directly examine subcellular trafficking and activation of FGFR and MAPK components.

Respective contributions of adhesion and protrusion to TVC induction

Although we have made some progress in unraveling the interactions between invasive protrusions and matrix adhesion, further studies are clearly warranted. In the current study, targeted perturbations of CDC42 activity indicate that localized protrusions are required for proper distribution of matrix adhesions (supplementary material Fig. S3; Fig. 7H). Additionally, disruption of Rap activity/matrix adhesion overrides enhanced induction by hyperactive CDC42 (Fig. 6). Furthermore, we have previously shown that disruption of protrusive actin dynamics leads to enhanced induction (Cooley et al., 2011). Thus, it appears that localized protrusions spatially confine rather than potentiate inductive FGF/MAPK signaling. These results suggest that adhesion directly enhances localized induction, whereas protrusion serves to modulate adhesion. However, targeted perturbations of Rap activity indicate that matrix adhesion is required for the formation of CDC42-enriched invasive membranes (Fig. 6; Fig. 7F). Additionally, constitutively active CDC42 partially restores induction in the RapS17N background with no corresponding restoration of matrix adhesion (Fig. 6I). Thus, it does not appear that adhesion acts as the sole direct modulator of localized induction. We therefore propose that protrusion and invasion cooperatively promote FGF/MAPK signaling (Fig. 7E). These cooperative interactions may involve reciprocal feedback between protrusion, adhesion and RTK signaling, as previously characterized in studies of migratory cell polarity (Hynes, 2002; Berrier and Yamada, 2007; Lock et al., 2008). Reciprocal feedback coordinates directed migration in complex embryonic environments (Gardel et al., 2010). We are interested in exploring whether similar feedback circuits mediate robust cell fate decisions in response to diffuse and dynamic inductive signals. Productive testing of these hypotheses will require a more comprehensive understanding of inductive signal polarization. In particular, it will be crucial to develop a reliable assay for examining localization of inductive signaling components.

Implications for vertebrate heart development

Widespread deployment of robust matrix adhesion complexes may obscure potential roles in cell fate patterning. Adhesive foci are protein complexes formed by dynamic interactions between hundreds of partially redundant components (Streuli and Akhtar, 2009). Owing to their resulting robustness, it may be hard to decipher the contribution of matrix adhesion complexes through gene knockout studies (as is often the case for morphogenetic processes) (Wieschaus, 1995). Additionally, perturbations that alter matrix adhesion may cause severe morphological abnormalities that obscure subtle alterations in cell fate. These caveats are relevant to studies of vertebrate heart development. Although knockout of matrix adhesion components can disrupt vertebrate cardiac

morphogenesis, these perturbations do not appear to impact heart progenitor specification. Our results suggest that refinement of inductive signaling can involve subtle and brief interactions with microenvironmental cues. Thus, high resolution, *in vivo* analysis will be required to dissect potential contributions of matrix adhesion in vertebrate heart progenitor specification.

Implications for stem cell biology

We are also intrigued by parallels between asymmetric division in *Ciona* founder cells and stem cells. Asymmetric stem cell division ensures that full pluripotency is only maintained in one daughter cell. In some cases, stem cells display regionalized adhesion to matrix proteins produced by an adjacent 'niche' (Ellis and Tanentzapf, 2010). As in founder cells, regionalized stem cell adhesive interactions can direct asymmetric division and differential fate specification (Yamashita, 2010). It is generally assumed that adhesion provides a structural cue, directing cell division geometry. However, there are indications that regional matrix adhesion directly polarizes signaling, as we have observed during *Ciona* TVC induction (Jones et al., 2006; Kloepper et al., 2008). Additionally, cell division geometry is assumed to play a passive structural role, ensuring the proper spatial relation between daughter cells and key inductive signals. A particularly novel insight provided by our study is the potential regulatory role of mitosis. Our results suggest that adhesion dynamics associated with cytokinesis polarize signaling. We anticipate that further insights into the complex interplay between adhesion, protrusion, mitosis and regional TVC induction will have profound implications for stem cell biology.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.085548/-/DC1>

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