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# Reiterative use of signalling pathways controls multiple cellular events during *Drosophila* posterior spiracle organogenesis

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ABSTRACT

Organogenesis proceeds in multiple steps and events that need to be coordinated in time and space. Yet the genetic and molecular control of such coordination remains poorly understood. In this study we have investigated the contribution of three signalling pathways, Wnt/Wingless (Wg), Hedgehog (Hh), and epidermal growth factor receptor (EGFR), to posterior spiracle morphogenesis, an organ that forms under Abdominal-B (AbdB) control in the eighth abdominal segment. Using targeted signalling inactivation, we show that these pathways are reiteratively used to control multiple cellular events during posterior spiracle organogenesis, including cell survival and maintenance of cell polarity and adhesion required for tissue integrity. We propose that the reiterative use of the Wg, Hh, and EGFR signalling pathways serves to coordinate in time and space the sequential deployment of events that collectively allow proper organogenesis.



## Introduction

Morphogenesis and organogenesis refer to processes adopted by cells to shape an embryo or organ. Paradigms to study the underlying molecular and cellular control includes gastrulation (Kolsch et al., 2007; Leptin, 2005), germ band elongation (Bertet et al., 2004; Zallen and Wieschaus, 2004), salivary gland morphogenesis (Myat and Andrew, 2000a,b), branching morphogenesis (Affolter and Caussinus, 2008; Metzger et al., 2008) as well as posterior spiracle organogenesis (Hu and Castelli-Gair, 1999; Lovegrove et al., 2006; Merabet et al., 2005; Simoes et al., 2006).

Posterior spiracles form under the control of the Hox gene *Abdominal-B* (*AbdB*), in the eighth abdominal segment A8, and constitute the opening of the larval respiratory system. The posterior spiracle comprises two morphologically distinct structures, the spiracular chamber, an internal multicellular tube connected to the tracheal system by the spiracular branch, and the stigmatophore, an external protrusion in which the spiracular chamber is located. The spiracular chamber is composed of about 90 ectodermal cells, first organised in a flat epithelium that following invagination undergoes massive changes to eventually form a three dimensional organ.

As for other organogenetic events, spiracular chamber organogenesis requires a progression through multiple steps. The first one, primordium specification, identifies within a field of equivalent cells, those that will later form the organ. This first step, which can first be visualised by Cut expression, occurs at early stage 11 and involves yet unknown intra-segmental positional cues restricting primordium specification to an antero-dorsaly located subset of AbdB expressing cells (Hu and Castelli-Gair, 1999). The second step consists in patterning the primordium, through localised expression of regulatory molecules such as transcription factors or signalling molecules that provide cells with positional information. This occurs within a short one hour time window (late stage 11), when Hox axial positional cues interfere with intra-segmental positional cues to confer A8-specific properties to Wingless (Wg) and EGFR signalling (Merabet et al., 2005). This axial refinement of signalling during the patterning step is crucial for proper ongoing morphogenesis. Lastly, according to their location within the primordium, cells will adopt specific behaviours such as proliferation, motility, shape and polarity changes, and will enter physiological differentiation to ultimately sculpt the organ form and function (Hu and Castelli-Gair, 1999). This last step, referred to as organ formation, starts at stage 12 and comprises two major phases. The first one, organ assembling, consists in the transition from an epithelium to a three dimensional structure, giving rise to a tube connected to the trachea. This phase involves cell invagination driven by apical constriction and relies on JAK/STAT signalling, that controls the transcription of *RhoGEF*, *crumbs* and *cadherins*, impacting on cytosqueletal reorganisation, cell polarity and cell adhesion (Simoes et al., 2006). The second phase, organ elongation, starts at early stage 14 and consists in dramatic cell elongation resulting from an expansion of basolateral membranes (Hu and Castelli-Gair, 1999). The molecular control of this process has not yet been elucidated.

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How the multiple cellular events that collectively define the specific features of posterior spiracles are coordinated, such that cells undergo specific transcriptional, morphological or physiological changes only when appropriate in time and at proper positions, still remains poorly understood. In this study, we report on the role of Wg, Hh and EGFR signalling during spiracular chamber organ assembling and elongation, after primordium specification and patterning. Targeted loss of function for EGFR, Hh and Wg signalling revealed that each pathway is required for the survival of specific cell sets. In addition, by impeding cells to enter apoptosis, we showed that EGFR and Hh pathways are also required to maintain proper cell polarity and adhesion, critically contributing to tissue integrity maintenance and required for proper organ elongation. Taken together with previous findings (Merabet et al., 2005), these results support reiterative use of signalling pathways for patterning, cell survival and cellular morphogenesis.

## Results

### *EGFR, Hh and Wg signalling inactivation affects different cell sets in the spiracular chamber*

To characterise the effects resulting from signalling pathway inactivation, we first aimed to refine the cellular topological map of the spiracular chamber at the end of embryogenesis (stage 15) using previously identified markers (Hu and Castelli-Gair, 1999): the Cut protein and the ems-Gal4 driver combined with a UAS-mCD8::GFP reporter (a combination hereafter referred to as ems/GFP), general yet distinct markers of spiracular chamber cells, and the grhD4-lacZ reporter gene which labels a subset of around 15 cells located in the proximal part of the spiracular chamber. Combining ems/GFP with Cut and with grhD4-lacZ in a triple staining experiment (Figs. 1A–A‴) identifies four distinct groups of cells. The first group, Cut positive only,

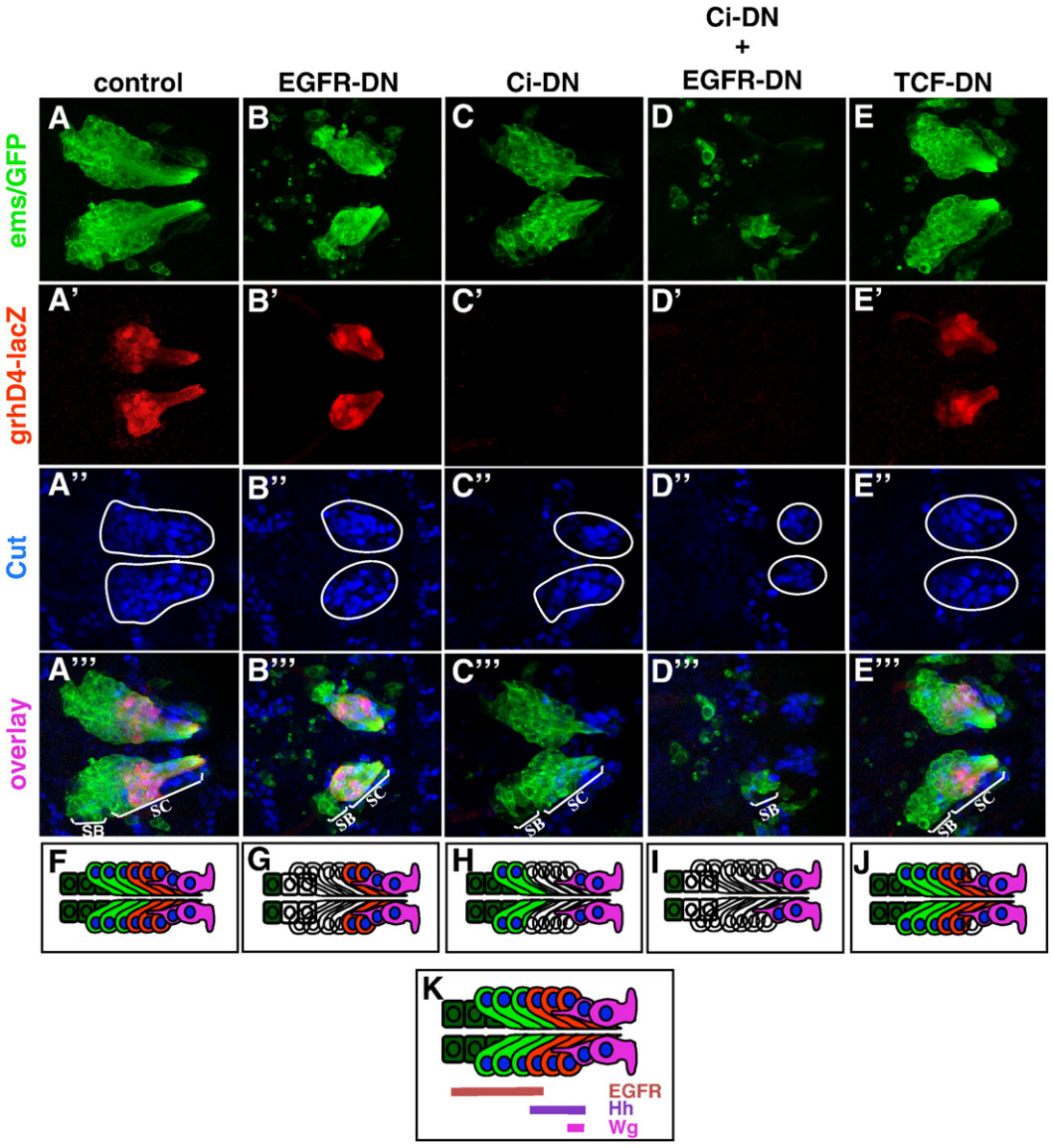



**Fig. 1.** Inactivation of signalling pathways affects different set of cells in the spiracular chamber. (A–E‴) Dorsal view of stage 15 embryos carrying the grhD4-lacZ reporter and ems-Gal4 driven mCD8::GFP, either alone (control, A–A‴) or in combination with EGFR-DN (B–B‴), Ci-DN (C–C‴), EGFR-DN + Ci-DN (D–D‴) and TCF-DN (E–E‴). (A–E‴) Spiracular cells are visualised with mCD8::GFP (green), grhD4-lacZ (red) and Cut (blue). Cut-positive cells belonging to posterior spiracle (spiracle chamber cells and spiracular hair cells) are circled in white in A″ to E″. In D″, circled cells correspond to spiracular hairs only. Brackets in A‴–E‴ indicate the position of the spiracular chamber (SC) and the spiracular branch (SB). (F–J) Schematic representation of the phenotypes observed. Spiracular hair cells are in pink; grhD4-lacZ-positive cells are in red; Cut-positive cells are represented with a blue nucleus; ems/GFP cells are the red cells + the green cells (light and dark green); spiracular branch cells are in dark green; uncoloured cells in F–J represent the cells lost by the inactivation of the signalling pathway. (K) Summary of EGFR, Hh and Wg signalling requirements.

is located close to the surface of the embryo. These cells (schematised in pink in Fig. 1F), correspond to the spiracular hair cells, the sensory organs of posterior spiracles. Slightly deeper in the organ, cells positive for Cut and ems/GFP can be resolved in two groups, based on the expression of the grhD4-lacZ marker (red for grhD4-lacZ-positive, and light green for the grhD4-lacZ-negative cells in Fig. 1F). Both groups correspond to spiracular chamber cells proper, previously described elongated "bottle" shaped cells (Hu and Castelli-Gair, 1999). The fourth group, located deeper in the organ, is only ems/GFP positive and corresponds to cells of the spiracular branch, the structure that connects the spiracular chamber to the tracheal dorsal trunk (dark green in Fig. 1F).

In order to investigate the function of EGFR, Hh and Wg signalling during spiracular chamber morphogenesis, we expressed dominant-negative (DN) forms of a critical component of each pathway: a DN form of the EGF receptor (EGFR-DN) and DN forms of the nuclear effectors cubitus interuptus (Ci-DN) and TCF (TCF-DN) to inactivate Hh and Wg pathways, respectively. In all the three cases, the DN impairs the pathway in signal receiving cells, and the phenotypic output is therefore expected to concern the domain of action of each signalling pathway. The DN forms were driven using ems-Gal4, conducting expression in all spiracular chamber and spiracular branch cells as well. This driver has been shown to be active just after primordium specification but prior to organogenesis, from late stage 11 on (Merabet et al., 2005). Inactivation of EGFR (Figs. 1B–B‴), Hh (Figs. 1C–C‴) and Wg (Figs. 1E–E‴) signalling result in distinct alterations. The most pronounced effect results from EGFR inhibition, affecting nearly all grhD4-lacZ-negative spiracular chamber cells as well as most of the spiracular branch cells (white cells in Fig. 1G), while Hh and to a lesser extent Wg inhibition affects the grhD4-lacZ-positive spiracular chamber cells (white cells in Figs. 1H, J). Inactivating simultaneously EGFR and Hh signalling results, with the exception of the spiracular hairs, in the loss of all spiracular chamber cells. The few remaining cells are exclusively GFP positive and therefore can be identified as spiracular branch cells (Figs. 1D–D‴, I). This further support that EGFR and Hh signalling affect distinct set of cells (Fig. 1K).

These results show that down-regulating each of the three pathways everywhere in the developing organ following organ specification and patterning results in defects confined to specific parts of the spiracular chamber and branch.

### *EGFR, Hh and Wg signalling are required for cell survival*

The loss of cells observed upon DN's expression suggests that signalling pathway inhibition induces cell death. In agreement with this, we noticed that a fraction of spiracular chamber and branch cells lacking EGFR, Hh or Wg signalling appeared fragmented (data not shown). To confirm that cell death follows pathway inactivation, we used an antibody against the cleaved caspase 3 (activated form of caspase 3) to detect apoptotic cells. Consistent with the previous finding that posterior spiracle formation does not require cell death (Hu and Castelli-Gair, 1999), control embryos only rarely exhibited dying cells in the developing spiracle chamber (Figs. 2A–A″). In contrast, apoptotic cells were consistently observed in signalling mutant spiracular chambers shortly after DN's expression, as soon as early stage 12 (Figs. 2B–B″, C–C″ and D–D″). Although difficult to estimate precisely (because apoptosis occurs over a 3-hour time window till stage 13), the number of apoptotic cells is clearly related with the strength of the phenotypes seen at stage 15 (Fig. 1): it is higher upon EGFR pathway inhibition compared to that of Hh or Wg (Figs. 2B–B″, C–C″ and D–D″).

To further support a role of EGFR, Hh and Wg signalling in preventing cell death, spiracular chamber cells of homozygous mutant embryos for null allele of *egfr*, *hh* or *wg* were also analyzed. Cell death (caspase staining) in spiracular chamber cells (Cut staining) were monitored at stage 12 (Figs. 2E–H″). Compared to wild-type embryos in which no cell death occurs in spiracular chambers (Figs. 2E–E″), we observed cell death in mutant embryos (Figs. 2F–H″). Notably, the amount of cell death observed correlates with the extent of cell death previously seen in the DN experiments ((Figs. 2B–D″). Finally, the restoration of appropriate posterior spiracle cell numbers in embryos simultaneously homozygous for null allele of *egfr*, *hh* or *wg* signalling, and for *Df(3L)H99* (White et al., 1994), that deletes the three death promoting genes *reaper* (*rpr*), *head involution defective* (*hid*) and *grim* (Figs. 2I–L″), further establishes that these signalling pathways prevent apoptosis.

These results suggest that the specific loss of cells in mutant spiracular chambers results from apoptosis rather than from cell relocation or transcriptional reprogramming. We therefore concluded that EGFR, Wg and Hh signalling are required for the survival of distinct groups of cells.

### *Cell loss following EGFR, Hh and Wg signalling inhibition does not impair spiracle tube formation*

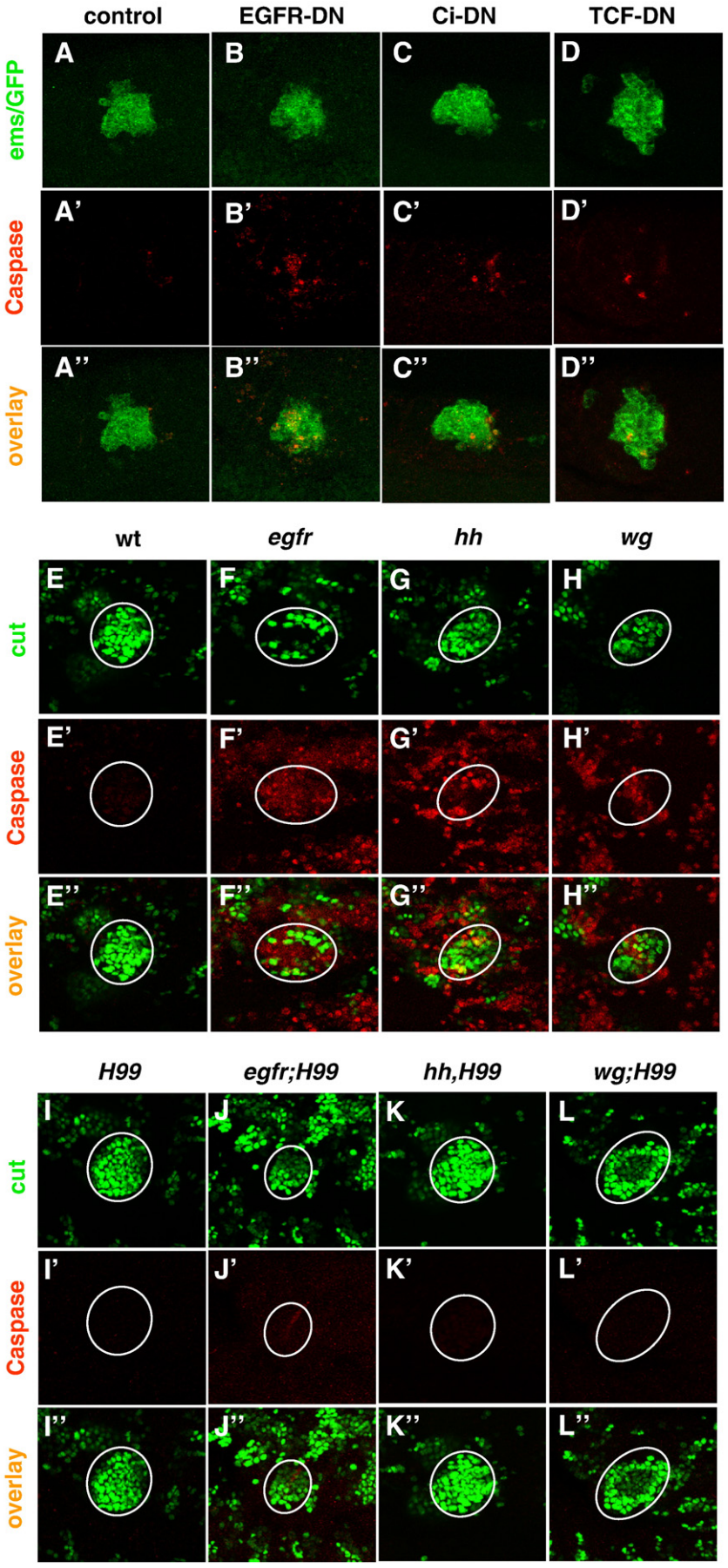
To evaluate how organogenesis proceeds upon EGFR, Hh and Wg pathway inactivation, we examined spiracular chamber tube formation, since the cells that are lost in signalling mutants normally contribute in its formation. To this end, we used an antibody against the protein Crumbs (Crb), which accumulates at high level at the apical side of cells surrounding the lumen of the spiracular chamber, and at significantly lower level in cells of the tracheal system, including the spiracular branch (Figs. 3A–A″).

We observed that impairing any of the three signalling pathways does not affect tube formation, even in the most severely affected EGFR mutant spiracular chambers (Figs. 3B–B″, C–C″ and D–D″). The transition between the spiracular chamber and branch is marked by a widening of the lumen, allowing the distinction between spiracular chamber and branch tubes (Fig. 3A′). Consistent with the extent of cell death, tubes are shorter in signalling mutants, especially in EGFR and Hh defective posterior spiracles. However, spiracular chamber and branch tubes form in all cases, indicating that surviving cells compensate the loss of cells and allow the formation of a continuous lumen between the spiracular chamber and the tracheal dorsal trunk.

### *EGFR and Hh signalling have a dual role in spiracular chamber morphogenesis*

EGFR, Hh and Wg signalling inhibition in spiracular chamber cells induces apoptosis. However, spiracular tube formation from surviving cells proceeds normally, suggesting that none of the three signalling pathways is critically required for organogenesis in cells that do not undergo apoptosis following pathway inactivation. We thus investigated the role of EGFR, Hh and Wg signalling further in cells that undergo apoptosis by expressing the caspase inhibitor P35 to block cell death. As expected since there is no apoptosis during posterior spiracle morphogenesis (Hu and Castelli-Gair, 1999), ems-Gal4 driven expression of P35 protein in control embryos does not affect spiracular chamber formation (Figs. 4A–A″, E–E″). Comparing mutant

**Fig. 2.** Inactivation of EGFR, Hh and Wg signalling induces cells death (or apoptosis) in the spiracular chamber. (A–L″) Lateral views of stage 12 embryos. (A–D″) are embryos expressing mCD8::GFP, either alone (control, A–A″) or in combination with EGFR-DN (B–B″), Ci-DN (C–C″), TCF-DN (D–D″) using ems-Gal4. Embryos shown in E–L″ are wild type in E–E″, homozygous mutant for $egfr^{f2}$ in F–F″, $hh^{21}$ in G–G″, $wg^{l-17}$ (H–H″) and respectively combined with *Df(3L)H99* deletion in I–L″. Spiracular cells are visualised in green either by ems/GFP in A–D″ or by a Cut staining in E–L″. Spiracular cells are circled in white in E–L″. Cells dying through apoptosis are labelled with an antibody against the cleaved caspase 3 (red) (A′–L″).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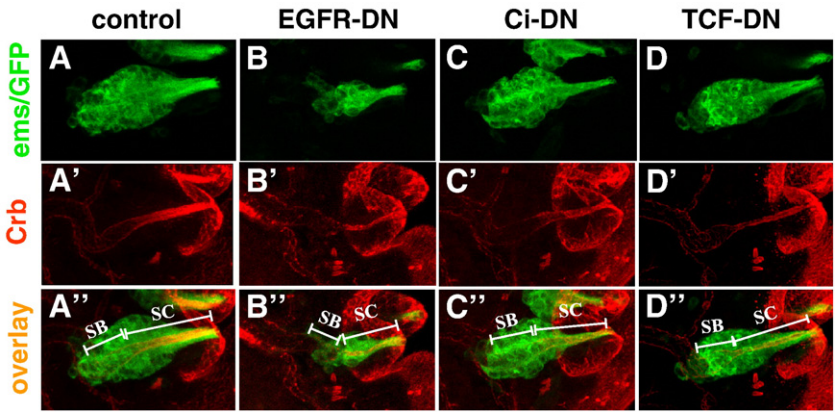



**Fig. 3.** Loss of EGFR, Hh and Wg signalling does not affect the behaviour of the spiracular chamber surviving cells. (A–D″) Semi-lateral view of stage 16 embryos. Spiracular cells expressing mCD8::GFP (green) alone (control, A) or with EGFR-DN (B), Ci-DN (C), TCF-DN (D) have elongated shape. The staining with Crb antibody (red) shows the formation of a continuous tube between the spiracle chamber and the tracheal system. Segments in A″–D″ indicate the length and position of the spiracular chamber (SC) and the spiracular branch (SB).

spiracular chambers in the absence (Figs. 1B, C, E) or presence (Figs. 4B, C, D) of P35 show an increase of ems/GFP-positive cells. This cell increase is not due to proliferation of non dying cells consequently to P35 expression since anti phospho-histone H3 staining did not reveal any cell division in the posterior spiracle (Fig. S1).

Scoring the number of grhD4-lacZ-positive cells reveals a significant increase when apoptosis is suppressed in mutant spiracular chambers for Hh and Wg signalling (Fig. 4I). This effect is by far less pronounced in EGFR mutant chambers, consistent with the previous observation that EGFR signalling mostly affects cells outside the grhD4-lacZ domain (Figs. 1B–B‴). Of note, the number of grhD4-lacZ-positive cells never reaches the value observed in wild-type controls, highlighting that apoptosis inhibition likely is incomplete, possibly reflecting insufficient levels of P35 expression owing the titration of the Gal4 protein by the presence of three UAS constructs in mutant genotypes.

Interestingly, spiracular chambers in which apoptosis and signalling pathways have been simultaneously inhibited show abnormal morphologies. The strength of the defect is correlated to the extent to which apoptosis takes place in the absence of P35, with the most dramatic phenotype accordingly occurring upon EGFR pathway inhibition. This includes a failure of the spiracular chamber to reach the final elongated shape, and the loss of regular cell arrangement, with some cells not appropriately integrated in the cell mass that constitutes the organ (Fig. 4B). These cells form an abnormal bulge in the spiracular chamber, and their position is consistent with being cells that would have undergone apoptosis in the absence of P35. Staining for Cut confirms that a large fraction of spiracular chamber cells have escaped apoptosis (Figs. 4B′–B″), and grhD4-lacZ expression in proximal most cells highlights that organ patterning is not significantly affected (Figs. 4F′–F″). Hh signalling inhibition by Ci-DN leads to a less pronounced, although qualitatively similar phenotype (Figs. 4C–C″ and G–G″). Finally, no discernable morphogenetic defect could be detected upon Wg signalling inhibition by TCF-DN (Figs. 4D–D″ and H–H″), suggesting that this pathway is only required for cell survival, as further supported by the apparently normal expression patterns of *ems*, Cut (Figs. 4D–D″) and Crb (Figs. 5D–D″).

We concluded that EGFR and Hh signalling have a dual role during spiracular chamber formation, first to prevent apoptosis of distinct cell sets, as Wg signalling does, and second, unlike Wg, to instruct cell behaviour during morphogenesis.

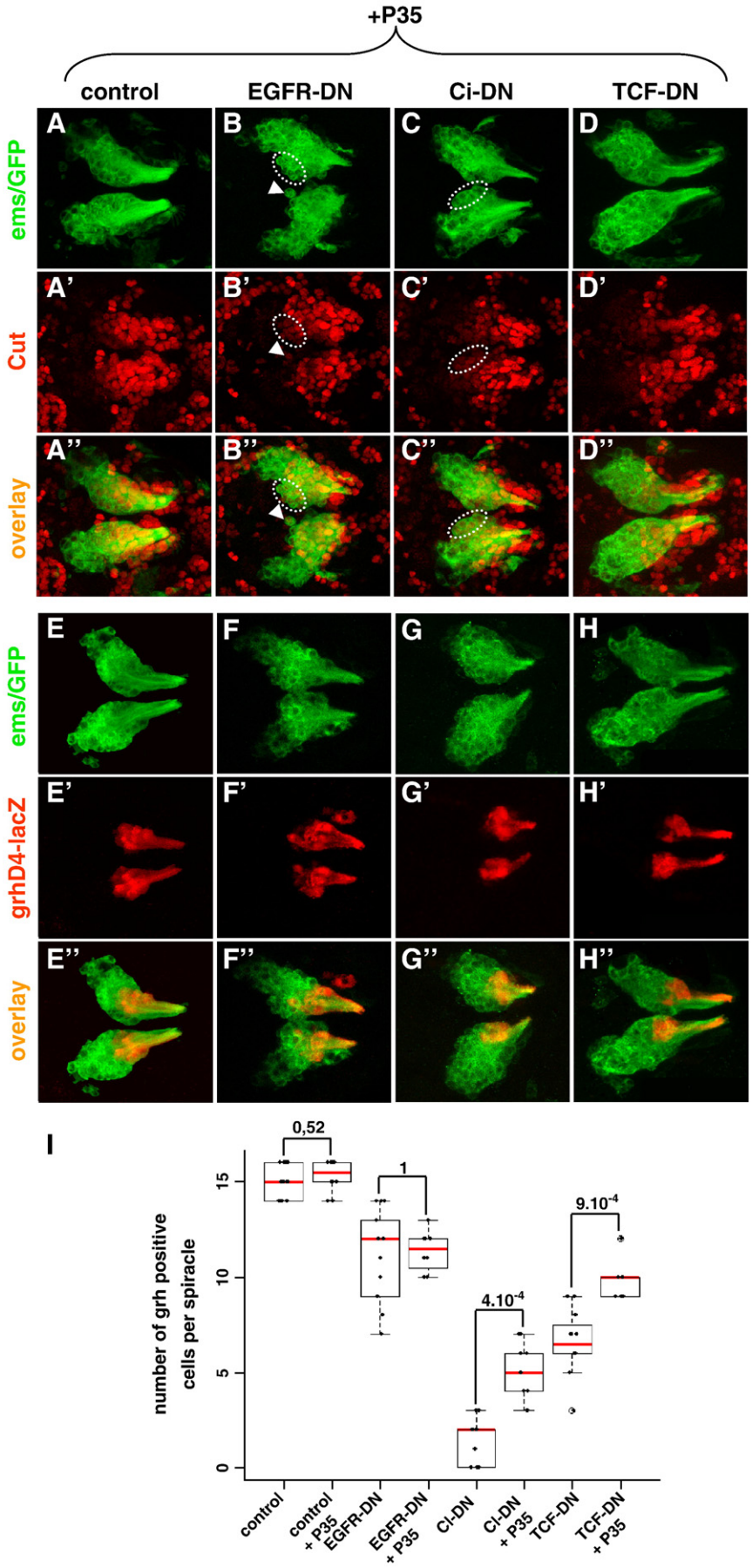
### *Early steps of spiracular chamber organogenesis are not compromised in EGFR and Hh deficient embryos*

We next aimed to identify which cellular events were affected upon simultaneous signalling and apoptosis inhibition. Driven by ems-Gal4, the expression of DN inhibitors starts at late stage 11 (Merabet et al., 2005). At that stage, most spiracular chamber cells have not yet undergone apical constriction and invagination (Hu and Castelli-Gair, 1999). These processes are not impaired by EGFR or Hh inhibition since, at stage 13, spiracular chamber cells have left the surface of the embryo and have invaginated with a normal bottle shape at appropriate proximodistal positions within the organ (not shown). Furthermore, staining for Crb shows that loss of EGFR or Hh signalling in the presence of P35 does not impair spiracular tube formation (Figs. 5B–B″ and C–C″). However, as in the situation where cell death is not inhibited (Fig. 3), these tubes are shorter compared to wild type (Figs. 5A–A″), suggesting that cells of abnormal morphology and organisation that have escaped apoptosis, localised in the characteristic bulges of EGFR and Hh mutant spiracular chambers (dashed circles in Fig. 5), do not contribute in the formation of the tube. Together with the abnormal morphology of EGFR and Hh mutant spiracular chambers at stage 16 (Fig. 4), the failure to identify defects in early steps of morphogenesis (apical constriction, invagination, transition to bottle shape) supports that these pathways are, after stage 11, critically required only for late steps of spiracular chamber organogenesis.

### *EGFR and Hh signalling are required for polarity maintenance and tissue integrity*

The round shape of signalling mutant spiracular chamber cells, as well as their occasional detachment from the developing organ (Fig. 6B′), suggest a role for EGFR and Hh signalling in cell adhesion and polarity. Spiracular chamber cells, like other epithelial cells, are held together by subapical adherens and more basal septate junctions,

**Fig. 4.** Inhibition of apoptosis uncovers a dual role for signalling pathways. (A–H″) Dorsal view of stage 16 embryos expressing mCD8::GFP (green) and the anti-apoptotic P35, alone (control, A–A″, E–E″) or in combination with EGFR-DN (B–B″, F–F″), Ci-DN (C–C″, G–G″), TCF-DN (D–D″, H–H″) driven by ems-Gal4. (A–D″) Maintenance of spiracular chamber cell identity is visualised by Cut expression (red). (A–D) P35 expression rescues cell death induced by signalling pathways inactivation; compare the GFP cell mass observed in B–D to the loss of GFP cell mass seen in Figs. 1B–E. Note the abnormal morphology of the spiracular chamber, especially for EGFR-DN (B) and Ci-DN (C); some cells are not well integrated to the structure (dashed circles in B–B″ and C–C″) and sometimes are detached from the organ (arrowhead in B–B″). However, these cells are still expressing Cut. (E–H″) Maintenance of spiracular chamber patterning is visualised by the grhD4-lacZ reporter gene (red). (I) Quantifications of the number of cell expressing grhD4-lacZ per spiracle are displayed using a boxplot representation. Numbers above the brackets indicate *p*-values. Number (*n*) of spiracles used for the quantification is between $7<n<14$.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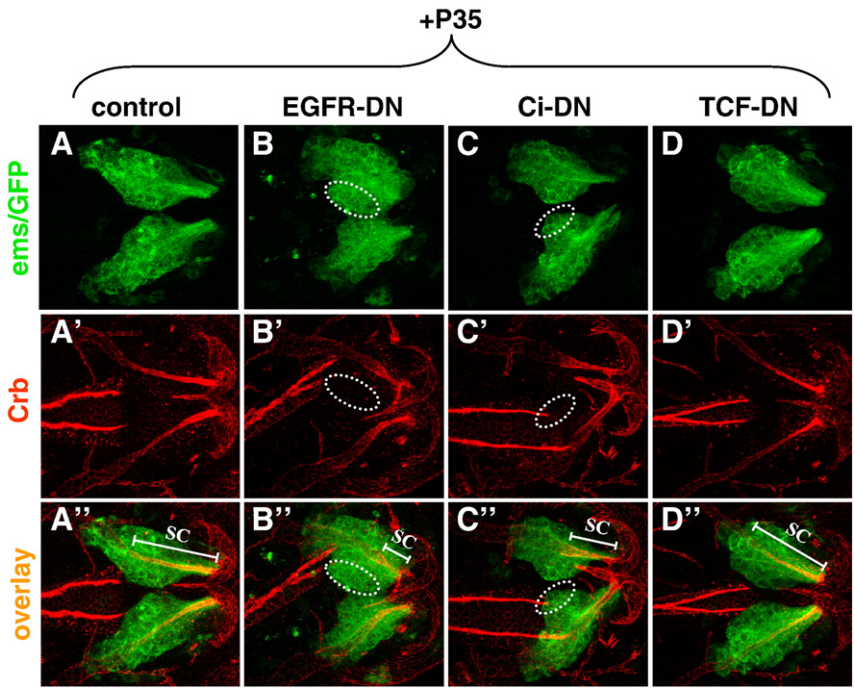


**Fig. 5.** EGFR and Hh, but not Wg signalling pathways inactivation, affect late steps of spiracle morphogenesis. Dorsal view of stage 15 embryos expressing in spiracle cells mCD8::GFP (green) and the anti-apoptotic P35, alone (control, A–A″) or in combination with EGFR-DN (B–B″), Ci-DN (C–C″) or TCF-DN (D–D″). Spiracular chamber with abnormal morphology are seen following EGFR-DN (B–B″) and Ci-DN (C–C″) expression, while no obvious phenotype are detected for TCF-DN (D–D″). Double staining with GFP and Crb (red) shows that spiracle cells are invaginating correctly but that cells localised in the abnormal bulged structures (dashed lines) do not contribute to tube formation, resulting in shorter spiracular chamber lumen. The segment in A″–D″ indicates the length and the position of the spiracular chamber (SC).

which provide cell polarity and organise the actin cytoskeleton (Bershadsky, 2004; Knust and Bossinger, 2002).

Checking the distribution of actin by an actin::GFP fusion indicates that actin is significantly enriched at the apical side of spiracular chamber cells, allowing to visualise the spiracular tube (Fig. 6A′). Such apical enrichment cannot be detected in EGFR and, to a lesser extent, Hh mutant cells that survive in the presence of P35 (dashed circle in Figs. 4B′, C′, and S2). Actin::GFP also labels, less intensely, non-apical parts of the cell membrane, allowing visualisation of cell shape, and therefore whether they participate in spiracular tube formation. Wild-type cells are elongated and have their apical side abutting the tube (Fig. 6A′), whereas signalling deficient cells do not display a bottle shape and do not project towards the spiracular tube (Figs. 6B′–C′ and S2).

We next looked at the distribution of DE-Cadherin (DE-Cad), an adhesion molecule localised at adherens junctions that is responsible for actin apical enrichment (Bershadsky, 2004; Gates and Peifer, 2005). In wild-type spiracular chamber cells, DE-Cad accumulates apically and delineates the spiracular tube (Fig. 6A″). In P35-rescued cells located in the characteristic bulges of EGFR and Hh mutant spiracular chambers, DE-Cad accumulation is no longer seen (Figs. 6B″, C″; cells in circle and Fig. S2). Two non-classical cadherins, Cad96C and Cad88C, are expressed in the spiracular chamber and cooperate with DE-Cad in posterior spiracle development (Lovegrove et al., 2006). To check whether these non-classical cadherins could also be required downstream of EGFR and Hh, we examined the expression of their encoding genes after simultaneous signalling and apoptosis inhibition. We found that expression of both genes is reduced in absence of EGFR or Hh signalling (Fig. S3), and combining EGFR and Hh loss results in a more complete reduction of *cad96C* and *cad88C* expression (Fig. S3).

We also examined the distribution of Discs large (Dlg), a protein that accumulates at the cell cortex and is enriched at septate junctions just below adherens junctions (Knust and Bossinger, 2002; Tepass et al., 2001). In stage 15 wild-type spiracular chambers, Dlg delineates the most apical part of basolateral membranes, immediately basal to the DE-Cad- and actin-enriched domain (Figs. 6D–D‴). As for DE-Cad and actin, we found that the simultaneous inhibition of EGFR (or Hh) signalling and apoptosis results in a complete loss of such Dlg accumulation in P35-rescued cells (dashed circle in Figs. 6E″, E‴ and F″–F‴).

We also made use of the *Df(3L)H99* deletion to analyse the effects of loss of EGFR and Hh signalling pathways. In homozygous embryos for *Df(3L)H99* and expressing DN form of EGFR or Ci, we observed an increase of ems/GFP-positive cells (Fig. S4), which correlates with the increase seen by blocking apoptosis through P35 expression (compare Figs. 1 to S4). Similar defects on DE-Cad and Dlg accumulation were seen in *Df(3L)H99* background (Fig. S4), and P35 expressing embryos (Figs. 6 and S2), ruling out that the defects seen results from P35 expression.

Finally, we aimed to trace the developmental dynamics of actin, DE-Cad or Dlg accumulation defects upon EGFR or Hh signalling and apoptosis inhibition. We found that none of the above-mentioned defects can be observed prior to stage 13 (not shown). We thus concluded that EGFR and Hh signalling are required for maintaining proper polarity and adhesion of spiracular chamber cells. Failure to do so results in a loss of tissue integrity and impairs the subsequent step of lateral membrane elongation that provides the organ with its ultimate and fully elongated shape.

## Discussion

### *Cell death and organogenetic plasticity*

Inhibition of EGFR, Hh and Wg signalling from early stage 12 onwards results in spiracular chambers lacking distinct sets of cells. These cells are located at different positions along the proximodistal axis of the organ: at the distal tip for Wg, distal for Hh and proximal for EGFR. This pattern of signalling requirement correlates with the patterns of expression of the Wg and Hh ligands and of Rhomboid (Rho), which identifies cells secreting an active form of the EGF ligand: Wg is only expressed in the spiracular hair cells at the distal tip

of the organ; Rho and Hh in large cell clusters within and immediately outside the spiracular chamber (Merabet et al., 2005).

Cell number reduction in signalling mutant spiracular chambers results from apoptosis, as revealed by the expression of the cell death marker caspase3, and of the significant cell rescue combining signalling inhibition by expression of the anti-apoptotic P35 molecule or the removal of the tree pro-apoptotic genes *rpr*, *hid* and *grim*. Cell death occurs a short time after signalling pathways have been inactivated, from early stage 12 onwards, as apical constriction driving cell invagination normally starts, a process not affected in our conditions of signalling inhibition. No morphological defect in the spiracular chamber can be detected before stage 14. Thus, cell death is not a secondary consequence of abnormal morphogenesis. Although our data do not address how EGFR, Wg and Hh signalling allow cells to survive, they establish that apoptosis inhibition by these pathways is required for proper spiracular chamber formation.

Spatially controlled cell death inhibition by signalling molecules have been previously reported for the EGFR pathway (Baker and Yu, 2001; Cabernard and Affolter, 2005; Cela and Llimargas, 2006). In posterior spiracle organogenesis, cell death inhibition, although in different groups of cells, in addition implies Wg and Hh signalling and may serve two purposes. First, it could modulate the final size of the organ in a precise manner by controlling the contribution of the distinct cell subsets that constitute the organ. Second, and not exclusively, it could couple the size of cell fields to be patterned with signal patterning cues themselves. In any case, spiracular chamber formation in signalling mutants still progresses through the main steps in the absence of P35: cells invaginate, elongate and reorganise around a lumen to form a tube in continuity with the tracheal system. The fact that surviving cells are able to resume a coherent behaviour to perform organogenesis while sets of neighbouring cells have been killed constitutes a remarkable example of organogenetic plasticity.

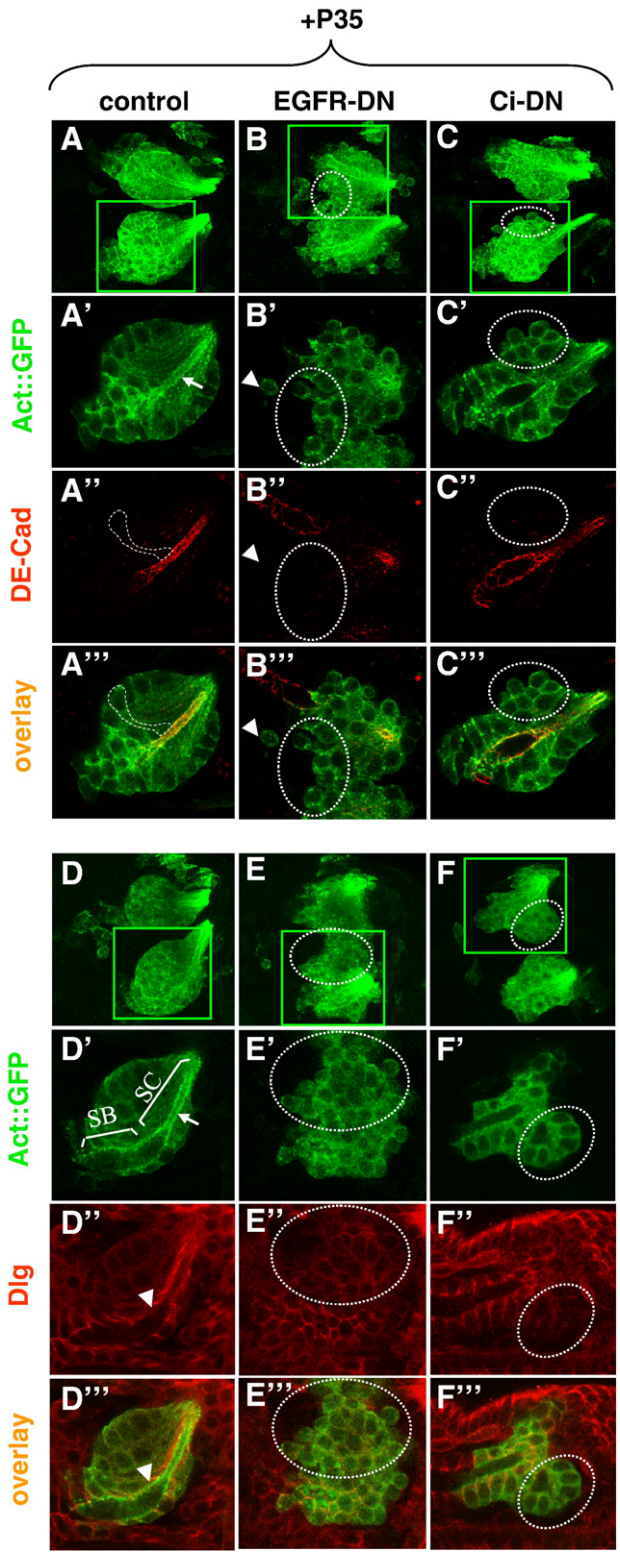


**Fig. 6.** Loss of cell polarity and adhesion in EGFR and Hh signalling mutant spiracular chamber. Dorsal view of stage 15 embryos expressing in posterior spiracular cells ems-GAL4 driver, act::GFP (green) and the anti-apoptotic P35, alone (control, A–A‴, D–D‴) or in combination with EGFR-DN (B–B‴, E–E‴) or Ci-DN (C–C‴, F–F‴). A–C and D–F are projections of confocal sections and A′–C‴ and D′–F‴ are single confocal sections of the green boxed area shown in A–C and D–F. (A–C‴) In control spiracle chambers, enrichment of actin (arrow) is seen at the apical surface of the lumen of the spiracle. Dashed lines in A″ and A‴ underline an elongated spiracular chamber cell. Note the accumulation of DE-Cad (red) and actin (green) at its apical surface. DE-Cad and actin accumulation is lost in embryos deficient for EGFR and Hh signalling (dashed circles in B′–B‴ and C′–C‴, respectively). The arrow head in B′– B‴ points the position of an isolated round shaped spiracular chamber cell without DE-Cad and actin accumulation. (D–F‴) Dlg enrichment (red) is disrupted in cell with abnormal morphology in embryos compromised for EGFR and Hh signalling (dashed circles in E′–E‴ and F′–F‴, respectively). The arrowhead in D″ and D‴ shows the normal Dlg (red) enrichment at the septate junction just below the apical accumulation of actin (green in D‴).

*Control of tissue integrity*

The involvement of regulatory molecules in cell survival can eventually mask other important functions. Previous findings have for instance shown that the role of the Forkhead transcription factor in salivary gland morphogenesis and of the PDGF/VEGF receptor in haematopoietic cell migration could only be revealed when apoptosis induced by their mutations had been blocked (Bruckner et al., 2004; Myat and Andrew, 2000a). The data reported here show that blocking apoptosis in developing spiracular chambers deficient for EGFR or Hh signalling, allowed to assign these pathways with a late function in spiracular chamber organogenesis, from stage 14 on.

EGFR and Hh pathways have been previously involved in mechanisms promoting epithelial cell invagination in *Drosophila*: EGFR through the control of the expression of the RhoGAP *crossveinless-c* gene, resulting in the apical enrichment of acto-myosin in tracheal cells (Brodu and Casanova, 2006); Hh through myosin II activation in cells of the eye morphogenetic furrow (Corrigall et al., 2007; Escudero et al., 2007). Our conditions of signalling inhibition did not reveal any role of EGFR and Hh pathways in the process of cell invagination during spiracular chamber morphogenesis. This is not due to the experimental set-up, as extensive apoptosis could be detected during the time window where cell invagination proceeds, proving that signal inhibition was effective. Previous work demonstrated that apical constriction leading to cell invagination during spiracular chamber organogenesis is instead controlled by JAK/STAT signalling (Simoes et al., 2006). Cytoskeletal and cell shape changes in response to signalling pathways

during organogenesis thus hardly obey a simple rule and may integrate combinatorial use of regulatory information, as exemplified by the cooperative use of Hh and TGF-β pathways for promoting cell invagination during eye morphogenetic furrow formation (Corrigall et al., 2007).

Our results rather support a role for EGFR and Hh signalling after cells have started to invaginate. Although affecting distinct cell subsets, both pathways impinge on the same cellular events, namely cell polarity and adhesion required for the maintenance of tissue integrity, as indicated by the loss of DE-Cad, Dlg and actin accumulation and the downregulation of *cad88C* and *cad96C*. This role of EGFR signalling is reminiscent of its function in maintaining tissue integrity in trachea, where it also impacts on cell adhesion through the control of DE-cadherin accumulation (Cela and Llimargas, 2006). Default in proper maintenance of tissue integrity results in cells that loose the proper balance between cellular adhesiveness and motility, a balance critical for proper tissue remodelling during organogenesis. This results in cells that do not maintain their proper position and shape in the organ, leading in extreme cases to dissociation from the organ. Consequently these cells do not proceed through the last step of spiracular chamber organogenesis, extension of baso-lateral membranes that provides the organ with its final elongated shape.

### *Spatio-temporal coordination of organogenesis*

Organogenesis can be subdivided in three main steps, primordium specification, primordium patterning and organ formation. Combining previous data (Merabet et al., 2005) and the results reported here indicate that EGFR, Hh and Wg signalling are involved in multiple events during all distinct steps of spiracular chamber organogenesis (Fig. 7). First, EGFR and Wg pathway activities are required from early stage 11 to maintain *ems* expression in the primordium (Merabet et al., 2005), whereas Hh signalling is dispensable. By mid-stage 11 and during the patterning step, Hh signalling together with the Hox protein Abdominal-B are needed to remodel Rho and Wg expression towards an A8-specific pattern (Merabet et al., 2005). Then, the three pathways are required for cell survival from stage 12 until stage 13 (this study). At that time, signalling by EGFR and Hh, but not by Wg, play important roles in the maintenance of tissue integrity (this study). EGFR, Hh and Wg signalling pathways are therefore reiteratively used during spiracular chamber morphogenesis.

We propose that the reiterative use of signalling activities, the spatial properties of which evolve in the course of spiracular chamber organogenesis (Merabet et al., 2005), may serve to coordinate the events that will collectively allow proper organogenesis. One key aspect of coordination is to couple patterning activities to cell behaviour. This is well exemplified by Hh signalling, whose function during the patterning step provides axial specificity to Wg and EGFR signalling, at stage 12 fulfils cell survival functions, and at stage 14 controls cell adhesiveness, polarity and tissue integrity. Another crucial aspect is to temporally and spatially coordinate the dynamics of cell behaviours. This appears particularly important in spiracular chamber organogenesis, as cells of the primordia will sequentially, depending on their position, adopt specific cell behaviours, instead of being all subject at the same time to the same changes. Deciphering how reiterative use of signalling pathways allows temporal coordination of organogenesis will require understanding how cells respond distinctly with regard to time to the same signalling cues.

## Materials and methods

### *Fly stocks*

The following fly strains have been used in this study: UAS-EGFR-DN (Bl 5364), UAS-Ci-DN (Hepker et al., 1997), UAS-TCF-DN (Bl 4784), ems-Gal4 (Merabet et al., 2002), UAS-P35 (Bl 5073), UAS-mCD8::GFP (Bl-5137), UAS-act::GFP (Verkhusha et al., 1999), *grhD4-lacZ* (Hu and Castelli-Gair, 1999), $wg^{l\text{-}17}$ (BL 2980), $hh^{21}$ (BL 5338), $egfr^{f2}$ (BL 2768) and *Df(3L) H99* (BL 1576).

### *Immunostaining and whole-mount in situ hybridization*

Embryos collections, immunodetections and in situ hybridizations of whole embryos were performed according standard methods. Primary antibodies used were: mouse anti-Cut (supernatant, DSHB) 1:20, mouse anti-Crb (concentrate, DSHB) 1:500, rat anti-DE-Cad (concentrate, DSHB) 1:500, mouse anti-Dlg (supernatant, DSHB) 1:50, rabbit anti-GFP (Molecular Probes) 1:500, mouse anti-GFP (Molecular Probes) 1:200, chicken anti-GFP (Aves labs, Inc) 1:1000, mouse anti-β-galactosidase (Promega) 1:1000, rabbit anti-β-galactosidase (MP Biomedical) 1:1000, rabbit anti-cleaved caspase 3 (Cell signalling) 1:100, rat anti-PH3 (Abcam) 1:1000, anti-digoxigenin coupled to biotin (Jackson) 1:500. Secondary antibodies coupled to Alexa 488,

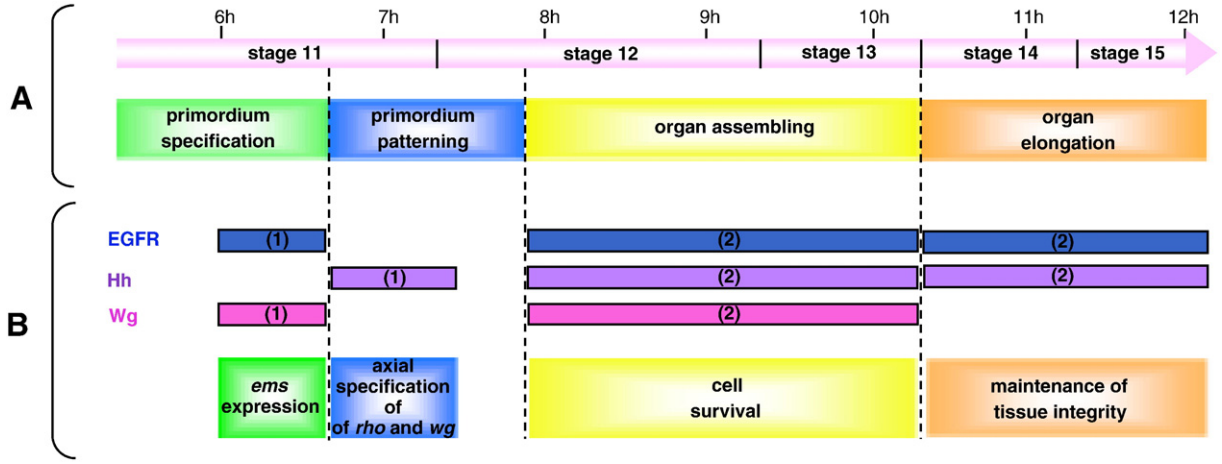


**Fig. 7.** Reiterative use of signalling pathways during spiracular chamber morphogenesis. (A) Correspondence between the four steps of posterior spiracle organogenesis, embryonic stages and time of development. (B) Temporal (blue, purple and pink boxes respectively for EGFR, Hh and Wg signalling) and qualitative requirements (boxes at the bottom) of EGFR, Hh and Wg signalling. Numbers within boxes refer to a previous study (1, Merabet et al., 2005) or this study (2).

Alexa 555 (Molecular Probes) or to biotin (Jackson) were used at a 1:500 dilution. When needed, the signal was amplified using the Tyramide Signal Amplification kit (NEN Life Science). Digoxigenin RNA-labelled probes were generated by in vitro transcription from plasmid containing a portion of *cad88C* or *cad96C* transcription unit (plasmid were kindly provided by Antonio Jacinto's lab). Embryos were mounted in Vectashield (Vector Laboratories). Images were collected at a 40× magnification by laser confocal scanning microscopy (Zeiss LSM510) and processed by using Zeiss LSM510 image browser and Adobe photoshop 7.0 softwares. Except when specified in Figs. 6, S2 and S4, images are projections of confocal sections.

*Boxplot data presentation and statistical analysis*

Representation was drawn using the R-Software. Boxplot depicts smallest value, lower quartile, median (red line), upper quartile and largest value for each genotype. Black points correspond to individual counts. Wilcox tests were performed ($p$-values establishment) to assess the significance of counts differences between genotypes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.04.001.

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