Cell movements controlled by the Notch signalling cascade during foregut development in *Drosophila*

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Summary

Notch signalling is an evolutionarily conserved cell interaction mechanism, the role of which in controlling cell fate choices has been studied extensively. Recent studies in both vertebrates and invertebrates revealed additional functions of Notch in proliferation and apoptotic events. We provide evidence for an essential role of the Notch signalling pathway during morphogenetic cell movements required for the formation of the foregut-associated proventriculus organ in the *Drosophila* embryo. We demonstrate that the activation of the Notch receptor occurs in two rows of boundary cells in the proventriculus primordium. The boundary cells delimit a population of foregut epithelial cells that invaginate into the endodermal midgut layer during proventriculus morphogenesis. Notch receptor activation requires the expression of its ligand Delta in the invaginating cells and apical Notch receptor localisation in the boundary cells. We further show that the movement of the proventricular cells is dependent on the *short stop* gene that encodes the *Drosophila* plectin homolog of vertebrates and is a cytoskeletal linker protein of the spectraplakin superfamily. *short stop* is transcriptionally activated in response to the Notch signalling pathway in boundary cells and we demonstrate that the localisation of the Notch receptor and Notch signalling activity depend on *short stop* activity. Our results provide a novel link between the Notch signalling pathway and cytoskeletal reorganisation controlling cell movement during the development of foregut-associated organs.

Key words: *Drosophila*, Cell movement, Notch, Short stop

Introduction

The Notch signalling pathway has been shown to mediate cell fate decisions through local interactions during animal development (for a review, see Artavanis-Tsakonas et al., 1999). Studies in the *Drosophila* wing have demonstrated that the range of Notch signalling is determined by the spatial and temporal expression pattern of its ligands, Delta and the transmembrane protein Serrate (Ser), and by the activity of the glycosyltransferase Fringe (Fng) (for a review, see Blair, 2000). Fng modulates ligand affinity of Notch and plays a major role in the Notch-dependent positioning of sharp compartment boundaries. It has been shown to modify the glycosylation state of the receptor in the Golgi complex, thereby lowering its sensitivity to Ser and enhancing its sensitivity to Delta (Ju et al., 2000; Brückner et al., 2000; Moloney et al., 2000). Ligand binding to the Notch receptor results in a proteolytic intracellular processing of Notch and gives rise to the Notch intracellular domain fragment (N^{ICD}). N^{ICD} is released from the membrane and translocates to the nucleus where it interacts as a transcriptional co-activator with Supressor of Hairless [Su(H)], a ubiquitously expressed DNA-binding protein. DNA-bound complexes containing of both Su(H) and N^{ICD} are thought to activate the transcription of Notch target genes in cooperation with other transcriptional activators (for a review, see Bray and Furriols, 2001). The genes of the *Enhancer of split* [*E(spl)*] locus which encode nuclear basic helix-loop-helix proteins, are primary target genes of Notch signalling that repress neural cell fate (for a review, see Greenwald, 1998).

Phenotypic analyses in both vertebrates and invertebrates revealed that apart from the well-documented involvement of Notch in cell fate decisions, both proliferation and apoptotic events can also be affected by Notch signalling. Notch activation appears to inhibit apoptosis in murine erythroleukemia cells (Shelly et al., 1999) and the involvement of Notch activation in proliferation has been demonstrated for wing and leg development in the fly (Go et al., 1998; de Celis et al., 1998). Furthermore, recent studies on neural crest cells in the mouse have suggested additional roles for the Notch signalling pathway during cell migration. Loss of *Delta-1* in mice causes severe disruption of neural crest migration and neural crest cells become randomly dispersed through the somites instead of following a restricted movement through the rostral portion of each sclerotome (De Bellard et al., 2002). However, the mechanism by which Notch controls cell migration is still rather elusive.

We provide evidence for an essential role of the Notch signalling pathway for the morphogenetic cell movements during the formation of the foregut-associated proventriculus organ in the *Drosophila* embryo. Notch signalling activity is required in two rows of boundary cells in the proventriculus primordium. These cells delimit a population of foregut epithelial cells that undergo a coordinated series of cell shape

changes and cell movement events leading to the invagination of the ectodermal foregut epithelium into the endodermal midgut layer. We further demonstrate that the *short stop* gene, which encodes a cytoskeletal crosslinker protein of the spectraplakin superfamily (Gregory and Brown, 1998; Strumpf and Volk, 1998; Röper et al., 2002), is essential for cell movement in the proventriculus primordium. *short stop* is transcriptionally activated in boundary cells in response to Notch and we provide evidence that its activity is required for Notch receptor localisation and Notch signalling. These results connect the Notch signalling pathway with the modulation of cytoskeletal organisation in key morphoregulatory cells that drive the formation of a foregut-derived organ in *Drosophila*.

Materials and methods

Drosophila stocks and analysis of mutants

The Oregon R strain was used as wild type. For mutant analysis we used N^{55e11}/FM7 (Kidd et al., 1983); $cdc42^{3}$/TM3 (R. Fehon); Dl^{9P}/TM3 (M. Muskavitch); $Su(H)^{AR9}$/CyO (A. Preiss); fng^{13}/TM3 (T. Klein); $kak^{65\text{-}2}$/CyO (Prout et al., 1997); and $shot^{k15606}$/CyO (Gao et al., 1999). Mutant alleles were kept on FM7*Act*GFP, CyO*wglacZ* and TM3*Act*GFP balancers. Notch signalling activity was detected by the Gbe-*Su(H)m8-lacZ* reporter construct (Furriols and Bray, 2001). Ectopic expression studies were performed using the Gal4 driver lines 14-3 *fkh*-Gal4 (Fuss and Hoch, 1998), *dri*-Gal4 (R. Saint) and hsGal4 (Bloomington Stock Centre). The *dri*-Gal4 driver mediates expression in the posterior boundary cells. 14-3 *fkh*-Gal4 drives expression from stage 10 onwards in the oesophagus and in the endodermal part of the proventriculus primordium. For heat-shock-induced expression, 0-3 hour egg collections were allowed to age for 13 hours at 25°C. The heat shock was performed three times for 20 minutes in a 37°C waterbath, interrupted by two 15 minute breaks at room temperature. Prior to fixation, embryos were allowed to age for 3 hours after the heat shock protocol. As UAS effector strains, we used UAS-Dl (M. Muskavitch), UAS-N^{ICD} (G. Struhl), UAS-N^{ECD} (T. Klein) and UAS-$Cdc42^{N17}$ (R. Schuh).

Immunostainings and in situ hybridisation

Embryos were staged and stained as described previously (Fuss and Hoch, 1998). As antibodies we used: anti-$Notch^{ICD}$ (1:10, mAbC17.9C6), anti-$Notch^{ECD}$ (1:10, mAb F461.3B), anti-Delta (1:5, mAbC594.9B); antiFas3 (1:5, mAb7G10¸ Hybridoma Bank, Iowa), anti-Dve (1:1000) (Nakagoshi et al., 1998), anti-Kakapo (1:300, T. Volk), anti-Forkhead (1:100, P. Carrera), anti-MHC (1:50; D. Kiehart) and anti-β-Gal (1: 100, Promega). Fluorescent labelling was performed with $Alexa^{543}$ and $Alexa^{488}$ coupled secondary antibodies (Molecular Probes) or Cy2, Cy3 and Cy5 coupled secondary antibodies (Dianova). Fluorescent images were recorded using a Leica TSP2 confocal microscope (Leica, Wetzlar, Germany) and images of multi-labelled samples were acquired sequentially on separate channels. Digoxygenin-labeled RNA antisense probes were generated by in vitro transcription of a *shot*/*kakapo* cDNA (kindly provided by N. Brown), *Su(H)* cDNA (F. Schweisguth) and a *Ser* cDNA (cDNA clone RE42104). For *Su(H)* and *fng* RNA expression, see also http://www.fruitfly.org/cgi-bin/ex/insitu.pl

Results

The proventriculus is a multiply folded, cardia-shaped organ that functions as a valve to regulate food passage from the foregut into the midgut of *Drosophila* larvae (Strasburger, 1932). It is derived from the stomodeum, which gives rise to the foregut tube and to parts of the anterior midgut in the early embryo (Campos-Ortega and Hartenstein, 1997). Cell shape changes are initiated at stage 12 when cell proliferation has been completed within the proventriculus primordium (Pankratz and Hoch, 1995; Campos-Ortega and Hartenstein, 1997). Anti-Forkhead (Fkh)/anti-Defective proventriculus (Dve) double immunostainings which specifically visualise ectodermal and endodermal cells, respectively (Fuss and Hoch, 1998), reveal that the first step of proventriculus morphogenesis involves the formation of a ball-like evagination at the ectoderm/endoderm boundary of the posterior foregut tube (Fig. 1A). The formation of this evagination is initiated by a local constriction of apical membranes at the ectoderm/endoderm boundary leading to an accumulation of membrane-associated markers such as Arm towards the luminal (apical) side (Fig. 1E). It is of note that the ectodermal part of the ball-like evagination localises in a mesoderm-free region, whereas the surrounding cells of the developing foregut and the midgut are covered by visceral mesoderm (Fig. 1I,J) (Pankratz and Hoch, 1995). At stage 14, a constriction forms at the boundary of the ectodermal and the endodermal cells (Fig. 1B,F,J). This results in the formation of the ‘keyhole’ structure that we have described previously (Fig. 1B) (Pankratz and Hoch, 1995; Fuss and Hoch, 1998; Bauer et al., 2002). From stage 14 onwards, cells from the anterior portion of the ectodermal keyhole part (in the mesoderm-free area) begin to move inwards into the endodermal part of the keyhole and a heart-like structure is formed (Fig. 1C,G,K). The ectodermal keyhole cells continue to move inward until late stage 17 (Fig. 1D,H,L) and give rise to the recurrent layer of the proventriculus; it links the outer endodermal layer (derived from the endodermal keyhole cells) and the inner layer of the proventriculus which is a continuation of the oesophagus (King, 1988). The cells at the tip of the invaginating ectodermal keyhole cells which derive from the most anterior region of the keyhole, are not covered by visceral mesoderm (Fig. 1L). It has been observed before that these cells assume a stretched appearance with long cytoplasmic extensions (Pankratz and Hoch, 1995). The different steps of proventriculus development are shown schematically in Fig. 1M-Q.

Notch signalling is required for cell movements in the proventriculus primordium

The Notch receptor ligands Delta (Fig. 2A-D) and Ser (not shown; *Ser* mutants do not show a proventricular phenotype) (B.F. and M.H., unpublished) are specifically expressed in the invaginating ectodermal keyhole cells from early stages of proventriculus development until the end of organogenesis. From stage 15 onwards, Delta becomes downregulated in the most anterior and the most posterior cells of the ectodermal keyhole domain (the latter are positioned directly at the ectoderm/endoderm boundary, see arrowheads in Fig. 2C,D). By contrast, Notch receptor expression is strongly elevated in these two cell rows from stage 13 onwards compared with the surrounding epithelial cells, as revealed by anti-Notch/anti-Dve double immunostainings (Fig. 2E-L). The Notch receptor continues to be upregulated in these two cell rows, which we designate as the anterior and posterior boundary cells, respectively, until late stage 17. The Notch receptor expression domain in the anterior boundary cells forms the tip of the ectodermal keyhole cells that invaginate into the endodermal part of the keyhole (Fig. 2G,H,K,L, designated ‘ac’), whereas

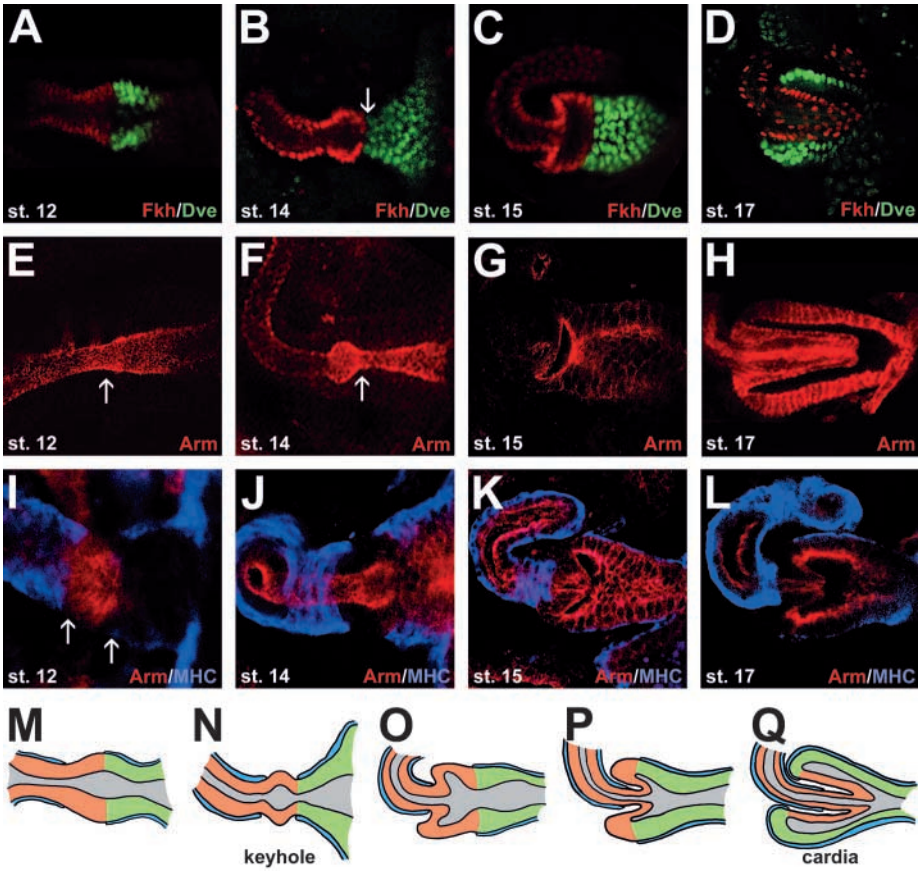

Fig. 1. Cell movement during proventriculus development. Developing proventriculi of stage 12 (A,E,I), stage 14 (B,F,J), stage 15 (C,G,K) and stage 17 (D,H,L) wild-type embryos. (A-D) Anti-Fkh (red)/anti-Dve (green) marking ectodermal and endodermal cells, respectively. A constriction separates the ectodermal and endodermal part of the keyhole (arrow in B, see also F). (E-H) anti-Armadillo staining. Note the concentration of Arm towards the apical side of the cells (arrow in E,F). (I-L) Anti-Arm(red)/anti-MHC (blue) immunostaining. The mesoderm-free region that lacks MHC expression is marked by arrows in I. (M-Q) Schematic representation of the different stages of proventriculus development and the cell movements resulting in the invagination of the ectodermal keyhole cells. Ectodermal cells in orange, endodermal cells in green and mesodermal cells in blue.

the Notch receptor expression domain in the posterior cells becomes localised at the rim of the developing proventriculus (Fig. 2G,H,K,L, designated 'pc'). *Su(H)*, which encodes the only known transducing transcription factor of the Notch signalling pathway, is rather ubiquitously expressed in the developing proventriculus; the glycosyltransferase Fng is expressed in domains anteriorly and posteriorly to Delta (data not shown; see also http://www.fruitfly.org/cgi-bin/ex/insitu.pl). The Notch-dependent genes of the *E (spl)* complex are not expressed during proventriculus development (Welch et al., 1999).

To determine the range of Notch signalling in the ectodermal keyhole cells, we used a *lacZ*-reporter construct carrying multiple Su(H)-binding sites from the *E(spl) m8* gene combined with binding sites for the transcription factor Grainyhead (Grh) (Furriols and Bray, 2001; Bray and Furriols, 2001). In cells, in which Notch signalling is active and Grh is expressed, Su(H) cooperates with Grh to yield high levels of reporter gene expression, whereas reporter gene expression is repressed in cells in which Notch is inactive (Furriols and Bray, 2001). Reporter gene expression in corresponding transgenic embryos reflects the range of Notch signalling. We used this construct previously to demonstrate that Notch signalling is restricted to the boundary cells that separate the dorsal from the ventral half of the hindgut (Fuss and Hoch, 2002). As shown in Fig. 2M-P, the Notch-dependent reporter construct is activated from stage 12 onwards until late stages of proventricular development in two domains that are localised directly adjacent to the Delta expression domain. Both Notch activity domains encompass the cells of the anterior and posterior boundary cells in which the Notch receptor is upregulated (compare the β-Gal pattern in Fig. 2P with Notch receptor expression in L).

Notch signalling is required for cell movements in the developing proventiculus

In *Delta*, *Notch*, *Fng* and *Su(H)* mutants, the early steps of proventricular development including the formation of the ball-like evagination at the ectoderm/endoderm boundary occur normally (Fig. 3A,D,G,J). However, the anterior boundary cells of the ectodermal keyhole region do not initiate cell movements to invaginte into the endodermal cell layer (Fig. 3B,E,H,K). Rather, the ectodermal keyhole cells arrest anteriorly and do not move inwards until late stages of embryonic development (Fig. 3C,F,I,L). Furthermore we find a significant collapse of the endodermal proventriculus rim (Fig. 3C,F, compare with wild type in Fig. 2D) suggesting defective function of posterior boundary cells in which the Notch receptor is expressed (Fig. 2K,L). Note that the number of ectodermal cells is not changed in both *Delta* and *Notch* mutants, indicating that no cell death has occurred. A very similar phenotype is also obtained in embryos in which Delta is ectopically expressed at a high level in the cells of the posterior foregut and the anterior midgut using the 14-3*fkh*Gal4 driver and the UAS-Dl effector line (Fig. 3M). This indicates that restricted expression of Delta in the ectodermal cells of the keyhole and its downregulation in the boundary cells may be necessary for the Notch signalling-dependent inward

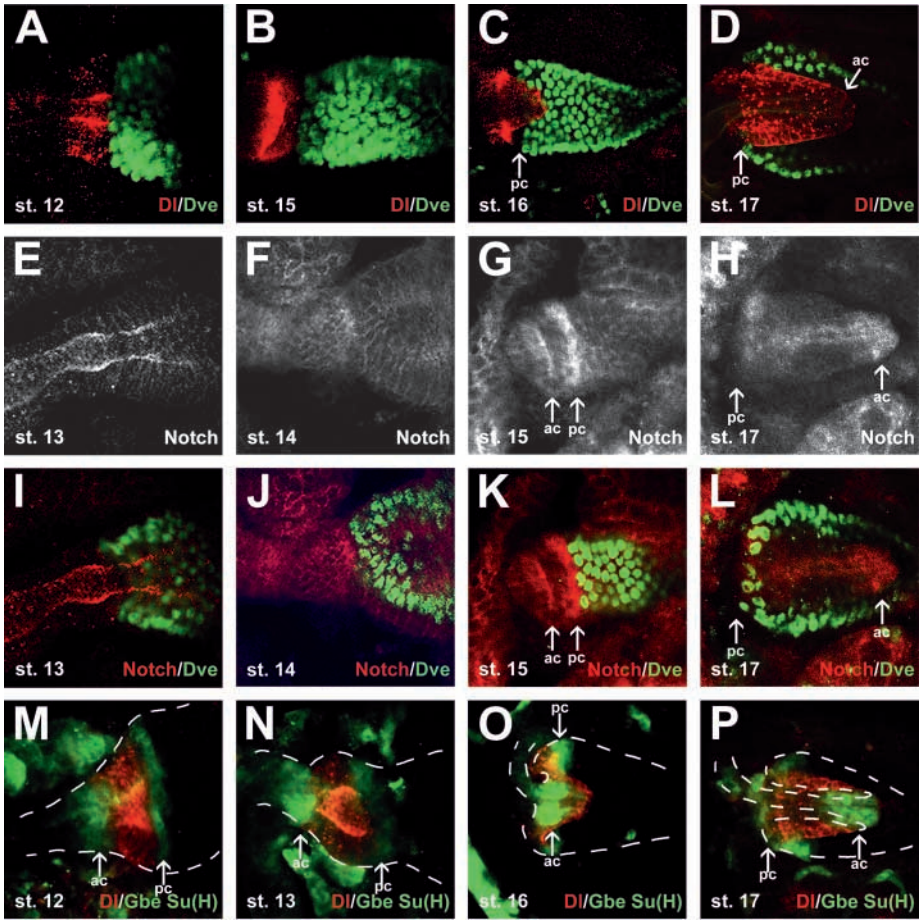

Fig. 2. Expression of members of the Notch signalling pathway during proventriculus development. (A-D) *Dl* expression in the ectodermal cells of the keyhole monitored by Anti-Dl (red)/anti-Dve (green) immunostaining at stage 12 (A), stage 15 (B), stage 16 (C) and late stage 17 (D). Delta localises to the ectodermal keyhole domain which moves into the endodermal cell layer. Arrows (C,D) mark the downregulation of Dl expression in anterior (ac) and posterior (pc) boundary cells. (E-L) Dynamic Notch receptor expression visualised in a single channel visualisation (E-H) and in an anti-Notch (red)/anti-Dve (green) immunostaining (I-L). (E,I) Stage 13; (F,J) stage 14; (G,K) stage 15; (H,L) late stage 17 wild-type embryos. The Notch receptor is upregulated in ac and pc. (M-P) Relative localisation Delta (green) and Notch signalling activity [Gbe-*Su(H)m8-lacZ;* red] during proventriculus development. The epithelial gut tube is surrounded by broken lines; ac and pc are highlighted by arrows. Notch signalling activity is restricted to the ac and pc, which are adjacent to the Delta expression domain.

movement. Notch-dependent reporter gene expression of the Gbe-Su(H)$_{m8}$ reporter is abolished in the anterior and posterior boundary cells when Delta is ectopically expressed in these embryos (B.F. and M.H., unpublished). When we ubiquitously express the Notch extracellular domain (N^{ECD}), which acts as a dominant-negative form of the Notch receptor, the ectodermal keyhole cells fail to complete the inward movement. By contrast, ectopic activation of Notch signalling by overexpressing the Notch intracellular domain (N^{ICD}) in the proventricular cells causes ectopic cell movements. However, we do not observe changes in endodermal or ectodermal cell fate in these embryos (Fig. 3O). In summary, these results suggest that the Notch signalling pathway controls cell movement of the proventricular epithelial cells via its localised activity in the anterior and posterior boundary cells.

The expression of the *Drosophila* spectraplakin *short stop* is controlled by the Notch signalling pathway in the posterior boundary cells

In a search for further genes controlling cell movement in the proventriculus, we identified the *short stop* (*shot*) gene as a key regulator of proventriculus morphogenesis. *shot* is allelic to *kakapo* (Gregory and Brown, 1998; Strumpf and Volk, 1998) and encodes a member of the recently named spectraplakin superfamily of cytoskeletal linker proteins (Röper et al., 2002). Shot is composed of a C-terminal microtubule-binding domain, a N-terminal actin binding domain and a plakin-repeat domain that may interact with transmembrane cell adhesion receptors (Leung et al., 1999; Röper et al., 2002). A role of *shot* has been shown for cytoskeletal organisation in tracheal cells (Lee and Kolodziej, 2002a; Lee and Kolodziej, 2002b), in neuronal support cells (Kuang et al., 2000), in muscle attachment cells (Prokop et al., 1998; Stumpf and Volk, 1998) and for the adhesion between and within germlayers in the embryo (Gregory and Brown, 1998).

Shot accumulates cortically on the apical side of all the ectodermal cells of the ectodermal keyhole domain that will subsequently move inwards, as shown by anti-Shot/anti-Dve antibody staining (Fig. 4A,B,E,F). In the adjacent endodermal cells, Shot is localised apically and also basally in a spot-like pattern at the interface to the overlying visceral mesoderm, reflecting most likely its requirement for the attachment of endodermal and mesodermal germ layers (Fig. 4A, arrowheads) (Gregory and Brown, 1998). From the keyhole stage onwards, we find a high level of Shot accumulation in the posterior boundary cells of the keyhole (Fig. 4C,D,G,H), whereas its expression in the anterior boundary cells (Fig. 4D,H) becomes localised to the tip of the invaginating cells. The accumulation of Shot in the posterior boundary cells is specific and not due to the fold of the epithelium, as other apical markers, such as Armadillo, are not upregulated in the posterior boundary cells (Fig. 4I). In amorphic *shot* mutants, initial formation of the ectodermal keyhole region is normal (Fig. 4J), but the inward movement of these cells into the endodermal keyhole domain

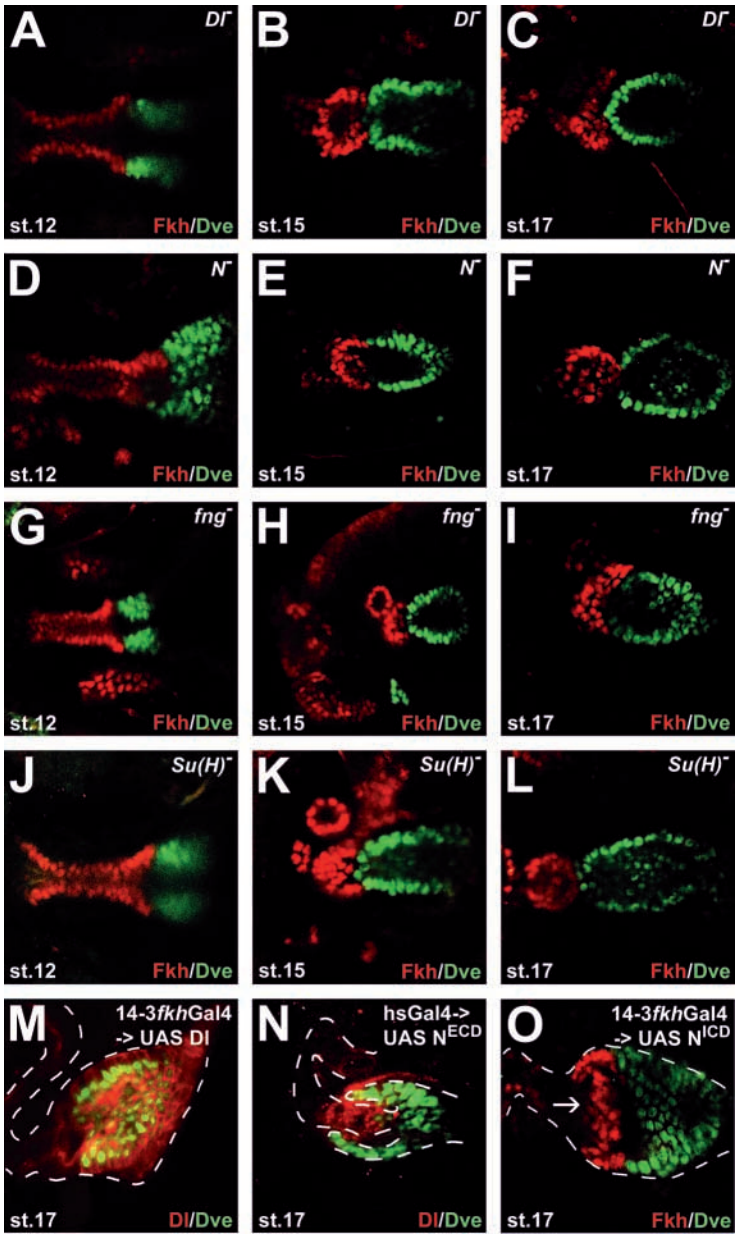

Fig. 3. Notch signalling is required for cell movements during proventriculus morphogenesis. Anti-Fkh(red)/anti-Dve (green) immunostaining of *Dl* (A-C), *Notch* (D-F), *fng* (G-I) and *Su(H)* (J-L) loss-of-function mutants at stage 12 (A,D,G,J), stage 15 (B,E,H,K) and stage 17 (C,F,I,L) of proventriculus development. Specification of the early proventriculus primordium is not affected in any of the mutants (compare with wild type, Fig. 1A), whereas cell movements leading to the keyhole structure at stage 15 (compare with wild type, Fig. 1C) and to the cardia structure at stage 17 (compare with wild type, Fig. 1D) do not take place, leading to block of invagination in mutants of the Notch signalling cassette. (M) Ectopic 14-3*fkh*-Gal4 mediated expression of the Notch ligand Dl causes a *Notch*-like phenotype, i.e. loss of invagination of ectodermal cells, as shown by anti-Dl (red)/anti-Dve (green) double staining. (N) Ectopic hsGal4 mediated expression of the Notch extracellular domain (N^{ECD}) also abrogates infolding of ectodermal cells at late stages of proventriculus development, visualised by anti-Dl(red)/anti-Dve(green) double staining. (O) Anti-Fkh (red)/anti-Dve (green) double staining showing that ectopic activation of the Notch signalling pathway causes ectopic cell movements (arrow). However, we do not observe changes in endodermal or ectodermal cell fate in these embryos.

fails to occur (Fig. 4K). Furthermore, the rim of the proventriculus is significantly reduced in size (Fig. 4L). The different steps in the manifestation of this mutant phenotype are very similar to *Delta*, *Notch*, *fng* and *Su(H)* mutants (compare Fig. 4J-L with Fig. 3A-L). Phalloidin stainings reveal an enrichment of actin cytoskeletal structures towards the apical sides of all the proventricular cells (Fig. 4M,N). Whereas in the anterior boundary cells, low levels of actin can be detected in the tip of the invaginating cells (Fig. 4N, ac), we find high levels of phalloidin staining on the apical side of the posterior boundary cells (Fig. 4M, pc, arrowheads). This indicates the presence of abundant actin cytoskeletal structures in an apical position in the posterior boundary cells, in which we also find Shot to be localised (Fig. 4M, compare with 4G). In amorphic mutants of the small GTPase Cdc42, which controls F-actin polymerisation in many developmental contexts (Nobes and Hall, 1999; Hall, 1998), the ectodermal keyhole cells fail to invaginate into the proventricular endoderm, resulting in a mutant phenotype that is reminiscent of *shot* and *Notch* mutants (Fig. 4O). Notably, ectopic expression of a dominant-negative form of Cdc42 in the posterior boundary cells using the *dri*-Gal4 driver causes a similar invagination defect of the ectodermal keyhole cells (Fig. 4P; Materials and methods).

As Shot accumulates at high levels on the apical sides of the posterior boundary cells, we tested whether this accumulation is dependent on Notch signalling. In amorphic *Notch* mutants, we still find basal levels of Shot expression both apically and basally in all the proventricular cells, as it is in wild-type embryos of the same stage. However, in *Notch* mutants, no upregulation of Shot occurs in the posterior boundary cells (Fig. 5B, compare with wild type in A; see arrowheads), arguing that either *shot* transcription or the accumulation/stability of the Shot protein may be dependent on Notch signalling. As shown above, Notch signalling is confined to the anterior and posterior boundary cells. To further test whether *shot* transcription is dependent on Notch signalling, we ectopically activated the Notch signalling pathway by expressing N^{ICD} using the 14-3*fkh*-Gal4 driver and the UAS-N^{ICD} effector lines (see Materials and methods). Ectopic activation of the Notch signalling pathway results in an ectopic activation of *shot* transcription, as determined by in situ hybridisation experiments using an antisense *shot* RNA probe (Fig. 5C,D). In summary, the lack and gain-of-function experiments provide strong evidence that the Notch signalling pathway directly or indirectly activates *shot* expression.

The cytoskeletal linker protein Shot is required for the localisation of the Notch receptor in the boundary cells

In the posterior boundary cells, high levels of Shot are localised at the apical cortex of the cells (Fig. 4G,H). It is known from vertebrate studies that the plakin domain of BPAG1e binds directly to the transmembrane protein BPAG2 (Hopkinson and Jones, 2000). To test whether the localisation of the Notch receptor is dependent on Shot, we analyzed Notch expression in *shot* mutants. From the early stages of proventriculus development onwards,

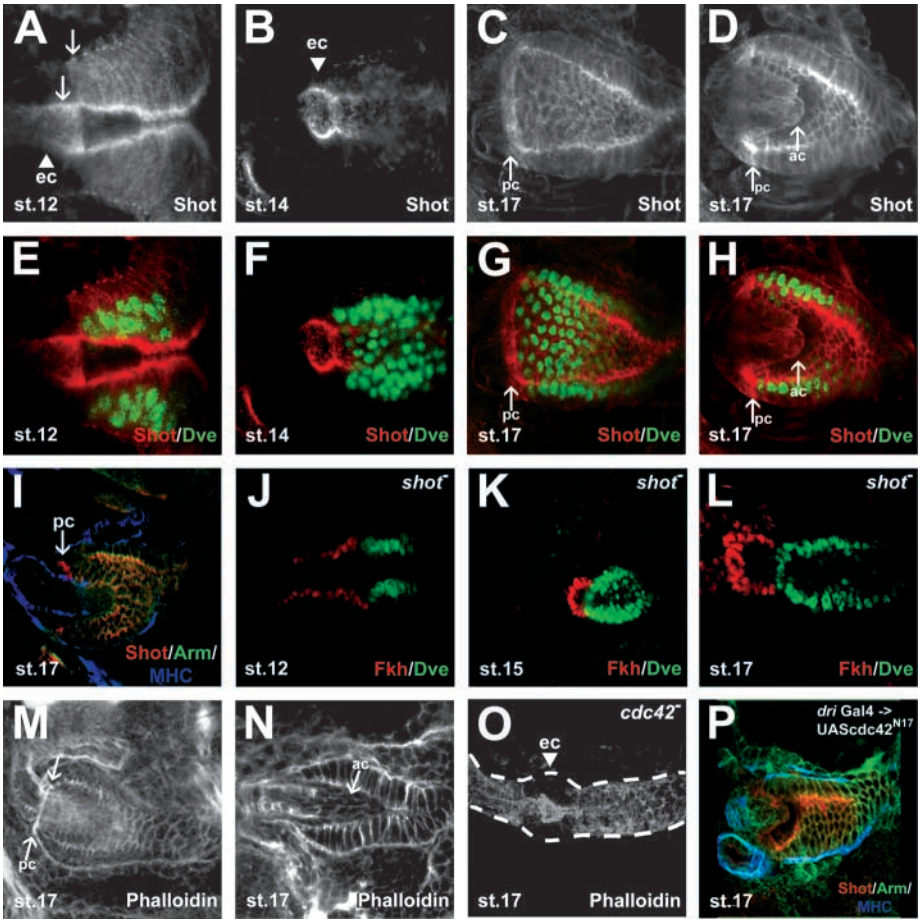

Fig. 4. Shot expression during proventriculus development. (A-H) Anti-Shot (red)/anti-Dve (green) antibody stainings of wild-type embryos of stage 12 (A,E), 14 (B,F) and 17 (C,G, tangential section; D,H, sagittal section). (A-D) Single channel visualisation of Shot expression. Shot localises to the apical side of the ectodermal (ec) keyhole domain (lower arrow in A) and to the apical and basal sides of the neighbouring endodermal cells that are covered by visceral mesoderm (upper arrow in A). During invagination, Shot protein is upregulated on the apical side of the posterior boundary cells (pc in C,D). Shot expression is reduced in the ac (D,H). (I) Anti-Shot (red)/anti-Arm (green)/anti-MHC (blue) immunostaining at stage 17 visualising uniform expression of Arm throughout the proventriculus epithelium and locally restricted elevation of Shot in the pc. (J-L) Anti-Fkh(red)/anti-Dve (green) immunostaining of *shot* mutants at stage 12 (J), stage 15 (K) and stage 17 (L) revealing the failure of ectodermal cells to invaginate and a collapse of the proventricular endoderm. (M-O) Phalloidin stainings visualising the actin cytoskeleton of stage 17 wild-type embryos (M,N) and a *cdc42* mutant embryo (O). In wild type, actin filaments accumulate on the apical side of pc (M) whereas lower levels of actin filaments are seen in the ac that move inward (N, arrow). (O) In *cdc42* mutants, cell movements leading to the keyhole structure are not initiated, the endodermal proventriculus epithelium is collapsed and ectodermal cells fail to invaginate. (P) Anti-Shot (red)/anti-Arm (green)/anti-MHC(blue) triple staining of a late stage 17 embryo in which a dominant-negative form of the GTPase Cdc42 was expressed in the posterior boundary cells. Note the failure of invagination.

Notch receptor expression is specifically reduced in the ectodermal cells of the keyhole region which undergo extensive cell movements, whereas there are basal levels of Notch expression in adjacent cells of the developing oesophagus or the proventricular endoderm (Fig. 5E,F; note that the ectodermal keyhole cells (ky) are still present in *shot* mutants). This effect is seen both with an antibody against the Notch intracellular domain (data not shown) and with an antibody against the Notch extracellular domain (Fig. 5E,F). To test whether Shot-dependent localisation of the Notch receptor is required for Notch signalling activity, we monitored the expression of the Gbe-Su(H)$_{m8}$ reporter construct in amorphic *shot* mutants. As shown in Fig. 5G,H, Notch dependent reporter gene expression is already reduced prior to the onset of the invagination movement in the posterior boundary cells of late stage 14 *shot* mutants (Fig. 5H); the reduction of Notch signalling in the posterior boundary cells is observed until late stages (Fig, 5I,J). These results suggest that Shot is crucial for proper Notch signalling in the posterior boundary cells.

Discussion

Previous work has shown that Notch signalling is an evolutionarily conserved mechanism to control cell fates through local cell interactions (Artavanis-Tsakonas et al., 1999). Our results suggest a crucial role of Notch for controlling morphogenetic cell movements within the proventriculus primordium. Furthermore, the activation of *shot* transcription in response to Notch signalling provides a novel link between the Notch signalling pathway and the modulation of cytoskeletal architecture during morphogenesis.

Notch signalling and the control of cell movement during proventriculus development

Immunohistochemical analysis demonstrates that the ligands of the Notch receptor, Delta and Serrate are expressed in the ectodermal keyhole cells that invaginate into the endodermal cell layer during proventriculus development. Their expression becomes downregulated in the anterior and posterior boundary cells in which the Notch receptor is elevated (Fig. 2C,D) and in which the Notch signalling pathway is activated, as demonstrated by the Notch-dependent Gbe-*Su(H)m8-lacZ* reporter construct (Fig. 2O,P). Whereas there is no proventricular phenotype in *Ser* mutants, the invagination movement of the ectodermal keyhole cells is defective in mutants of other components of the Notch signalling pathway, such as *Notch*, *Delta*, *fng* or *Su(H)* (Fig. 3). This strongly suggests that the boundary cells play a crucial role for cell

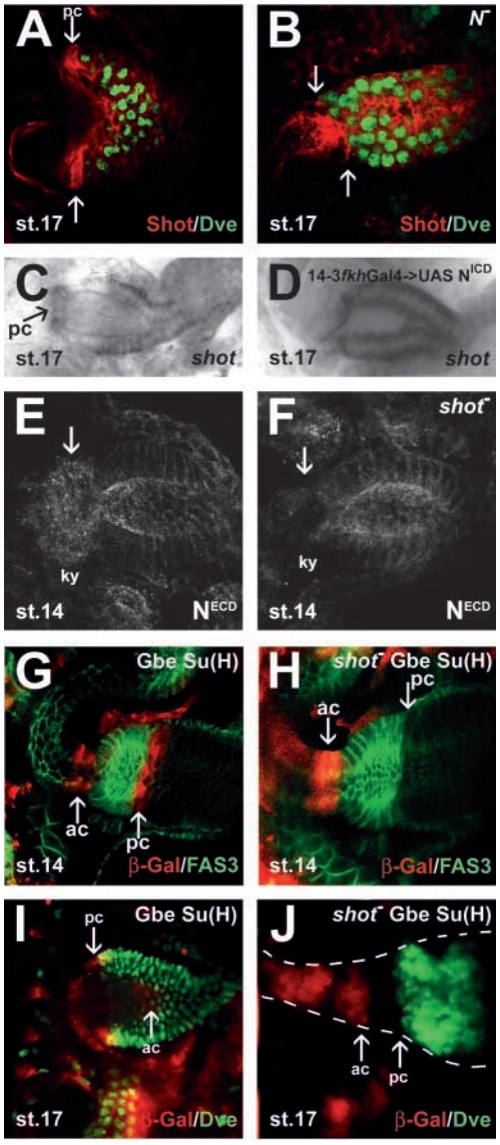

Fig. 5. *shot* is a Notch target gene and required for Notch signalling. (A,B) Anti-Shot (red)/anti-Dve (green) immunostaining of stage 17 wild-type (A) and *Notch* (B) mutant embryos. Upregulation of Shot expression in the posterior boundary cells (pc) does not occur in *Notch* mutants (arrow). (C) *Shot* mRNA expression in a wild-type embryo of stage 17 and in an embryo in which the Notch pathway has been ectopically activated (D). Note the ectopic activation of *shot* transcription as compared with wild type. (E,F) Anti-Notch^{ECD} immunostaining of the proventriculus primordium of stage 14 wild-type (E) and *shot* mutant embryos (F). Arrows in E and F indicate the keyhole. Notch receptor expression is strongly reduced in the ectodermal keyhole (ky) domain in *shot* mutants. (G,H) Anti-β-Gal(red)/anti-Fas3 (green) double staining of late stage 14 Gbe-*Su(H)m8-lacZ* embryos in a wild-type (G) and a *shot* mutant embryo (H); note the loss of reporter gene expression in pc. (I,J) Anti-βGal(red)/anti-Dve (green) double staining of stage 17 Gbe-*Su(H)m8-lacZ* embryos in wild-type (I) and *shot* mutant background (J). The activity of the Notch signalling pathway is strongly reduced in the posterior boundary cells (pc).

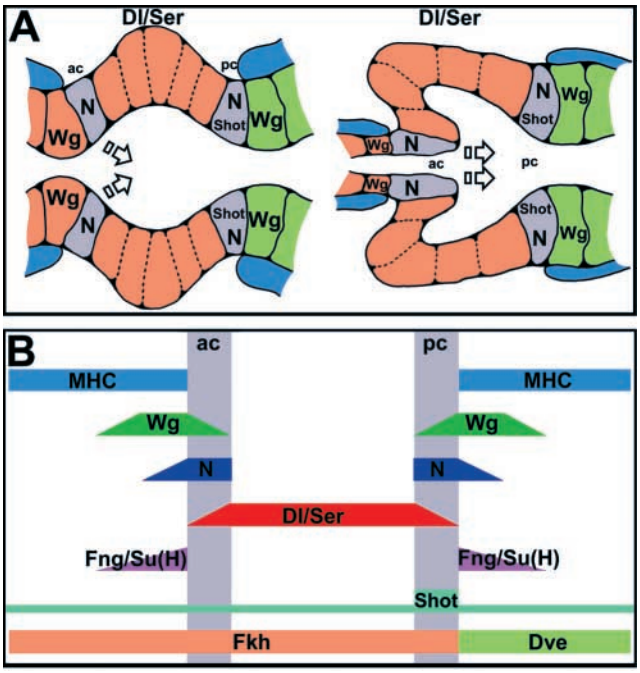

Fig. 6. Model of Notch signalling controlling cell movement in the proventriculus. (A) Schematic illustration of the proventriculus primordium in late stage 13 (left) and stage 15 (right), highlighting the expression domains of proventriculus regulators and the cell movement events. Mesoderm in blue; ectoderm in orange; endoderm in green; ac, anterior boundary cells; pc, posterior boundary cells. Arrows highlight the inward movement of the ac. Note that the number of cells in the mesoderm-free region is about 10. For a better overview of the localisation of gene activities in the proventriculus primordium, only maximum gene activities are highlighted in the cells. The expression domains of regulators of proventricular development are shown in B. For further information, see text.

movement during proventriculus development. A schematic model of the activities of the regulators during proventriculus morphogenesis is shown in Fig. 6. We do not know whether the cell movements are driven by the anterior boundary cells, dragging the oesophageal cells behind or whether the major force for the inward movement is contributed by the ectodermal foregut cells changing their shapes from a cuboidal to a more stretched appearance. The latter is known to occur during mid and late stages of embryogenesis when the foregut and the hindgut elongate dramatically increasing their size by two- to threefold (Skaer, 1993; Lengyel and Liu, 1998). It has been shown for dorsal closure that multiple forces contribute to cell sheet morphogenesis (Kiehart et al., 2000; Hutson et al., 2003). A similar scenario may apply for proventriculus morphogenesis. Genetic mosaic studies have revealed that the activity of the Notch receptor occurs in cells that are adjacent to the ligand-expressing cells (Heitzler and Simpson, 1991). Therefore, the downregulation of Delta in the boundary cells may be a prerequisite for Notch signalling and cell movement, which would be consistent with our observation that a *Notch*-like proventriculus phenotype is induced when Delta expression is maintained in the anterior and posterior boundary cells (Fig. 3M).

Recent studies on neural crest cells in the mouse also have suggested a role for the Notch signalling pathway during cell migration (De Bellard et al., 2002). The neural crest cells in vertebrates give rise to a wide range of cell types, including

nerve cells, pigment cells, as well as skeletal and connective tissue (Bronner-Fraser, 1986). These cells constitue a migratory cell population that leaves the dorsal neural tube to migrate along specific tracks to their final destinations in the periphery of the body. In *Delta1* knockout mice, the local expression of Ephrin receptors and ligands which are guiding molecules (Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999) is reduced in the caudal region of the sclerotome, as well as in neural crest-derived peripheral ganglia (De Bellard et al., 2002). A connection of Notch signalling with the modulation of cytoskeletal architecture has not been shown in these mutants. From our loss-of-function experiments, we cannot totally exclude an alternative view that Notch signalling may determine the fate of the boundary cells rather than directly controlling cell movement. However, when we ectopically activate the Notch pathway by misexpressing N^{ICD} in the proventricular endoderm, this does not result in a change of cell fates of the endodermal cells towards ectodermal boundary cell fates (Fig. 3O). Furthermore, the link between Notch signalling and the activation of *shot* which is a known cytoskeletal regulator, provides good evidence for a more direct role of Notch in controlling cell movements rather than determining cell fates.

The spectraplakin Shot may be involved in Notch receptor localisation and is required for Notch signalling

Our results further demonstrate that the *shot* gene which encodes a member of the spectraplakin superfamily of cytoskeletal linker proteins, is directly or indirectly transcriptionally regulated by the Notch signalling pathway (Fig. 5A-D). Members of the spectraplakin superfamily such as Shot in flies or dystonin/BPAG1 or MACF1 in mammals share features of both the spectrin and plakin superfamilies and produce a large variety of giant proteins of up to almost 9000 amino acids in length (Röper et al., 2002). These proteins contain motifs interacting with all three elements of the cytoskeleton, the actin, the microtubules and the intermediate filaments, and they contribute to the linkage between membrane receptors and the cytoskeletal elements. *shot* is strongly expressed during embryogenesis at the muscle attachment sites, which are the most prominent sites of position-dependent integrin adhesion (Gregory and Brown, 1998). An essential role for Shot has been shown for muscle-dependent tendon cell differentiation (Strumpf and Volk, 1998; Prokop et al., 1998). In the *shot* mutant tendon cells, Vein, a neuregulin-like factor that activates the EGF-Receptor signalling pathway, fails to be localised properly at the muscle-tendon junctional site; Vein is dispersed and its level is reduced (Strumpf and Volk, 1998). In these cells, Shot is concentrated at the apical and basal sides. Similarly, our results place *shot* both upstream and downstream of Notch signalling during proventricular development. In the posterior boundary cells, *shot* transcription is activated in response to Notch signalling; Shot protein, in turn, is required in the posterior boundary cells for Notch receptor localisation and/or stability as receptor expression and Notch signalling activity in the posterior boundary cells are affected in *shot* mutants (Fig. 5G-J). This indicates a feedback loop, as we have suggested previously for Crumbs-dependent localisation of the Notch receptor in the boundary cells of the hindgut (Fuss and Hoch, 2002). It is not clear how *shot* expression is regulated in the ac cells, in which it may require additional inputs from other yet unknown signalling pathways. Further molecular and biochemical experiments will have to demonstrate whether there exists a direct interaction between the Notch receptor and the cytoskeletal Shot protein.

Apical localisation of the actin cytoskeleton in the posterior boundary cells

In the tracheal system, Shot is required for the formation of the RhoA-dependent F-actin cytoskeleton in the fusion cells and to form the lumenal connections between tracheal branches (Lee et al., 2000; Lee and Kolodziej, 2002a). It has been suggested that Shot may function downstream of RhoA to form E-cadherin-associated cytoskeletal structures that are necessary for apical determinant localisation. Our analysis of the actin cytoskeleton using phalloidin staining reveal a strong apical localisation of F-actin filaments in the posterior boundary cells in which Shot also accumulates apically to a high level. By contrast, the density of the actin cytoskeleton is reduced in the anterior boundary cells that move inwards and in which the contribution of Shot for Notch signalling activity seems minor (Fig. 4M,N). A stabilised cytoskeletal architecture in the posterior boundary cells may be required to provide stiffness/tension that may enable the inward movement of the anterior boundary cells. Our lack- and gain-of-function results suggest that the small GTPase Cdc42 that is one of several known cytoskeletal regulators (Hall, 1998), may play a major role to control cytoskeletal architecture during the inward movement of the proventricular cells (Fig. 4O,P). These results are consistent with the idea that Notch signalling controls cytoskeletal organisation via the cytoskeletal linker protein Shot and they suggest a role for Cdc42 in this process, the specific involvement of which, however, has to be studied in more detail.

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